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Abstracts

– Plenary Lectures –

O-1

Far-field optical nanoscopy

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The resolution of a far-field optical microscope is usually limited to $d = \lambda/(2n\sin\alpha) > 200$ nm, with $n\sin\alpha$ denoting the numerical aperture of the lens and λ the wavelength of light. We will discuss lens-based fluorescence microscopy concepts that feature a resolving power on the nanoscale. All these concepts share a common basis: exploiting selected (pairs of) states and transitions of the fluorescent marker to neutralize the limiting role of diffraction. Specifically, the fluorophore is switched on and off, that is, between a bright and a dark state, to detect the emission of adjacent features sequentially in time. The first viable concept of this kind was Stimulated Emission Depletion (STED) microscopy in which the fluorescence ability of the dye is switched off by stimulated emission. In the STED microscope, the extent of the region in which the molecule is able to fluoresce follows $d \approx \lambda/\left(2n\sin\alpha\sqrt{1+I/I_s}\right)$, meaning that fluorophores that are further away than d can be separated. I is the intensity that drives a fluorophore from the bright fluorescent state to the dark ground state by stimulated emission. I_s depends (inversely) on the lifetime of the states. For $I/I_s \to \infty$, it follows that $d \to 0$, meaning that the resolution can be molecular). Altogether, far-field optical 'nanoscopy' is a fascinating development in optics with high relevance to the many areas of sciences, in particular the life sciences. Since it has already been a key to answering important questions in biology, and owing to its simplicity and commercial availability, we expect far-field fluorescence 'nanoscopes' to enter most cell biology and many nanoscience laboratories in the near future.

O-2

***In vivo* molecular sensing: fluorescence beyond labeling**

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Fluorescent molecules are powerful reporter tools that have much extended the impact of optical microscopy, particularly thanks to the flexibility of genetically encoded tags. Detection can now target single molecules even in the complex environment of intact live cells offering unprecedented insight on biological processes in real time in live cells and tissues.

Fluorescent labels, however, can do more that this. Our increased ability to tailor molecule and, more in general, nanosystem properties allows us to design, produce and exploit *intelligent* tags that can actually *analyze* the cellular environment. Today multifunctional nanosystems can be produced that provide a signal dependent on the value of a specific biochemical parameter. Importantly these nanosystems can target specific subcellular domains and have the ability to be used also in the case of live organisms.

Recent results will be discussed that highlight the impact of nanobiotechnology in this context with a particular emphasis given to methods suitable for *in vivo* studies that can be transferred to the biomedical world.

O-3

Mass spectrometry and its role in structural biology

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Standard proteomics techniques are unable to describe the stoichiometry, subunit interactions and organisation of assemblies since many are heterogeneous, present at low cellular abundance and frequently difficult to isolate. We have combined two existing methodologies to tackle these challenges: tandem affinity purification (TAP) and nanoflow ESI-MS. We use methods designed to maintain non-covalent complexes within the mass spectrometer to provide definitive evidence of interacting subunits based on the masses of complexes and subcomplexes generated by perturbation both in solution and gas phases. Structural models will be presented for three oligomeric protein complexes of unknown structure: the yeast exosome and the human U1snRNP and eIF3 complexes. These models will then be examined within the context of their function.

Recent developments in mass spectrometry have added a further dimension to our studies of protein complexes: that of their collision cross-section. Using ion mobility mass spectrometry we have been able to add spatial restraints to our models validating our models with measurements of collision cross–sections.

Very recently we have had a considerable breakthrough which has enabled us to preserve intact membrane complexes in the gas phase . This enables us to establish lipid and nucleotide binding and to define the stoichiometry and post translational modifications within the intact transmembrane regions of a number of complexes.

O-4

GFP: Lighting up life

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Since its introduction as a biological marker, the *Aequorea victoria* Green Fluorescent Protein (GFP) has had a strong impact in biology, being used in an ever-increasing variety of ways. I will review a bit of the history of GFP and show how having a genetically-encoded marker that can be visualized in living tissues and does not require additional cofactors continues to inform the research in my own laboratory on the development and differentiation of touch-sensing neurons in the nematode *Caenorhabditis elegans*. A short list of uses in just our lab includes the use of GFP in promoter and protein fusions to 1) characterize spatial and temporal gene expression patterns; 2) characterize the subcellular distribution of proteins; 3) identify mutations affecting specific cells or processes; 4) label cells so they can be identified in culture or isolated by fluorescence-activated cell sorting; 5) label cells for electrophysiological characterization; and 6) develop additional biological methods. The development of GFP as a biological marker is a particularly good example of the importance of basic research on non-traditional organisms.

Abstracts
– Plenary Lectures –

O-5

Divergence in protein polymers: implications for evolutionary mechanisms

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Most proteins in the cell exist as part of higher-order assemblies, and biological insights into function require knowledge of the quaternary structures formed. We have developed new techniques for looking at helical polymers in electron cryomicroscopy, surmounting the problems caused by structural variability and heterogeneity. Applications of these methods to actin filaments, bacterial plasmid segregation protein ParM, bacterial and archaeal pili, and bacterial flagellar filaments shows how relatively conserved building blocks (protein monomers) can form dramatically different quaternary structures (polymers). This suggests that small changes in sequence, with relatively little impact on tertiary structure, can be greatly amplified by large changes in quaternary structure. The large changes in quaternary structure provide a mechanism for the evolutionary divergence of organisms that has not been fully appreciated.

O-6

Watching proteins function in real time via time-resolved X-ray diffraction and solution scattering

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To generate a deeper understanding into the relations between protein structure, dynamics, and function, we have developed X-ray methods capable of probing changes in protein structure on time scales as short as 150 ps. This infrastructure was first developed on the ID09B time-resolved X-ray beamline at the European Synchrotron and Radiation Facility, and more recently at the ID14B BioCARS beamline at the Advanced Photon Source. In studies of ligand-binding heme proteins, a picosecond laser pulse first photolyzes CO from the heme, then a suitably delayed picosecond X-ray pulse passes through the protein and the scattered X-rays are imaged on a 2D detector. When the sample is a protein crystal, this "pump-probe" approach recovers time-resolved diffraction "snapshots" whose corresponding electron density maps can be stitched together into movies that unveil the correlated protein motions that accompany and/or mediate ligand migration within the hydrophobic interior of the protein. When the sample is a protein solution, we recover time-resolved small- and wide-angle X-ray scattering patterns that are sensitive to changes in the size, shape, and structure of the protein. Scattering studies of proteins in solution unveil structural dynamics without the constraints imposed by crystal contacts; thus, these scattering "fingerprints" complement results obtained from diffraction studies. *This research was supported in part by the Intramural Research Program of the NIH, NIDDK*

O-7

Grabbing the cat by the tail: Discrete steps by a DNA packaging motor and the inter-subunit coordination in a ring-ATPase

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As part of their infection cycle, many viruses must package their newly replicated genomes inside a protein capsid. Bacteriophage φ29 packages its 6.6 mm long double-stranded DNA into a 42 nm dia. x 54 nm high capsid using a multimeric ring motor that belongs to the ASCE (Additional Strand, Conserved E) superfamily of ATPases. A number of fundamental questions remain as to the coordination of the various subunits in these multimeric rings. The portal motor in bacteriophage phi29 is ideal to investigate these questions and is a remarkable machine that must overcome entropic, electrostatic, and DNA bending energies to package its genome to near-crystalline density inside the capsid. Using optical tweezers, we find that this motor can work against loads of up to ∼55 picoNewtons on average, making it one of the strongest molecular motors ever reported. We establish the force-velocity relationship of the motor. Interestingly, the packaging rate decreases as the prohead fills, indicating that an internal pressure builds up due to DNA compression attaining the value of ∼6 MegaPascals at the end of the packaging. We show that the chemical energy of ATP is converted into mechanical work during phosphate release. Using ultra-high resolution optical tweezers, we determined the step size of the motor and established the coordination of the polymerases around the ring. We propose a comprehensive model of the operation of this motor.

Abstracts
– Membrane microdomains and signalling –

P-8

Short- and long-term effects of perinatal hypoxia in neuronal and glial P2X7 receptor

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Glutamate is the major excitatory neurotransmitter released from most neurons as well as glial cells. Perinatal hypoxia-ischemia can disrupt synaptic function leading to accumulation of extracellular glutamate with subsequent excitotoxicity linked to cellular Ca^{2+} overload and neuronal death.
Since we previously demonstrated that P2X7 activation can induce neuronal glutamate release by exocytosis and through P2X7 itself, here we teste if neuronal and glial P2X7 activity could be associated to short- and long-term effects of perinatal hypoxia. Experiments were performed measuring intracellular calcium $[Ca^{2+}]_i$ dynamic and [3H]D-aspartate ([3H]D-ASP) release in synaptosomes and gliosomes from rats born by natural birth or caesarean delivery followed by global anoxia.
The data show that perinatal anoxia significantly influenced the functionality of P2X7 receptor, whereas did not affect the depolarising agents (4-aminopyridine and ext. high K^+) action. In glial particles it decreased the Ca^{2+} influx induced by the synthetic P2X7 selective agonist BzATP and enhanced the [3H]D-ASP release in both early phase and in adulthood. Instead, only in adult synaptosomes the BzATP-evoked $[Ca^{2+}]_i$ level decreased, while [3H]D-ASP release was increased.
This work is supported by Mariani Foundation, Milan (Italy).

O-9

Synapsin Knock-Out mice as an *in vitro* model of human epilepsy studied with Multi-Electrode Arrays

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Mutant mice lacking synapsins (Syn), a family of synaptic vesicles (SV) proteins implicated in the regulation of neurotransmitter release and synapse formation, are epileptic. The attacks appear after the third month of age and severity increases with age. Several mutations of Syn genes have been found in families of patients with epilepsy. We used Micro-Electrode Arrays (MEAs) to study spontaneous and chemically evoked epileptiform activities in cortico-hippocampal brain slices obtained from wild-type (WT) and SynKO mice. 6-months old SynKO mice show sporadic ictal (IC) events in the entorhinal cortex. A potassium channel blocker, 4-aminopyridine (4AP), elicits IC and inter-ictal (I-IC) events in both WT and SynKO slices. In the hippocampus of young SynKO (15-days old) mice, 4AP induces I-IC events at higher frequencies than in WT mice. Also the frequency of IC events, mainly observed in the cortex, is higher in SynKO. The analysis of adult (1-year old) mice, revealed a clear age-related aggravation, which paralleled the increase in the severity of the epileptic phenotype observed *in vivo*. Many slices from adult SynKO mice showed an IC event, while WT slices were refractory at this age to experience IC activity. SynKO mice are useful to study how neuronal network hyperexcitabilty due to mutations in SV proteins leads to the development of epileptiform activity. MEAs proved themselves to be useful tools to characterize the epileptic signals *foci* and patterns of propagation.

O-10

Imaging of mobile stable lipid rafts in the live cell plasma membrane

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The organization of the cellular plasma membrane at a nanoscopic length scale is believed to affect the association of distinct sets of membrane proteins for the regulation of multiple signaling pathways. Based on *in vitro* results, conflicting models have been proposed which postulate the existence of stable or highly dynamic platforms of membrane lipids and proteins. Here we directly imaged and further characterized lipid rafts in the plasma membrane of living CHO cells by single molecule TIRF microscopy. Using a novel recording scheme for "Thinning Out Clusters while Conserving Stoichiometry of Labeling"[1], molecular homo-association of GPI-anchored mGFP was detected at 37°C and ascribed to specific enrichment in lipid platforms. The mobile mGFP-GPI homo-associates were found to be stable on a seconds timescale and dissolved after cholesterol depletion. Having confirmed the association of mGFP-GPI to stable membrane rafts, we attempted to use an externally applied marker to test this hypothesis. We used Bodipy-GM1, a probe that was recently reported to be enriched in the liquid-ordered phase of plasma membrane vesicles. When applied to CHO cells at different surface staining, we found that also Bodipy-GM1 homo-associated in a cholesterol-dependent manner, thus providing further evidence for the existence of membrane rafts. [1] Appl Phys Lett 87, 263903 (2005).

P-11

Structural determinants essential for nanodomain formation and function of the HIV receptor DC-SIGN

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DC-SIGN is a trans-membrane protein expressed on antigen presenting cells and recognizes pathogens like HIV-1, Hepatitis C virus and Ebola. By electron microscopy and near-field optical nanoscopy, we demonstrated that at the plasma membrane DC-SIGN is organized in well-defined nanodomains of 100-180 nm in diameter. Intensity–size correlation analysis revealed remarkable heterogeneity in the nanodomains molecular packing density. We constructed and characterized several DC-SIGN mutated forms lacking specific molecular domains. By immunogold labeling and spatial point pattern analysis, we show that the extracellular neck domain is essential for DC-SIGN nanoclustering. Finally, we present a model that describes the probability of a cell to have a certain number of receptors joining the contact site in the initial encounter with an external object. Monte Carlo simulations subsequently define the parameters that are determinant in the object-cell encounter. Our results show that receptor nanoclustering is of particular importance for binding objects of sizes comparable to the nanocluster size, indicating that the nanoscale spatial organization of DC-SIGN is optimized for binding to virus-sized objects.

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– Membrane microdomains and signalling –

P-12

Specificity of a HIV fusion inhibitor towards phosphatidylcholine gel phase membranes

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Since the efficacy of HIV fusion inhibitors was previously reported to be related to an ability to interact with membranes, we studied the interaction of the HIV fusion inhibitor sifuvirtide, a 36 aa negatively charged peptide, with lipid vesicles. Since this peptide has aromatic residues, fluorescence spectroscopy techniques were used with no need for attached probes. Results showed no significant interaction with both zwitterionic fluid phase and cholesterol-enriched membranes; however extensive partition to fluid phase cationic membranes were observed. In the DPPC gel phase, however, an adsorption at the surface of these membranes was detected by using a differential quenching approach with lipophilic probes, as well as by FRET. Moreover, the interaction with gel phase membranes seems to be specific towards PC vesicles, since no significant interaction was retrieved for membranes composed by shingomyelin and ceramide. Besides fluorescence, atomic force microscopy and zeta-potential were used to further investigate this issue. Our results show a selectivity and specificity of the peptide towards rigid domains, where most of the receptors are found, and help explain the importance of the interaction with membranes in the improved efficacy of sifuvirtide compared to other fusion inhibitors, by providing a local increased concentration of the peptide near the fusion site on both cellular and viral membranes.

P-13

The interaction of the Bax C-terminal domain with membranes

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The C-terminal domain of the pro-apoptotic protein Bax (Bax-C) acts as a membrane anchor during the translocation to the membrane of this protein leading to programmed cell death. We have used static and MAS-NMR techniques to show that the interaction of Bax-C with membranes is modulated by the presence of a negatively charged phosphatidylglycerol. The width of the resonance peaks were considerably more increased by Bax-C, in the presence of phosphatidylglycerol. Bax-C substantially decreased the T_1 relaxation times of phosphatidylglycerol and those of phosphatidylcholine when mixtured with phosphatidylglycerol but they were not decreased when phosphatidylcholine was the only phospholipid present in the membrane. ^{13}C-MAS-NMR showed that T_1 values were decreased when Bax-C was incorporated and, when phosphatidylglycerol was also present, the decrease in T_1 affected considerably more to some carbons in the polar region. These results indicate that Bax-C interacts differently with the polar part of the membrane depending on whether phosphatidylglycerol is present or not, suggesting that an electrostatic interaction of Bax-C with the membrane determines the membrane disposition of this domain. Fluorescence spectroscopy showed that the Trp residues of Bax-C were located in a microenvironment more hydrophobic when phosphatidylglycerol was present.

O-14

Measuring diffusion by spatial-cross-correlation

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Fluorescence correlation spectroscopy (FCS) has emerged as a very powerful method to study the motions of proteins both in the interior and exterior of the cell. It provides information at the single molecular level by averaging the behavior of many molecules thus achieving very good statistics. Single particle tracking (SPT) is also a highly sensitive technique to measure particle movement. However, the FCS method suffers in spatial resolution while the SPT technique only allows for the tracking of isolated molecules. Here we propose a change of paradigm in which using spatial pair cross-correlation functions we can overcome this limitation. Our method measures the time a particle takes to go from one location to another by correlating the intensity fluctuations at specific points on a grid independently on how many particles are in the imaging field. Therefore we can trace the average path of the particles. For example, our method could be used to detect when a protein passes the nuclear barrier and the location of the passage. This information cannot be obtained with the FRAP (fluorescence recovery after photobleaching) technique or the image correlation spectroscopy method.

P-15

Semiconductor based biosensors: applications in radiation biology

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High Electron Mobility Transistor (HEMT) structures were used to bridge the gap between the analysis of biological reactions and biophysical characterization. The combination of nanotechnological measurement approaches with biological reactions provides new possibilities for living cell examinations after exposure to ionizing radiation and basically during the irradiation experiments itself. In this transdisciplinary approach experimental data and handling of biological material enables the identification and specification of systems properties of biological responses to ionizing radiation at different hierarchical levels.
GaN/AlGaN-heterostructures form a HEMT with a gate very sensitive to pH-value changes and potential changes in general. To record cell membrane potentials and ion fluxes during and after irradiation experiments living cells are cultivated on the functionalised biocompatible chip surface.
Here, we present results of X-ray stimulated cell responses grown on GaN-chip surfaces. We recorded transistor signal changes of 0.13 μA within 60 s caused by an irradiated cell monolayer. To measure cell potentials, not only after irradiation experiments but also during the irradiation itself expands the examination restrictions in an enormous way.

Abstracts

– Membrane microdomains and signalling –

P-16

ESR and microcalorimetry studies on interaction of resveratrol and piceatannol with DMPC membranes

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Resveratrol (trans-3,4',5-trihydroxystilbene), a phytoalexin present in grapes and its analogue piceatannol (trans-3,4,3',5'-tetrahydroxystilbene) are biologically active compounds and possess potential chemopreventive and anticancer properties. The activity of resveratrol and piceatannol can be mediated by membrane effects since structure of lipid membrane domains may play an important role in cell signalling pathways.

Drugs interactions with DMPC bilayers was investigated using a combination of ESR spectroscopy and differential scanning calorimetry. Spin probes used in EPR experiment were located in different part of lipid bilayer. Study was performed at temperatures below and above phase transition temperature (T_m). EPR spectra were simulated and displayed with GHOST condensation method. The values of ϑ and φ (the main and asymmetry cone angles of wobbling spin probe, respectively) were taken for free rotational space parameter (Ω) calculation. The decrease of Ω values was observed in the presence of both compounds and the effect was more pronounced in lipid gel phase. Order parameter and correlation time were also determined and presented in form of GHOST patterns. Using this approach the differential influence of studied compounds on membrane heterogenity was revealed.

P-17

Structural and dynamic properties of caveolin-1 and -2 fragments at the membrane interface

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Caveolins are major protein components of caveolae, microdomains of the plasma membrane involved in a large number of biological functions, including signal transduction, cholesterol homeostasis and transport. The consensus topological model of caveolin-1 includes a small central intramembrane region (102-134) flanked by two cytosolic amphiphilic domains (82–101 and 135–150) which probably constitute in-plane membrane anchors. We investigated the interaction of the cav-1(94-102) juxta-membrane segment with various membrane mimics, using fluorescence, CD and NMR. This segment partitioned better in DPC and in DM/anionic lipids micelles than in DM micelles and this partitioning was coupled with the formation of an amphipathic α-helix. This amphipathic helix was located in an average shallow position, in the polar head group region of the DPC micelle, as shown by fluorescence data and intermolecular NOEs, with the aromatic doublet W98-F99 probably pointing towards the inside of the micelle on average. The peptide encompassing the homologous sequence of cav-2 was also localized to the DPC micelle polar head group region, in which it adopted a more stable helical conformation than cav-1(94-102). These data brings experimental support for the role of this segment as an interfacial membrane anchor.

O-18

Modulation of the properties of membrane microdomains as a control mechanism in cellular physiology

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This presentation will outline the molecular-physical rationale of how membrane microdomains may modulate the behaviour of membrane receptor systems as a controlling mechanism in cell signaling. A number of external factors that modulate these properties will be indicated that have a bearing on controlling cellular behaviour. Some of key questions will be considered such as the factors that control the assembly and disassembly of microdomains, the size and number-density of the microdomains and the lifetime that they exist within the membrane. The technical challenges that these questions identify will also be outlined with some possible solutions. Throughout this presentation, correlations will be made between theory and experiment as well as between model membrane systems and real cellular systems.

O-19

Exploring the nanoscale: Dynamics of lipid rafts revealed by STED fluorescence spectroscopy

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The study of molecular dynamics at the single-molecule level with fluorescence far-field optics offers new detailed insights into scientific problems, especially in living cells. Unfortunately, the resolution of common far-field techniques is limited to about 200nm in the lateral direction by diffraction. In recent years, several concepts such as stimulated emission depletion microscopy (STED) have been successfully applied to overcome the diffraction barrier by exploiting the photophysical properties of fluorescent labels. We present the combination of high resolution STED microscopy with different fluorescence fluctuation techniques providing the unique ability to study molecular dynamics with high spatial (<40nm) and temporal resolution (<1ms) in living cells. Using fluorescence correlation spectroscopy (FCS) and general single-molecule analysis, we were able to explore single-molecule dynamics in up to 70-fold reduced focal volumes on two-dimensional samples such as lipid membranes with excellent signal-to-noise ratios. Special attention is drawn to inhomogeneous lipid diffusion on the plasma membrane of living cells[1]. By extending the available spatial scale of standard single-molecule fluorescence far-field spectroscopy techniques, our experiments outline a new way of approaching scientific problems.

[1]Eggeling, C., Ringemann, C. et al. Nature (2009) 457, 1159-1163.

Abstracts
– Membrane microdomains and signalling –

P-20

Selective changes of hMOP receptor compartmentalization by distinct agonists as revealed by vrFRAP

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We report fluorescence recovery after photobleaching (FRAP) measurements performed at variable spot radius for T7-EGFP-hMOP receptors on SH-SY5Y neuroblastoma cells in the presence of ligands. Two different agonists, DAMGO and morphine, caused markedly different changes to receptor diffusion as compared to the basal state. Like receptors in the absence of ligand, receptors bound to morphine exhibited diffusion confined to joint semi-permeable domains, but with smaller domain size and diffusion coefficient. This effect was inhibited by pertussis toxin, suggesting that this dynamic behaviour is associated with early steps of signaling. In the presence of DAMGO, half of the receptors displayed free long-range diffusion and the other half were confined to smaller isolated domains. Hypertonic sucrose buffer suppressed this effect which we attribute to receptor entry into clathrin-coated pits. It is likely that the observation of distinct receptor dynamics in the presence of DAMGO and morphine involves the agonist-selective phosphorylation of the receptor.

P-21

AFM Study of Protein Redistribution upon Lipid Domain Formation in Supported Lipid Bilayers

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In a recent study we showed that the melting behavior of Supported Lipid Bilayers (SLBs) on mica can be influenced by the solution ionic strength and by the SLB preparation temperature [1]. By changing these parameters we could control the coupling between the two bilayer leaflets obtaining a coupled or decoupled melting behavior. Thus, we could provide evidence that the SLB model system is also suited for the study of lipid/protein interactions which had been questioned in the past. Then we investigated the mutual interactions between the membrane lipids (POPE:POPG 3:1) and the KcsA potassium ion channel by studying KcsA proteins reconstituted in SLBs. In particular, we studied the melting behavior of the SLB and the ion channel distribution relative to the different membrane phases by temperature controlled atomic force microscopy (AFM). By decreasing the temperature we found that the proteins underwent diffusion so to be excluded from the growing solid ordered regions. Further, the ion channels tended to accumulate at the domain boundaries or they aggregated in the liquid disordered phase. Both effects have been suggested to affect protein function. When we started from a low temperature at which the membrane was mainly in the solid ordered phase the membrane melting processes started in the vicinity of the included ion channels.
[1] Seeger et al., Biophys.J. (2009), in print

O-22

Membrane potential dynamics of living cells in response to femtosecond laser irradiation

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The ultrashort pulsed near-infrared femtosecond laser has had a large impact in biomedical research fields and in microscopy, where it has enabled new imaging methodologies. At high intensities, the focused beam of a femtosecond laser has been used to irradiate specific locations inside a cell, often beneath the cell membrane, exploiting the inherent penetration and localized absorption that comes from the multiphoton absorption physics. This has been applied in photobleaching, photouncaging, laser surgery and other experiments where the light is used not merely for observation but is instead an integral tool to interact with the dynamics of cells, to probe and perturb the cell condition. In this talk I will discuss biological and mechanical effects that can be generated by short exposures to femtosecond laser irradiation, such as calcium waves, membrane hyperpolarization, and cell contraction. This talk will concentrate on the changes in membrane potential that can occur when the cell is subjected to focused femtosecond laser beams. Both depolarization and hyperpolarization of the membrane potential could be evoked, depending on the laser parameters and on the position of the laser focus. These results have implications for the use of laser beams in microscopy, optical gene transfection, and laser nanosurgery.

P-23

Separating hydrostatic pressure from cellular strain: development of an *in vitro* model system

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Endothelial cells (ECs) line blood vessels & are constantly subjected to haemodynamic and mechanical stresses and strains. These stimuli are known to influence ECs, modifying their morphology, intracellular signalling & gene expression. Most reported systems exposing EC to mechanical forces *in vitro* alter pressure & strain simultaneously, making it impossible to distinguish the two potentially independent stimuli. This distinction is particularly relevant when examining the interaction of haemodynamic forces on microvascular ECs, which are exposed to low hydrostatic pressure but significant strains.
This research aims to create an *in vitro* system that can independently examine the effects of pressure and strain, over a range experienced by ECs in the microvasculature.
Human ECs are seeded (12x10ˆ4cells/cmˆ2) on to the inner surface of compliant 4mm diameter tubing. Which is mounted on a perfusion rig inside a sealed, fluid-filled chamber. A continuous sinusoidal cyclical strain of 5-10% is created by a pump attached to the external chamber. Lumenal pressure is generated using two hydrostatic pressure heads. Validation experiments show that pressure & substrate strain can be independently varied & controlled over a physiological range. The system is now being used to investigate the effects of pathophysiological haemodynamic abnormalities on EC function.

Abstracts
– Membrane microdomains and signalling –

P-24

FRET-sensitized acceptor bleaching reveals the large-scale co-clustering of ErbB1 and ErbB2

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According to the classical model ErbB receptor tyrosine kinases form homo- and heterodimers upon ligand stimulation leading to their activation and cell proliferation. However, it has recently been shown that dimers and larger clusters of both ErbB1 and ErbB2 are present even in the absence of stimulation. In the present study we used the FRET-sensitized acceptor bleaching (FSAB) technique to quantitate the ratio of ErbB1 and ErbB2 in heteroclusters. In this method a photostable donor excites a photolabile acceptor by FRET, and the acceptors within FRET distance to the donor will get photobleached and the fraction of acceptor molecules in the vicinity of donors can be determined.
During prolonged excitation of the donor the FRET efficiency decreased to zero long before all of the acceptors were bleached indicating that only a fraction of acceptors is in the vicinity of donors. In quiescent cells 40% of ErbB1 colocalizes with ErbB2 in large-scale clusters, while 10% of ErbB2 coclusters with ErbB1. Upon EGF (ligand of ErbB1) stimulation the fraction of ErbB2 forming heterocluster with ErbB1 increased, while that of ErbB1 forming heterocluster with ErbB2 decreased. The fraction of ErbB1 and ErbB2 in heteroclusters was independent of the expression level of the receptors. In contrast, the FRET efficiency depended on the donor and acceptor ratio as expected. The FSAB technique can be used to quantitate the composition of large-scale protein clusters.

P-25

High resolution mapping of LFA-1 association with lipid rafts

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Lymphocyte function-associated antigen-1 (LFA-1) is a leukocyte specific integrin that mediates migration across the endothelium and is also involved in the formation of the immunological synapse. Association of LFA-1 into specific cholesterol enriched microdomains, called lipid rafts, is thought to regulate its activity. These 20 to 200 nm lipid rafts are abundantly present. However, these length-scales are not available for conventional light microscopy, showing the need for high resolution techniques. Near-field scanning optical microscopy (NSOM) is a shearforce based scanning probe technique that uses a sub-wavelength aperture probe to locally excite fluorophores. Here, we have used two-colour excitation/detection NSOM, with a resolution below 100 nm, in aqueous conditions to map with nm accuracy the LFA-1 association with lipid-rafts on cell membranes. High resolution NSOM imaging showed that LFA-1 and raft markers are pre-organised in different but 50-100 nm proximal nano-compartments. This well-defined pre-assembling of lipids and receptors might constitute a prominent mechanism exploited by the cell to rapidly and efficiently aggregate distinct nano-domains into larger functional cell surface assemblies.

P-26

The aggregation behavior of Janus Kinase 3

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The four members of Janus kinase family, JAK1-3 and TYK2, provide the interconnecting link for a complex matrix of extracellular and cytoplasmic receptor-ligand pairs in cytokine signaling. Here we show first results of our attempt to reconstitute a functional fluorescently tagged Interleukin-4 receptor (IL4R) signaling complex at the plasma membrane. The IL4R type I consists of the IL4R alpha chain (IL4Ra) bound by JAK1 and the co-receptor IL2R gamma chain (IL2Rg, common gamma chain) bound by JAK3. In agreement with literature, we see that cytoplasmic fractions of JAK3 do not exchange with receptor bound fractions as shown by fluorescence recovery after photobleaching (FRAP). In addition, co-expression of JAK3 and IL2Rg leads to aggregation phenomena at the plasma membrane as indicated by the occurrence of fluorescent speckles. We further characterized the aggregation behavior of JAK3-EGFP, and mutants thereof, with fluorescence correlation spectroscopy (FCS) in the cytoplasm. The biophysical data are discussed with respect to downstream signaling. For this we studied IL4-induced and JAK3-mediated target gene activation with a Signal Transducer and Activator of Transcription 6 (STAT6) -responsive luciferase assay.

Abstracts
– Chromatin, nucleosomes and molecular machines –

O-27

Chromatin changes in leukemia: an imaging approach to study deregulated transcription in cancer

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The alteration of the physiological transcriptional program is one of the constant features of cancer cells. However, the characterization of the chromatin changes at single-gene level requires going beyond the diffraction limit affecting conventional fluorescence microscopy. The ability of molecular biology techniques to obtain a detailed view of the chromatin status at a sub-promoter resolution has to pay instead the price of averaging over a cell population. We report here the application of an approach based on high-resolution cytometry, chromatin immuno-precipitation and transcriptional profiling (DNA microarray) for the characterization of the transcriptional and chromatin changes induced by the oncogenic transcription factor PML/RARα. The presentation will focus on the imaging protocol employed to observe the effects on the chromatin status and the extent of the deregulation induced on transcriptional activity in Acute Promyelocytic Leukemia cells. This multiple-approach examination provides a further step towards the comprehension of the hierarchy of chromatin modifications leading to the establishment of a malignant transcriptional program.

P-28

The DNA-gate of gyrase is predominantly in the closed conformation during DNA supercoiling

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DNA gyrase catalyzes the introduction of negative supercoils into DNA *via* a strand-passage mechanism. In the first step, the gate-DNA binds to gyrase, is cleaved, and a covalent DNA-gyrase complex is formed. A second DNA segment, the transfer-DNA, is passed through the gap, and the gate-DNA is re-ligated. Strand passage requires opening of a transient protein interface at the cleavage site, the so-called DNA-gate, by ∼2 nm. The intermediate cleavage complex presents an inherent danger of DNA breaks and genome instability. Cleavage complexes have consistently been detected in very low amounts, but a recent study predicted frequent opening of the topoisomerase II DNA-gate. We present a single molecule FRET study that monitors both the conformation of DNA bound to the DNA-gate of gyrase, and the conformation of the DNA-gate itself. DNA bound to gyrase adopts two conformations, one slightly, one severely distorted from B-DNA geometry. Distortion requires cleavage, but not ATP or a transfer-DNA. The DNA-gate of gyrase is mainly in the closed conformation, in agreement with <5% of cleavage complexes in equilibrium. Importantly, gyrase with an open DNA-gate is also scarcely populated during the relaxation and supercoiling reactions. Presumably, distortion of the gate-DNA unlatches the DNA-gate, and prepares it for transient release by the transfer-DNA, thus providing a strict coupling of gate-opening to strand passage.

P-29

Nano-scale analyses of the chromatin decompaction induced by histone acetylation

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Acetylation of histone tails is one of the key factors involved in maintenance of chromatin dynamics and cellular homeostasis. The hallmark of active chromatin is the hyper-acetylation of histones, which appears to result in a more open chromatin structure. Although short nucleosomal array had been studied, the structural dynamics of relatively long acetylated chromatin remains unclear. Using atomic force microscopy (AFM), we have analyzed the detailed structure of long hyper-acetylated chromatin fiber. Hyper-acetylated chromatin fibers isolated from nuclei that had been treated with Trichostatin A, inhibitor of histone deacetylase, were found to be thinner than those from untreated nuclei. The acetylated chromatin fibers were easier to be spread out of the nuclei by a high-salt treatment, implying that hyper-acetylation facilitates the release of chromatin fibers from the compact heterochromatin regions. Chromatin fibers reconstituted *in vitro* from core histones and linker histone H1 became thinner upon acetylation. In the AFM images, the gyration radius of the nucleosomal fiber became larger after acetylation, suggesting that the acetylation increases long-range repulsive interactions between nucleosomes. Based on these data, we consider a simple coarse grained model, which explains that acetylation of histone-tails induces chromatin de-compaction by altering the electric charge distribution in nucleosomes.

P-30

Molecular crowding affects diffusion and binding of nuclear proteins in heterochromatin

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In the nucleus of eukaryotes, the genetic material is organized into functional membraneless compartments, the two most prominent being heterochromatin and nucleoli. Because these two structures contain large amounts of DNA, proteins or RNA in comparison to bulk euchromatin, they should be densely crowded at the molecular level. *In vitro*, crowded microenvironments have been shown to alter protein behavior by inducing volume exclusion, slowing down diffusion and enhancing association, but whether these effects are relevant *in vivo* remains unclear. We probed the behavior of fluorescently labeled inert tracers and chromatin interacting proteins in heterochromatin and nucleoli in comparison to euchromatin. We establish that size-dependent volume exclusion and hindering of diffusion occurs in dense nuclear compartments. In addition, we determine that binding of three generic chromatin-interacting proteins is enhanced in heterochromatin compared to euchromatin. Finally, we show that these effects of nuclear crowding are best explained by a fractal model of chromatin organization, which allows us to derive a set of chromatin structural parameters that provide a comprehensive framework to predict nuclear protein dynamics. Taken together our results show that fractal crowding has a key role in determining nuclear protein dynamics.

Abstracts

– Chromatin, nucleosomes and molecular machines –

P-31

Interaction between proteins from linker region of nucleosome in presence/absence of DNA in solution

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Interactions in the linker region of the nucleosome play a key role in the structural organization of the chromatin. The most fascinating and least understood is the interplay between non-histone chromatin protein HMGB1 and a linker histone H1. It is known that both H1 and HMGB1 bind the linker region of the DNA *in vivo*. However it is still a matter of debate as to whether these proteins assist each other or compete upon binding. The main attention in this work is paid to the investigation of the interactions between the HMGB1 and H1 proteins in physiological environment.
Using circular dichroism (CD) spectroscopy we have studied the interactions between HMGB1 and H1 at various HMGB1/H1 ratios (R). It has been shown that there is a CD-detectable interaction between the proteins H1 and HMGB1 at $R < 1$. We have demonstrated that the interaction between these proteins results in changes of their secondary structure. CD indicates that the structural impact of the unordered fragments decreases while the net α-helicity of the proteins increases upon the interaction. We have also shown that large higher order structures are formed in solution. In this work we have also discussed the DNA-binding properties of the HMGB1 and H1 proteins.
The work was supported by a RFBR grant (09-08-01119) and the Government of Staint-Petersburg.

O-32

A universal description for salt-(in)dependent oligocation-induced DNA condensation

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DNA is rigid negatively charged polymer and in solution exists in extended conformation. *In vivo*, volume occupied by DNA must be reduced to fit to tiny space of cell nucleus. To condense DNA, DNA-DNA electrostatic repulsion must be cut off that is achieved by interaction with cationic ligands. In binding to DNA, oligocations compete with salt cations (K^+, Na^+, Mg^{2+}). Description of salt dependence of oligocation-induced DNA condensation is still lacking. We studied DNA condensation by model oligocations, ε-oligo(L-lysines), with variation of charge from +3 to +31. Combination of light scattering, UV-monitored precipitation assay and isothermal titration calorimetry allowed covering wide range of DNA (C_{DNA}) and salt (C_{KCl}) concentrations. Salt dependence of DNA condensation efficiency of the ligand, EC_{50} (ligand concentration at the transition midpoint) displays two regimes: salt-independent at low C_{KCl} and salt-dependent at higher C_{KCl} (steep increase of EC_{50} with C_{KCl}). Simple formula describing EC_{50} as function of ligand charge, C_{DNA} and dissociation constant of ligand-DNA complex (K_d), was proposed. In the salt-independent regime EC_{50} is defined by C_{DNA}. Salt-dependence of EC_{50} is rooted in the variation of K_d with C_{KCl} earlier described in ligand-DNA binding studies. Importance of our findings for description of chromatin is discussed.

O-33

Chromatin dynamics: simulations and single molecule fluorescence studies

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With a coarse-grained model that approximates amino acids and DNA nucleotides by single beads we simulate nucleosomal DNA dynamics on a 10 μs time scale. We observe transient opening ('breathing') of one linker DNA end from the histone core; without histone tail - DNA interactions, both linker DNA arms breathe with increasing frequency. Similar events were seen by others in single molecule fluorescence on immobilized nucleosomes, and by ourselves in free solution single molecule FRET.
A larger-scale Brownian dynamics model, in which DNA is viewed as a wormlike chain and the histone core as a cylinder, serves to simulate DNA unrolling from the histone core. Comparison of the force-extension curves with experiments yields a histone/DNA binding energy of 2.1 kT per base pair. The folding of chromatin in the nucleus and the diffusional transport of proteins is simulated in a discrete-lattice model. This is complemented by intranuclear mobility measurements of GFP mono- to tetramers; these penetrate the cell nucleus more or less homogeneously, however, the dependence of the diffusion coefficient on molecular mass is much higher than that free in solution. Thus, larger proteins 'feel' the chromatin network more strongly, producing anomalous diffusion behavior. Such mobility measurements lead to a 'mobility image' of the fluorescent marker in the cell.

O-34

Nucleic acid-protein interactions: one-at-a-time

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Single-molecule approaches can be used to achieve a fundamental understanding of biological processes occurring at the nanometer (nm) scale, in the millisecond (ms) time domain and at the piconewton (pN) force level. We will describe experiments using various single molecule approaches to follow the interactions of individual nucleic acid-protein interactions. http://www.cbp.pitt.edu/faculty/leuba/index.html

Abstracts
– Chromatin, nucleosomes and molecular machines –

P-35

Nitroxides induce apoptosis through caspase-3 activation and collapse of mitochondrial potential

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Nitroxides are new class of antioxidants that have been proved to show high reactivity toward free radicals. They act as superoxide dismutase mimics dismutating superoxide anions, but can also exert pro-oxidative properties. In view of their possible dual activity nitroxides could be of great importance in medicine.
We have investigated pro-apoptotic activity of pyrroline and pyrrolidine nitroxides Pirolid (PD) and Pirolin (PL) in human breast cancer cells. In cancer, it is the failure of malignant cells to undergo apoptosis that is crucial. Using microplate fluorescence methods, we estimated kinetics of changes in mitochondrial transmembrane potential and caspase-3 activity in breast cancer cells MCF-7 treated with Pirolin or Pirolid. These features are connected with induction of apoptosis in some type of cancer cells. We observed steady-state increase in caspase-3 activity up to 12 h of post-incubation that was followed by a decrease in the enzyme activity at 24 h. Caspase-3 activation was considerably greater in cells treated with Pirolid. Both nitroxides also caused notable decrease in mitochondrial transmembrane potential, which suggest that they can induce apoptosis in breast cancer cells through mitochondrial pathway.

P-36

Biophysical properties and mechanisms of phage DNA ejection

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To determine the requirements for phage adsorption on bacterial cell and for the realizing resources of following DNA ejection thermodynamic and hydrodynamic methods were employed. The temperature, bacterial membrane fragments and receptors had been chosen as such external factors. The phages with short and long tail, both contractile and noncontractile have been studied. Our viscometric studies of the phage DNA ejection induced by receptor by the example of *T5* phage and its receptor *FhuA* have shown that the minimum protein-to-phage ratio necessary for complete DNA release is 300 to 1. The viscometric study of *DDVI* phage DNA ejection induced by membrane fragments obtained from its host cells has shown that the environmental conditions play significant role in ejection process. Both methods show that the thermally induced phage DNA ejection for all investigated by us phages have shown that this process is nonenthalpic. Finally from our experimental results we conclude that the start of the DNA ejection process from the phage particle occurs without additional energy from either a physical or chemical source.
We thank GNSF for the financial support.

O-37

Structural organization of supramolecular complexes of DNA with chromosomal proteins HMGB1 and H1

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A combination of UV and IR absorption and circular dichroism spectroscopy together with atomic Force Microscopy was applied to investigate the structure and formation of large supramolecular DNA-protein complexes. This combination of techniques was used to overcome limitations of UV-CD spectroscopy due to considerable light scattering in such solutions. Based on the analysis of FTIR and UV circular dichroism spectra and AFM data the interaction of DNA with high-mobility group non-histone chromatin protein HMGB1 and linker histone H1 was studied.
It is believed, that HMGB-domain proteins perform both structural and regulatory functions in chromatin. However, the particular mechanisms of it functioning remain unclear/ Our data show that histone H1 facilitated binding of HMGB1 to DNA by interacting with the sugar-phosphate backbone and binding of Asp\Glu amino acid residues of HMGB1. Acting together, HMGB1 and H1 stimulated the assemblage of supramolecular DNA-protein structures. The organization of the ternary complexes is modulated by the interactions between HMGB1 and H1 molecules. The DNA-proteins interactions in the presence of metal ions were different, causing prominent DNA compaction and formation of large intermolecular complexes.
The work was supported by RFBR (grant 09-08-01119).

P-38

Structural changes of HMGB1 chromosomal protein upon binding to DNA

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HMGB1 is a nonhistone chromosomal protein. Data regarding the structure of the HMGB-proteins obtained so far are rather different. Thermodynamic experiments reveal predominant α-helical structure of the proteins only at temperatures below +5°C, i.e. under physiological conditions they are mainly disordered. Despite a lot of experimental data biological role of HMGB1 still remains unclear. It is believed that the proteins perform structural functions in chromatin and participate in various regulatory processes in cell.
Using circular dichroism spectroscopy and DNA melting analysis we have shown that HMGB1 changes its structure upon binding to DNA. It was shown that at room temperature only about 25% of amino acid residues form α-helices, while in DNA-HMGB1complex the degree of the α-helicity of the protein increases to approximately 50%. Based on the data obtained we estimate the size of HMGB1 binding site as 80-100 b.p. We have also demonstrated that despite of strong DNA-bending properties of HMGB1 its binding to DNA results in increase of the double helix termostability.
The authors are grateful for the financial support from the Russian Foundation for Basic Research (grants 09-08-01119, 07-04-01072) and the Government of Saint-Petersburg.

Abstracts

– Chromatin, nucleosomes and molecular machines –

P-39

Different decondensed states of nuclei reveal nucleosome-like structures in the histoneless liquid crystalline chromosomes of the dinoflagellates

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The largest known genomes are encoded by the liquid crystalline chromosomes (LCC) of the dinoflagellates. There is a small amount of lysine-rich basic nuclear "histone-like" proteins associate with DNA with the ratio of less 0.1 in LCC, previous ultrastructure and biochemistry study of dinoflagellate chromosomes suggest that they are absence of nucleosome and there is no connection between dinoflagellate and others eukaryotic chromosome. The DNA packing in these amazing chromosomes still remains enigmatic. In this study, the different decondensed states of isolated dinoflagellate nuclei were controlled by a simple EDTA chelating method. The hydrodynamic characteristics of decondensed processes were measured using microcalorimetry techniques. The native chromosome structures of different decondensed states observed by CLSM with crossed polarizer, AFM and TEM. The results revealed both macroscopic and ultramicroscopic distinctive features of the dinoflagellate chromosomes. Our data suggest that there is similar fundamental granular nucleosome like substructure in dinoflagellate system which exists both in prokaryotes and eukaryotes. The present study was supported by grant CERG HKUST6421/06M from the Research Grant Council of Hong Kong to JTYW.

O-40

Nucleosome dynamics dependent on salt environment, H1, Topo II, and histone tails

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The most fundamental structure of the chromosome is a nucleosome that forms "beads-on-a-string" fiber with the width of 11 nm. It has been widely assumed that nucleosomes array forms a 30 nm fiber structure, and that the ∼30 nm fiber is converted into one-step higher-order structure in interphase nuclei. In order to monitor the chromatin dynamics with nanometer-scale resolutions, we developed a chromatin reconstitution system where nucleosomes can be formed on long (∼100 kb) plasmids and the structural dynamics induced by the changes in chemical environment and the presence of chromosomal proteins can be analyzed. Well-spread beads-on-a-string fibers in 50 mM NaCl were converted to partial aggregations in 100 mM NaCl. Namely, the nucleosome-nucleosome interaction is strongly affected by the salt environment. When histone H1was added into the reconstituted nucleosome at the molar ratio of 1 against the core histone octamer, the fibers with 20∼30 nm width were formed. With tailless-octamars prepared by a mild treatment with trypsin, nucleosomes were efficiently reconstituted as with the case for intact octamars. However, salt-induced partial aggregation and H1-induced ∼30 nm fibers could not be detected in the tailless samples. These results suggest that the histone-tail plays critical role for the higher-order assembly of the nucleosomes. In conclusion, our *in vitro* system provided clues for uncovering the underlying physical properties of chromatin formation.

Abstracts
– Live cell imaging –

O-41

A generalized quantitative FRAP method with no restriction on the size of the photobleached area

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A FRAP model based on the assumption of ‘a small amount of photobleaching’ and hence a linear photobleaching process has been developed. This approach facilitates the data analysis for a wide range of region of interest (ROI) sizes on a confocal laser scanning microscope. A data analysis program was written to allow for the simultaneous analysis of FRAP traces collected at various ROI sizes. This global analysis by linking common model parameters has several benefits: a) less stringent conditions with respect to the signal-to-noise ratio, b) higher model discrimination power, and c) the possibility for an intrinsic determination of an instrumental parameter obviating the need for an extrinsic calibration. Additionally, attention has been devoted to estimate the variance of the individual pixel values. This allows for a proper weighting in the global analysis approach. Validation experiments were performed on a Bio-Rad MRC1024 confocal microscope with as a test sample, a 2000 kDa FITC-dextran solution in HEPES buffer (5 mg/ml) to which 56% (w/w) sucrose was added to increase the viscosity. FRAP experiments were carried out for disks of various sizes (from $\sim$1.5 μm radius and higher).

P-42

Towards intravital two photon microscopy study of lymphocytes mobility in lymphonodes

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During the last 30 years the edge between optical fluorescence based microscopy and the world of bio-medical research has become thinner and thinner and Two Photon Laser Scanning Microscopy (TPLSM) is one of the most powerful tool for immunological and medical research. One of the most limiting step in intravital microscopy is the preparation of the animal model and the number of animals to be sacrificed to get good statistics. Alternative routes must be searched.
We employ TPLSM to explanted lymphnodes. Two photon excitation and a non descanned detection mode allow to increase, respectively, the excitation and detection efficiency while the explanted organs are kept very close to the condition they experience in live animals by means of an home-made temperature controlled box surrounding the microscope and a system for the flux of physiological fluids.
Experiments performed on explanted lymphonodes, kept under constant flux of CO_2-O_2 saturated buffers and at 36 ^oC, agree with literature for what concern T-cell homing and motility. This seems to confirm that T-cells behavior in explanted organs mantained in physiological conditions is very similar to that observed in live animals. We then believe that our TPLSM microscope would allow to study cell behaviors in *in-vivo* with high efficiency and a little technical effort.

P-43

Intracellular delivery and fate of peptide-capped gold nanoparticles: towards cellular biosensors

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Gold nanoparticles (NPs) have extraordinary optical properties that make them very attractive single molecule labels. Although understanding their dynamic interactions with biomolecules, living cells and organisms is a prerequisite for their use as *in situ* sensors or actuators. While recent research has provided indications on the effect of size, shape, and surface properties of NPs on their internalization by living cells, the biochemical fate of NPs after internalization has been essentially unknown. Here we show that peptide-capped gold NPs enter mammalian cells by endocytosis. We demonstrate that the peptide layer is subsequently degraded within the endosomal compartments through peptide cleavage by the ubiquitous endosomal protease cathepsin L. Preservation of the peptide layer integrity and cytosolic delivery of NPs can be achieved by a combination of cathepsin inhibition and endosome disruption. This is demonstrated using a combination of distance-dependant fluorescence unquenching and photothermal heterodyne imaging. These results prove the potential of peptide-capped gold NPs as cellular biosensors. Current efforts focus on in-vivo labeling of NPs, nanoparticle-based real-time sensing of enzyme activity in living cells, and the development of photothermal microscopy for single nanoparticle imaging in living cells.

P-44

In vivo study of mouse experimental melanoma by combination of confocal and nonlinear microscopy

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The second harmonic generation (SHG) imaging along with confocal laser scanning microscopy in reflectance mode can be applied to imaging unstained tissues *in vivo*, so it can be considered as a fast and non-invasive tool for *in vivo* studies. Murine B16F10 melanoma cells after subcutaneous inoculation in syngeneic mice were let to develop into tumor up to 15-20 mm in diameter. Microscopic images were taken before and after microwave hyperthermia treatment (MWHT). The microscopic images were acquired by 1-photon imaging in reflectance mode, SHG imaging and 2-photon imaging of tissue autofluorescence. The evaluation of changes in the images after MWHT of the tumor demonstrated changes in the architecture and organization in both the tumor capsule and tumor mass.
The presented study was supported by the Academy of Sciences of the Czech Republic (grant IAA500200510, Institutional Research Concepts No.AV0Z50200510, AV0Z50110509), and Ministry of Education, Youth and Sports of the Czech Republic (research program LC06063).

Abstracts
– Live cell imaging –

O-45

Local BDNF delivery by means of laser tweezers to stimulate hippocampal neurons signalling

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Brain derived neurotrophic factor (BDNF) secretion occurs from both dendrites and axons and is regulated in an activity-dependent manner. Its effects, mediated mainly by TrkB receptor, are partially contrasting since BDNF can induce survival, growth, differentiation or cell death. This dichotomy can be ascribed to the differences between neuron types, to the activation of different pathways, or to the fine regulation of its local availability. In this work we show a novel application of optical tweezers for localized delivery and stimulation of neurons and discuss the experimental results obtained so far. We use 1.5 μm diameter silica beads functionalized with BDNF to stimulate precise domains of hippocampal rat neurons and transporte them to the site of stimulation by means of optical tweezers. We demonstrate that BDNF bound to the beads preserves its biological activity and is able to induce the translocation of the transcription factor c-Fos, which is downstream to TrkB pathway, to the nucleus. Moreover, stimulation with BDNF-coated beads of pyramidal neurons can lead to the localized increase of calcium levels in dendrites.

P-46

Spatio-temporal analysis of membrane lipid remodeling during phagocytosis

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The constant threat posed by pathogens and cell debris is tackled by phagocytosis, the process through which cells engulf and destroy dangerous material. The signaling, targeting and trafficking during phagocytosis is dependent on cytoskeleton rearrangements and membrane remodeling. It is becoming increasingly evident that lipids play an important role and can affect the phagocytic response. They assemble microdomains which can act as signaling platforms and confer charge and curvature to the membrane surface promoting electrostatic attraction and retention of proteins. Little is known about the mechanism(s) regulating membrane lipid remodeling during phagocytosis. Here we used fluorescently labeled biosensors based on K-ras and H-ras proteins to obtain spatio-temporal information on phagosomal membrane lipid remodeling during FcReceptor-mediated phagocytosis. The results show that cell activation by cytokines modulates the kinetics of anionic lipids thus affecting the membrane charge and the recruitment of cytosolic proteins to the phagosomal membrane. Our data emphasize the fundamental role of lipids in the generation and transduction of signals in phagocytosis, and we believe this can be extrapolated to many important processes in a cell.

O-47

Determining stoichiometry of molecular complexes in live cells

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In this presentation, we report a method for determining both the presence and the stoichiometry of protein complexes at pixel resolution and apply it to disassembling focal adhesions. The method is derived from fluorescence fluctuation methods that have single molecule sensitivity and is based on our previously described N&B (Number and Brightness) method that measures the number and brightness (aggregation state) of fluorescent molecules in every pixel of a confocal microscope image. The new method exploits the correlation of fluorescence amplitude fluctuations for two colors and detects the presence of molecular complexes and their stoichiometry. While the original N&B method was developed for one color, i.e., a single molecular species, the new method, ccN&B, extends the analysis to two colors and introduces the concept of cross-variance. This method is similar in concept to the two-color PCH analysis. However, the covariance-based ccN&B method also generates pixel resolution maps of protein complexes and can be used on commercial confocal microscopes. The method is highly sensitive and has relatively high temporal resolution. We have applied this method to adhesion complexes in cells. In addition of their structural role, to link the extracellular substratum to actin filaments, they also serve as signaling centers that regulate many cellular processes including their own assembly and turnover, migration, gene expression, apoptosis, and proliferation.

O-48

Heterochromatin protein 1 in DNA damage response - recruitment or dissociation from repair sites?

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We studied recruitment of DNA repair proteins to damage sites in live cells, by microscopy approaches, using a new method of inflicting local, sublethal damage in nuclei of live cells. Oxidative damage, which was inflicted by exciting DNA-intercalated ethidium with focused green light, triggered recruitment of base excision repair enzymes. Surprisingly, an epigenetic regulator, heterochromatin protein 1 (HP1) (Zarebski et al., 2009) was recruited to damage as well. HP1 is a constitutive component of heterochromatin, and plays an important role in transcriptional repression and regulation of euchromatic genes, however it was not known to be required for repair of oxidative damage. The finding of HP1 recruitment is particularly puzzling, since in another study HP1 was shown to dissociate from chromatin as a result of DNA damage (Ayoub et al. 2008). Technical aspects of live cell imaging that may explain these contradictory results will be discussed.

1. Ayoub N, Jeyasekharan AD, Bernal JA, Venkitaraman AR. HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. Nature 2008;453(7195):682-686.
2. Zarebski M, Wiernasz E, Dobrucki JW. Recruitment of heterochromatin protein 1 to DNA repair sites. Cytometry, in press.

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Quantitative imaging and the homeostasis of *Plasmodium falciparum* infected erythrocytes

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Plasmodium falciparum (*Pf*) causes the most lethal form of malaria in humans. Early research exposed two paradoxes: 1) during its intraerythrocytic cycle *Pf* permeabilizes the host cell so much that a comparably permeabilized healthy red blood cell (RBC) would lyse prematurely, and 2) *Pf* digests far more hemoglobin than needed for its metabolism. A model of the homeostasis of a *Pf* infected RBC suggested a common explanation of both puzzles: excess hemoglobin digestion is required to reduce the colloidosmotic pressure within the host cell thus ensuring its osmotic stability to the end of the *Pf* asexual cycle.
We investigated these predictions with direct measurements of [Hb] and volumes of parasitized RBCs. Reliable volume and morphological data was obtained by confocal microscopy and quantitative surface reconstruction. Furthermore, we developed a new FRET-based method to measure hemoglobin molecular crowding by exploiting the reduction in fluorescence lifetime of a donor fluorophore loaded in the RBC cytosol.
FRET imaging techniques are powerful tools for probing the biophysics of living cells. These tools provided a first validation of the colloidosmotic hypothesis and a deeper understanding of the homeostasis of the intraerythrocytic stage of *Pf*.

P-50

Imaging dynamics of DC-SIGN at the plasma membrane of HIV-1-stimulated dendritic cells

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Understanding how viruses interact with host receptors is essential for developing new antiviral strategies. Dendritic cells (DCs) can efficiently capture and take up HIV-1 through multiple attachment factors, such as the C-type lectin DC-SIGN. However, the initial interactions between HIV-1 and this receptor on DCs are not fully understood yet. In this work, we have used single-molecule EPI-TIRF microscopy in combination with fluorescently labelled HIV-1 virus like particles (VLPs) and DC-SIGN-specific antibodies to image the dynamic interaction between HIV-1 and DC-SIGN nanoclusters at the plasma membrane of DCs. By tracking individual trajectories of DC-SIGN on the cell surface of living DCs we have found heterogeneity in the modes of motion of DC-SIGN: some clusters are immobile whereas others move very quickly, with a few ones showing a directed motion on the cell membrane. To investigate how such motion might be correlated to DC-SIGN function as virus attachment factor, we have developed glass platforms functionalized with HIV-1 VLPs to locally stimulate living DCs. These virus platforms have allowed us to measure DC-SIGN diffusion rates and motion modes over the cell surface of stimulated DCs and represent a powerful tool for studying the dynamic interactions between HIV-1 and the DC membrane.

P-51

Evaluation of synaptic vesicle pools at synapse expressing human epileptogenic synapsin mutations

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Several mutations have been discovered in the Synapsin I (Syn) gene in families with epilepsy, but the mechanism inducing the epileptic phenotype is unknown. Syn is a protein associated with synaptic vesicles (SVs) that control SV trafficking and neurotransmitter release. The Syn mutations subject of this study are a non sense (ns-1) and of two missense (ms-2, ms-3). Syn knockout (KO) hippocampal neurons were transfected with the either wild type (WT) or mutated Syns. All Syns presented a common punctate pattern of expression at the level of axonal arborizations but, while ns-1 Syn targeted to synapses as WT-Syn, ms-2 and ms-3 Syns reached the presynaptic terminal less efficiently. We next set up a live cell imaging experiment using synaptophysin-pHluorin and evaluated the effects of ns-1, ms-2 and ms-3 Syns on the size of SV pools. Restoring WT-Syn in Syn I KO terminals led to a increase of all SV pools. Restoring ms-2 and ms-3 Syns resulted in a phenotype not significatively different from Syn I KO background whereas restoring ns-1 Syn caused a decrease of all SV pools as compared either with WT-Syn or Syn I KO background. These data suggest an alteration in the subcellular distribution and function of Syn in patients carrying ms-2 e ms-3 mutation and a more severe effect on synaptic activity in patient carrying ns-1 mutation.

P-52

FCS in live *Bacillus subtilis* cells: an *in vivo* study of transcriptional regulation

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Fluorescence correlation spectroscopy (FCS) is a useful technique for characterizing the mobility and concentration of fluorescent molecules both *in vitro* and *in vivo*. We utilize two-photon FCS to characterize the concentration and mobility of fluorescent molecules within living cells of *Bacillus subtilis*. Autocorrelation functions were measured in bacteria expressing green fluorescent protein (GFP) under the *lac* promoter in both nutrient rich and nutrient poor culture medium. Although considerable heterogeneity was evident from cell to cell, on average, both intracellular concentration and mobility were found to be dependent upon culture medium. We also investigated bacteria expressing GFP under control of native promoters for involved in the regulation of the carbon metabolic cycle in *Bacillus subtilis*. The GFP concentration, which should be related to promoter activity, was investigated for single cells and cell populations under different metabolic conditions. Some photobleaching was observed during the course of the measurements as a decrease in the average fluorescence intensity. This is due to the small size of the bacteria (∼1 fL) and low basal expression levels of GFP (∼100 nM) in the absence of IPTG. Methods to take this into account during data analysis are discussed.

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O-53

Intracellular calcium and chloride concentrations in neuronal cells – a Fluorescence Lifetime Imaging (FLIM) approach

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The determination of ion concentrations in cells – in particular in neurons – is very important for understanding cell function and life. Ca^{2+} is an ubiquitous messenger in almost all cell types, Cl^{-} has several roles, e.g. plays an important role in some neuronal signaling pathways including olfaction, nociception and vision. Fluorescence lifetime imaging (FLIM) is of advantage over intensity based fluorescence microscopy, when comparisons between micro-domains of one cell or between different cells of one cell type are performed. Several (organic chromophores and fluorescent protein based) Ca^{2+}- and Cl^{-}-sensors have been tested in culture cells with respect to their applicability in FLIM studies. The Ca^{2+}- and Cl^{-} concentration in rodent olfactory sensory neurons, dorsal root ganglion neurons and neurons of the retina is investigated by time-resolved FLIM with two-photon excitation.

O-54

Retrovirus induced remodeling of the host cell actin architecture

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Retrovirus budding is a key step in the virus replication cycle. Yet, despite substantial progress in the structural and biochemical characterization of retroviral budding, the underlying physical mechanism remains poorly understood, primarily due to technical limitations preventing visualization of bud formation in real time. Using atomic force-, fluorescence- and transmission electron microscopy we find that both HIV and Moloney murine leukemia virus (MLV) remodel the actin cytoskeleton of their host cells and utilize the forces it generates to drive their assembly and budding. Highly dynamic actin-filamentous structures which varied in size over the duration of budding appeared to emanate from the assembled virion. These actin structures assemble simultaneously or immediately after the beginning of budding, and disappear as soon as the nascent virus is released from the cell membrane. Analysis of sections of cryo-preserved virus infected cells by TEM reveals similar actin filaments structures emerging from every nascent virus. Substitution of the nucleocapsid domain implicated in actin binding by a leucine-zipper domain resulted in budding of virus-like particles that was not accompanied by remodeling of the cell's cytoskeleton. Notably, budding of viruses carrying the modified nucleocapsid domains was an order of magnitude slower than that of the wild type. The results of this study show that retroviruses utilize the cell cytoskeleton to expedite their assembly and budding.

O-55

Fast 3D chromatin dynamics studied in living yeasts using a novel lab on chip technology

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We present a novel Lab-on-Chip technology for 3D particle tracking yeast abased on V-shaped mirrors, which are used to observe fluorescent specimens from multiple vantage points, providing stereo-images that can be recombined for 3D reconstruction.

Our technology is based on V-shaped mirrors, which are fabricated by wet etching of silicon wafers, and used as optical and fluidic components. After rigorous optical optimization of the device in vitro, the Lab-on-Chip is applied to study chromatin dynamics in vivo using budding yeasts as a model system. Yeasts cells are visualized using GFP fused to the associated repressor protein. We confirm earlier observations that telomeric sequences, i.e. located close to chromosome ends, accumulate at the nuclear periphery, whereas genes found midway along chromosome arms are mostly present in the nuclear lumen. The dynamics of these sequences is followed in 3D with an unpreceded temporal resolution of 10 ms, showing that chromosome dynamics is non linear in the short time regime and switches to a linear regime at larger timescales. Notably, this behavior is reminiscent of universal responses observed in polymer solutions, and is related to the confinement and the structure of chromosomes. This technique shows a great potential for studying dynamic processes in small living organisms.

O-56

HIV budding and release

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HIV-1 assembly and release occur at the plasma membrane of infected cells and are driven by the Gag polyprotein. Using a combination of wide-field and total internal reflection fluorescence microscopy, we have investigated assembly of fluorescently labeled HIV-1 at the plasma membrane of living cells with high time resolution. Gag assembled into discrete clusters corresponding to single virions. After their initial appearance, assembly sites accumulated at the plasma membrane of individual cells over 1-2 hours. Using a photoconvertible fluorescent protein, we determined that assembly was nucleated by membrane bound Gag molecules, while both membrane-bound and cytosol derived Gag polyproteins contributed to the growing bud. Assembly kinetics were rapid and three phases are observed. In phase I, the number of Gag molecules at a budding site increases following a saturating exponential with a rate constant of $\sim 5 \times 10^{-3}$ s^{-1}. Hence, Gag assembly is complete in $\sim$ 200 s. In Phase II, a plateau in fluorescence intensity is observed with no exchange of Gag protein. The fluorescence intensity decays in phase III. This decay, in some cases, corresponds to the release of a virion. The time scale from the onset of assembly to release of extracellular particles was measured to be $\sim$1,500+/-700 s.

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O-57

Activity correlation imaging: visualizing function and structure of neuronal populations

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Understanding a neuronal network relies on knowledge about both the function and the structure of its neurons. While he simultaneous observation of hundreds of neurons is possible by densely staining brain tissue with functional dyes, the low contrast of these stainings does not allow the identification of neuronal processes from the raw fluorescent images. However, as neurons are known to exhibit complex temporal patterns of activity, fluctuations in signal intensity over time could be exploited to generate contrast in densely stained tissue. We demonstrate that the uniform calcium signals within individual neurons of the olfactory bulb can be exploited to visualize the morphology and projection patterns of these cells in tissue slices. As different neurons exhibit distinct time patterns, it is possible to generate a high-contrast multi-color visualization of the network's active neurons, solely based on the specificity of temporal fluctuations of a single-color calcium dye. It is thus possible to use other spectral channels for additional labelling, for example using cell-type or protein specific markers. The ability to map function and structure of neuronal populations online opens up a number of intriguing applications, such as selecting cells or cell pairs with certain functional or projection profiles for targeted recordings, ablation or stimulation.

P-58

Evaluation of cell damage after photodynamic and sonodynamic treatment

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Photodynamic therapy (PDT) and sonodynamic therapy (SDT) is a new, combined therapy for treating cancer. The basis of the therapy is to administer a small amount of photosensitizer and sonosensitizer, which are selectively taken up by cancer cells, and then expose the body to light and ultrasound to activate these sensitizers. When the sensitizers absorb light of an appropriate wavelength, it may cause their excitation with subsequent energy transfer to oxygen; the oxygen then becomes highly reactive in cancer cells and produces reactive oxygen species (ROS). The resulting damage to organelles within malignant cells leads to tumor ablation. SDT uses an agent that is sensitive to ultrasound, allowing deeper penetration and destruction of abnormal cells. Changes in human melanoma cells were evaluated using fluorescence microscope and atomic force microscopy.We focused on obtaining pictures of the topography and pictures involving elastic properties of cell surface. The production of ROS was investigated with the molecular probe CM-H2DCFDA, and morphological changes in cells were evaluated using fluorescence microscope. The quantitative ROS production changes in relation to phthalocyanine concentration, irradiation doses and ultrasound intensity were measured by a fluororeader.

P-59

Action potential facilitates permeation of methyl viologen into chloroplasts of a plant cell

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Characean algae, close relatives of higher plants, represent a convenient model for studying the effect of propagating electrical signals, action potentials (AP) on photosynthesis. Illuminated *Chara corallina* cells produce coordinated spatial patterns of chlorophyll fluorescence (Chl Fl) and extracellular pH. Photosynthesis is higher in the cell regions adjacent to acid zones compared to alkaline zones. Under physiological conditions, the electrically induced AP differentially and reversibly suppresses photosynthesis in the alkaline and acid regions. In this work we examined the effects of an artificial PSI acceptor, methyl viologen (MV), on Chl Fl with Imaging-PAM technique. MV is a divalent cation and poorly permeates through biological membranes. The presence in the medium of MV had no effect on Fl as well as on $P700^+$ absorbance signals until the application of a single excitatory stimulus. Once an AP was generated in the presence of MV, it induced irreversible inhibition of native (NADP-dependent) electron flow and a strong non-photochemical Fl quenching all over the cell. This indicates that AP redirects electron flow from the main pathway to the artificial acceptor. We concluded that AP generation opens access for permeation of MV from the medium to the chloroplast stroma across two membrane barriers, plasmalemma and chloroplast inner membrane. We suggested that MV might enter *Chara* cell via plasmalemma Ca^{2+}-channels activated during AP.

O-60

Molecular rotors measure intracellular viscosity

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Viscosity is one of the main factors which influence diffusion in condensed media. In a cell viscosity can play a role in several diffusion mediated processes, such as drug delivery and signalling. Previously, alterations in viscosity in cells and organs have been linked to malfunction; however, mapping viscosity on a single-cell scale remains a challenge.
We have imaged viscosity inside individual cells using novel fluorescent probes, called molecular rotors, in which the speed of rotation about a sterically hindered bond is viscosity-dependent.[1,2] This approach enabled us to demonstrate that viscosity distribution in a cell is highly heterogeneous and that the local microviscosity in hydrophobic cell domains can be up to 100× higher than that of water.[1]
We have also shown that the intracellular viscosity increases dramatically during light activated cancer treatment, called Photodynamic therapy (PDT).[2] We have demonstrated the effect of such viscosity increase on intracellular reactions by directly monitoring the rates of formation and decay of a short lived toxic intermediate, crucial in PDT, called singlet molecular oxygen, in light perturbed cells.[2]

1. M.K. Kuimova *et al J. Amer. Chem. Soc.,* **2008**, *130*, 6672
2. M. K. Kuimova *et al*, *Nature Chem.*, **2009**, *1*, 69

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P-61

A novel scanning microwave microscope for investigating living cells at the nanometric scale

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We present a novel near-field scanning microwave microscope (SMM) capable of providing surface impedance measurements of samples with nanometric resolution. The instrument is the integration of a microwave Vector Network Analyzer (VNA) and a scanning probe microscope (AFM/STM). A key point is that our software, controlling and synchronizing both the instruments, creates simultaneously images of the sample at several frequency points. This can be used to extract several features of the sample depending on the frequency. Moreover, close frequencies show the same features, added to random noise. Exploiting this redundancy of information, we have achieved remarkable results. We have been working on the optimization of this system for biological applications, to detect functional characteristics of cells generating a variation of their dielectric properties. This instrument offers the possibility of performing local impedance measurements on a single live cell and, if correctly calibrated, it provides also quantitative information (e.g. absolute measurements of membrane permittivity). The system was demonstrated to work on Saccharomyces Cerevisiae. A better model for a full test of the potentialities of the new technique is given by excitable cells, characterized by a greater variability of dielectric properties. A challenging objective could be directly imaging ion channels.

P-62

Monitoring gene expression via novel nucleic acid and delivery methods

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Application of single-molecule and high-resolution fluorescence methods to monitor gene expression in living cells increase the demand on novel probes and delivery methods.They require fluorophores with high photostability and quantum yield and highly-efficient delivery methods that ensure the minimum interference with cell processes such as metabolism and signal transduction.
We used a novel class of dendrimers with varying generations and branching factors and different number of positive charges due to different moieties and functional groups.These different properties were tested for their efficiency to transfect eukaryotic cells with fluorescent oligonucleotides (ODNs).Different parameters (temperature,concentration of dendrimers, ratio of dendrimers and ODNs) were evaluated and optimised.We utilised these established optimal conditions to deliver a modified concept of SmartProbes to mammalian cells targeting mRNAs involved in signal pathways.We tested different mRNA targets and we optimised the fluorescence signal by varying a range of parameters,namely the fluorescent label and the intrinsic properties of the SmartProbe (length of the loop and stem, conformation and number of guanosines).In the near future,we plan to use these probes for monitoring gene expression levels using Diffusion Imaging Microscopy.

P-63

Multiplexed FRET for live cell studies of protein-protein interactions

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Fluorescence resonant energy transfer (FRET) is a popular approach to studying molecular interactions. Fluorescence lifetime imaging (FLIM) provides a robust read-out of FRET but has largely been restricted to fixed cells owing to relatively long acquisition times. We present a high-speed wide-field multidimensional fluorescence microscope for FLIM FRET in live cells on second timescales for high content analysis and studying fast dynamics. The system uses a Nipkow disc for optical sectioning and a time-gated image intensifier for wide-field FLIM. A spectrally selected supercontinuum excitation source facilitates versatile real-time FLIM of live cells transfected with fluorescent proteins. For cell signalling studies we have developed a multiplexed FRET approach. Ras activation at the plasma membrane is demonstrated using FLIM to read out TagRFP-Raf RBD interaction with mPlum-H-Ras. Simultaneously, a spectral ratiometric read-out is used for a second FRET (CFP/YFP Cameleon) probe to monitor the downstream calcium flux. To further develop multiplexed FRET as a tool for imaging multiple components of cell signalling networks, we are working to include polarisation-resolved imaging for steady-state and time resolved fluorescence anisotropy measurements.

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Investigation of the dynamics of redox elements in live cells by using fluorescence ratio microscopy

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Oxidative stress or signaling events can affect cellular redox environment, which act simultaneously as regulator and indicator of key cellular functions in both physiological and pathological settings. By using a redox-sensitive protein (rxYFP), employed ratiometrically, it is possible to generate high resolution redox maps of cells. The spatial distributions of oxidized and reduced elements have been discriminated in human embryonic kidney cells by the deconvolution of the histograms of redox maps. By transfecting cell with Glutaredoxin V (GRX-V), a significant shift towards more reduced state with respect to that recovered from non-transfected cells is observed. Despite such large differences, a common behaviour in the spatial distribution of reduced and oxidized couples can still be observed: oxidized population shows a pronounced localization on the cell borders, near the plasma membrane, while reduced population appears as a collection of well separated spots homogeneously distributed thoroughly the inner part of the cell with a mean dimension of 2 um. Furthermore we observe that the role of GRX-V consists in causing a shift towards reduced values of the highly reduced region, while leaving unaltered the redox-balance of the intracellular side of the plasma membrane.

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O-65

Do retinal rod outer segments carry out oxydative phosphorylation?

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Visual transduction in vertebrate retinal rod Outer Segments (OS) is very energy demanding. However, ATP supply in OS, that are devoid of mitochondria, is still puzzling. By a proteomic analysis we identified in purified bovine OS disks, proteins involved in vision, as well as the respiratory chain complexes I to IV and F_1F_o-ATP synthase, whose activity was comparable to that of mitochondria and sensitive to specific inhibitors. Rhodamine 123 fluorescence quenching experiments showed the presence of a proton potential difference across disks. Disks consumed oxygen. Confocal laser scanning and transmission electron microscopy showed that Cytochrome *c* Oxydase and ATP synthase are localized on disks. Mitochondrial vital dyes stained OS *ex vivo,* and disks. Rhodopsin and MitoTracker fluorescence co-localized in OS. Data, suggestive of an aerobic metabolism in OS, point to the existence of "mitochondrial inner membrane-like membranes" ectopically producing ATP through oxidative phosphorylation, with respect to mitochondria. New scenarios open on the patho-physiology of many retinal diseases associated to mitochondrial dysfunction.

O-66

Imaging cellular communication in insulin secretion *ex vivo* and *in vivo*

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The islet of Langerhans is the functional unit responsible for glucose-stimulated insulin secretion (GSIS), and thus plays a key role in blood glucose homeostasis. The importance of the islet is demonstrated by the proven ability of islet transplants to reverse Type I diabetes pathologies in human patients. We are interested in understanding the multicellular mechanisms of islet function, and their role in the regulation of blood glucose under normal and pathological conditions. In many ways, the islet appears to function as a syncytium, which exhibits synchronous behavior of membrane action potentials, Ca^{2+} oscillations, and pulsatile insulin secretion across all β-cells in the islet. In other ways, the islet works as individual cells, especially in the regulation of gene transcription. Using our unique quantitative optical imaging methods and novel microfluidic devices, the *dynamics* of these molecular mechanisms can be followed quantitatively in living cells within intact islets. These investigations utilize transgenic and tissue-specific knock-out mouse models with demonstrated phenotypes, as well as traditional biochemical and molecular biological approaches.

P-67

Second harmonic generation microscopy reveals sarcomere contractile dynamics of cardiomyocytes

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Cardiomyocytes, like other striated muscles, exhibit strong inherent second-order nonlinear optical properties that make them exemplar for live cell dynamic studies with second harmonic generation (SHG) microscopy. Laser scanning SHG microscopy with an incident wavelength around 1μm, enables fast imaging for extended periods of time with negligible tissue damage. Strong SHG signal originates from the anisotropic (A-) bands which are comprised of regularly arranged myosin molecules, while actin molecules, the main constituent of the isotropic (I-) bands, produce negligible SHG. Consequently, the alternating bands along the myofibril are clearly visualized, therefore, enabling the determination of individual sarcomere lengths and the study of sarcomere length dynamics during macro-scale contractions. SHG intensity is shown to positively correlate to sarcomere length, which leads to the development of real-time inherent force sensors for *in vivo* myocytes. Rich sarcomere dynamics can be observed during myocyte contraction, which can be used for medical diagnostic purpose of muscular degenerative diseases.

P-68

Nuclear trafficking of HIV-1 pre-integration complexes in living cells

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HIV-1 to efficiently complete a replication cycle has to integrate its genome into the host cellular DNA.After HIV-1 enters target cells,neosynthesized viral DNA forms along with other proteins the pre-integration complex (PIC).PICs are then transported into the nucleus where integration,catalyzed by the viral integrase,takes place.HIV-1 viral particles engineered to incorporate integrase fused to EGFP have proven effective to study PICs within nuclei of infected cells.In this study we report the live imaging analysis of nuclear PIC dynamics obtained by time-lapse microscopy.Intranuclear trajectories of IN-EGFP-labeled PIC were collected in three dimensions and examined by both mean squared displacement (MSD) and cage diameter (CD) analysis.In CD the maximum distances measured between two positions occupied by a PIC in a time window of 2 minutes were calculated while in our MSD analysis 5-minute long trajectory segments were considered.Remarkably,MSD revealed the presence of an underlying active transport mechanism.To test the possible role of actin filaments,PIC nuclear trafficking was analyzed in cells treated with latrunculin B (actin polymerization inhibitor).Preliminary results suggest that the disruption of actin function impairs the active nuclear movement of PICs.

Abstracts
– Live cell imaging –

P-69

Involvement of pendrin in volume and pH control analyzed through the fluorescence properties of EYFP

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The pendrin (SLC26A4) gene is responsible, when mutated, for the Pendred syndrome, a recessive disorder characterized by sensorineural hearing loss often accompanied by thyroid disfunctions. Pendrin is an anion exchanger. The way it works and its role in different tissues, owing to the lack of known isoforms, is matter of wide research and debate. We focused on a still unexplored pendrin function, that is most important in the inner ear: cellular volume control and Cl^- fluxes regulation. We used HEK cells over-expressing wild type pendrin or a mutated isoform together with the EYFP. We challenged cells with hypo-osmolar solutions and followed their volume variations in time. Taking advantage of the confocal optical sectioning we independently measured cell volume and fluorescence intensity. In this way, given the dependence of EYFP fluorescence from $[Cl^-]$ and pH (measured with SNARF5) we could estimate at the same time Cl^- fluxes, volume and pH variations. The contemporary measurements of the three variables, not yet reported in living cells, allowed to assess the role of pendrin in volume regulation and evidenced its participation to Cl^- fluxes as compared to the mutated isoform or controls.

P-70

Efficient evaluation of FRET in image cytometry with acceptor photobleaching and ratiometric methods

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Fluorescence resonance energy transfer (FRET) is a powerful technique that can be applied to study nanoscale intra- and intermolecular events and interactions of molecules in situ in biological systems. A robust, easy to use, self-controlled FRET method, independent of donor and acceptor concentration and stochiometry, is acceptor photobleaching FRET, which requires only simple image mathematics. Another approach with more complicated calculations is the intensity-based ratiometric method, which is not based on destroying the acceptor fluorophores, making it applicable for following molecular interactions in live cells. As the need for using FRET in image cytometry for evaluating molecular interactions increases, we have undertaken to develop softwares for these methods involving the calculation and usage of all correction factors needed to obtain reliable energy transfer efficiencies. Correction possibilities of the acceptor photobleaching method include unwanted photobleaching of the donor, fluorescent photoproduct of the acceptor after photobleaching, cross-talk of unbleached acceptor into the donor channel and partial photobleaching of the acceptor. In the case of intensity-based ratiometric FRET, we can correct for all channel cross-talks and calibrate the FRET efficiency calculations. Both programs provide registration and semi-automatic processing, and they are freely available.

P-71

Advanced neuroimaging with diffractive optical elements

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Studying neural systems is complicated by the large number and small size of its cellular elements. The traditional way of exploring neuronal function by electrical monitoring with micropipettes is increasingly replaced by molecular imaging. Fluorescent molecules allow optical monitoring of neuronal signaling with cellular and often subcellular resolution.
In addition to monitoring activity, analyzing the functional properties of individual neurons or neuronal populations requires their controlled activation. Optical approaches are well-suited, including molecular photolysis of inert precursors and light activation of engineered proteins that control ionic membrane currents.
To fully utilize optical techniques for exploring neural systems requires more than adequate spatial resolution to distinguish neuronal elements and sufficient temporal resolution to induce and/or monitor neuronal signaling. Because of the non-linear and non-stationary nature of the studied system it is necessary to access many neuronal sites simultaneously.
In modern imaging, wide-field illumination and image-formation are often replaced by patterned excitation and non-imaging photon collection. In many instants, diffractive illumination schemes can substitute for conventional reflective or refractive designs. In particular, the availability of programmable diffractive optical elements has made this an attractive alternative, since they permit highly versatile microscope designs and support a significant increase in the overall spatio-temporal resolution.
I will present an advanced imaging approach using diffractive optical elements to analyze structure and function of live neurons in brain slices and intact cortex.

P-72

Unmixing using lifetime-excitation multidimensional confocal fluorescence microscopy data

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Fluorescence imaging provides a powerful tool to probe biological systems, enabling the high resolution investigation of the localization, interaction and biochemical modification of biomolecules. Multidimensional imaging, in particular, is a growing application of confocal and other forms of fluorescence microscopy. It seeks to measure not only fluorescence intensity in three spatial dimensions, but also other features of fluorescence emission, such as lifetime and fluorescence spectra. Although multi-dimensional fluorescence microscopy has been demonstrated before, little attention has so far been paid to the problem of data interpretation and representation when dealing with such large datasets.
We present here an instrument capable of recording fluorescence lifetime and excitation data by combining TCSPC for lifetime determination and a supercontinuum excitation source for extracting excitation spectra. These technologies permit the acquisition of 2D and 3D images with lifetime and excitation spectrum information at every pixel. We demonstrate a method whereby the multidimensional datasets acquired with this instrument are processed to yield biologically relevant parameters, (e.g. unmix fluorophores in a multiply labelled sample). Our method relies on a global analysis approach and uses AB plots for displaying multidimensional data. We demonstrate the instrument & processing method on dye and multiply labelled biological samples.

Abstracts
– Live cell imaging –

P-73

Investigation of post-thaw damage of S.cerevisiae yeasts using fluorescent dyes 2-DAB and 3-DAB

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We investigate applicability of the fluorescent probes 2-DAB and 3-DAB available from SETA BioMedicals (www.setabiomedicals.com) to study the post-thaw damage of *Saccharomyces Cerevisiae* yeast cells. The leaving cells stained with these dyes have bright fluorescence in the yellow and red region, respectively. However, the freezing followed by post-thawing causes cell damage, which results in a change of fluorescence intensity. In the native living cells the dyes are preferably localized in the cell membranes and organelles which are highly fluorescent. Partially damaged cells have even brighter fluorescence as compared to the living cells. However, their cell ultra-structure is not well-distinguished in the fluorescence mode. Ultimately destroyed cells are almost non-fluorescent: the cell membranes are not visible in the fluorescent mode and their organelles are only weakly fluorescent. The number of intensively fluorescing cells with partially destroyed membranes, which were obtained by freezing to –20 °C, is lower compared to those frozen at –196 °C. 2-DAB and 3-DAB enable not only to distinguish damaged and undamaged cells, but also allow quantitative estimation of the extent of damage in membranes by cryogenic effects.

P-74

LANCL2 is the abscisic acid receptor in human granulocytes and in rat insulinoma cells

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Abscisic acid (ABA) is a plant hormone regulating fundamental physiological functions in plants, such as response to abiotic stress. Recently, ABA was shown to be produced and released by human granulocytes, by insulin-producing rat insulinoma cells and by human and murine pancreatic β cells. ABA autocrinally stimulates the functional activities specific for each cell type through a receptor-operated signal transduction pathway, sequentially involving a pertussis toxin (PTX)-sensitive receptor/G-protein complex, cyclic AMP, CD38-produced cyclic ADP-ribose and intracellular calcium. Here, the ABA receptor on human granulocytes and on rat insulinoma cells is identified as the lanthionine synthetase C-like protein LANCL2. Co-expression of LANCL2 and CD38 in the human HeLa cell line reproduces the ABA-signaling pathway. The PTX-sensitive G protein coupled to LANCL2 is identified as G_i by transfection of $CD38^+$/$LANCL2^+$ HeLa with a chimeric G protein ($G\alpha_{q/i}$). Identification of the mammalian ABA receptor will enable the screening of synthetic ABA antagonists as prospective new anti-inflammatory and anti-diabetic agents.

P-75

Motile plaques involved in stress fiber assembly revealed by high-speed SPM for living cells

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Stress fibers, which are contractile actin cables aligned in a highly-ordered manner, are important for cell migration, mechanical support of plasma membranes and extracellular matrix organization. Although stress fibers are dynamically disassembled and assembled again in cells, it is poorly understood how actin filaments are organized into cables with highly-ordered alignment for stress fiber assembly. For investigation of actin cytoskeletal dynamics during actin cable formation, we performed time-lapse observation of actin cytoskeleton in lamella of living fibroblasts by using scanning probe microscopy (SPM). High-speed SPM observation revealed that motile plaques defined front-side ends of new actin cables and that preexisting mesh-form actin networks were remodeled into new actin cables. Directional order analysis of movement of plaques and pharmacological experiments clarified that plaques were driven by myosin-II-based retrograde actin flow, indicating that plaques are associated with actin cytoskeleton. Immunofluorescence experiments showed that plaques were localized on foci of vinculin, a component of cell-substratum adhesions, suggesting that plaques can bind to extracellular substrata via vinculin. Based on these results, we propose a model for actin cable formation that motile plaques initiate remodeling of preexisting actin networks into actin cables aligned in a direction of actin flow by associating with extracellular substrata.

O-76

Protein kinetic evaluations by two photon photoactivation in living cells

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Particle tracking inside the cell largely benefits of the ability to spatially and temporally mark specific structures to follow their "signalling" over a "dark" background as made possible since the advent of the photo-activatable markers. In terms of spatial confinement of the photo-activation process, the use of multiphoton excitation provides several favourable aspects compared to single photon confocal microscopy in photomarking biological structures to be tracked: the confined excitation volumes, of the order of magnitude of subfemtoliter, due to the non-linear requirements provide a unique control of the excitation and consequently photoactivation in the 3D space. In this context photoactivation experiments can be used to assess quantitative information about the binding kinetics of a macromolecule expressed in different cellular compartments. In this work we extended to photoactivation procedures and models originally developed for the quantitative analysis of FRAP experiments and we evaluated, for different proteins of medical interest (Rac-paGFP), the diffusive behaviour in the cytoplasm and the binding kinetics at the large endosomes. The results are compared with standard photobleaching experiments, in order to evidence the gained sensitivity obtained with photo-activatable proteins.

Abstracts

– Live cell imaging –

P-77

Time lapse and flow cytometry data integrated in a common cell cycle proliferation model

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We present a cell cycle simulation tool, connecting the basic proliferation process to the cell population data obtained by time lapse and flow cytometry. The computer program is a general framework within which cell cycle progression can be interactively modeled at the desired level of complexity, including G1/S/G2M cell cycle phases with variable duration, G0 phase, cell subpopulations belonging to different generations or differentiation stages, distinct block and cell death parameters for each phase. In this way, we achieved a detailed rendering of cell proliferation of normal or tumor cell populations, additionally including cytostatic and cytotoxic effects of treatments. The program gives as output simulated measures, reproducing those obtained by absolute cell counting, growth inhibition tests, flow cytometric DNA histograms and cell cycle percentages, pulse continuous-labeling studies, time-lapse intermitotic times and generation-wise analyses. Each technique provides a piece of information related to the underlying proliferation process, catching only some of the phenomena in play, and the measures often cannot be univocally interpreted. We used the cell cycle simulator to fit together time lapse and flow cytometry time courses measures, to achieve a detailed reconstruction of the cell cycle proliferation of an ovarian cancer cell line in vitro after X-ray exposure.

P-78

Mapping diffusion by raster image correlation spectroscopy (RICS) with analog detection

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Raster Image Correlation Spectroscopy (RICS) allows for mapping local translational diffusion coefficient(s) D. RICS applicability was recently extended to confocal laser scanning microscopes (CLSM) equipped with one-photon laser excitation and analog detection [Brown C. et al *J. Microsc.* 2008, Gielen E. et al *Langmuir* 2009]. To better understand the reproducibility and accuracy of RICS analysis of the top membranes of giant unilamellar vesicles (GUV) and cellular membranes, the influence and the constraints imposed by instrumentation and samples on the retrieved D values was simulated similar to Brown C. et al. The dependence of the parameter recovery on scan speed choice and detection noise, and on the total number of particles and mapping brick size was explored. Experiments show that in our Zeiss LSM 510 META CLSM the correlated detection noise along the fast x-scan axis ($\psi = 0$) is considerably larger than reported for other CLSMs. Near the GUV perimeter a drop in mapped D-values occurs. RICS (LFD, UCI, USA) and our Matlab software gave similar simulation and D mapping results. This work was supported by the Research Council of the UHasselt, tUL, the K.U.Leuven (GOA/2006/02), a Ph.D grant of IWT-Vlaanderen and by IAP P6/27 Functional Supramolecular Systems (BELSPO).

P-79

Cytomechanical modifications induced by drug-loaded carriers uptake by breast cancer cells

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The novel opportunities offered by the nanotechnologies have attracted great interest in the development of novel biomaterials for targeted drug delivery in cancer research. The desired features of pharmaceutical drug delivery for intravenous administration are their small size, biodegradability, high drug content, prolonged circulation in the blood and the ability to target required areas. In this work we have compared efficacy of different type of carriers having complementary properties for pharmaceutical delivery in cancer therapy. Drug nano-colloids encapsulated by combination of layer by layer (LbL) techniques and ultrasonication, phytochemical encapsulated artificial oleosomes and drug-loading clay/carbon nanotubes have been used for uptake into breast cancer cells. Analysis of viscoelastic response of neoplastic cells induced by cargo-carriers uptake has been carried out by a combination of high resolution optical and scanning force microscopy techniques. Furthermore, the effects of drug-reservoir carriers on the cytoskeleton (re)organisation of neoplastic cells were further investigated by confocal microscopy using different fluorescent probes.

P-80

Unusual diffusion characteristics of chloroplast thylakoid membrane proteins

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The thylakoid membrane is a structured network in higher plants which is organised into stacked granal thylakoids that are interconnected by single 'stromal' lamellae. We have studied the mobility of a resident protein of the stromal lamellae, Hcf106, part of the Tat protein translocase. Hcf106-green fluorescent protein fusion (GFP) was targeted into thylakoids and studied using photobleaching approaches. We show that small regions fail to recover significant levels of Hcf106-GFP fluorescence within 3 minutes after photobleaching. Autofluorescence from the photosystem II light-harvesting complex (LHCII) in granal stacks likewise fails to recover over this time scale. Although the thylakoid membrane is a single continuous entity, these data show that both Hcf106-GFP and LHCII are constrained within this network. Since the Hcf106 homologue, TatB, is highly mobile in the *Escherichia coli* plasma membrane, we believe that the stromal lamellae take the form of distinct domains that are effectively constrained by boundaries within the thylakoid network.

Abstracts
– Multiscale simulation –

P-81

Self-assembly and equilibration of bola-lipids membranes studied by molecular dynamics

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Bola-lipids consist of two monopolar, twin-tailed lipids that are held together by chemical linkage between one or both ends of the tails from one lipid and the corresponding ends from the other one. Membranes formed by these lipids or by their mixtures with monopolar lipids are known to have additional mechanical stability while retaining membrane fluidity. This is traditionally attributed to the fact that, in the membrane phase, the bola-lipids have predominantly spanning configuration (the two polar heads are positioned at opposite membrane-water interfaces) in detriment to looping configuration (both head groups are located in the same membrane-water interface). We perform molecular dynamics simulations, using the coarse grained MARTINI forcefield. We start with self-assembly simulations of bola-lipids followed by bilayer equilibration. An artificial pore is created in the membrane that significantly increases the flip-flop mobility of the lipids and hasten equilibration. The membrane properties are characterized (area per lipid, thickness, order parameter, pressure profile) with emphasis on the spanning/looping ratio. Our study can help designing new artificial membranes, with higher stability under extreme conditions.

P-82

Molecular dynamics simulation of CB1 and CB2 cannabinoid receptors in solution

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Cannabinoid receptors are an important class of G protein coupled receptors. In particular CB1 and CB2 have received considerable interest because they mediate a variety of physiological responses in the Central Nervous and Immune Systems. Their tertiary structure is still unknown. Experimental information suggests the presence of both, an active and an inactive conformation.
CB1 and CB2 share a common structural framework consisting in a seven transmembrane α-helix bundle connected by three extracellular and three intracellular loops. However, the knowledge of structural differences between both receptors may serve to design new ligands to activate/deactivate selectively only one of those receptors.
In the present research, we report a multi-nanosecond Molecular Dynamics simulation of both receptors in solution, starting from a structure obtained by homology modelling using the X-ray determined bovine rhodopsin protein.
We look for differences in the behaviour of CB1 and CB2 during the simulation process to shed light about those structural features which can be important for ligand selectivity. In this sense, we observed that CB1 tend to present a more flexible and opened helix bundle than CB2. Thus, it is expected than the former receptors would present less steric hindrance for ligand binding.
We expect these results will be useful to design more selective ligands.

P-83

Logic estimation of the optimum source neutron energy for BNCT of brain tumors

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Boron Neutron Capture Therapy (BNCT) is a promising method for treating the highly fatal brain tumor; glioblastoma multiform. It is a binary modality; in which use is made of two components simultaneously; viz. thermal neutrons and Boron-10.
A new concept was adopted for estimating the optimum source neutrons energy appropriate for different circumstances of BNCT. Four postulations on the optimum source neutrons energy were worked out, almost entirely independent of the RBE values of the different dose components. Four corresponding conditions on the optimum source neutrons energy were deduced. An energy escalation study was carried out investigating 65 different source neutron energies, between 0.01 eV and 13.2 MeV. MCNP4B Monte_Carlo neutron transport code was utilized to study the behavior of these neutrons in the brain. The deduced four conditions were applied to the results. A source neutron energy range of few electron volts (eV) to about 30 keV was estimated to be optimum for BNCT of brain tumors located at different depths in brain. The results were discussed.

P-84

Detection of functional modes in protein dynamics

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Proteins frequently accomplish their biological function by collective atomic motions. Yet the identification of a collective motions related to a specific protein function from, e.g. a molecular dynamics trajectory, is often non-trivial. Here, we propose a novel technique termed `functional mode analysis' that aims to detect the collective motion that is directly related to a particular protein function. Based on an ensemble of structures, together with an arbitrary `functional quantity' that quantifies the functional state of the protein, the method detects the collective motion that is maximally correlated to the functional quantity. The functional quantity could, e.g., correspond to a geometric, electrostatic, or chemical observable, or any other variable that is relevant to the function of the protein. Two different correlation measures are applied. First, the Pearson correlation coefficient that measures linear correlation only; and second, the mutual information that can assess any kind of interdependence. Detecting the maximally correlated motion allows one to derive a model for the functional state in terms of a single collective coordinate. The new method is illustrated using various biomolecules, including a polyalanine-helix, T4 lysozyme, Trp-cage, and Leucine-binding protein.

Abstracts
– Multiscale simulation –

P-85

Refinement of protein model structures using biasing potential replica exchange simulations

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Comparative protein modeling of a target protein based on sequence similarity to a protein with known structure is widely used to provide structural models of proteins. Frequently, the quality of the target- template sequence alignment is non-uniform along the sequence: parts can be modeled with a high confidence, whereas other parts differ strongly from the template. In principle, molecular dynamics (MD) simulations can be used to refine protein model structures but it is limited by the currently accessible simulation time scales. We have used a recently developed biasing potential replica exchange (BP-Rex) MD method (Kannan, S. Zacharias, M. *Proteins* 2007, 66, 697-70) to refine homology modeled protein structure at atomic resolution including explicit solvent. In standard Rex-MD simulations several replicas of a system are run in parallel at different temperatures allowing exchanges at preset time intervals. In a BP-RexMD simulation replicas are controlled by various levels of a biasing potential to reduce the energy barriers associated with peptide backbone dihedral transitions. The method requires much fewer replicas for efficient sampling compared with standard temperature RexMD. BP-RexMD simulations on several test cases starting from decoy structures deviating significantly from the native structure resulted in final structures in much closer agreement with experiment compared to conventional MD simulations.

O-86

Protein mechanics, function and recognition

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Although experiments which can directly probe the mechanical properties of proteins have only been performed recently, it is clear that the complex folds of polypeptide backbones lead to very heterogeneous mechanical behavior. This heterogeneity is likely to play an important role in protein function and interaction and it would be useful to be able to predict what mechanical (and dynamical) properties will result from a given structure. We have been using coarse-grain elastic network models to investigate this question and have found that these models are able to link specific mechanical properties to a number of functional features including enzymatic active sites, folding nuclei and changes in behavior due to point mutations. These models are also adapted to looking at complex formation between proteins and how specific recognition is achieved, while being fast enough to be applied to very large numbers of interactions.

O-87

The role of water in Zn(II)-Abeta(1-16) complexes

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Beta-amyloid (Aβ) peptides are the main component of amyloid fibrils detected in the brain of Alzheimer patients. Fibrils display an abnormal content of Cu and Zn ions whose binding to Aβ-peptides has been recently studied by X-ray Absorption Spectroscopy [1] and interpreted in terms of ab initio simulations. In order to perform such simulations it is of the utmost importance to find a compromise between the need of having a realistic description of the actual physical system and the difficulty of dealing with too many atoms and electrons. What is usually done is removing solvent (water molecules), thus studying the system in the so called "gas-phase". In this work we investigate the relevance of water in the Zn-$A\beta_{1-16}$ coordination mode. Relying on a combination of Classical [2] and Quantum Chemistry [3] methods we find a significant difference in the Zn coordination geometry depending on whether water is present or not. This information is exploited in building a full model system for subsequent Car-Parrinello simulations where two Aβ peptides in water are in interaction in the presence of Zn.

[1] V. Minicozzi et al. (2008) *JBC* **283**, 10784; [2] D. van der Spoel et al. (2005) *J Comp Chem* **26**, 1701; [3] T. Vreven et al. (2006) *JCTC* **2**, 815

P-88

Evidence for proton shuffling in a thioredoxin-like protein during catalysis

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Proteins of the thioredoxin (Trx) superfamily catalyze disulfide-bond formation, reduction and isomerization in substrate proteins both in prokaryotic and in eukaryotic cells. All members of the Trx family with thioldisulfide oxidoreductase activity contain the characteristic Cys-X-X-Cys motif in their active site. Here, using Poisson-Boltzmann-based protonation-state calculations based on 100-ns Molecular Dynamics simulations, we investigated the catalytic mechanism of DsbL, the most oxidizing protein known to date. We observed several correlated transitions in the protonation states of the buried active-site cysteine and a neighboring lysine coupled to the exposure of the active-site thiolate. These results support the view of an internal proton shuffling mechanism during oxidation crucial for the uptake of two electrons from the substrate protein. Intramolecular disulfide-bond formation is probably steered by the conformational switch facilitating interaction with the active-site thiolate. A consistent catalytic mechanism for DsbL, probably conferrable to other proteins of the same class, is presented. Our results suggest a functional role of hydration entropy of active-site groups .

Abstracts
– Multiscale simulation –

P-89

Multiresolution modelling of drug and hormone permeability through a lipid bilayer

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Traditional atomic-level (AL) modelling of biomembranes is time-consuming, and hence limited in the range of systems and phenomena that can be simulated. To alleviate this problem, we designed a coarse-grain (CG) representation where each lipid molecule, in reality consisting of more than 100 atoms, is modelled with only 10 CG sites. Our CG technique proves two orders of magnitude less demanding of computational resources than traditional AL methodology. A unique feature of our approach is that the CG potentials are directly compatible with standard AL models, thus facilitating the simulation of multiresolution systems, where the "chemically-sensitive" components (e.g., the solutes in membrane permeation studies) are modelled atomistically, while the surrounding environment is coarse-grained.
In this contribution, we present a summary of our multiscale methodology, together with its application to the permeation of large molecules – drugs and steroid hormones. The calculated permeabilities are compared to the available experimental measurements and AL simulation data. Molecular-level insights regarding the permeation mechanism are obtained and rationalised.

P-90

Conformational study on a Myelin Basic Protein fragment: molecular dynamics simulations in membrane

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Myelin basic protein (MBP) is a multifunctional protein of the central nervous system whose principal role is in maintaining the compactness and integrity of the myelin sheath, the multilamellar membrane wrapped around nerve axons. However, MBP also interacts with other proteins such as cytoskeletal and signalling proteins, adapting its structure to the different roles. MBP is a candidate autoantigen in the human demyelinating disease multiple sclerosis. This study investigated at atomic detail the conformation of a highly conserved central fragment of MBP, constisting of two consecutive regions with different relevant functionalities. The first one is associated with the membrane and comprises the primary immunodominant epitope in multiple sclerosis; the second one was predicted to be a ligand for SH3-domains of signalling proteins. Molecular dynamics simulations were perfomed in the presence of dodecylphosphocholine micelle, starting from a structure extrapolated from experimental data (Harauz and Libich, *Curr. Protein Pept. Sci.*, 2009). The results confirm the experimetal hypothesis, showing, in the micelle, a stable alpha-helix anchored to the membrane for the first region and, for the proline-rich second one, a poly-proline type II helix pointing outwards, ready to interact with the signalling proteins.

P-91

The static modes: a new tool for the prediction of induced-fit biomolecular flexibility

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We present a new competitive approach for the treatment of biomolecular flexibility to provide an alternative to the limitations of current methodologies such as molecular dynamics and normal mode analysis. This method, called Static Mode method, is based on the "induced-fit" concept and is aimed at mapping the intrinsic deformations of a biomolecule subject to any external excitations: direct mono or multi-site contact, electrical etc... The algorithm allows obtaining a set of deformations, each one corresponding to a specific interaction on a specific molecular site, in terms of force constants contained in the energy model. Such a process can be used to explore the properties of single molecular intrinsic flexibility, as well as to predict molecular docking or molecule/surface interactions. From a modelling point of view, the interaction problem can be expressed in terms of reactive sites between the interacting entities, the molecular deformations being extracted from the pre-calculated Static Modes of each separated ones. The first applications of our method have focused on the intrinsic flexibility of biomolecules like nucleic acids and proteins. More recently, this new methodology allowed us to investigate the folding of the region 1-16 of the Amyloid β-peptide, via the docking of a zinc ion on the reactive sites of the molecule.

O-92

Multiscale simulations of membrane proteins

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Coarse-grained molecular dynamics simulations may be used to probe the interactions of membrane proteins with bilayers and their component lipids on an extended (~1 μs) timescale (*1*). Conversion to atomistic resolution allows more detailed protein/lipid interactions to be examined. This multiscale approach will be examined via three examples: (i) interactions of a small ion channel toxin (VsTx1) with lipid bilayers; (ii) interactions of large monotopic enzymes with lipid bilayers; and (iii) interactions of PIP_2 with the Kir6.2 potassium channel.
(1)Lindahl, E., and Sansom, M. S. P. (2008) Membrane proteins: molecular dynamics simulations. *Curr. Opin. Struct. Biol. 18*, 425-431.

Abstracts
– Multiscale simulation –

O-93

Membrane aoration by antimicrobial peptides combining atomistic and coarse-grained descriptions

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Antimicrobial peptides (AMPs) comprise a large family of peptides that include small cationic peptides, such as magainins, which permeabilize lipid membranes. Previous atomistic level simulations of magainin-H2 peptides show that they act by forming toroidal transmembrane pores. However, due to the atomistic level of description, these simulations were necessarily limited to small system sizes and sub-microsecond time scales. Here, we study the long-time relaxation properties of these pores by evolving the systems using a coarse-grain (CG) description. The disordered nature and the topology of the atomistic pores are maintained at the CG level. The peptides sample different orientations but at any given time, only a few peptides insert into the pore. Key states observed at the CG level are subsequently back-transformed to the atomistic level using a resolution-exchange protocol. The configurations sampled at the CG level are stable in the atomistic simulation. The effect of helicity on pore stability is investigated at the CG level and we find that partial helicity is required to form stable pores. We also show that the current CG scheme can be used to study spontaneous poration by magainin-H2 peptides. Over-all, our simulations provide a multi-scale view of a fundamental biophysical membrane process involving a complex interplay between peptides and lipids.

O-94

On the structural and dynamic determinants of the specific recognition of collagen by collagenase

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Controlled degradation of collagen is an important process in tissue remodeling and wound healing. Collagenase cleaves fibrillar collagens about three quarters of the distance from the amino-terminus. Even though the determination of the cleavage site and the collagenase structure took place decades ago, the mode of action of collagenase on collagen is not clear. To understand the mechanism of collagenase activity on collagen, the structure, stability and dynamics of collagen, its conformation around the cleavage site and the possibilities of conformational rearrangements between the two domains in collagenase was explored using MD simulation. The results of principal component (PCA) and normal mode (NM) analysis of the collagen and collagenase suggests that the C-terminal domain of collagenase recognizes the collagen, and then the N-terminal catalytic domain undergoes rearrangement on the substrate (with the help of linker regions). The SDA software for carrying out Brownian dynamics simulations of protein-protein diffusional association with the help of biochemical constraints is being used to predict the mode of interaction of collagen and collagenase. Different conformations obtained from PCA and NM analysis are being used for the docking. The predicted structures of the complex will help us to understand how collagenase recognizes and binds collagen specifically. *Acknowledgment*: DAAD, Germany, CSIR-SRF India, and the Klaus Tschira Foundation.

O-95

Bridging atomistic and meso- scale with minimalist models for bio-molecules

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The processes of life involve a variety of events that occur on different scales, ranging from a few Å/ps of the triggering steps of the biochemical reactions, up to their macroscopic effects in cells and organs. Intermediate steps involve the structural rearrangement of the bio-molecules (∼nm/10-100ns), their aggregation, folding (∼10nm-μm/ μs-ms), internal cell diffusion and dynamics (μm–mm/ms–hours), evidently requiring a multi-scale modeling approach.

Here the multi-scale approaches are first briefly illustrated with a particular attention to the issue of matching the different resolutions, which is essential to achieve a coherent descripition. The focus is then fixed on the Coarse Grained (CG) models, typically spanning the nm-μm and μs-ms scale, and in particular on their minimalist – simplest and computationally cheapest – versions[1,2]. A parameterization strategy that combines accuracy and predictive power within these models is presented here and applications are shown to relevant cases including the proteins involved in the HIV replication, the Green Fluorescent Proteins and examples of macromolecular complexes.

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P-96

Ion transport and energy transduction of P-type ATPases studied by simulations

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P-type ATPases actively transport cations across the membrane. The basic mechanisms of ion transport and energy transduction are supposed to be the same in all P-type ATPases. Both reaction mechanisms were studied computationally for the Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase. The Ca^{2+} transport of the Ca^{2+}-ATPase is associated with a proton countertransport from the SR to the cytoplasm. Binding of the protons is thought to take place at acidic residues in the binding sites. The protonation of acidic ligands were analyzed in different enzyme states of the Ca^{2+}-ATPase by multiconformation continuum electrostatic calculations. Glu^{771}, Asp^{800} and Glu^{908} are prime candidates for the proton countertransporting residues and are likely to receive and release their proton via the same path. The Glu^{309} instead might serve as a proton shuttle between Ca^{2+} binding site I and the cytoplasm. The reaction cycle of P-Type ATPases is physiologically initiated by the binding and hydrolysis of ATP but can also be induced experimentally by voltage jumps across the membrane. We simulated the applied electric field by an "ionic capacitor" and studied the impact on different enzyme states of the Ca^{2+}-ATPase and the Na^{+}/K^{+}-ATPase by a combination of MCCE and MD. A selective activation of specific helices in response to the electric field is observed.

Abstracts
– RNA world –

O-97

A structure-based approach for targeting HIV-1 genomic RNA Dimerization Initiation Site

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All retroviral genomes consist in two homologous single stranded RNAs. HIV-1 Dimerization Initiation Site (DIS) is a conserved hairpin in the 5' non-coding region of the genomic RNA and essential for viral infectivity. The DIS initiates genome dimerization by forming a kissing-loop complex, further stabilized into an extended duplex upon interaction with the NCp7 nucleocapsid protein.
X-ray structures of the DIS kissing-loop and extended duplex revealed similarities with the bacterial 16S ribosomal RNA A-site, which is the target of aminoglycoside antibiotics. As a result, aminoglycosides also bind the HIV-1 DIS as shown by our X-ray structures of the DIS kissing-loop bound to aminoglycosides. Using fluorescence, UV-spectroscopy and microcalorimetry, we further characterized HIV-1 DIS/aminoglycosides interactions. We found that the affinity of aminoglycosides for the DIS was higher than for their natural target, the 16S A site. They strongly stabilize the DIS kissing-loop, blocking its conversion into the duplex form. Finally, we also solved X-ray structures of the DIS duplex bound to aminoglycosides, revealing an important conformational change following drug binding. These structures show that it is possible to target the HIV-1 DIS dimer before and after the NCp7-assisted RNA maturation with the same molecule. Altogether, our studies create the basis for a rationally driven design of novel potential drugs targeting the HIV-1 genome.

O-98

Single molecule studies of spliceosomal RNAs U2 and U6

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Splicing is an essential step in the maturation reaction of mRNA, in which intervening introns from exons. The spliceosome is a dynamic assembly of 5 small nuclear RNAs and >150 proteins that catalyzes splicing. U2 and U6 are the only 2 snRNAs strictly required for splicing. Major conformational changes are expected to take place during spliceosomal assembly and catalysis.
We have developed a single-molecule fluorescence assay to study the structural dynamics of a protein-free U2-U6 complex from yeast. Our previous data have revealed a 2-step large amplitude conformational change of the U2-U6 complex. The 1*st* step is a Mg^{2+}-induced conformational change where helix III and the U6-ISL are in close proximity in low Mg^{2+} and separated in high Mg^{2+}. The 2*nd* step corresponds to the formation of the highly conserved helix IB.
We now examine the role of the highly conserved bases in the folding dynamics of the U2-U6 complex. The data show that U80 and the ACAGAG loop play an important role in stabilizing the interaction between helix III and the U6-ISL. We hypothesize that U80 flips out of the U6-ISL and interacts with the ACAGAG loop to bring them in close proximity. Our results raise the interesting possibility that this interaction plays an important role in bringing the 5' splice site and the branched A into close proximity of U80, which may be critical for catalysis.

P-99

Comparison of DNA and siRNA binding and nuclease protection by non-viral vectors for gene delivery

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The biggest obstacle for the success of gene therapy is delivery. With the discovery of RNAi, the use of siRNA to regulate gene expression as potential therapy has attracted much attention recently. In contrast to DNA, siRNA delivery does not require nuclear entry. With one less barrier to overcome, the delivery of siRNA seems to be easier. It is frequently assumed that comparable delivery strategy could be used for both DNA and siRNA. In fact the two types of nucleic acids are fundamentally different with distinct properties, which impact their delivery strategies. In the present study it was found that most non-viral delivery vectors including polymers, peptides and lipids were generally more efficient in binding with DNA than siRNA as shown in gel retardation assay. The inferior siRNA binding is possibly due to the rigid structure of siRNA, resulting in weaker electrostatic interaction with the cationic vectors. Surprisingly, it was observed that all the vectors studied offered better nuclease protection for siRNA than DNA despite poorer siRNA binding. Either the nuclease protection for siRNA is easier to achieve due to its small size, or the gel retardation assay did not truly reflect the binding efficiency as the weaker siRNA complexes may dissociate during electrophoresis. Not only the delivery strategy, the results between DNA and siRNA study must also be carefully adapted and interpreted.

O-100

Structure and mechanism of the Varkud Satellite ribozyme

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The Varkud Satellite (VS) ribozyme is the largest of the nucleolytic ribozymes, and the only one for which there is no crystal structure.
We have determined the overall architecture of the complete VS ribozyme using small-angle X-ray scattering in solution. The substrate stem-loop docks into the tertiary fold of the ribozyme, allowing an intimate loop-loop interaction to occur. This brings two key nucleobases A756 and G638 into close proximity of the scissile phosphate, and we believe that these nucleobases are involved in general acid-base catalysis of the phosphoryl transfer reactions. This is supported by functional group substitution, and the pH dependence of the reaction rate for the natural and modified RNA.
Although possessing totally different folds, the functional elements of the VS and hairpin ribozymes are topologically and mechanistically very similar if not identical. This has probably arisen by convergent evolution. Other nucleolytic ribozymes have diversified to employ hydrated metal ions and even small molecules to participate in genera acid-base catalysis. By contrast, the larger ribozymes seem to have adopted a different, metalloenzyme, catalytic strategy. Why these different groups have evolved different catalytic chemistries is an interesting challenge to our understanding of biocatalysis.

Abstracts
– RNA world –

P-101

Surface-Enhanced Raman Scattering to probe Hairpin Ribozyme Catalysis

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The existence of “RNA world” as an early step in the history of life increases the interest for the characterization of these biomolecules. The studied hairpin ribozyme is a self-cleaving/ligating motif found in the minus strand of the satellite RNA associated with tobacco ringspot virus. Surface-enhanced Raman spectroscopy (SERS) was successfully used to detect sub-picomole quantities of nucleic acids. SERS takes advantage of the strongly increased Raman signals generated by local field enhancement near metallic (typically Au and Ag) nanostructures. SERS spectra of DNA or RNA are strongly dominated by Stockes modes of adenine. Through an interaction of adenyl residues with silver colloid, adenyl Raman signal is 10^6 times increased compared to Raman scattering. In controlled conditions, SERS signal is proportional to the amount of free residues adsorbed on the metal surface. Upon RNA cleavage, residues are unpaired and free to interact with metal. In the present study, we proposed to follow the cleavage reaction of hairpin ribozyme (HPR85) using the SERS signal of the liberate adenyl residues. As the SERS signal is proportional to the adenyl residues, reactivity of HR85 was monitored by measuring the Raman intensity of the fragment liberated during the cleavage of hairpin. The results obtained were compared with the electrophoresis method performed on the same sample and similar results were obtained.

O-102

Structural basis for the encapsidation process of turnip yellow mosaic virus

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Formation of hairpins with internal loops with C·C and C·A mismatches in the 5' untranslated region is a common characteristic among the plant viruses belonging to the Tymovirus genus. Turnip yellow mosaic virus possesses two such hairpins, HP1 and HP2. HP1, and in particular the presence of the C·C and C·A mismatches in its internal loop, is important for initiation of the encapsidation of the viral genome. The encapsidation occurs under acidic conditions at the neck of invaginations of the chloroplast membrane.
We have now determined the 3D structures of revertants involved in the evolutionary pathway using NMR spectroscopy. These structures reveal how the grooves in the hairpin become increasingly positively charged in successive generations of evolution. The similarity between the CCCA mutant and the wild-type hairpin (HP1) is striking and explains why this mutant gives rise to a viable virus. In addition, a characteristic tilt of the backbone is observed upon occurrence of a protonated C·C base pair.
Both the positively charged major groove and the kink in the RNA backbone appear to be crucial for interactions with the viral capsid. At neutral pH, the structure of HP1 resembles the Watson–Crick base paired mutant which explains why encapsidation only occurs under acidic conditions.

P-103

Structure and conformational dynamics of a unique DEAD box helicase

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DEAD box helicases couple ATP hydrolysis to RNA structural rearrangements. *T. thermophilus* Hera consists of a helicase core and a C-terminal extension (CTE) with a putative RNase P motif. Crystal structures show that the CTE is bipartite, forming a highly flexible dimerization motif with a novel fold and an additional RNA-binding module. We provide a first glimpse on the orientation of an RNA-binding domain outside the helicase core. In a structure-based model for the complete Hera dimer, the RNA-binding sites of the helicase cores face each other, allowing for inter-subunit communication. The plasticity of the dimerization motif allows for drastic changes in the juxtaposition of the helicase cores within the dimer. In single molecule FRET experiments we identified fragments of the 23S rRNA and RNase P RNA as substrates for Hera. Both substrates switch the Hera core to the closed conformation and stimulate the intrinsic ATPase activity. RNA binding is mediated by the CTE, but does not require the putative RNase P motif. ATP-dependent unwinding of a short helix in 23S rRNA suggests a specific role for Hera in ribosome assembly, in analogy to the *E. coli* and *B. subtilis* helicases DbpA and YxiN. In addition, the specificity of Hera for RNase P RNA may be required for RNase P RNA folding or RNase P assembly. Simultaneous action of two Hera subunits on the same RNA molecule may be important for efficient RNA remodeling *in vivo*.

P-104

Mechanism of the chaperone properties of the Hepatitis C Virus core protein

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The core protein of Hepatitis C virus is a multifunctional protein of 179 aa, consisting of a hydrophilic N-terminal domain with three basic domains (BD1-BD3) responsible for the interactions with RNA and a hydrophobic C-terminal domain. The N-terminal domain exhibits nucleic acid chaperone properties similar to those of the nucleocapsid protein from HIV. Here, we characterized the mechanism of the chaperone properties of a peptide E corresponding to the BD2 and BD3 clusters of the N-terminal domain. To this end, we monitored the promotion by this peptide of the annealing of dTAR, the DNA analogue of the transactivation response element to its complementary sequence, cTAR DNA, taken as models. The annealing involves two second-order kinetic components that are activated by at least three orders of magnitude by peptide E. This activation was correlated with the ability of peptide E to destabilize the lower half of dTAR stem. Using, cTAR and dTAR mutants, the two kinetic components were assigned to two pathways which are connected with the fast annealing of the terminal bases of cTAR to dTAR and slow extended duplex formation, limited kinetically by the nucleation of central segments of cTAR and dTAR stems.

Abstracts
– Interaction and recognition of DNA –

P-105

Tunable nanoconfinement structures for DNA manipulation

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Nanostructures, such as nanochannels [1] or nanoslits [2], have been successfully used to confine and stretch DNA molecules, offering interesting opportunities of investigation on conformational changes induced by confinement, physical and biological properties, etc. The integration of these nanostructures on lab-on-chip systems has shown their great potential for applications such as DNA sieving or single molecule manipulation [3], [4].
Arrays of nanochannels fabricated, using a Focused Ion Beam (FIB) on a silicon master, are replicated using elastomeric materials, such as poly(dimethylsiloxane) (PDMS), and soft-lithography techniques. The cross-section of these flexible polymeric nanoconfinement structures can be reversibly and dynamically tuned, in order to vary biomolecules transport characteristics and confinement conditions of trapped DNA molecules. Moreover, these nanochannels, with tunable cross-section, are used to study and exploit the sieving mechanism of "entropic recoil" [5] for the separation of long DNA chains.

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P-106

Modeling of the joint binding of different types of ligands with DNA

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The influence of ligand, irreversibly binding with DNA, on the isotherm of adsorption of ligand binding with DNA reversibly has been modeled theoretically. The isotherm of adsorption of EtBr on DNA in presence of *cis*-DDPt has been considered at low concentrations of *cis*-DDPt and EtBr.. The isotherm of adsorption of EtBr on DNA has linear form in Scatchard coordinates at low degrees of occupation. The comparison with experimental isotherm permits to estimate the parameters of EtBr binding with DNA. With taking into account the pseudo-ring structures formation with partially molten regions we consider two types of binding regions, linear and ring at low degrees of occupation. The isotherm of adsorption of EtBr on DNA and also variation of the number of bounded EtBr molecules are calculated with taking into account these two types of binding regions. It was shown that at low concentrations *cis*-DDP, bounding with DNA, changes the isotherm of ligands adsorption. The linear in Scathcard coordinates isotherm of adsorption transforms into non-linear isotherm. The degree of transformation depends on the fraction of DNA in the ring regions, on the ratio between number of EtBr binding cites in the ring and linear regions, and also on the binding constants for these regions. It was shown that low concentrations of *cis*-DDP affect the dependence of dispersion on the concentration of ligands, changing both the magnitude of maximum and its position.

P-107

DNA accessible surface area and indirect protein-DNA recognition: study by bioinformatical approach

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Revealing the mechanisms of protein-DNA recognition is essential for understanding the regulation of many cellular processes. There is growing evidence that recognition through sequence-specific contacts (direct readout) can be enhanced by recognition via DNA sequence-dependent deformability (indirect readout). The role of changes in DNA accessible surface area (ASA) in distorted DNA configurations in complexes with proteins is not fully understood yet, even though such changes and related changes in polarity of DNA surface are among key factors of indirect readout. To fill this gap we have developed a publicly-available Internet database of protein-DNA complexes, which integrates the data on DNA conformational parameters with information on ASA of DNA atoms in the minor and major grooves, ProtNA-ASA. The database has been used to analyze the effect of changes in DNA backbone configuration on ASA of DNA atoms in major and minor grooves. We observe that sugar puckering and conformation of torsion angle γ affect the accessibility of DNA atoms in both grooves to a noticeable extent. We also report sequence specific preferences of the nucleotides for structural domains of γ. These results can shed new light on the mechanisms of indirect protein-DNA recognition.

O-108

Towards multiprotein nanochips using nanografting and DNA directed immobilization of proteins

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The development of arrays for biomolecular recognition for a broad range of applications in biomedical diagnostics is receiving a constantly increasing attention. The design of efficient protein biochips, however, requires the optimization of protein immobilization protocols for improving the device sensitivity. Moreover, innovative platforms for in-vitro detection in highly diluted, small volume samples need to be developed, for which standard micro-fabrication techniques are not suitable. Therefore, the development of nano-scale platforms for protein and antibody detection is essential.
We report here on a novel approach for the fabrication of multiple protein nanosensors using Atomic Force Microscopy based Nanografting and DNA-directed immobilization (DDI), which takes advantage of the specific Watson-Crick hybridization of oligonucleotide-modified proteins to surface-bound complementary oligomers. Using Nanografting, single-stranded DNA nano-structures with well defined local environments were fabricated on a flat surface. DNA-protein conjugates were then anchored on the engineered binding sites by DDI in a single chemical operation and detected by the corresponding topographic height increase of the relevant patches. Immunological assay were used to demonstrate the biochemical functionality of the immobilized proteins, proving the specificity of biomolecular recognition of our nanodevices, in the micro-molar to pico-molar concentration range.

Abstracts
– Interaction and recognition of DNA –

P-109

Use of MD simulation to identify the critical radiation-induced lesions of a DNA binding protein

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A key step in the regulation of gene expression, DNA structuring and DNA repair is the binding of some proteins to specific DNA sequences. We have shown previously that ionizing radiation destabilizes such DNA-protein complexes mainly through damage to the protein. For the typical lactose operator - repressor system we have shown by fluorescence measurement and mass spectrometry that upon irradiation, all the four tyrosine residues of the DNA binding domain (called headpiece) are oxidized into 3,4-dihydroxyphenylalanine (DOPA). A circular dichroism study revealed a global conformational change and the destabilization of the headpiece. To decide which lesion is critical for the induction of these effects, a molecular dynamics simulation study was undertaken in parallel with a site-directed mutagenesis one. Each tyrosine residue of the headpiece was replaced by another amino-acid that mimics the damaged tyrosine. The most common amino-acid used in site-directed mutagenesis being alanine, we have replaced one tyrosine (7, 12, 17 or 47) in the NMR-based structure of the headpiece from PDB databank (1LQC) by an alanine. The conformational stability of each Tyr→Ala mutant has been studied by Molecular Dynamics simulation (MD) and compared to that of the native sequence and of the different Tyr→DOPA mutants.

P-110

Description of non-specific DNA/protein interaction and facilitated diffusion

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We present a dynamical model for simulating non-specific DNA protein interactions, which is based on the "bead-spring" model for DNA with elastic, bending and Debye-Hückel electrostatic interactions, and where the protein interacts with the DNA chain through electrostatic and excluded-volume forces. We study the properties of this model using a Brownian dynamics algorithm that takes hydrodynamic interactions into account and obtain results that partially agree with experiments and predictions of kinetic models. For example, we show that the protein samples DNA by a combination of three-dimensional diffusion in the solvent and one-dimensional sliding along the DNA chain. This model evidences the presence, in a certain range of values of the effective protein charge, of facilitated diffusion, i.e. a combination of the two types of diffusion that leads to faster than 3-dimensional diffusion sampling of DNA. Moreover, the analysis of single sliding events shows that the number of base pairs visited during sliding is comparable to those deduced from single molecule experiments. In contrast to kinetic models, which predict an increase of the number of different base pairs visited by the protein proportional to the square root of time, our model however suggests that this number increases linearly with time until it reaches a value that is close to the total number of DNA base pairs in the cell (published in J. Chem. Phys. 130, 015103 (2009)).

P-111

Exploring DNA orientation in flow

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DNA is one of the most important biomolecules. In order to undertake its biological role the DNA needs to fold and unfold for which its structure and flexibility are crucially important. We have studied the characteristics of DNA in flow to probe its structure. Flow aligned Linear Dichroism (LD) is a technique that uses light polarised parallel and perpendicular to an orientated sample. It can be used to measure how aligned a sample is, and the orientation of any interacting molecules. To create this alignment, the sample is placed between two concentric cylinders where one is spun to create a shear flow. The longer and more rigid the molecule is the better the orientation and the signal. As the sample is in flow there are other factors than need to be considered when using LD. These include viscosity and temperature. There are many methods to measure the viscosity of a sample of DNA at varying temperatures. These include viscometer, rheometer and dynamic light scattering. The effects temperature has on viscosity and the sample itself need also to be considered. The findings of all these studies have been reported and show the significance that viscosity and temperature have on DNA LD measurements. Possible applications of using LD to study DNA have also been discussed, showing the importance of the use of LD and in the results shown.

O-112

Mechanism of the nucleic acid chaperone properties of the HIV-1 nucleocapsid protein

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The nucleocapsid protein NCp7 of HIV-1 is characterized by two conserved zinc fingers and plays crucial roles in the virus, through its binding to nucleic acids. NCp7 is required for efficient proviral DNA synthesis, by promoting the initiation of reverse transcription and the two obligatory strand transfers. Using fluorescence techniques as well as FCS and NMR, we investigated the chaperone properties of NCp7 on the primer binding sites (PBS) and transactivation response elements (TAR) sequences involved in the two obligatory strand transfers. We showed that NCp7 binds mainly to the (-)PBS loop, which results in an extension of the loop and a destabilization of the upper base pair of the stem. These structural changes chaperone a kissing complex with the complementary (+)PBS loop and its further conversion into an extended duplex. In contrast, the NCp7-promoted annealing of cTAR-TAR results from the destabilization of the bottom of cTAR stem, which favors the invasion of the TAR stem. By developing new fluorescence methodologies to site-specifically characterize these interactions, we further showed that NCp7 slows down the ps to ns conformational fluctuations of its nucleic acid targets and freezes the local mobility of the bases contacted by the zinc fingers.

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– Interaction and recognition of DNA –

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A theory of the mechanisms on the simultaneous binding of two aromatic drugs to DNA

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It has long been recognized that certain combinations of DNA-binding aromatic drugs, $X+Y$, lead to synergistic biological effects. Considering X as a basic ligand and Y as an added ligand, the change of the integral biological response of X in the presence of Y has been interpreted in terms of two mechanisms: the interceptor and protector action of Y on X. This mechanisms have been characterized by two criteria, R_D and A_D, reflecting the removal of X from DNA by Y (*Biophys.Chem., 2008, Vol.132, pages 148-158*). In this work we develop the theory of the interceptor-protector action of a mixture of two biologically-active DNA-binding aromatic drugs. The theory is based on solution of a general system of mass balance equations in the three-component system X-Y-DNA with respect to the two factors, R_D and A_D. The outcome is a set of expressions enabling estimation of the change in biological response of X on addition of Y as a function of equilibrium parameters under different restrictions. The results are in good agreement with known *in vitro* data on Caffeine+Antibiotic action in leukemia cell lines.

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Ultrasonic cleavage of double stranded DNA

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We report the effects of 22 kHz ultrasound irradiation of double-stranded DNA solutions under conditions of transient cavitation. A new method was developed for studying these effects which is based on combination of two procedures: ultrasound irradiation of the solutions of double-stranded DNA fragments and subsequent analysis of the high resolution denaturing gel electrophoresis data. Statistical treatment of the data on the mobility of 3'-end-labelled restriction fragments with known sequence allowed us to discover the phenomenon of sequence specific ultrasonic cleavage of DNA. Our analysis results in the following conclusions: 1) all steps with 5'-ward cytosine [5'-d(CpN)-3'] have significantly higher cleavage rates than others; the intensity of cleavage diminishes in the order CpG > CpA $\approx$ CpT > CpC; 2) the cleavage rates of all 16 steps depend on the type of flanking base pairs; 3) the cleavage rates of the complementary base pair steps are not identical. Thus, subtle sequence specific conformational and physical-chemical variations modulate the reaction of sugar-phosphate single bonds on the ultrasound exposure.

P-115

Partition of Gibb´s free energy of drug-DNA complexation

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We report a computation methodology, which leads to the ability to partition the Gibb's free energy for the complexation reaction of aromatic drug molecules with DNA. Using this approach it is now possible to calculate the absolute values of the energy contributions of various physical factors to the DNA binding process, whose summation gives a value that is reasonably close to the experimentally-measured Gibb's free energy of binding. Application of the methodology to binding of various aromatic drugs with DNA provides an answer to the question 'What forces are the main contributors to the stabilization of aromatic ligand-DNA complexes'?

O-116

Molecular mechanisms of the mammalian high mobility group protein AT-hook 2 recognizing AT-rich DNA

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The mammalian high mobility group protein AT-hook 2 (HMGA2) is a nuclear protein associated with mensenchymal cell development and differentiation. Disruption of its normal expression pattern is directly linked to oncogenesis and obesity. Our laboratory has utilized a variety of biochemical and biophysical methods to investigate the molecular mechanisms of HMGA2 recognizing AT-rich DNA. Using a PCR-based SELEX procedure, we discovered that HMGA2 is a sequence-specific DNA-binding protein and recognizes the following two sequences: 5'-ATATTCGCGAWWATT-3' and 5'ATATTGCGCAWWATT-3', where W is A or T. Using a double-label EMSA assay, we found that HMGA2 binds to AT-rich DNA as a monomer. Using isothermal-titration-calorimetry, we demonstrated that HMGA2 binds to AT-rich DNA with very high binding affinity whereby the binding of HMGA2 to A-tracts is entropy-driven and to alternate AT sequences is enthalpy-driven. This is a typical example of enthalpy-entropy compensation in which the hydration difference between HMGA2-DNA complexes is a main reason for the compensation. Interestingly, the binding of HMGA2 to different AT-rich sequences is accompanied with a large negative heat capacity change indicating an important role of solvent displacement and charge-charge interaction in the linked folding/binding processes.

Abstracts
– Interaction and recognition of DNA –

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Side-by-side and end-to-end attraction of double-stranded DNA

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Genomic DNA is densely packed inside cell nuclei and viral capsids. Such close packing suggests that electrostatic repulsion between negatively charged DNA in the condensed states is balanced by counterion-induced attraction. Several theoretical models have been proposed to explain DNA attraction, however, specific microscopic mechanisms are not known. Here, we report all-atom molecular dynamics simulations of the effective force between double-stranded DNA in side-by-side and end-to-end orientations. In the side-by-side orientation, the DNA molecules were found to form a bond state in the presence of magnesium ions. In the bond state, the DNA molecules contact each other via their negatively charged phosphate groups, bridged by magnesium ions. The maximum attractive force in the side-by-side orientation is about 40 pN per DNA turn. In the end-to-end orientation, a strong ($>$ 60 pN) attractive force was observed at short ($<$ 0.8 nm) end-to-end distances regardless of the electrolyte concentration. The presence of a phosphate group at the 5'-ends of the fragments was found to direct DNA end-to-end self-assembly and produce bound states resembling a continuous DNA molecule. The computed potentials of the mean force suggest that the end-to-end attraction, rather than being mediated by counterions, is likely caused by hydrophobic and van der Waals interactions between terminal nucleobases of the fragments.

P-118

Thermodynamic analysis of 1,4-interstrand cross-links formed by a trinuclear Pt complex BBR3464

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Multinuclear platinum complexes represent a new class of anticancer agents, distinct in DNA binding and antitumor activity from their mononuclear counterparts. BBR3464 as a first representative of this class has undergone Phase II clinical trials for treatment of ovarian and lung cancers. The structure of this trinuclear Pt drug consists of two *trans*-$PtCl(NH_3)_2$ units bridged by a *trans*-$H_2N(CH_2)_6NH_2Pt(NH_3)_2NH_2(CH_2)_6NH_2$ tetraamine linker. The main lesions formed by multinuclear Pt complexes in DNA are long-range intra- and interstrand cross-links (CLs) bridging two guanines separated by up to four base pairs. Since interstrand CLs can prevent DNA strand separation interfering with critical cellular events they represent a serious obstacle in cell survival. In order to contribute to understanding the biological effects of DNA interstrand CLs of BBR3464, we analyzed the effect of the single, site-specific 1,4-interstrand CL formed by this metallodrug between two guanine bases on opposite strands in the 3'-3' and 5'-5' direction on the thermal stability and energetics of short DNA duplexes. The results demonstrate that 1,4-interstrand CLs of BBR3464 in both 3'-3' and 5'-5' directions exist as two distinct conformers that are not interconvertible and affect thermodynamic stability of the DNA differently.

P-119

Toward rapid DNA sequencing – *ab initio* study of nucleotide sandwiched between Au(111) plates

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Recently a new technique for DNA sequencing based on measurement of transversal conductive properties of a single strained DNA molecule has been proposed. Such a method would allow single-base resolution by measuring the electrical current perpendicular to the DNA backbone. Until now, it is still not clear if the electrical signals obtained for the four nucleotide can be clearly distinguished by a hypothetical experimental setup. Several factors – like the influence of the pentose group or the presence of water – may influence quite strongly the electrical signatures of the four bases. Therefore, in order to obtain a working device, the understanding and detailed description of the conduction mechanisms through DNA bases connected to a metallic electrode is essential.
The goal of theoretical studies in this field is to describe the electric signatures of DNA bases from a theoretical point of view. Our study is focused on the detailed description of the electronic structure of DNA's base pairs "squeezed" between two Au plates. While such a geometrical model closely mimic the sequencing devices proposed in the literature, it allows us to compute meaningful physical properties such as density of states, charge transfer and orbital localization – by using "ab initio" methods. The results allow us to give qualitative prediction over the potential use of such a device in the DNA's sequencing technology.

P-120

A Raman spectroscopic study on the influence of Mn^{2+} ions on DNA structure

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The influence of Mn^{2+} ions on the structure of natural calf thymus DNA was studied by Raman spectroscopy. Measurements were done at room temperature and pH 6.2 $\pm$ 0.2, in the presence of the physiological concentration of 150 mM Na^+ ions, and in the presence of Mn^{2+} concentrations that varied between 0 and 600 mM. No condensation of DNA was observed. DNA backbone conformational changes were not detected in the whole concentration range of Mn^{2+} ions as judging from the Raman spectra. No evidence for DNA melting was identified. Binding of manganese(II) ions to the charged phosphate groups of DNA, stabilizing the double helical structure, is indicated in the spectra. As judged from the marker band of dC near 785 cm^{-1}, altered nucleoside conformations in dC residues are supposed to occur, in the Mn^{2+} concentration range of 400-600 mM. Binding of divalent ions to N7 of guanine and, possibly, in a lesser extent to adenine was observed as judging from the Raman marker bands at 1336, 1376, 1490 and 1578 cm^{-1}.

Abstracts
– Interaction and recognition of DNA –

P-121

Comparing the native and an irradiated lactose repressor-operateur complex by MD simulation

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The function of the *E. Coli* lactose operon requires the binding of a protein, the tetrameric repressor, to a specific DNA sequence, the operator. The formation of this complex involves the interaction of at least one protein dimer with the operator sequence. This occurs via the DNA-binding domains (called headpieces) of the two constitutive monomers. We have previously shown that upon irradiation with gamma rays the complex is destabilised mainly because the repressor losses its DNA binding ability. Radiation-induced lesions were identified that may be responsible for this deleterious effect: all tyrosine residues of the headpieces are oxidized into 3,4-dihydroxyphenylalanine (DOPA). In order to unravel the mechanisms leading to the observed destabilization of the operator-repressor complex, we compare by MD simulations two complexes: 1- the native complex formed by a dimer of two headpieces and a fragment of DNA with the operator sequence and 2- the damaged complex in which all tyrosines are replaced by DOPA. Analysis of these trajectories shows a loss of stability and binding energy as well as changes in the structure of the damaged complex with respect to the native one. By comparing precisely the evolution of the two complexes we can explain how the oxidation of the tyrosine residues of the headpieces into DOPA may trigger the destabilization of the complex.

P-122

Ultrasonic cleavage of nicked DNA

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Nicks represent the most common damage in DNA which occurs naturally in living cells. Structural properties of nicked DNA fragments have been an object of numerous studies due to its special role in reparation processes. Here we report experimental results covering ultrasound irradiation of nicked DNA solutions. Several single-stranded nicks were produced into one strand of dsDNA fragments by the nicking enzyme Bst9I. We have quantitatively estimated the ultrasonic cleavage rates in nicked DNA fragments with known sequences using the polyacrylamide gel electrophoresis. Computer analysis of the cleavage pattern in the 3'-end labeled and primarily intact strand reveal cleavage enhancement in the regions of about 10 b. p. up and down the nicks which were initially produced into complementary strand. The intensity of cleavage near the nicks is (in average) about 20 times higher than cleavage in the same sites of the intact dsDNA fragments. At the same time, the cleavage rates in positions beyond the regions of the nick markedly grow weak even comparing to the sequence-specific cleavage of intact double-stranded DNA fragments. Thus, the presence of the nick serves as an expressive structural indignation, which exceeds modulation of the structure caused by the base-pair sequence and is capable of absorbing mechanical stresses applied to the nearby sites of the molecule.

P-123

Design of a microfluidic devices for the detection of oligonucléotides by SERS

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Designing fast and efficient analytical tools allowing the detection of free DNA or RNA at very low concentration within small volumes, without specific molecular labeling, remains a major issue of significance to perform diagnostic or to detect pathogen agents. Our strategy is to use Surface-Enhanced Raman Scattering (SERS) to probe selectively the spectral signature of each base in polynucleotides. SERS takes advantage of the strongly increased Raman signals of species when adsorbed on adapted silver colloids. We already demonstrated that adenyl Raman signal of pA in presence of silver colloids is 10^6 times enhanced compared to bulk signal. We plan to use nanoliter droplets of uniformed size that form spontaneously in microchannels when two immiscible fluid streams merge. These tiny droplets are almost ideal reactors as they create homogeneous control condition. We will design an optimized channels network platform that result in droplets production hosting both nucleotides and silver colloids: internal fluids recirculation provide fast and efficient mixing, favoring base adsorption on silver nanoparticles. SERS will be used to determine the chemical composition of the droplets.

P-124

DNA interactions of antitumor doxorubicin and its more potent analog 2 pyrrolinodoxorubicin

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Doxorubicin (DOX) is an anticancer antibiotics with a four-membered ring system containing an anthraquinone chromophore, and an aminoglycoside. It has good anticancer activity against a wide spectrum of tumors and is one of the most extensively used antitumor chemotherapeutic compounds currently in clinical use. Interestingly, conversion of DOX to 2pyrrolinodoxorubicin analog (p-DOX) exhibits 500-1000 times higher toxic effects in human breast cancer cell line (Nagy, A. et al., PNAS, 93 (1996) 2464). Molecular mechanisms responsible for this enormously enhanced cytotoxicity have not been entirely clarified. There is good evidence that a key component of the mechanism of action of DOX is its intercalation into DNA and the formation of DOX-DNA adducts. Therefore, we have examined in detail, using the methods of molecular biophysics, DNA interactions of p-DOX in cell-free media and compared these results with the same studies focused on the parental DOX. We find distinct differences between DNA interactions of DOX and p-DOX and suggest that these differences are responsible at least in part for different biological effects of these two anticancer drugs.

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Amplification of oligonucleotide sequence recognition using bioresponsive hydrogels

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We describe development and characterization of oligonucleotide functionalized hydrogels for amplifying the molecular recognition signal occurring on hybridization between di-oligonucleotides. The 50 μm radius hemispherical hydrogels were integrated on a high resolution interferometric fiber-optic readout platform supporting determination changes in the optical length of the hydrogel with 2 nanometer resolution. The hydrogels were designed with hybridized di-oligonucleotides grafted to the polymer network as network junctions in addition to the covalent crosslinks or oligonucleotides grafted to the network chains. The probe oligonucleotide destabilizing the junction point by displacement hybridization yielded an optical signal about five times as large as for binding within the hydrogel design with a comblike grafted oligonucleotide. The optical signal was found to be dependent on the concentration of the probe, the sequence and matching length between the probe and sensing oligonucleotide. Concentration sensitivity applied as specific label-free detection of oligonucleotide is estimated to be in the nanomolar region. The current design support detection in excess of $1x10^{12}$ sequences. Amplification of the molecular recognition employing the developed oligonucleotide imprinted hydrogel for label-free sensing of probe oligonucleotide sequences or taking advantage of the oligonucleotide sequence designed as aptamers for determination of other types of molecules are discussed.

P-126

Recognition of nucleic acids by anticancer ligands: kinetics and thermodynamics via MD simulations

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The minor groove of DNA is the target of several anticancer agents, which interfere with replication and translocation processes, leading to cell death. The molecular recognition event is a key step to achieve detailed knowledge of the interactions behind selectivity and affinity of a ligand towards a particular nucleic acids sequence. Recognition is a multi-route process which can involve many steps before the formation of the most stable adduct. In particular, many studies have pointed out the importance of events like sliding along the groove and dissociation (which is a relevant step in the translocation among different sequences) for the affinity and the specificity of minor groove binders. Despite this, no studies on the dynamics of this event were reported in the literature. In this contribution I present our recent work on the subject. Umbrella sampling and metadynamics were used in the framework of classical MD to characterize mechanisms andfree energy profiles of molecular recognition routes by the antitumoral agents anthramycin, duocarmycin and distamycin. Our results are in very good agreement with the available experimental data, and provide insights on the influence of factors like size, charge and flexibility on the molecular recognition process.

P-127

Raman spectroscopy of DNA modified by new antitumor nonclassical platinum complexes

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Platinum anticancer agents (cisplatin, carboplatin, oxaliplatin) are in widespread clinical use especially against testicular, ovarian and head and neck carcinomas. There is a large body of experimental evidence that DNA is the critical target for the cytostatic activity of cisplatin. Platinum complexes form several types of adducts, which occur in DNA with a different frequency and differently distort the conformation of DNA. Clinically ineffective trans isomer of cisplatin (transplatin) also covalently binds to DNA bases. The trans geometry in dichloridoplatinum(II) complexes was activated by various ways. The replacement of at least one amine ligand by planar amine ligand represents example of such activation. Raman spectroscopy is powerful technique for examining both structural and thermodynamic properties of nucleic acids in solution. Interactions of cis- and trans-Pt(II) complexes having nonplanar heterocyclic amine ligand such as piperidine, piperazine and 4-picoline with DNA have been investigated by laser Raman spectroscopy. Raman difference spectra reveal that the Pt(II) complexes induce great structural changes in B-DNA and indicate disordering of B-DNA backbone, reduction in base stacking and base pairing and specific metal interaction with acceptor sites on purine residues.
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O-128

Local conformation of confined DNA studied using emission polarization anisotropy

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In nanochannels with dimensions smaller than the DNA radius of gyration, DNA will extend along the channel. We investigate long DNA confined in nanochannels with dimensions down to 50*50 nm, using fluorescence microscopy. Studies of the statics and dynamics of the DNA extension or position in such confinements as a function of *e.g.* DNA contour length, degree and shape of confinement as well as ionic strength has yielded new insight into the physical properties of DNA with relevance for applications in genomics and fundamental understanding of DNA packaging in *e.g.* viruses. Our work extends the field by not only studying the location of the emitting dyes along a confined DNA molecule but also monitoring the polarization of the emission. We use intercalating dyes whose emission is polarized perpendicular to the DNA extension axis, and by measuring the emission polarized parallel and perpendicular to the extension axis of the stretched DNA, information on the local spatial distribution of the DNA backbone can be obtained. We will discuss results in shallow (60 nm) and deep (180 nm) channels and describe how the technique can be used to investigate non uniform stretching of a single DNA molecule.

Abstracts
– Imaging and spectroscopy –

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G-quadruplexes: combining theory with experimental spectroscopic methods

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Guanine-rich oligonucleotides can form unique tetrameric structures with four coplanar guanine bases, known as G-quadruplex motifs. The G-quadruplexes have been found *in vivo* in the terminal parts of telomeres and other genomic regions. Ligands, specifically binding to the G-quadruplex regions inhibit telomerase activity and thus can play an important role in the cancer therapy. Most recently, the potential use of these structures has been tested in biosensors and nanotechnology industry. In the present work we use the infrared spectroscopy, including the relatively novel technique of the vibrational circular dichroism (VCD), in a combination with molecular dynamics and quantum chemistry computational methods to investigate the structure and spectroscopic response of the quadruplexes formed by the $d(G)_8$ and self-associated dGMP. We obtained a good agreement between the computed and experimental spectra, confirming that the proposed geometrical models are realistic. The VCD technique appears especially convenient for the studies as it can detect the liquid-crystalline phases of the G-quadruplexes by an anomalous enhancement of the signal.

P-130

The influence of potassium and sodium ions on the aggregation process of antibiotic amphotericin B in aqueous solution

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Amphotericin B (AmB) is a polyene antibiotic that has been widely used for treatment of systemic fungal infections. The main mechanism of biological mode of action of AmB is considered to be associated with formation of ionic membrane pores or channels in the lipid membranes.
The aim of this work was to study the influence of the K^+ and Na^+ ions on the aggregation process of AmB in aqueous medium. The analysis of electronic absorption and fluorescence spectra of AmB shows that the increasing K^+ concentration have influence on the level of aggregation of the drug much more than the same amount of Na^+ ions. This effect is especially noticed at neutral pH values. The RLS technique was used to study aggregation of AmB in solution, in the environment of the K^+ and Na^+ ions. The application of this technique makes it possible to study the electronically coupled chromophores, especially molecular aggregates. The results of the ATR-FTIR and Raman spectroscopic studies also support this conclusion. These results provide a better understanding of the interaction between K^+ and Na^+ ions and antibiotic which has not been previously considered to be significant for biological action of AmB.

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Emodin uptake study in U-87MG cells using optically trapped surface-enhanced Raman scattering probes

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Emodin (1,3,8-trihydroxy-6-methylantraquinone) is a photosensitizing pigment present in plants of herbal laxatives. Emodin inhibits nuclear transcription factor-κ*B* activation and induces free radical production in human mononuclear cells resulting in its antiviral and anti-cancer activity.
The uptake and distribution of emodin inside the U-87 MG cell line is studied by combining optical tweezers and surface-enhanced Raman spectroscopy (SERS). SERS greatly enhances the spectrum of an otherwise weakly scattering material which is achieved by attaching nano-sized silver colloids to micron-sized dielectric beads.
The distribution of emodin in the cell is studied by simultaneously trapping and exciting the SERS bead and scanning it across the membrane while recording the emitted light. Secondly, the beads are statically placed inside the cell and excited at certain intervals in order to track the migration of emodin through the membrane. The results give new insight in to the metabolic pathways of emodin and demonstrate a new imaging and detection technique that is fast and less invasive than current standards.
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P-132

Tracing T-cells by paramagentic nanoparticles in the brain of the rat model of ALS

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Amyotrophic lateral sclerosis (ALS) is a devastating neurological disorder affecting upper and lower motoneurons. Since immune disbalance is known to be an important manifestation of the disease we were particularly interested in following the labeled immune cells in the familial ALS rat model, hSOD-1^{G93A}. A T2- or T1- weighted MRI protocol was used with a mini surface coil placed over the skull of the anesthetized animal in a 1.5 T wide bore magnet. In order to compare this approach to standard high field small animal imaging a 7.0 T MRI system was also used. There was a congruence of images of lesions in the brainstem at the two field strengths.
It was confirmed with Gd-DTPA contrast that the blood brain barrier (BBB) is compromised at the interbrain level. In order to study immune cell infiltration rats were i.v. injected with magnetically labeled antibodies against helper CD4+ or cytolytic CD8+ killer T cells. By combined T1, T2 and T2* weighted imaging CD4+ lymphocyte infiltration was observed in the brainstem-midbrain region while the CD8+ cells were more confined to the brainstem region. Comparison of MRI of labelled CD4+ vs. CD8+ lymphocytes reveals the relevant cellular inflammatory mechanism in ALS. The appearance of the MRI signal from the latter T cell type points to the mechanism of BBB disruption as suggested from a recent study on the role of CD8+ T cells in a model of multiple sclerosis.

Abstracts

– Imaging and spectroscopy –

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Fast-Acquisition Multispectral FLIM by Parallel TCSPC

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Currently used TCSPC FLIM systems are characterised by high counting efficiency, high time resolution, and multi-wavelength capability. The systems are, however, restricted to count rates on the order of a few MHz. In the majority of applications, such as FRET or autofluorescence, the photostability of the samples limits the count rate to much lower values. The limitation of the count rate is therefore no problem. However, if FLIM is used for ion concentration measurements or imaging of chlorophyll in plants the available count rates can exceed the counting capability of a single TCSPC channel. We therefore developed a FLIM system that uses eight fully parallel TCSPC channels. By using a polychromator for spectral dispersion and a multi-channel PMT for detection we obtain multi-spectral FLIM data at a rate of several frames per second. We will demonstrate the application of the system to dyamic changes of the fluorescence lifetime of chlorophyll in living plants.

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Three-dimensional SHIM and 2PEM to study collagen arrangement and crimping pattern

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Ligaments have been generally described as multifascicular structures with collagen fibre bundles cross-connecting to each other or running straight and parallel with crimps. A different collagen array and crimping pattern in different ligaments may reflect a different mechanical role. Aim of this study was to relate the 3D collagen arrangement and crimping pattern by backward and forward second harmonic imaging microscopy (SHIM) and 2-photon excitation microscopy (2PEM). SHIM on a laser-scanning system is a powerful and unique tool for high-resolution, high-contrast, three-dimensional studies of tissue architecture. Although it is a coherent process the multiple scattering through the tissue give us the capability to acquire signal in both backward and forward direction [1]. SHIM and 2PEM were combined in a dual-mode nonlinear microscopy to find out collagen fibre arrangement and crimping pattern. Both polarization dependence and differences between forward and backward signals allowed to yield information on local structure[2].

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P-135

Calyx of Held: STED nanoscopy of a glutamatergic terminal

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The calyx of Held, a large glutamatergic synaptic terminal in the auditory brainstem circuit has been increasingly employed to study presynaptic mechanisms of neurotransmission in the central nervous system. A highly detailed model of the morphology and distribution of cytoskeleton, synapsin, synaptic vesicles, calcium sensors, mitochondria, the presynaptic membrane and its active zones is derived by colocalization analysis of these different key elements of synaptic transmission in the rat brain. The various cellular components are visualized with subdiffraction resolution by stimulated emission depletion (STED) microscopy. Imaging individual structural elements exhibit a focal plane resolution of <50 nm inside 3 μm thick tissue sections.

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Role of fructose in the seasonal adaptation of Picea omorika (Pančić) Purkinye to cold

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Fructose, due to its high antioxidative capacity, represents a significant component of non-enzymatic defense of some plants against cold-provoked oxidative stress. In the present study, we have investigated role of fructose in seasonal adaptation of Picea omorika (Pančić) Purkinye to cold. This endemic coniferous species is exposed to subfreezing temperatures that range from -10 to -30°C during the autumn/winter and high temperatures exceeding 30°C during the summer. Characteristic EPR signal of free or weakly bound Mn^{2+} was used as an indicator of oxidative status of needles, since cold-related oxidative damage leads to Mn^{2+} release from photosystem II. It was observed that prooxidative conditions developed in the autumn, at the beginning of cold season, which corresponded to significant increase of fructose level. Total SOD, as well as MnSOD activity also rose significantly higher in the autumn. Observed activation of antioxidative system (non-enzymatic and enzymatic) led to adaptation of needles to cold, as oxidative status during winter was decreased and similar to the status of needles in cold-free seasons.

Abstracts
– Imaging and spectroscopy –

P-137

A synchrotron based IR spectroscopy detection of apoptosis in U-87 MG cells induced by hypericin

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Synchrotron based Fourier transform infrared (SR-FTIR) microspectroscopy was applied to investigate apoptotic death of U-87 MG cells induced by the photosensitizer hypericin (Hyp), in using different transport systems (Hyp alone vs. Hyp/LDL complexes) and incubation protocols. The differences between IR spectra of non-treated and Hyp treated cells are mainly manifested in the positions of amide I and amide II vibrational bands of proteins. These vibrational shifts are attributed to the protein structure changes from dominantly alfa-helix, in the non-treated cells, to beta-sheets and random coil structures, which prevail 4h and 24h after photodynamic treatment, respectively. The observed conformational changes of proteins can be explained as the consequences of the processes leading to apoptosis as was verified by flow cytometry experiments. The results confirm suggestion that IR spectroscopy can be successfully applied for the detection of early apoptotic processes.
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Polarized transient absorption to resolve electron transfer between tryptophans in DNA photolyase

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Photoactivation of DNA photolyase comprises electron transfer through the chain FADH°-W382-W359-W306. Photo-excited FADH° abstracts an electron from the tryptophan residue W382 in ~30 ps (monitored by transient absorption spectroscopy). The subsequent electron transfer steps (from W359 to W382$^{\circ+}$ and from W306 to W359$^{\circ+}$) are difficult to resolve experimentally, because electron transfer between chemically identical species does not give rise to net absorption changes. To overcome this difficulty, we make use of the fact that polarized excitation (pulse laser) induces a preferential axis (that of the excited flavin transition) in the system (photoselection), and that W359 and W306 form different angles with that axis (known from the crystal structure). Thus, polarized detection should allow distinguishing between them. Using polarized "classical" transient absorption on a nanosecond time scale and the pump-probe technique on a picosecond scale, we demonstrate the feasibility of the method and provide evidence that electron transfer from W306 to W359$^{\circ+}$ is faster than the 30 ps time constant of the initial electron transfer from W382 to excited FADH°.

P-139

Biospectroscopic probes for real time measurement of hydrogen-deuterium exchange

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Isotopic exchange has long been used for the analysis of biomolecular structure and dynamics. Hydrogen-deuterium exchange rates depend on pH, temperature and biomolecular environment. This is due to hydrogen bonding, low solvent accessibility, and steric blocking.
Time resolved measurement of hydrogen-deuterium exchange for subsequent 2D correlation spectroscopic analysis can, then, be very useful to obtain structural information from the said viewpoint. We have developed a microdialysis quartz cell for use in conjunction with Raman spectroscopy to investigate hydrogen-isotope exchange reactions of biomolecules. The system requires only 40 μl volumes of the initial substrate and perturbing effluent solutions. We have obtained a D_2O efflux rate of $k_d = 0.38 \pm 0.008$ min^{-1} with the greatest MWCO (18 kDa) used here, which involves that an exchange rate of 2.5 min^{-1} is the limiting rate that could be resolved with the said cell system. Analogous results have been obtained using an infrared biospectroscopic microdialysis probe. The use of the method described here has the advantage of avoiding sample dilution (and subsequent signal loss) involved in the known stop flow methods.
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P-140

Scattering effects on non linear imaging of thick biological samples

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Non linear optical scanning microscopy became a useful tool for living tissue imaging. Biological tissues are highly scattering media and this leads to an exponential attenuation of the excitation intensity as the light travels into the sample. While performing imaging of biological scattering tissues in two photon excitation (2PE) regime, the localization of the maximum 2PE intensity was found to shift closer to the surface[1] and the imaging depth limit appears strongly limited by near surface fluorescence[2]. In this work we computed illumination and photobleaching intensity distribution[3] in order to characterize the effects induced by scattering. Simulations of 2PE illumination and photobleaching intensity profiles have been performed for different scattering coefficients and at different focus depth. Furthermore imaging of fluorescent immobile sample (polyelectrolyte gel) allowed to perform an experimental test on thick turbid media. Results confirm that under these conditions no photobleaching effects due to scattering occur close to the surface.
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Abstracts
– Imaging and spectroscopy –

O-141

Spectrally Assigned Localization Microscopy (SALM): A new approach in nuclear genome biophysics

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Here we report on "Spectral Precision Distance/Position Determination Microscopy (SPDM) with Physically Modifiable Fluorochromes ($SPDM_{Phymod}$) to analyse the spatial distribution of single nuclear proteins and DNA sequences at the macromolecular optical resolution level. Like other methods of "Spectrally Assigned Localization Microscopy" (SALM), $SPDM_{Phymod}$ is based on labelling 'point like' objects (single molecules) with different spectral signatures, spectrally selective registration and high precision localization monitoring by far field fluorescence microscopy. The intranuclear spatial location of single molecules was determined up to a density up to ca. 1000 molecules/μm^2 of the same type, and distances down to 15 – 30 nm were nanoscopically resolved.

*P.Lemmer et al. (2008) SPDM – Light Microscopy with Single Molecule Resolution at the Nanoscale. Applied Physics B **93**: 1-12.*
*R. Kaufmann et al. (2009) SPDM – Single Molecule Superresolution of Cellular Nanostructures. (2009) Proc. SPIE, Vol. **7185**, 71850J ; DOI:10.1117/12.809109.*

P-142

A XAS study of the sulphur environment in human neuromelanin and its synthetic analogues

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Neuromelanin is a complex molecule accumulating in the catecholaminergic neurons that undergo a degenerative process in Parkinson's Disease. It was shown to play an either protective or toxic role depending on whether it is present in the intraneuronal or extraneuronal milieu. In the present study X-ray Absorption Spectroscopy is employed to investigate the sulphur binding mode in natural human neuromelanin, synthetic neuromelanins and in certain structurally known model compounds, namely cysteine and trichochrome C. Based on comparative fits of human and synthetic neuromelanin spectra in terms of those of model compounds, the occurrence of both cysteine- and trichochrome-like sulphur coordination modes is recognized and the relative abundance of these two types of structural arrangement is determined. Data on the amount of cysteine- and trichochrome-like sulphur measured in this way indicate that among the synthetic neuromelanins those produced by enzymatic oxidation are the most similar ones to natural neuromelanin.

P-143

Interkingdom signalling in *pseudomonas aeruginosa*

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Quorum sensing is the process through which some bacterial species coordinate cell-cell communication. *Pseudomonas aeruginosa;* the pathogen responsible for over 90% of chronic lung infections and the leading cause of mortality in cystic fibrosis patients, expresses two major classes of quorum sensing molecules characterized as N-acyl homoserine lactones and 2-alkyl-4-quinolones. These compounds, in addition to their signalling roles have also been found to possess virulent properties, not only against competing species of bacteria such as *Staphylococcus aureus* but also eukaryotic cells such as T-lymphocytes. The process of bacterial quorum signalling molecules influencing eukaryotic cell activity is termed 'interkingdom signalling'. To date it has been suggested that the Quorum sensing molecules outlined above act on eukaryotic cells through interactions with an as yet unidentified plasma membrane or cytosolic receptors. This project is directed towards developing an understanding of how these compounds elicit eukaryotic response through a combination of membrane based interactions at physiologically relevant concentrations. Particular emphasis is placed on studies of ligand binding with membrane microdomains in and the consequent downstream signalling are also considered. This work is significant as it will not only lead to a better understanding of *Pseudomonas* infection, but may also lead to the discovery of new classes of agents for the treatment of infective diseases.

P-144

Controlling molecular fluorescence in laser-scanning microscopy

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Molecular fluorescence has been an indispensable tool in modern day optical imaging. One of the state-of the-art challenges in fluorescence microscopy is having better depth resolution as embodied by the confocal and multi-photon laser-scanning microscopic techniques. However, each technique bears its own limitation in having sufficient out-of-focus signal for the former while the low non-linear photon absorption cross-section for the latter. For confocal microscopy using one-photon excitation, we have shown how the clever choice of pulsed illumination instead of continuous-wave excitation leads to a gigantic enhancement in fluorescence that also has immediate applications in microscopy. Moreover, single-photon illumination with ultrafast pulses leads to a novel way of achieving axial resolution along with numerous other advantageous applications *e.g.* reduced photo-bleaching of the chromophore. On the other hand we have thrown new insight demonstrating that the use of mode-locked laser pulses in multi-photon microscopy induces severe solvent-induced photo-thermal damage and prescribed methods to get rid of it. Besides, the use of pulse pair excitation in multi-photon microscopy leads to probe and control the dynamics of fluorophores which has crucial role in selective excitation of fluorophores from quantum control perspectives. All these cutting edge research works will be presented in addition to our recent work on application of laser pulse shaping in multi-photon microscopy.

Abstracts
– Imaging and spectroscopy –

O-145

Fluorescence imaging for a MVAC chemotherapy resistance predicative test in human bladder cancer

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Multidrug resistance is a well known phenomenon which limits effectiveness in treating malignancy with chemotherapy by modifying the internalization and/or externalization flow of the drugs through the cancerous cells. Combined chemotherapies, such as MVAC, are therefore currently used in bladder cancer treatment. However, about 30% of patients do not respond this chemotherapy because of inherent or acquired drug resistance.
We developed a non invasive predicative test on urinary cells to estimate the chemotherapy effectiveness before treatment, based on the fluorescence emission of MVAC. We first studied the MVAC photophysical properties in solution and using five cell lines: a drug sensitive cancer cell line MGH-u1S, its multidrug resistant subline MGH-u1R, a not tumorigenic cell line SV-HUC-1, its tumorigenic counterpart MC-SV-HUC T-2 and a cell line from transitional cell carcinoma T24.
The results revealed a penetration and localization of the drug depending of the cell line type, allowing us to find a specific fluorescence signature for the identification of MVAC resistant cells. Similar data have been obtained for cytospined fixed culture cells and patients urinary cells.

P-146

3D Correlative Light-Electron Microscopy approach to study cellular and molecular events

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Correlative light/electron microscopy (CLEM) is becoming increasingly frequent in molecular and cellular biophysics. We successfully applied the method to analyze the 3D structure of rough and smooth Russell bodies used as model systems. The major advantages of this approach are the following: (i) the ability to correlate several hundreds of events at the same time, (ii) the possibilitˆ to perform 3D correlation, (iii) the potential to immunolabel both endogenous and recombinantly expressed proteins at the same time and (iv) the effective combination of the high data analysis capability of FLM with the high precision-accuracy of transmission electron microscopy in a CLEM hybrid morphometry analysis. We have identified and optimized critical steps in sample preparation, defined routines for sample analysis and retracing of regions of interest, developed software for semi/fully automatic 3D reconstruction and defined preliminary conditions for an hybrid light/electron microscopy morphometry approach. The relevance of the presented approach lies in two important key elements, namely: the development of optical nanoscopy methods and the potentiality for exploring different correlative frameworks like optical nanoscopy vs. optical microscopy adding scanning force microscopy techniques.

P-147

Modulating the response of single neurons and neuronal networks with biophysical stimuli

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During differentiation, cell processes create connections with other cells to form tissue capable of performing complex tasks. Biophysical constraints provide necessary inputs for cellular organization in living organism[1]. To better understand how biophysical conditions influence tissue development, it is necessary to bridge the gap between experiments on single cells and complex tissues[2,3]. To achieve this goal we pair optical tweezers with electrophysiology measurements[4]. By adopting neuronal networks as a biological model, neuronal signal transmission can be recorded either by patch-clamp electrophysiology or microelectrode arrays (MEAs). Dissociated neurons will be cultured on MEAs to record neuronal network activity at different sites of the network while applying spatio-temporally defined biophysical stimuli to individual neurons.

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P-148

Labeling of the isolated plant cell walls with CdSe Quantum dots

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Quantum dots (QDs) are semiconductor nanoparticles with increasing application as fluorescent markers in biology.We investigated structure of the cell walls of different species complexed with CdSe QDs using fluorescence microscopy, fluorescence spectroscopy and FTIR techniques. In the experiments we used the cell walls isolated from three distinct plant species: *Arabidopsis thaliana*, *Acer sp.* and *Picea omorika*. We studied both unlabeled and CdSe-labeled cell walls. Fluorescence spectroscopy and microscopy were used for detection of QDs alone or complexed to the cell walls. Emission spectra were deconvolved using the Nelder–Mead algorithm in Matlab 6.5. We calculated approximate probability distribution (APD) for positions of spectral component maxima. There was certain difference between unlabeled cell walls and those complexed with QDs. The FTIR spectra also show some difference between the complexed and pure cell walls. The results show that structure was changed, but not significantly in reaction with CdSe QDs. These results are promising in context of use of QDs as labels in cell wall studies. The characterization of the complex of cell wall structure with QDs is a part of the study of nanoparticles application in investigations of plant materials.

Abstracts
– Imaging and spectroscopy –

P-149

Simultaneous measurements of solvent dynamics and functional kinetics in a light-activated enzyme

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Solvent fluctuations play a key role in controlling protein motions and functions. Here, we have studied how the reaction catalyzed by the light-activated enzyme protochlorophyllide oxidoreductase (POR) couple with solvent dynamics (G. Durin et al, Biophysical J. (2009) 96, 1902-10). To simultaneously monitor the catalytic cycle of the enzyme and the solvent dynamics, we designed temperature-dependent UV-visible microspectrophotometry experiments, using flash-cooled nano-droplets of POR. The temperature-dependant formation of the first two intermediates in the POR reaction were measured, together with the solvent glass transition temperature (T_g) and the build-up of crystalline ice. We find that formation of the first intermediate occurs below T_g and is not affected by solvent dynamics, whereas formation of the second intermediate occurs above T_g and is influenced by solvent dynamics. These results suggest that internal protein motions drive the first step of the POR reaction whereas solvent slaved motions control the second step. We propose that the concept of solvent slaving applies to complex enzymes such as POR.

P-150

Immobilization of liposomes in a sol-gel matrix: a fluorescence confocal microscopy study

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Immobilization of liposomes shows interesting applications in protein biology, membrane biophysics and biosensor technology. Previous fluorescence spectroscopy works revealed that the entrappement of zwitterionic phospholipid liposomes in a silica sol-gel matrix alters the thermodynamic properties as well as the fluidity of the lipid bilayer. Interactions between the polar head of phospholipids and the porous surface of the host matrix could be responsible of such behaviour. In order to get more insight into this possibility we have immobilized, for the first time, giant unilamelar vesicles (GUVs) and the shape and size of these structures as well as the possible existence of lipid domains have been visualized through fluorescence confocal microscopy. This technique allows for direct observation of the effect of encapsulation on an individual liposome, in contrast to the averaged information given by macroscopic spectroscopic techniques. Liposomes composed of pure POPC or DOPC as well as mixtures DOPC/DPPC were labelled with the fluorescent probes Bodipy, Rhd-PE and Rhd-DOPE. Preliminary results shows that only the smallest GUVs (6-10μm) survive to the encapsulation process but often with a slight loss of its sphericity, probably due to pressures suffered during the matrix gelation. However no change in the gel/fluid phase proportion has been observed for immobilized DOPC/DPPC GUVs, regarding to solution.

O-151

Translational biophysics: multimode imaging for preclinical and clinical applications

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Our focus is where light and patient meet, and improvements yielding better outcomes. Surgery is moving towards minimally invasive intervention, where biophotonics represents a major area of hope and growth. The translation of useful laboratory-derived knowledge into clinical practice has been hampered by the difficulty of detecting, characterizing and monitoring molecules and cells in the human body, especially dynamically. *Advanced biophotonic imaging* is best suited for studying such entities, but has been lagging in clinical acceptance, in spite of major advances, and a clear need for the kind of resolution (spatial, temporal, spectral) and specificity that it alone can offer. Biophysics-based new strategies are needed to address this challenge.
The development of biophysical methods for translational medicine will be reviewed, with emphasis on our recent advances. Our approach is a *multimode* one - combining methods to achieve early, quantitative detection of abnormalities. With imaging fulfilling its dual role of better describing anatomy *and* physiology, intrasurgical histopathology-equivalent molecular and cellular imaging is achievable *in vivo*, as is a closer spatio-temporal connection between imaging and intervention.
Some application areas to be covered: cancer (early detection by spectral reflectance/autofluorescence; progression quantitation by OCT; nano- and targeted chemotherapy assessment *in vivo)*; neurobiology (imaging fast calcium transients and Alzheimer's plaques); hyperspectral Mie scattering imaging for *in situ* displasia; design and use of an advanced multimode imaging endoscope with *in vivo* delineation of Hirschsprung's disease for better intervention; monitoring of stem cell fate *in vivo*.

P-152

Multilayered photoresist system as a ghost model for biological samples in confocal microscopy.

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We have realized a bilayer photoresist system as a model to perform optical characterization in the VIS-IR range of multilayered complex biological specimen. Our goal is to obtain structural and spectroscopic reference parameters from the identification of reflectance pattern features within a novel project granted by MIUR (SKINTARGET, IDEAS FIRB). Our model is composed by a 1300 nm thick layer (Shipley photoresist 1813) with a refractive index n= 1.6-1.8, wavelength dependent, and a 600 nm tick HSQ (hydrogen silsesquioxane) layer with a refractive index n around 1.38, both spin-coated over a glass substrate.
We present the analysis of reflectance pattern that can be obtained by a confocal laser reflective system as compared to the expected values as coming from analytical calculations, using a matrix approach, or a microscopic electromagnetic finite element analysis. Interfacial roughness and consequent optical scattering are analyzed using a neural network approach.

Abstracts
– Imaging and spectroscopy –

P-153

Seeing more in total internal reflection fluorescence (TIRF) microscopy

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Total internal reflection fluorescence (TIRF) microscopy is an effective widefield imaging tool that selectively excites a very thin sample layer within the evanescent excitation field at the glass-water interface.
Lateral resolution of standard TIRF microscopy is limited to approx. 240nm using green emission, which can be insufficient for a large class of biological investigations. Additionally, the evanescent excitation field is prone to light scattering, creating out of focus blur in the final image.
We present several techniques that address these mentioned shortcomings of standard TIRF microscopy. Using evanescent standing waves, the lateral resolution in TIRF microscopy can be extended by a factor of 2.5, reaching 90nm. We further show techniques to reduce the blur induced by light scattering of the evanescent field. Finally, we demonstrate optical sub-sectioning capabilities in TIRF microscopy by acquiring several images with different penetration depth of the evanescent field and applying suitable post processing algorithms. Thereby the obtainable z-resolution exceeds the classical limit of widefield microscopy, and object structures lying within the evanescent field can be reconstructed.

P-154

Super-resolution imaging of DNA through single molecule switching of intercalating cyanine dyes

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A growing trend in far-field super resolution fluorescence microscopy involves the replacement of photoactivatable fluorophores by common dyes such as Cy, Atto or Alexa [1]. It has been shown that these dyes can blink in useful timescales for single-molecule based imaging by adding suitable buffers. This strategy greatly simplifies the sample preparation and imaging scheme, enabling its application to a wider range of biological systems.
We have explored if a similar approach might be useful to study DNA topology using intercalating cyanine dyes such as YOYO-1. There are two main advantages of this approach: i) DNA labelling with intercalating dyes is straightforward, and ii) the free dye in solution is essentially non-fluorescent, greatly reducing the fluorescence background. We show that YOYO-1 can blink in the absence of oxygen and in the presence of cysteamine, which allows its application to nanoscale imaging. We exemplify its use by imaging λ-DNA and a pUC19 plasmid. We also explore the compatibility of several intercalating dyes with biological systems such as enzymes or cells. Our results suggest that DNA intercalating dyes are a promising option for fluorescence super-resolution studies of DNA topology.

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P-155

Morfo-functional asymmetry of the olfactory receptors of the honeybee Apis mellifera L

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Lateralization, i.e. the different functional specialisation of the left and right side of the brain, has been documented in many vertebrate and, recently, invertebrates species. In the Honeybee *Apis mellifera* L. olfactory memory seems to involve at first the use of the right antenna. The present study investigated physiological and anatomical differences between left and right antennae of honeybees. Electroantennographic responses (EAG) were recorded from the left and right antennae of 16 honeybees to Linalool, a floral volatile compound, and IsoAmyl Acetate, an alarm pheromone, at 5 doses (from 0,25 to 2500 μg). The number of sensilla on the left and right antennae was recorded by Scanning Electron Microscopy (SEM). Each antenna segment, from 14 insects, was observed from 4 different viewpoints in order to image the whole antenna surface and compute the number of sensilla. The tested compounds induced higher EAG responses on the right than on the left antenna at every dose. SEM showed that the placoidea olfactory sensilla were slightly more abundant on the right antenna surface than on the left one. Results suggest an asymmetry in the peripheral odour perception mechanism in the honeybee *A. mellifera.*

P-156

Amphotericin B – cooper complexes

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Amphotericin B (AmB) is one of the main polyene antibiotics widely used to treat deep-seated fungal infections. The mechanism of biological action of AmB is most probably directly related to the ability of the drug to form hydrophilic pores in the membrane core, thus affecting physiological transport of ions.
The effects of AmB–Cu^{2+} complexation are demonstrated by the electronic absorption and fluorescence spectra. The absorption spectra of AmB in water (pH=7) after the injection of water solution of copper(II)sulfate display a complex structure with hypsochromic- and bathochromic-shifted bands indicative of formation of molecular aggregates of the drug. Formation of the electronically coupled chromophores of AmB, especially aggregates, was analyzed at different Cu^{2+} concentrations by the RLS (Resonance Light Scattering) technique. Intensity of the fluorescence emission spectrum (characteristic of the dimeric form of AmB) decreases after the AmB–Cu^{2+} complex formation. This effect of the formation of the AmB aggregated structures by AmB–Cu^{2+} are different from the spontaneous molecular aggregation process, as deduced from the spectroscopic analysis.

Abstracts

– Imaging and spectroscopy –

P-157

Muscle structure and GABAergic innervations in the limbs of barnacle cyprid

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Balanus amphitrite is a sessile crustacean that settles at the larval stage of cyprid. In this stage we studied the GABAergic innervation of limb striated muscular fibers, by immunohistochemistry. The second harmonic generation (SHG) and 2-photon excitation (2PE) microscopy were used to set out the muscle structure and its relationship with nerve terminal. Sections were observed at a multimodal nonlinear microscope composed by the Leica CLSM. The laser system used is a Ti:Sapphire Chameleon-Ultra (Coherent Inc, Santa Clara, CA, USA), tunable between 690 nm and 1040 nm and characterized by a pulse width of 140 fs delivered at a repetition rate of 90 MHz by means of a home-built set-up (Bianchini P. and Diaspro A. J Biophotonics 2008 1:443-50). The z- stacks were performed in order to obtain the 3-dimensional distribution of the muscular fibers. In the posterior ganglion GAD immunoreactive (IR) motor neurons were arranged in 12 clusters near the emergence of the limb nerves. GABA and GABAbR1 IR neuromuscular junctions (NJ) were localized in the limb muscle fibers; VGAT IR cells surrounded each limb muscles. These results suggest that GABA plays a key role in the regulation of limbs movement. The SHG was very useful to outline the relationship between nerve terminals and limbs muscle fibers.

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Biophysical identification of orf10 from clavulanic acid biosynthesis cluster as a CYP450

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Streptomyces clavuligerus produces the clinically important β-lactamase inhibitor clavulanic acid. Biosynthesis related genes reside in three gene clusters, one of these, named clavulanic acid gene cluster, includes most of the known clavulanic acid biosynthetic enzymes. The penultimate step along clavulanic acid biosynthesis remains unclear. Required transformation involves at least two events: oxidative deamination and double epimerization of ($3S$, $5S$)-clavaminic acid into ($3R$, $5R$)-clavaldehyde. Downstream the known part of clavulanic acid cluster lays *orf10*, a putative gene encoding a tentative cytochrome P450-like protein which knockout has been proven deleterious to clavulanic acid biosynthesis. Should such protein exist, it would be candidate to fulfill the clavulanic acid pathway missing step. In this work, *orf10* encoded protein is characterized aiming to place it as a real P450. For this task, molecular cloning and recombinant expression of *orf10* were accomplished. Purified protein was submitted to spectroscopic measurements such as circular dichroism and electron paramagnetic resonance which indicate P450 features, including catalytic relevant heme iron redox states and homolytic peroxide scission mechanisms. Further, peroxide reaction adducts were characterized by spin trapping.
This work is supported by FAPESP.

O-159

LEDGF/p75 switches from a dynamic to a tight chromatin interaction upon binding to HIV-1 integrase

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Human transcriptional co-activator LEDGF/p75 is hijacked by HIV-1 integrase (IN) during the replication of HIV. Little is still known about the molecular complex of these two proteins in the living cell. In this work we first studied the cellular chromatin interaction of eGFP-tagged LEDGF/p75 with tunable-focus fluorescence correlation spectroscopy (TF-FCS) and show that LEDGF/p75 is in equilibrium between a free Brownian motion and a very slow movement on the chromatin. Being dependent on the size of the laser focus, this slow movement represents a continuous association-dissociation-reassociation process that is governed by diffusion. Concentration-dependent continuous photobleaching measurements (CP) furthermore revealed the existence of high-affinity chromatin binding sites. Next, we co-expressed mRFP-tagged IN and confirmed its intracellular interaction with LEDGF/p75 by fluorescence cross-correlation spectroscopy (FCCS). Interestingly, CP and fluorescence recovery after photobleaching (FRAP) indicated that the affinity of this complex for chromatin is exceptionally high. By two-photon fluorescence lifetime imaging (2P-FLIM) we verified if the cellular stoichiometry was altered when the proteins were expressed together. We believe that this work is useful for the understanding and targeting of HIV-replication.

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Dynamic of the interaction of photoactive drug hypericin with LDLs and U87 glioma cells

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The natural photosentizer hypericine (Hyp) exhibits potent properties for tumor diagnosis and photodynamic therapy. Evidences of Hyp release from LDLs prior to passive diffusion within cells are addressed in this study. Fluorescent properties of Hyp have been used for dynamic studies of its interaction with low-density lipoproteins (LDLs) and U87 glioma cells. Subsequent non-specific staining of intracellular membranes compartment were observed by mean of co-localization fluorescent imaging studies. It was shown, that monomers of Hyp are only redistributive forms. Increasing of Hyp concentration leads to the formation of non-fluorescent aggregates within LDLs as well as within the U87 cells, and can preclude its photosensitizing activities. In all experiments, hydrophobic character of the molecules appears as the driving force of its redistribution process.
Acknowledgment: This work was supported by the Slovak Res. and Dev. Agency contracts No. LPP-0337-06. We also kindly thank the synchrotron SOLEIL for using the detection system of the disco beamline.

Abstracts
– Imaging and spectroscopy –

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Present opportunities and future developments in soft x-ray transmission and emission microscopy

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Soft X-ray transmission and emission imaging and spectromicroscopy are bridging the gap to other microscopy techniques in terms of lateral resolution, penetration depth and chemical sensitivity. The novel soft X-ray spectromicroscopy approach of the TwinMic instrument at the Elettra synchrotron radiation facility is combining several imaging modes for morphology characterization, such as scanning, projection and full-field imaging with several contrast techniques including brightfield, darkfield, differential phase and interference contrast at sub-100 nm lateral resolution. Complementary chemical information is provided by chemical imaging and micro-spectroscopy using the photon-in/photon-out X-ray absorption and X-ray fluorescence. Unique for TwinMic is the low-energy X-ray fluorescence setup operated at 280 – 2200 eV photon energies, which allows simultaneous analysis of the morphology, *and* the distribution of light elements on cellular and sub-cellular level. In the presentation the principles of the methods used in the TwinMic instruments the performance and potentials of the instrument will be demonstrated by several examples of applications in the field of human, animal and plant biology, biophysics and chemistry, physiology and genetics. The potential impact of microscopy techniques using free-electron X-ray lasers on biophysics will be illustrated by the DiProI project at Fermi@Elettra.

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Site-directed spin labeling study of the light-harvesting complex CP29

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The topology of the long N-terminal domain of the photosynthetic light-harvesting complex CP29 was studied using electron spin resonance (ESR). CP29 is a minor antenna complex of the Photosystem II (PSII), a multisubunit protein complex. Wild-type CP29 protein containing a single cysteine at position 108 and nine single cysteine mutants were produced, allowing to label different parts of the domain with a nitroxide spin label. In all cases the apoproteins were either solubilized in detergent or they were reconstituted with their native pigments (holoproteins) in vitro. The spin label ESR spectra were analyzed in terms of a multi-component spectral simulation approach, based on hybrid evolutionary optimization and solution condensation. These results permit to trace the structural organization of the long N-terminal domain of CP29. We took the crystal structure of light-harvesting complex II (LHCII), major antenna complex of PSII available in PDB as a starting point and constructed a model for CP29 based on ESR data.

P-163

Mechanical properties of polymeric membranes probed by AFM

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Many biological cell functions are dependent on the mechanical properties of the membrane. Polymeric membranes that mimic native cell membranes are valuable research tools which can be used to better understand the physics of biological membranes. We have investigated free standing artificial membranes prepared from polybutadiene-*b*-polyethylene oxide (PB-*b*-PEO). The membranes were prepared from vesicles ruptured on porous silicon substrates. These polymeric membranes were studied by confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM). Force indentation curves were performed on the membranes and theoretical models were used to extract elasticity constants from the results. The study of polymeric membranes can give insight to the function of biological membranes, furthermore polymeric membranes can be used to create new hybrid systems by incorporating biological (lipids, proteins), artificial (polymers, dyes) and inorganic (nanoparticles) components.

P-164

Preparation of giant unilamellar vesicles containing lipopolysacharide under physiological conditions

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Giant unilamellar vesicles (GUVs) are very useful model membrane systems to study many aspects of lipid-lipid and lipid protein interactions, particularly employing fluorescence microscopy related techniques (Bagatolli 2006). The use of this model system can be particularly useful to study aspects related with the lateral structure of bacterial membranes using the aforementioned approach (i.e. fluorescence microscopy). Bacterial cells have a size close to the resolution limit of optical microscopy and details about the organization of their membranes are not easy to achieve using such technique. Recently a new electroformation method to prepare GUVs composed of compositionally complex mixtures under physiological conditions was developed in our laboratory (Montes 2007). In the present work we further extended this electroformation method to prepare GUVs composed of bacterial lipid extracts and lipopolysaccharide (LPS). In our experiments we used E.coli lipid extract to prepare small vesicles containing various LPS species, from smooth strains and rough mutants. SUVs were used as starting point to electroform GUVs using various types of buffers with high ionic strength. The successfully obtained bacterial-GUVs were used to study the interaction of these membranes with known LPS-binging proteins (lung surfactant protein D, SP-D) and peptides (polymyxin B). Our results remark the usefulness of this particular bilayer models to perform studies mimicking bacterial membranes.

Abstracts
– Imaging and spectroscopy –

P-165

Cholesterol-rich domains and apoptosis studied by new fluorescent membrane probes

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Herein, we have developed different membrane probes binding spontaneously to the outer plasma membrane leaflet and showing sensitivity to membrane properties such as surface charge and phase state. The first two types of probes were based on 3-hydroxyflavone fluorophore. One of them showed a high sensitivity to the surface charge and to the phase state of lipid bilayers, while the other was only sensitive to the surface charge. The third type, which was based on Nile Red fluorophore, was sensitive only to the phase state. Surprisingly, the probes that were sensitive only to the surface charge did not respond to apoptosis, while the other two types of probes showed significant spectroscopic response to it. Moreover, the latter exhibited response to cholesterol depletion, which was similar to that observed on apoptosis. Thus, according to our data, the intact living cells present a remarkable fraction of the cholesterol-rich domains, while apoptotic loss of the transmembrane asymmetry decreases it dramatically. These probes represent a new tool for quantification of surface charge and cholesterol-rich domains in cell membranes.

P-166

Rocking, tumbling, and sliding: Real-time nanomotion of a membrane-bound virus

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The interaction of a virus with its receptors in the plasma membrane is decisive for its infection of cells. Optical studies have revealed that after binding, virus particles move laterally on the membrane, but the complexity of the cellular environment and the drawbacks of fluorescence microscopy have prevented access to the molecular dynamics of early virus-host couplings. Here, we examine a model system, in which single Simian viruses (SV40) interact with their GM1 ganglioside receptors in supported lipid bilayers. We employed scattering interferometry and single molecule fluorescence localization to visualize the vectorial rotational motion of virions. At low receptor concentration, we observed sliding and tumbling of single virions during rapid lateral diffusion. In contrast, at increased receptor concentration the virions repeatedly underwent periods of standstill, reminiscent of their behavior prior to endocytosis. By an unprecedented combination of millisecond time and nanometer spatial resolutions, we revealed that during these immobile periods, the virions rock back and forth among nanoscopic spots separated by 9 nm. Our insights, together with the structure of the viral capsid, suggest aggregation of receptors in nanodomains and recurrent swap of binding between receptor molecules and neighboring viral protein pentamers.

P-167

The wavelength dependence of the luminescence for different sized AuNP by 2-photon CLSM

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Gold nanoparticles (AuNP) with different sizes can exhibit original luminescent properties if excited with pulsed near-infrared laser light which makes them a suitable object to be detected in biological tissues. The main obstacle is to distinguish between the object of interest (emitted light) and the autofluorescence from the sample which limits the scope of application of AuNP. Therefore, our aim is to characterize the luminescent properties of such nanoparticles regarding their excitation and emission. In this study, the excitation and emission spectra of AuNP with different sizes below 50 nm in an excitation range of 720 nm to 900 nm were investigated.

Our study shows the emission spectra curves of AuNP are broadband spectrum and vary with the changing of excitation wavelength from 720 nm to 900 nm. The results also suggest the minimum laser power necessary to trap the AuNP depends on particle size and excitation light wavelength and a maximum power above which the particles are destroyed. In all the experiment above, to avoid the simultaneous effects from slide and cover slip is a must.

P-168

Fluorescence anisotropy and AFM used as tools to characterized porin´s reconstituion in LUV´s

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A major requirement to perform structural studies with membrane proteins is to define efficient reconstitution protocols that assure, not only, a high incorporation degree in preformed liposomes, but also a protein directionality and topology that mimics its in vivo conditions. For this kind of studies, protein reconstitution in membranes systems via a detergent-mediated pathway is usually successfully adopted, since detergents are generally used in the initial isolation and purification of membrane proteins. In this study we report the reconstitution of OmpF in preformed DMPC and E. coli liposomes using two different techniques for detergent removal: (1) exclusion chromatography and (2) incubation with detergent adsorbing beads. The incorporation degree was determined by bicinchoninic acid assay and fluorescence anisotropy was used to determine OmpF effect on the structural order of membrane lipids. These results show that protein insertion in membranes depends both on the technique used to remove detergent and on the lipids used to prepare the liposomes. Moreover more anisotropy and atomic force microscopy studies will allow a better characterization of bacterial model system membranes.

Abstracts
– Imaging and spectroscopy –

P-169

Insights on the mechanism of electron transfer in Complex I

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Complex I (NADH Dehydrogenase) plays a central role in cellular energy production, transferring two electrons from NADH through a series of iron-sulfur clusters (FeS) to ubiquinone (coenzyme Q); the electron transfer is coupled to the translocation of protons across the membrane. The FeS center N2 is the last acceptor in the electron-transfer chain, but the mechanism through which the enzyme couples the $1e^-$ reduction of the FeS centers to the $2e^-$ reduction of ubiquinone ($Q \rightarrow SQ \rightarrow QH_2$) is unclear [1]. In our experiments, submitochondrial particles were treated with different inhibitors, in the absence or in the presence of different quinone analogues, and NADH addition initiated the electron transfer. We assess by EPR (Electron Paramagnetic Resonance) spectroscopy the relative abundance of the reduced N2 center and of the semiquinone radical, and coupled EPR data to enzymatic activity assays and to fluorescence measurements on the effect of inhibitors on Reactive Oxygen Species (ROS) production. We identify two different classes of inhibitors showing different effects on ROS production. Moreover the redox state of the complex has shown to depend both on the inhibitors and on the quinone analogues. A possible mechanism of the electron transfer, that can explain the experimental findings, will be presented.

[1] U. Brandt , *Ann. Rev. of Biochem.* **2006**, 75, 69-92.

P-170

Patterning of bio-molecules: methods for characterization of neuron-substrate interfaces

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We investigated how to use and improve micro printing techniques to obtain molecules patterns on cell culture substrates. With micro contact printing (μCP), we generated geometrically defined depositions of Poly-D-Lysine (PDL) using poly-dimethylsiloxane stamps and optimized neuronal culturing conditions. Rat and mice hippocampal neurons grown on those patterns showed to be alive and functional.
We then applied μCP to study axonal development by combining cell culture assays with Atomic Force Microscopy (AFM) to investigate in more detail the molecule deposition on the surface and to measure morphological changes in the growth cone (GC) during the early phases of neurite development. We found distinct shapes of the GCs depending on whether they were growing on L1 adhesion molecule patterned by indirect-μCP or on PDL coated surfaces.
We also attempted to transfer such patterns on Multi Electrode Arrays in order to constrain neuronal cell bodies on the electrode area and to improve electrophysiological recordings from neuronal networks. Other patterning techniques were therefore explored using a nano-drop printing system. Patterned surfaces were analyzed with AFM and Scanning Electron Microscopy to combine different approaches aimed to the improvement and characterization of the printing techniques.

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Molecular detection via hybrid protein-silicon photonic systems

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Due to its porosity and unique optical properties porous silicon (PSi) is an attractive template to develop biomaterials and biosensors. Porous silicon microcavity (PSiMc) structures were prepared then functionalized for covalent protein attachment of glucose oxidase (GOX) or solubilized bacteriorhodopsin (BR). Functionalization and protein infiltration was monitored by specular reflectometry sensitive to change in refractive index, when a molecule is attached to the large internal surface of PSi. Protein infiltration into the porous scaffold was confirmed by EDX spectroscopy and the structures were imaged by biphoton microscopy. Second harmonic generation and enhanced two-photon excited fluorescence emission from porous silicon was observed when resonantly exciting the structures. In addition, when the microcavities were infiltrated with GOX or BR, the proteins acted as a very efficient internal two-photon-excited fluorescence emitter, hence protein infiltration enabled the in-depth visualization of the porous structure by taking advantage of the optical sectioning capacity inherent to the non linear optical microscopy technique.

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Effect of HLA-II on the distribution of HLA-I on the surface of lymphoid cells

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Receptor clustering and changes in their cell surface distribution have often pivotal role in the ligand receptor interactions. HLA I and II have a key role in the immune response since they display fragmented pieces of an antigen on the host cell's surface. Previous studies indicated a non-random distribution of HLA I molecules on the cell surface. In present study we analyzed the role of individual molecules on the pattern formation of HLA I.
We have established a stable transfected HLA II-deficient BLS-1 human B cell line expressing HLA-DQ6 and determined the expression levels of HLA-DQ6, HLA I, HLA II and ICAM 1 proteins. On HLA-DQ6 transfected cells expression of HLA I decreased. Homo- and heteroassociations of HLA I, HLA II and ICAM 1 molecules were assessed on EBV-transformed lymphoblast JY, BLS-1 human B and HLA-DQ6 transfected BLS-1 cell lines by flow and image cytometric FRET methods. No notable difference was found in the heteroassociation of ICAM 1 with HLA I and HLA II on JY and HLA-DQ6 transfected cells. However, HLA-DQ6 nucleofected BLS-1 showed significant decrease in energy transfer efficiency between HLA I and HLA II antigens (using HLA I as donor and HLA II as acceptor, and vice versa). Homoassociation of HLA I also decreased significantly on transfected BLS-1 cells. These findings indicate that HLA-II influences the distribution of other cell surface receptors.

Abstracts
– Imaging and spectroscopy –

O-173

Measurement of *in vivo* binding reactions with Fluorescence Correlation Spectroscopy (FCS)

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The interpretation of in-vivo binding rate measurements allows inferring the molecular interactions that regulate cellular functions. Fluorescence Recovery After Photobleaching (FRAP) is a widely used tool to quantify binding reactions in vivo. However the lack of "golden standards" for these measurements requires the measured binding rates to be validated with other techniques. We present a new Fluorescence Correlation Spectroscopy (FCS) method to measure the in vivo bound fractions and residence times for molecules that interact with an immobile substrate. We apply this method to measure binding of mutants of the transcription factor VBP (vitellogenin binding protein) to the DNA. Comparison of FCS with FRAP results in comparable estimates of the measured diffusion constants and bound fractions. However, FCS provides an estimate of the VBP residence time at the DNA, while FRAP does not. This limitation in the analysis of VBP is due to the larger photobleaching volume used in FRAP, if compared to the observation volume of an FCS experiment. In sum, we present a method to measure binding rates with FCS. Substantial agreement of this method with FRAP is shown. However, further validation on tightly bound molecules will be necessary to assess if FRAP and FCS agree in the measurement of residence times.

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A biophysical study of equine herpesvirus-1 entry into cells

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Equine herpesvirus-1 (EHV-1) can cause respiratory disease in young horses, varying degrees of paralysis and abortion during the later stages of pregnancy. Furthermore, there has been a recent increase in the number of outbreaks involving paralysis of Thoroughbred horses in training. Virus disseminates rapidly after initial infection via cell associated viraemia. Controlling this may prove crucial in combating the pathogenicity of the disease.
We have investigated the cellular interactions of EHV-1. Initially, binding of EHV-1 to fluorescein phosphatidylethanolamine (FPE)-labelled phospholipid vesicles of various compositions was examined. A variety of microscopy techniques were then employed to study the events surrounding the binding of virus to equine peripheral blood mononuclear cell (PBMC) membranes. Confocal microscopy images have highlighted possible colocalisation of EHV-1 with membrane 'rafts'. Total Internal Reflection Fluorescent microscopy was then used to identify viral binding at the membrane with high contrast images, able to observe single virus particles. Establishing the viral entry pathway into PBMCs would allow drugs that target this process to be employed, reducing clinical viraemia.

P-175

Characterization of Hepatitis B antigen particles by atomic force microscopy

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Each year, over one million people die from hepatitis B virus-related chronic liver disease, including cirrhosis and hepatocellular carcinoma. The major surface antigen of Hepatitis B virus (HBsAg) is a cysteine-rich, lipid-bound protein with 226 amino acids. Recombinant HBsAg produced in yeast can self-assemble into 22-nm immunogenic spherical particles that are used in licensed Hepatitis B vaccines (protein/lipid ratio is 60/40 in mass). HBsAg size and shape have been mainly investigated by transmission electron microscopy after negative staining of the particles. However, under these conditions, no details of the particle surface can be obtained because of the shadowing effect due to the uranium salts.
Here we describe new structural insights of HBsAg particles using Atomic Force Microscopy (AFM) performed under physiological conditions. We applied atomic force microscopy to define structural details of the surface organization with a resolution in the nanometer range. As expected, the diameter of HBsAg particles is 26,8 ± 2,5 nm in average. The surface of these particles clearly shows the presence of protuberances that most probably correspond to proteins. Indeed, reduction and alkylation induces the disappearance of the protuberances. The number of the protuberances estimated from AFM micrographs is about 70 per spherical particles.

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Fast beam scanning stimulated emission depletion (STED) microscopy with continuous wave lasers

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Conventional fluorescence microscopy suffers from a resolution limit imposed by the diffraction of light. Stimulated emission depletion (STED) microscopy overcomes this resolution barrier without being limited by the wavelength by taking advantage of the photophysics of the observed sample into the image formation process, and has proven to be a powerful approach for exploring relevant biological issues.
The outstanding resolution of STED microscopy is achieved by drastically minimizing the spatial extent of the focal region from which fluorescent molecules can emit signal in the sample. So far, mainly complex and relatively expensive lasers systems providing pulsed beams have been used to inhibit the fluorescence in the outer area of the focal region. Here we report on the development of a new setup based on a fast beam scanning confocal microscope using compact turn-key and inexpensive continuous wave lasers. The great potential of this simple configuration is demonstrated for a selection of commonly used fluorescent markers.

Abstracts
– Imaging and spectroscopy –

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Transport Mode of Photoactive Drug Hypericin into Cells Influences the Mode of Cell Death

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Correlation between sub-cellular distribution of photoactive drug Hypericin (Hyp), determined by applied delivery systems (Hyp *vs* Hyp/LDL), and mode of the cell death is addressed in this study. Co-localization of Hyp with mitochondria, lysosomes and Golgi apparatus in U-87 MG glioma cells was determined by confocal laser scanning microscopy in using organelle specific fluorescent dyes as well as by time resolved FRET experiments. Flow cytometry experiments were realized to study a photodynamic effect of Hyp (598 nm/4 Jcm^{-1}) on cells. Significant differences in the proportional representation of live, apoptotic and/or necrotic cells were observed for different types of delivery systems of Hyp 24 hours after Hyp ($5x10^{-7}$ M) photoactivation. Conclusions: i) Sub-cellular distribution of Hyp depends on using delivery systems, ii) the mode of cell death depends more on concentration of Hyp inside cells, than on different type of delivery systems (for non selective wide-field cell illumination), iii) fluorescence lifetime is sensitive parameter to study sub-cellular distribution of Hyp.
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P-178

The regulation of the formation of cytoskeletal protein complexes by actin-binding proteins

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In living cells various groups of proteins are associated to supramolecular actin filament structures, often in a nucleation factor dependent manner. For example, actin structures associated with formins can bind tropomyosin and profilin, while those polymerised by the nucleation of the Arp2/3 complex bind cofilin and myosin I. The molecular mechanisms underlying the regulation of the formation of these protein complexes is still ambiguous.
We have shown recently that formins can bind the actin filaments and change their conformational state. Subsequent binding of other actin-binding proteins, such as tropomyosin and myosin, can reverse these changes. It appears that the reversal effect assumes that the actin-binding protein binds the filaments in a well-defined and specific binding site.
The altered conformational state of the actin filaments observed after the binding of these proteins provides a possible explanation for the modified affinity of the filaments for other-actin binding proteins. Based on the results available so far we assume that the affinities are modified differently by different nucleation factors, and the conformational changes introduced to actin by actin nucleation factors can serve as the molecular bases for the regulation of the formation of actin based intracellular protein complexes. Experiments are currently in progress to test and further corroborate the existence of such regulatory mechanisms in living cells.

P-179

Dynamical behavior of ribose and deoxyribose supercooled water solutions

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Ribose and 2'-deoxyribose are probably the most widespread monosaccharides in nature. They can be extensively found in ribonucleic acid (RNA) and 2'-deoxyribonucleic acid (DNA), respectively, where they form, together with a nitrogenous base and a phosphate group, a peculiar building-block structure called nucleotide. In the present work, the relaxation dynamic of ribose and deoxy-ribose water solutions at different concentrations has been studied by broadband dielectric spectroscopy and differential scanning calorimetry in the temperature range of 150-250K. Two relaxation processes are observed for all the hydration levels; the slower (process I) is related to the relaxation of the whole solution whereas the faster one (process II) is associated with the reorientation of water molecules in the mixture. As for other polymeric water solutions, dielectric data for process II indicate the existence of a critical water concentration above which water mobility is less restricted. Moreover, according with these results, ATR-FTIR measurements of the same sugar solutions showed an increment in the intensity of the OH stratching sub-band close to 3200 cm^{-1} as water content increases.

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Mechanical properties of Zona Pellucida hardening

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We have investigated the changes in the mechanical properties of the zona pellucida (ZP), a multilayer glycoprotein coat that surrounds mammalian eggs, that occur after the maturation and fertilization process of the bovine oocyte by using atomic force spectroscopy. The response of the ZP to mechanical stress has been recovered according to a modified Hertz model.
Zp of immature oocyte's shows a pure elastic behaviour. Mature and fertilized oocyte's ZPs evidence, instead, a transition from a purely elastic behaviour, which occurs when low stress forces are applied, towards a plastic behaviour has been observed. The high critical force necessary to induce deformations, that well supports the non-covalent long interactions lifetimes of polymers, increase after the cortical reaction.
AFM images show that oocytes' ZP surface appear to be composed mainly of a dense, random meshwork of non-uniformly arranged fibril bundles More wrinkled surface characterize marure oocytes with respecto to immature and fertilized oocytes
From a mechanical point of view, the transition of the mature ZP membrane toward fertilized ZP, through the hardening process, consists in the recovery of the elasticity of the immature ZP, while maintaining a plastic transition that, however, occurs with a much higher force with respect to that required in mature ZP.

Abstracts
– Imaging and spectroscopy –

P-181

Vibrations of DNA with light and heavy counterions in low-frequency Raman spectra

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Stability of the DNA double helix is determined by Na^+ counterions, neutralizing the negatively charged phosphate groups of the macromolecule backbone. But under spatial conditions they may be replaced by much heavier ions, for example Cs^+. To determine the influence of heavy counterions on internal dynamics of the double helix the conformational vibrations of Na- and Cs-DNA are studied. For this purpose the model of conformational vibrations of DNA with counterions is used [Perepelytsya S.M., Volkov S.N., Eur.Phys.J.E. 24, 261, 2007]. As the result the frequencies and amplitudes of vibrations for *B*-DNA with Na^+ and Cs^+ counerions are calculated. The frequencies of internal modes of the double helix are about 110, 79, 58 and 15 cm^{-1}. The frequencies of ion-phosphate modes are about 180 and 110 cm^{-1} for Na- and Cs-DNA respectively. The calculated amplitudes of vibrations show that light counterions not disturb the DNA internal dynamics, but heavy counterions make move all structure elements of the double helix. Using the valence-optic approach the intensities of the DNA vibrations in Raman spectra are calculated. The calculations show that the ion-phosphate mode in Cs-DNA spectra is prominent, in contrast to Na-DNA spectra, where it has very low intensity. Obtained results describe the intensity increase of the band 100 cm^{-1} in Cs-DNA spectra that was observed in [Bulavin L.A., *et al.*, `arXiv:0805.0696v1`].

O-182

FRET microscopy: red fluorescent proteins as an acceptor

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FRET microscopy is widely used to study protein-protein interactions in living or fixed specimens. Currently, most commonly used visible fluorescent proteins for live-cell FRET studies are the Cerulean and Venus variants of the cyan and yellow fluorescent proteins. Even though this FRET pair appears to be ideal for monitoring protein-protein interactions, the most commonly used fixed laser wavelengths do not excite Cerulean at peak absorption. Recently, we characterized an ideal donor, the monomeric teal fluorescent protein (mTFP), which is excitable using the commonly available 457(8) nm argon laser line (Day et al., J. Biomed. Opt. June/July, 2008). We used Teal as a donor for various red fluorescent proteins as acceptors including tdTomato, mKO2, mOrange2, mTagRFP, mKate. We have employed a “FRET standard” genetic construct to minimize variability in separation distances and positioning of the fused donor and acceptor FPs. Using spectral FRET imaging and fluorescence lifetime measurements in living cells expressing the fused proteins, we have characterized both sensitized acceptor emission and the change in the donor lifetime distribution as a result of quenching for each of the fused FP pairings. Our results indicate that some red FPs are better acceptors than others in terms of quenching the Teal donor and sensitizing the emission of the acceptor indicating a FRET event.

P-183

Monitoring of oxygenation transition and protein ordering in red blood cells using Raman tweezers

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Optical tweezers are used to controllably apply forces to red blood cells and the resulting chemical and structural changes are monitored using Raman spectroscopy. The forces are applied *in vivo* and mimic that which the cell undergoes mechanically as it passes through vessels and smaller capillaries. The first result presented will be spectroscopic evidence of a transition between the oxygenation and deoxygenation states of hemoglobin that is caused by the stretching of the red blood cell. The transition is due to mechanically induced enhancements of hemoglobin-membrane and hemoglobin neighbor-neighbor interactions. The latter, lesser known effect is further studied by modeling the electrostatic binding of two of the protein structures using molecular dynamics methods.
Secondly, polarized Raman spectroscopy is utilized to study the packing and ordering of the hemoglobin proteins in the red blood cell as it is stretched. Depolarization ratios for a number of heme group modes change, indicating that the applied force additionally packs and orders the proteins inside the cell which further demonstrates the role of cell deformation in the oxygen transport kinetics.
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P-184

Site-specific interactions of a lytic peptide through surface-selective fluorescence spectroscopy

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Novel time-resolved spectroscopic methods have been used to investigate the interactions between a fluorescently-labelled mutant of the peptide melittin and supported lipid bilayers, formed by self-assembly at a silica-water interface via vesicle deposition. Time-resolved evanescent wave-induced fluorescence spectroscopy (TREWIFS) is a surface-selective technique in which the evanescent field from a pulsed laser source is used to photoexcite fluorescent species at an interface. The resulting fluorescence decay kinetics, measured using time-correlated single-photon counting, report on the micro-domains experienced by those fluorescent species at an interface. Extending TREWIFS to time-resolved evanescent wave–induced fluorescence anisotropy measurements (EW-TRAMS) provides dynamic rotational information of a fluorescent species, reporting on its mobility at an interface.
Presented here are TREWIFS and EW-TRAMS data obtained from the fluorescence of an Alexa 430-labelled melittin mutant interacting with a dipalmitoylphosphocholine bilayer at room temperature, physiological pH and ionic strength. The results provide new insights into the conformation, location and motion of cytolytic peptides interacting with cell membranes.

Abstracts
– Imaging and spectroscopy –

P-185

Annular pupil filter to improve spatial high-frequency signal to noise ratio in linear and non linear microscopy

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Shot-noise significantly affects and deteriorates the imaging capabilities of typical two-photon excitation and confocal laser scanning microscope, especially in biological applications where the detected signal can be remarkable slight[1]. In particular, shot-noise substantially influences the spatial high-frequency range inducing a remarkable reduction of the optical transfer bandwidth.
The insertion of an annular filter on the microscope objective lens in the illumination light pathway is here proposed to retrieve the high frequencies information loss[2].The electromagnetic interference effect induced by the filter insertion, gives a redistribution of the optical transfer function. In particular, the microscope frequency response in filter scheme exhibits an enhancement of signal to noise ratio at the high frequencies able to recover the high frequencies hampered by shot-noise[3].

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P-186

A modified law of light absorption is relevant for biological molecules

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Despite the vast application of Lambert-Beer law in biology, this empirical law cannot accurately describe the light absorption process of molecules with a long-lived excited state. This family of molecules includes most fluorescent molecules, which are very important in biology.
Lambert-Beer law is in fact only valid at very low intensity of incident light and very low concentration of chromophore. If the system doesn't meet these conditions, it falls into a nonlinear regime when it is affected by a phenomenon which we call ”dynamic photobleaching”: the depletion of chromophores from the first layers, due to their transition to the excited state, leads to a sub-exponential propagation of light in the medium [1]. This phenomenon leads to the necessity of a new formula for the light absorption dynamics which depends on the lifetime of the excited state of the chromophore. The predictions of the theory were successful in describing the absorption dynamics of azobenzene [2], but now they have been tested also on a biologically relevant molecule like chlorophyll. The results indicate that the absorbance is affected by the intensity of the incident light, and it is therefore a non reliable way of determining, for example, the concentration of the molecule.

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P-187

Resolving the quaternary structure of plague F1 capsular antigen

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Most Gram-negative pathogens express multi-subunit fibres on their surfaces that mediate host cell attachment, biofilm formation, invasion of host defenses and protection against phagocytosis. Here we have studied Capsular antigen fraction 1 (or Caf1), secreted through the conserved chaperone/usher pathway by the plague agent *Yersinia pestis* [1].
Caf1 is highly immunogenic and is used in a recombinant subunit vaccine against plague. Recent immunological studies indicated vaccines containing polymeric Caf1 have higher protective efficacy than vaccines containing its monomeric variant. This difference in protective efficacy was attributed to the quaternary structure. However the quaternary structure of Caf1 has not been characterized [2].
In our study we have used transmission electron microscopy of negatively stained specimen and linear dichroism spectroscopy to determine the quaternary structure of recombinant Caf1. Whereas electron microscopy revealed morphology of Caf1 fibres bound to the surface of a carbon coated grid, linear dichroism gave the orientation of subunits in flow aligned Caf1 fibres in solution. Our results show recombinant Caf1 comprises extended linear fibres and circularized fibres, both of which have high degree of conformational freedom.

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P-188

Coherent white light confocal fluorescence imaging and FLIM for FRET

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We report on the application of a simple white light source based on the supercontinuum generation from commercial photonic crystal fibres to confocal fluorescence microscopy and fluorescence lifetime imaging (FLIM) microscopy. The coherent white light can be tuned by varying the wavelength and intensity of the pump, a Ti:Sapphire laser. There are several advantages jn the use of SC sources: spatially coherent white radiation, tuning ranges of approximately 400 nm, high brightness, a robust compact system (potentially all-fibre) and relatively low cost. Being pulsed, SC sources are suitable for FLIM.
We have used this system for measuring Foerster Resonance Energy Transfer (FRET) in order to study interactions between ions channels and proteins of membrane within lives cells

Abstracts
– Imaging and spectroscopy –

O-189

Calyx of Held: STED nanoscopy of a glutamatergic terminal

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The calyx of Held, a large glutamatergic synaptic terminal in the auditory brainstem circuit has been increasingly employed to study presynaptic mechanisms of neurotransmission in the central nervous system. A highly detailed model of the morphology and distribution of cytoskeleton, synapsin, synaptic vesicles, calcium sensors, mitochondria, the presynaptic membrane and its active zones is derived by colocalization analysis of these different key elements of synaptic transmission in the rat brain. The various cellular components are visualized with subdiffraction resolution by stimulated emission depletion (STED) microscopy. Imaging individual structural elements exhibit a focal plane resolution of <50 nm inside 3 μm thick tissue sections.

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Differential polarization laser scanning microscopy. Anisotropy in biological samples

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Differential polarization (DP) spectroscopy provides unique information on the anisotropic organization of biological samples [Tinoco et al. 1987 Ann Rev Biophys Biophys Chem 16:319]. However, anisotropy is often a microscopic property. The DP-LSM, constructed in our laboratory enables us to image the main DP quantities: linear and circular dichroisms (LD&CD), also in fluorescence detection (FDLD&FDCD, confocal), linear and circular anisotropy of the emission (r&CPL, confocal), linear birefringence (LB), and the degree of polarization of fluorescence emission (P, confocal). DP images revealed: strong LB with large local variations in chloroplasts [Garab et al. 2005 Eur Biophys J 34:335]; strong anisotropy of F-actin filaments that depends on key proteins (FDLD&r) [Gorjánácz et al. 2006 J Str Biol 154:27]; siginificant variations in P of a lipid probe in lymphoid cell membranes, correlating with domain structure [Gombos et al. 2008 Cytometry 73A:220]; quantitative 3D data on the fiber-laminate structutures of plant cell walls (FDLD&r) [Steinbach et al. 2008 Cytometry 73A:202]. Further applications include: periodic structure of isolated amyloids, anisotropy variations in cell walls related to drought resistance, and strong anisotropy of 'artificial chlorosomes', nanorods of synthetic porphyrins. DP-LSM might thus represent a novel tool in the better understanding of highly organized molecular macroassemblies.

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Surface generated fluorescence detection by supercritical angle confocal microscopy

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A two channel confocal microscope for surface and simultaneously in solution fluorescence detection is presented. The microscope's core element is a parabolic mirror objective that collects the fluorescence above the critical angles for total internal reflection (TIRF) of the water/glass interface. An aspheric lens incorporated into the parabolic mirror is used for diffraction limited focusing and collecting the fluorescence at low angles with respect to the optical axis. This low angles excitation approach is technically straightforward and gives an advantage over high numerical objectives that require very high angles for TIRF illumination. By separating the collection of the fluorescence into supercritical and subcritical angles, two detection volumes highly differing in their axial resolution are generated at the interface. The surface selectivity of the detection volume is obtained on the basis of the dipole emission profile near a dielectric interface. Its angular distribution is highly anisotropic and consists of a superposition of traveling and evanescent waves, which both are detected using the parabolic mirror objective. Unlike with objective TIRF microscopy, the parabolic mirror objective achieves easily diffraction limited excitation/detection volume at the water/glass interface. The objective optical performance is shown by measuring the actin cytoskeleton of cultured cells, FRET energy transfer within adsorbed clustered proteins and single molecules detection.

P-192

Label-free high-throughput detection systems for systems biology approaches to disease diagnosis

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The goal of biomarker studies is to develop simple non-invasive tests that identify disease states. Focus is beginning to shift from identification of individual biomarkers to identification of biomarker panels comprising multiple targets of different molecular species. There are, however, no current technologies available that allow for a comprehensive and simultaneous analysis of the expression levels of multiple cellular components, i.e. proteins and RNA. Surface plasmon resonance (SPR) polaritons are surface electromagnetic waves that propagate in a direction parallel to the interface between a metal surface and an external medium e.g., liquid. These oscillations are very sensitive to any change of this boundary, a phenomenon that has been exploited to facilitate label-free, real-time detection of biological interactions, e.g. protein binding interactions. We are utilising the power of SPR to develop technologies that facilitate diagnostic procedures for complex diseases such as Alzheimer's Disease and Chronic Obstructive Pulmonary Disease, through identification and detection of patterns of biomolecules indicative of disease. Our approach will facilitate better disease characterisation, improve early detection strategies and aid drug discovery.

Abstracts
– Imaging and spectroscopy –

O-193

Blind image restoration in 4Pi microscopy with space-variant point-spread-function

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Fluorescence far-field microscopy is a indispensable tool in modern life science. However, the resolution of its standard variants is limited by diffraction to ∼200nm in the focal plane and ∼600nm along the optical axis. To overcome this limit, a new class of super-resolution microscopy techniques has been designed. One example is 4Pi microscopy. To obtain isotropic resolution, 4Pi uses interference of the wave fronts from two opposing lenses. The point spread function (PSF) of 4Pi system is characterized by multiple maxima/sidelobes, which replicate the object in the image. Therefore image restoration is mandatory to render unambiguous imaging. The situation is further complicated because the positions and the relative heights of the multiple maxima/sidelobes of the PSF depend on the phase difference (PD) between the two wave fronts at the focus. If the refractive index of the sample varies in space, this PD becomes a function of position and 4Pi image formation process looses its shift invariance. The PD function (PDF) may not even be known *a-priori* and must then be estimated from the image, leading to a blind image restoration problem. Here, we propose a maximum *a-posteriori* based method to solve the problem. We either assumed a mathematical model for the PDF that depends on a small number of parameters or allowed for an arbitrary PDF but introduced a smoothing constraint.

O-194

Confocal microscopy at high resolution and efficiency by image inversion interferometry

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Fluorescence confocal microscopy, an indispensable tool of modern biology, allows the imaging of live fluorescent specimen with high lateral as well as axial resolution. Through the introduction of a sufficiently small confocal pinhole, the lateral resolution can be enhanced compared to that of a wide field microscope. However, this gain in resolution comes at the cost of a decrease in detection efficiency, as light blocked by the pinhole is lost.
We present a method for improving the lateral resolution (extending work of Sandeau et al. [1]) and detection efficiency of scanning microscopes by adding an interferometer with image inversion in one of its arms to the detection pathway [2]. This surpasses the lateral resolution achievable in a conventional confocal microscope (closed pinhole) while increasing the detection efficiency substantially.
Point spread function measurements for a UZ-Interferometer (UZI) are shown. The light in this setup follows three-dimensional U- and Z-shaped paths and relies on reflections off planar surfaces only in order to achieve image inversion. We achieved an interference contrast of 91% for white light, and excellent agreement with theoretical predictions.
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P-195

Towards reconstitution of the interleukin-4 receptors in artificial membranes

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Interleukin-4 (IL-4), a small soluble protein, is a principal regulatory cytokine, playing an important role during the maturation and clonal expansion of antigen specific B-cells in mammals. At the plasma membrane, IL-4 is recognized by a receptor that consists of two single spanning transmembrane proteins: a high affinity IL-4R alpha chain, and a low affinity IL-2R gamma chain. It is still controversial by which molecular mechanism the signaling complex is fully activated: dimerization of chains, large conformational change upon IL-4 binding or a combination of both. Moreover, the influence of the lipid environment in which the activation takes place is poorly characterized.
To address these issues we aim to reconstitute the receptor component in artificial membrane systems to study the various mutual interactions by means of fluorescence-based techniques, mainly fluorescence correlation spectroscopy. Due to reduced background in a chemically defined system this may provide details not yet accessible in the living cell.

Abstracts
– Biomaterials and drug delivery –

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Use of lipoamino acids for nasal delivery of therapeutic proteins

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Most of the therapeutic proteins are clinically administered through an intravenous injection several times a day / week. Because the repeated injections are not convenient and cause pain in patients, an alternative route of administration is desirable such as oral or nasal. Unfortunately, proteins are easily degraded by proteolytic enzymes in the gastrointestinal tract and, therefore, have a low bioavailability when administered via oral route. Physica Pharma has gained experience in forming sprayable solutions combining lipoamino acids (LAA) and Small Therapeutic Organic Compounds with the aim to improve their intranasal absorption. In the present work we investigated the ability of LAA to complex large molecule such as Human Erythropoietin, Human growth hormone and salmon calcitonin in order to form easily sprayable colloids. These three proteins are used respectively to treat anaemia, growth problem and hypercalcemy. Circular dichroism and dynamic light scattering were used to further characterize the LAA-Protein colloids. A specific molar ratio of LAA versus protein was found for wich the proteins keep their secondary structure and have an overall isotropic size slightly increased. Molecular dynamics show that proteins are indeed coated with LAA. Such a complex is shown to pass very easily through a culture of nasal cells growth at confluence.

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Planar patch clamp for neuronal networks

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The technique of choice to measure the electrophysiological activity of neuronal cells is the so called patch-clamp method because of its precision and sensitivity; this procedure could play a major role also in the investigation of the behavior of a biological neuronal network and so represents an important tool for understanding its functionality and for screening the effects of drugs and compounds on it. The final aim of this project is the development of a planar patch clamp device suited to measure simultaneously the electrical activity of cultured neurons associated in a network. This device will be made of polymeric disposable material and will include a microfluidic perfusion system. In order to create a smooth micro sized pore in a thin polymeric membrane we exploited the prototyping capabilities of Focused Ion Beam etching and we extended the air moulding technique by combining it with soft moulding, obtaining micro structured substrates with the requested features. We also subjected our substrates to a chemical treatment capable of rendering its surface stable and hydrophilic and we verified that it makes them suitable for neuron culturing.

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Dynamics of interaction of hypericin with low-density lipoproteins

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Fluorescence spectroscopy and stopped-flow technique were utilized for the study of the kinetics of incorporation of hypericin (Hyp), a natural photosensitizing pigment, into low-density lipoproteins (LDL). Triphasic kinetics of Hyp association with LDL was observed when solutions of Hyp and LDL were mixed together. The most rapid phase of Hyp incorporation is completed within tens of msec, while the slowest one lasts 10-20 min. The most of Hyp molecules are incorporated into LDL in the slowest phase. The kinetics of the incorporation of Hyp into LDL particles pre-loaded with Hyp were also investigated. The observed decrease of the lifetime and total intensity of Hyp fluorescence with the increase of the incubation time of Hyp with Hyp/LDL complex is a sign of the formation of aggregates and the dynamic quenching of singlet excitation state of Hyp inside LDL. To study the kinetics of a transfer of Hyp molecules between LDL particles, the time evolution of the stopped-flow and time-resolved fluorescence experiments were investigated after the mixing of the complex Hyp/LDL=200:1 with appropriate amounts of free LDL. For each final Hyp/LDL ratio the increase of the lifetime and total intensity of Hyp fluorescence was observed. The half-time of this process is similar to that one of the slowest phase of Hyp incorporation into free LDL.

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Efficient escape from endosomes determines the superior efficiency of multicomponent lipoplexes

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Designer multicomponent lipoplexes have recently emerged as especially promising transfection candidates, since they are from 10 to 100 times more efficient than binary complexes usually employed for gene delivery purposes [1-3]. Here, we show, for the first time, that after internalization binary complexes of lower transfection potency remain in compact perinuclear endosomes, while multicomponent systems have intrinsic endosomal rupture properties that allow plasmid DNA to escape from endosomes with extremely high efficiency [4]. Endosomal rupture results in an extraordinarily homogeneous distribution of unbound plasmid DNA throughout the cytoplasm and in the nucleus [4].

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Abstracts
– Biomaterials and drug delivery –

P-200

Cardiotoxic effects of pentavalent antimony in guinea-pig

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Pentavalent antimony (PA) (Glucantime, Sanofi-Aventis or Glaxo) are the mainstream agents of choice for Leishmaniasis treatment. In therapeutic doses the PA treatment has cardiac side effects like electrocardiographic (ECG) alterations, that include QT segment prolongation, T wave flattening or inversion, inversion of ST segment, P, R and T waves amplitude reductions, torsade de pointes arrhythmias and sudden death by cardiac arrest. The objective of this study was to characterize the arrhythmogenic potential of PA. We used 30 guinea-pig to assess the chronic effects of PA therapeutic dose on corrected QT interval, QT dispersion, ventricular action potential (AP) amplitude and duration at 30% and 90% of maximal repolarization and survival rate. Guinea-pig received daily 30mg/kg PA or saline for 15 days. Eight lead ECG were recorded before and in the last treatment day. At the end the animals were killed and the left ventricle papillary muscles excised for AP recording with the intracellular microelectrode technique. Our results of chronic PA treatment showed significant increase of QRS complex duration, QT interval duration, QT dispersion and incidence of T wave flattening or inversion and arrhythmias. The AP analysis demonstrated prolongation at 90% duration. The treatment was lethal in 30% of the animals. We concluded that PA is a proarrhythmic drug that upon chronic use may causes arrhythmias and mortality by disturbances in the ventricular repolarization process.

O-201

Nanomechanics of Rubella virus shells: How soft viruses are?

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Rather than being just pathogens, from the view point of biotechnology and materials science viruses can be regarded as nanocontainers in which nucleic acids have been enclosed in a protective self assembled protein cage. In some viruses an additional lipid-protein envelope wraps the capsid shell. The capsid is constituted of several copies of one single protein subunit or just a few, arranged in a regular fashion showing icosahedral symmetry. The mechanical properties of the cage are important for understanding the infection mechanism involving the release of the encaged genome. By means of Atomic Force Microscopy, it becomes possible to probe such material properties by nanoindentations of single viral particles. It is very interesting to learn how strong or brittle a virus can be. Here we have studied the mechanical properties of empty Rubella virus particles (RLPs) due response of external applied forces. We found that RLPs are extremely soft comparable to that of some rubber materials. A peculiarity of the rubella virus is that the capsid is considerably smaller than the surrounding shell filling only a fraction of the lumen provided by the envelope. The envelope in Rubella has a distinguishable response on the material properties of the virus. Deformation and fracture of the capsid requires comparatively larger forces. Our results indicate that the pH is a major factor influencing on rubella particle material properties. This can be related to the infection mechanism.

P-202

Multi-scale estimation of water soluble diffusivity in polysaccharide gels

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Diffusion properties in gels play important role in food and biotechnological applications. An attractive goal is to design gels in such a way that the active molecules are delivered by the material according to specific release sequences. The transport of macromolecules within polymeric gels depends: on the obstruction effects of the surrounding gel strands, on the molecular interactions between the gel and the solute, on the interactions between the solute molecules themselves and the interactions between the solute and the solvent. Physical polysaccharide gels were evaluated in this work to probe diffusion over both microscopic and macroscopic distance scales. Physical gels from agar and starch were investigated by high and low resolution NMR techniques in order to characterize their structures. Obstruction effects of the surrounding gel strands were considered by studying diffusion of glucose. Local diffusion, due to Brownian motion, was quantified by low resolution NMR spectroscopy. The Fickian diffusion coefficient was measured by modelling experimental concentration-distance curves obtained by means of a two-compartment diffusion-cell. Diffusion coefficients depend on the viscoelastic properties of the gel matrix and on water-polysaccharide interactions.

O-203

Soft matter for biomedicine: two case studies

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In this study we describe two systems based on soft matter designed for the drug delivery and for the replacement of synovial fluid in osteoarticular pathologies.
(i) a new class of temperature sensitive hydrogels PVA/Poly(MA_mNiPAAm$_n$) shaped as microspheres obtained with a water-in-water emulsion photocrosslinking reaction. Microparticles of PVA/Poly(MA_mNiPAAm$_n$) with m:n theoretical molar ratios equal to 1:0; 1:4, 1:8 have been studied in terms of average size and responsiveness to temperature characterized by confocal laser scanning microscopy (CLSM), dynamic light scattering (DLS) and differential scanning microcalorimetry (DSC).
(ii) a physical network based on hyaluronic acid with a small extent (degree of substitution: 1%) of hydrophobic moiety grafted on the backbone, HYADD4, has been characterized in order to account for the influence of thermal treatment on the stability of the hydrogel. Dynamic light scattering (DLS) and small angle neutron scattering (SANS) have been used for dynamic-structural characterization of HYADD4 hydrogels. Diffusion of macromolecular probes has been studied by fluorescence recovery after photobleaching (FRAP) to study the mesoscopic texture of the hydrogel and molecular dynamic (MD) simulations were used to approach the time evolution of the physical junction points and of chains clusters.

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Pyropheophorbide-a-methyl ester: DMPC liposome vectorization and biophysical properties for PDT

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PPME is a second generation photosensitizer (PS), and a promising candidate for photodynamic therapy (PDT) treatment. We have previously demonstrated that PPME can be easily and efficiently encapsulated in DMPC liposomes, used as PS-vector. We therefore compared the photophysical and photochemical properties of free and encapsulated PPME incubated with human carcinoma cells. Absorption and fluorescence microspectroscopy as well as FLIM analysis allow evaluating the aggregation state of PPME inside the different cellular organelles and the extracellular medium. Confocal microscopy established undoubtedly the colocalization of PPME, by robust probabilistic exclusion method, within mitochondrion (central siege of apoptosis). After PS activation (4h and 24h), the balance apoptosis-necrosis was double-estimated by FACS device and fluorescence confocal microscopy. Quantification of hydroxyl radical was purchased by spin trapping-ESR spectroscopy and quenching technique. All these techniques required peculiar settings because of the fluorescence activity of PPME. These results allow to ascertain that the vectorization of PPME affords a better cellular penetration and a monomeric state of PPME. The presence of PPME inside mitochondrion orientated the cellular death in apoptosis 4h following PS-activation. But necrosis is the major actor 24h after treatment.

P-205

Towards a gene-delivering engine: Integration of viral (phi29) portal proteins in lipid membranes

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We consider the integration of the portal protein of the bacteriophage virus ϕ29 into lipid bilayers of giant unilamellar vesicles (GUVs) membranes with the aim of constructing a functional cargo-device able to transport DNA and later translocate it outwards. Our nano-engineering plan consists of growing GUVs from bilayer membranes built up from proteoliposomes previously prepared by extrusion. We have designed two alternative chemical routes for integrating the portal protein in the lipid bilayer, the first considering the native protein and the second a mutant modified with a hydrophobic belt made of histidine-tags. In our contribution we will present details on the different nano-engineering strategies and experimental evidence about the integration of the portal protein in the membrane with an orientation adequate to allow for functional DNA translocation.

O-206

Protein-based “epoxy-like” physical hydrogels for stem cell transplantation

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Stem cell transplantation has emerged as a promising therapy for multiple injuries and diseases; however, cell survival after transplantation is often poor and unpredictable. We hypothesize that co-injection of stem cells encapsulated within an optimized physical hydrogel will enhance viability. Whereas, current physical hydrogels require a shift in environmental conditions (e.g., pH, temperature) to initiate the sol-gel phase transition during encapsulation, our newly designed molecular-recognition gels do not require environmental triggers. Instead, these “physical epoxy-like” gels consist of two components that undergo a sol-gel transition upon mixing due to specific hydrogen bonding. The gel viscoelasticity is predictably tuned through precise variation in the molecular-level design of the two components, created using recombinant protein techniques. The design of the two components is based on simple polymer physics considerations and utilizes bio-mimimcry. Adult neural stem cells or mesenchymal stem cells are encapsulated within these gels with high viability at constant physiological conditions. The gels promote the growth and differentiation of neural progenitors into neuronal phenotypes, which adopt a 3D-branched morphology. The gels are further optimized for use in the central nervous system by tethering neuroprotective peptides to the gel through molecular-recognition sites. These peptides are released-on-demand by cells through the action of proteolytic enzymes.

P-207

Biophysical investigation of PLGA nanoparticles and their interaction with DNA

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We have studied the thermodynamical properties of chitosan-coated nanoparticles (cNP) and non-coated nanoparticles (NP) and have gained some insights about PLGA nanoparticles’ properties using supersensitive differential microcalorimetry. The experiments show that in a wide pH interval the changes in transition temperature did not take place. It was shown that such nanoparticles could be used in acidic surrounding for drug transfer. Stability and their other properties are less depended on either the particles were in bidistilled or deionized water, or the suspension of particles were located in buffer.
To determine the interaction of PLGA nanoparticles with DNA. In the case of DNA presence in cNP solution the calorimetric experiments show that the heat absorption peak is constricted, what biophysically means that interaction between them takes place. For more exact determination the contribution of cNP in spectrum, we have compared the spectra of pure DNA with the spectrum of the same concentration DNA plus cNP. The optimal ratio for DNA loading onto the cNP was found to be 7:1.

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P-208

Study of magnetic PLGA nanospheres containing anticancer drug Paclitaxel

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The aim of this study was to develop biodegradable and biocompatible Paclitaxel loaded magnetic PLGA nanospheres (NPs) suitable for biomedical applications. Biodegradable poly(D,L-lactic-*co*-glycolic acid) (PLGA) was used as a capsulation material and the magnetic fluid was used as a magnetic carrier. Incorporation of magnetic particles and drug in the PLGA polymer matrix was confirmed by infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). The release of the drug from the prepared NPs to the surroundings under the different conditions was studied also.
The prepared magnetic PLGA NPs with encapsulated Paclitaxel (spherical shape, size 200 - 250 nm) have good stability in the presence of high NaCl concentration at 25°C, the toxicity of prepared samples declared 3 times higher value of lethal dose LD_{50} in comparison with pure Paclitaxel (LD_{50} = 33mg/kg) and showed the significant response to external magnetic field which is useful from the point of view to achieve pharmaceutically acceptable drug delivery systems for tumour treatment.

O-209

Selective drug delivery and novel drug approaches by polyelectrolytes

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The use of polyelectrolytes in the past was mainly related to targeted drug delivery and nanoparticle preparations for medical applications. But rarely the polyelectrolytes were investigated for the own features as a drug.
In our present work we use a special physical feature of cancer cell membranes as a target for a specific polycation. We found that the polycation is selectively up-taken by cancer cells (leukemia, hepatocarcinoma, cancer stem cells) while normal cells remain unaffected.
Another interesting application of polyelectrolytes are in form of a multilayer coating on the surface of nanogold particles. For this topic, two new approaches were developed for a drug delivery through the blood brain barrier. In the first case, high amounts of creatine were bound to the gold particles and delivered as protective agents for ischemic stroke (Viota et al., J Colloid Interface Sci. 2009, 332(1):215-23). In the second case coated multifunctional gold particles were prepared as a drug for neurodegenerative disease on the basis of protein aggregates. The use of gold as core for coated nanoparticles offers the possibility to study our systems as "theranostics", system which are modified to recognize selectively diseased cells and carry the moieties to treat or destroy the malfunctioning cells. Possible treatments can be local hyperthermal therapy by using the particles as amplifier and enhancer or photodynamic therapy with gold as a drug.

P-210

Glucose scavenging activities of PAMAM dendrimers

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Glycation is a spontaneous, non-enzymatic modification of biomacromolecules with hexoses, mainly glucose. In terms of its pathophysiological relevance, it targets predominantly proteins, but also nucleotides and phospholipids, and is of major importance to both physiology (ageing) and pathology (diseases with a metabolic background, like diabetes).
Earlier we demonstrated *in vivo* that the administration of PAMAM G4 to diabetic rats resulted in a significantly reduced blood glucose, as well as the early (HbA_{1c}) and late (AGEs) protein glycation products. In this study we investigated the ability of PAMAM G2 (16 surface NH_2 groups) and G4 (64 NH_2) to inhibit glycation of proteins in plasma, and a model protein - BSA. PAMAM G2 and G4 competed chemically with protein NH_2 for the binding of glucose, and hampered protein glycation. In a high-glucose medium they underwent an excessive glycation themselves. This modification was more effective in PAMAM G2, in which surface NH_2 were more mobile and accessible. PAMAM modification with glucose rendered these dendrimers less polycationic in aqueous solutions. PAMAMs neither affected BSA conformation nor formed stable complexes with a protein. We conclude that PAMAMs are very effective glucose scavengers. Thus, even less toxic PAMAMs of lower generations, like G2, may appear useful in further medical applications as the agents attenuating the detrimental effects of sever hyperglycaemia on biomacromolecules.

P-211

Active substrates to study mechanotransduction

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Cellular processes imply an important coordination of interactions with the extracellular medium. Accumulating evidences demonstrate that cell functions can be modulated by physical factors such as the mechanical forces acting on the cells and the ExtraCellular matrix, as well as the topography or rigidity of the matrix. These extracellular signals can be sensed by mechanosensors on the cell surface or in the cell interior to induce various cell responses. We have developed an original approach based on micro-fabricated substrates of PolyDimethylSiloxane (PDMS) to study cell migration. We used a closely spaced array of flexible micropillars (diameter∼1?m) to map the forces exerted by cells on their substrates. In this case, the micropillars act as passive force sensors. Here, we propose to analyze the cell response to an external applied stress by a well-controlled actuation of the substrate. To do so, we used magnetic pillars. Such substrates allow us to modify dynamic adhesion conditions of cells and to better understand the coupling phenomena between mechanical sensing and biochemical activity of a living cell. Using polyacrylamide hydrogels doped with ferromagnetic iron oxide particles or ferrofluids, we can make magnetic pillars with diameters of 5 to 10 microns while a magnetic field can be locally applied with a magnetic needle. Such a technique will be helpful to study the mechanical response of cells to an external force or to local changes in their microenvironment.

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Gene delivery with chitosan: influence of chain length on intracellular trafficking and dissociation

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Chitosan, a cationic polysaccharide presenting low cytotoxicity, is a promising nonviral gene delivery vector. Chitosan complexes DNA into nanoparticles, and the complexation and cell transfection efficacy are function of the chitosan/DNA ratio and of the intrinsic properties of chitosan, i.e. degree of polymerization DPn, charge density, and molecular structure. Nanoparticles formed by shorter chitosan (DPn31-41) mediated higher transgene expression than nanoparticles based on longer chitosans. The purpose of this work is to relate the DPn of linear chitosan with the cellular uptake, intracellular trafficking and dissociation of chitosan-pDNA complexes, measured by confocal laser scanning microscopy and fluorescence correlation spectroscopy (FCS).
Cells were incubated with oligomers (DPn 31 and 88) either free or complexed with plasmid-DNA. Both chitosan oligomers seem to penetrate cell nucleus in association with or free from pDNA. 24 hours after incubation, accumulation of oligomers in cell nucleus is similar for free and complexed DPn31, whereas it increases for complexed DPn88 compared to free, indicating a possible delayed dissociation of the complexes based on DPn88 in the nucleus and suggesting a dissociation of pDNA-DPn31 in cytoplasm. These data are consistent with previous studies which suggested that longer chitosan chains led to tighter complexes, and hence to a delayed dissociation process and lower transfection efficiencies compared to shorter chitosans.

P-213

Growth enhancement and adhesion control of PC12 on micropatterned ns-TiO_2 thin film

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Cluster assembled nanostructured titanium dioxide (ns-TiO_2) has been explored as novel substrate for in vitro cell culture. In this work, we report micropatterned ns-TiO_2 thin film as putative microdevice for neuron culture and growth. In additions, we show a simple scheme of molecular patterning of Bovine Serum Albumin (BSA) as cell anti-adherent substrate complementary to ns-TiO_2micropattern which favors a selective spatially confined adhesion of neurons. BSA was drop coated and physisorbed over glass coverslip covered with a thin conducting layer of indium tin oxide (ITO) and then PMMA was spin coated over it using standard protocols. Later, using combinations of e-beam lithography and pulsed microplasma cluster source (PMCS), a thin layer of ns-TiO_2 was deposited over micropatterned PMMA. Further, lift off process enabled us to generate complementary micropatterns of hydrophobic BSA (cell repellent) and hydrophilic ns-TiO_2 (cell adhesive). Cell culture studies have confirmed that PC12 cells like to grow on ns-TiO_2 substrate and not on BSA layer. The technique offers a novel approach for neuronal cell assay applications.

P-214

Effect of nanopatterned substrate on neuronal growth cones activity

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In the last20years an increasing interest has been address to explore, at the level of the single cell, the physical interaction between neurons and micro-and nano-patterned surfaces which mimic the biological environment and can induce specific biological behavior. A consistent number of substrates have been tested (nano-grooves, pillars, GaP nanowires) but the effect of nano-topographical features at subcellular level e.g. branching and pathfinding of growth cones(GC)is still unexplored. Using nanoimprinting lithography we fabricated gratings on glass with grooves of variable pitch, depth and width all in the nm range. Embryonic stem cell derived neurons were seeded on nanostructured and on flat control glasses. We investigate GC morphology with nm resolution by AFM. A significant effect on sub-cellular architecture was observed.On nanopatterned substrates72% of GC were branched with a large number of *long and thin* filopodia(average length and height4.3μm and 75nm)while on control only34%of GC were branched with an higher percentage of *long and thick* filopodia (average length and height 6,5μm and 300nm). On the contrary we did not observe a significant influence of the nanopatterning on the alignment and elongation of neurites. In both cases the distribution of angles between axon and filopodia showed a preferential direction at30°. In conclusion, the tested nanopatterns do not influence the neurite directions but do enhance the GC morphology and explorative activities.

P-215

Biophysical study of Polyethylenimine-DNA complex used in DNA transfection

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Success of human gene therapy depends upon the development of delivery vehicles or vectors, which can selectively deliver therapeutic genes to target cells with efficiency and safety. Many cationic polymers have been used to condense DNA by electrostatic interaction into small particles (Polyplex), for protecting the DNA from degradation and enhancing uptake via endocytosis. Polyethylenimine (PEI) appears to be one of the most advanced delivery system that can condense DNA efficiently forming PEI-DNA Polyplex complex. The physicochemical properties of different molecular weights of PEI, such as condensation ability, buffer capacity, time kinetics, FTIR and surface charges of the PEI-DNA complexes may be important factors to obtain a higher transfection efficiency of the polycation vectors. Our intent in this study was to characterize PEI-DNA complexes to see whether these physicochemical properties have any influence on their disposition characteristics and cellular uptake process. We found that PEI-DNA complexes, obtained by the 25 k PEI at N/P ratio > 4, were more stable in the presence of tissue culture medium & serum, and did not dissociate in Nacl easily.
Key words: Polyethylenimine, Polyplex, Polycations, DNA, transfection, gene delivery.

Abstracts
– Biomaterials and drug delivery –

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Physical characterization of nanocarriers for drug delivery

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Nanoparticles used as nanocarriers for pharmaceuticals can improve solubility and bioavailability of problematic drugs and protect labile or toxic molecules. Fundamental parameters like drug encapsulation efficiency and release kinetics are tuned by the physico-chemical features of the drug/carrier complex. A challenging aspect of the pharmaceutically-oriented issues resides in the relatively low number of molecules allowed to build up nanosystems with different properties and morphology, according to the specific drug and to the intended therapy. We have studied: 1) soybean-lecithine/chitosan nanoparticles for Progesterone and Tamoxifen delivery; 2) solid-lipid nanoparticles (Compritol 888 ATO, Poloxamer 188, Tween 80 and chitosan) for Cyclosporine-A delivery via ophthalmic formulation. Both void carriers and drug-loaded nanoparticles have been studied, to understand how the structure of the carriers can be modified by the active molecules. Several non-invasive physical techniques have been used to achieve a detailed knowledge of the systems: a) dynamic light scattering for the dimensional distribution of the nanocarriers, b) zeta potential to determine surface charge, c) cryo-Tem for morphological analysis d) X-ray scattering (in small angle configuration) and DSC to access information on the inner structure.
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Chitosan-arabic gum nanoparticles as potential vehicles for peptide delivery

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Chitosan (CS) – arabic gum (GA) nanoparticles were produced by an ion-ion interaction process, using different weight ratios of polysaccharides. According to zeta potential (ZP) measurements, particles are ionically stable. ZP values ranged from 30 to 70 mV for CS:GA ratios of 1:3.3 and 1:0.8, respectively. CS:GA 1:5 yielded uncharged nanoparticles and aggregates, showing that, at this ratio, the negative charges of GA neutralize the positive charges of CS. The particle diameters ranged from 300 to 500 nm, as measured by Dynamic Light Scattering (DLS), and displayed a slight tendency for decreasing as the GA concentration increased. Transmission Electron Microscopy (TEM) images showed numerous spherical nanoparticles. Peptides were encapsulated within the nanoparticles, by mixing them with chitosan solution prior to adding GA. This system can protect short peptides from rapid metabolization, prolonging their blood half-life.

P-218

Nanoporous alumina fabricated using unconventional acids for enhanced biomolecular physisorption

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In the last decades the available porecell sizes of porous alumina have been extended, but operating conditions to obtain some pore diameters still have to be optimized, particularly below the 50 nm limit. In this range the use of biocompatible porous alumina thin films could find applications in biosensors, after functionalization by appropriate physisorbed biomolecules. While pore ordering and film growth rate are mainly influenced by the electrical parameters of voltage and current, respectively, the typical pore size primarily depends on the electrolyte. In the search for alternative anodization conditions, we have investigated several acids that have never been used before, namely gallic, lactic, propionic, and glycolic acid. The anodizations were carried out in galvanostatic conditions at fixed concentration and durations, by varying the current. Atomic force microscopy was used to test the oxidized surface morphology. In particular, lactic and propionic acids demonstrated feasible. Lactic acid gave best results for $\sim$30 mA/cm^2 current density, corresponding to roughly constant $\sim$15 V potential, with resulting pore diameter in the 4060 nm range, whereas propionic acid performed best for $\sim$5 mA/cm^2, corresponding to $\sim$43 V, resulting in 7090 nm pore diameter. Both kind of films looked lightgray, different from the yellowish oxalic acid porous alumina, which can be a hint of lower ion contamination.

P-219

Material surface properties greatly influence DNA purification and PCR yield in microsystems

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Modern microchip platforms integrate DNA purification, target amplification by PCR and DNA detection in a single device. Combination of these processes minimizes sample loss and contamination problems as well as reduces analysis time and costs. Different strategies are available to perform DNA extraction on a chip. Here we exploited amino-coated materials as a tool for specific binding of DNA through the electrostatic interaction between amine groups and nucleic acids. We analyzed the ability of different treated substrates to selectively absorb/desorb the genomic DNA with the aim to purify DNA from unwanted components. Amino-coated substrates were characterized by AFM, XPS, fluorescence microscopy and absorption spectroscopy to define the surface chemical and morphological properties. The distribution of the DNA adsorbed on materials was homogeneous and the eluted DNA was tested for PCR. Same materials were analyzed for their compatibility with PCR and the use of different enzymes and reagents or proper surface treatments was employed. We established the best conditions for DNA amplification in silicon/pyrex microdevices depending on the type and the fabrication method used and on the quality of reagents more than on the passivation treatment or increment in standard Taq polymerase concentration.

Abstracts
– Biomaterials and drug delivery –

O-220

A new brain-targeted analgesic peptide: from biophysics to in vivo proof-of-concept

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Kyotorphin (KTP) was found in 1979 in bovine brain. Despite revealing remarkable analgesic properties, analgesia was only induced after central delivery. This limited ability to cross the blood-brain barrier (BBB) combined with the unknown mechanism of action largely confines its pharmacological use. To surpass these problems, we designed new KTP derivatives: KTP-RC and KTP-RC-Lipogen. Biophysical studies were carried out using fluorescence methodologies to characterize the peptides' interaction with biomembrane model systems. Partition coefficient quantification showed a clear preference of the derivatives towards fluid zwitterionic and anionic membranes. Moreover, a relationship between anionic lipid percentage and in-depth insertion in membrane was established. Additionally, studies with fluorescent probe di-8-ANEPPS revealed different membrane-interaction profiles of morphine and KTP-derivatives, suggesting distinct actions between them. The analgesic efficacy of the compounds was studied in vivo after systemic administration in models of acute and tonic pain. Unlike KTP, both KTP-RC and KTP-RC-Lipogen displayed high efficacy from doses as low as 1.67 and 0.85 mg/100g, respectively, indicating BBB crossing. The observed correlation between higher partition/insertion in the membrane and enhanced analgesic action proved the biophysical rationale to be a powerful strategy for early screening in CNS drug development.

P-221

Polysaccharide nanoparticles for antioxidant and peptide delivery

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The work comprises the design of polysaccharide based nanoparticles for drug delivery. Chitosan/arabic gum and arabic gum/maltodextrin nanoparticles were prepared by polyelectrolyte complexation and spray drying [1], respectively. Dynamic light scattering characterization established that arabic gum/maltodextrin nanoparticles are more polydisperse (diameters ranging from 8 to 400 nm) than chitosan/arabic gum particles (diameters of approximately 500 nm). Scanning electron microscopy measurements demonstrated that the particles are spherical and have a smooth surface. The systems are highly stable to forces up to 80 nN as observed by atomic force microscopy. Due to their nature they are hydrophilic and biodegradable. The nanoparticles were used to entrap short peptide sequences and antioxidants and were proved to be efficient in maintaining the biological activity of the molecules.

[1] I. Ferreira, S. Rocha and M. Coelho, Encapsulation of antioxidants by spray-drying, *Eng. Trans.* **11** (2007), pp. 713–717.

P-222

Effect of powder air polishing on nanocomposite dental materials measured by atomic force microscopy

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In dentistry, airpolishing with glycine and bicarbonate powders is widely used to remove accumulated plaque. However, the resulting teeth and gingival surface roughness, which is a risk factor for further plaque accumulation, has to be considered. In this in vitro study the effect of the above mentioned technique on the surface of a novel nanocomposite dental material is evaluated by means of Atomic Force Microscopy (AFM). Square specimens (0.5×0.5 cm^2 size) were airpolished either with glycine or bicarbonate, using different application times (2, 8, 30 sec) and distances (2, 4, 6 mm). Four specimens were evaluated for each timedistance combination, and checked versus untreated specimens (controls). The specimens were imaged with Tappingmode AFM at two different scan sizes (3×3 and 30×30 μm^2) in two different regions for each sample. The surface roughness was measured as the RMS of the feature heights. For the 2 sec2 mm group airpolishing resulted in an increased surface roughness as compared to the controls; however, glycine was associated with a lower roughness than bicarbonate (glycine: 238±91 nm; bicarbonate: 436±75 nm; controls: 14±14 nm; $p<0.0001$ for treated specimens vs controls, $p<0.001$ for glycine vs bicarbonate, ANOVA test). Similar results were obtained for all the other timedistance combinations.

P-223

Protein adsorption on PEG brushes studied by neutron reflectometry

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Protein adsorption on surfaces is responsible of several unwanted effects in technological and pharmaceutical applications: fouling of contact lenses, clotting in blood containing devices, inflammation around artificial organs for instance. Those phenomena can be repressed with certain types of polymer brushes, in particular PEG (polyethylene glycol) brushes, and a better understanding of the mechanism of adsorption could lead to improvements in the design of biomaterials. We have developed a theory describing this mechanism and carried out measurements to confirm the theoretical predictions [*Langmuir* 2007, 23, 10603]. Three cases are predicted: primary adsorption at the grafting surface, secondary adsorption at the outer edge of the brush, and ternary adsorption within the brush itself. We prepared samples with different grafting densities and different degrees of polymerization and used different protein size and concentrations. Neutron reflectivity experiments, able to determine the structure and composition of material interfaces with a fraction of nanometer resolution, were carried out and, by using deuterated proteins (Anti-Freeze Protein, Dihydrofolate Reductase and Myoglobin) on different PEG compositions and grafting densities, it was possible to locate the proteins in the brush and to distinguish between the different kinds of adsorption : primary adsorption is dominant for short brushes and ternary adsorption for long brushes.

Abstracts
– Biomaterials and drug delivery –

P-224

Slowdown of 1-40 β-peptide aggregation by addition of two synthetic biocompatible polymers

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Fibril deposit formation of amyloid β-protein (Aβ) in the brain is a hallmark of Alzheimer disease (AD). Fibrils formation is triggered by molecular conformational changes and protein-protein interactions involving partially unfolded regions of different Aβ peptide molecules. Increasing evidence suggests that toxicity is linked to diffusible Aβ oligomers, which have been found in soluble brain extracts of AD patients, rather than to the insoluble fibres. New therapeutical approach, based on searching molecules capable of regulating the peptide aggregation, is currently developing. Here, we study the effects on the aggregation of Aβ 1-40 peptide of two different synthetic polymers with structure similar to that of a protein (α,β-polyasparthylhydrazide – PAHy and α,β-polyasparthylhydrazide-polyethyleneglycol – PAHy-PEG). Static and Dynamic Light Scattering measurements showed that the aggregation kinetic is slowed down by the presence of both polymers. Optical Microscopy revealed the presence of aggregates of different dimension in all samples. Transmission Electron Microscopy allowed to establish that all aggregates are made of fibers, as confirmed by Fluorescence Spectroscopy measurements on thioflavine binding.

P-225

Polycationic carbon nanotubes for gene delivery applications

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Functionalized carbon nanotubes[1] (CF-CNTs) have a promising future as vectors for drug delivery[2] and for neuronal cell growth and communications[3]. It has been demonstrated that CNTs can be employed in drug delivery systems. The toxicological properties of CNTs result strictly correlated with nanotube solubility[4]. In this study we focused our attention on multi-walled CNTs (MWCNTs) because they are easy to manipulate and we covalently functionalize them in different ways to increase their solubility.. We grew different derivatives of a dendrimer on MWCNTs surface with positive charges at the periphery, in order to study the different solubilisation properties and correlate them to the biological activity in gene therapy [5].

[1]S.Campidelli, et al., *J. Am. Chem. Soc.* **2006**, *128*, 12544. Sang-Keun Oh, et al., *Langmuir.* **2003**, *19*, 10420. [2]A. Bianco, et al., *Curr. Opin. Chem. Biol.* **2005**, *9*, 674. [3]G. Cellot, et al., *Nat. Nanotech.*, ***2009***, ***4***, 126· [4]N. Lewinski, et al., *Small.* ***2008, 4, 26.*** [5]F.M· Toma, et al. *submitted.*

P-226

Action of ferritin, a nanoparticle model, on ROS formation and glutamate uptake in synaptosomes

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Nanoparticles are currently used in medicine as agents for targeted drug delivery and imaging. However it has been demonstrated that nanoparticles induce neurodegeneration in vivo and kill neurons in vitro. The cellular and molecular bases of this phenomenon are still unclear. We have used the protein ferritin as a nanoparticle model. Ferritin contains iron particles (Fe^{3+}) with size 7 nm and a protein shell. We investigated how ferritin influences uptake and release of [^{14}C]glutamate and free radical formation as monitored by fluorescent dye DCFDA in rat brain synaptosomes. We found that even a high concentration of ferritin (800 μg/ml) did not induce spontaneous [^{14}C]glutamate release. In contrast the same concentration of this protein inhibited [^{14}C]glutamate uptake two fold. Furthermore ferritin induced intrasynaptosomal ROS (reactive oxygen species) formation in a dose- dependent manner. This process was insensitive to 30 μM DPI, an inhibitor of NADPH oxidase and to 10 μM CCCP, a mitochondrial uncoupler. These results indicate that iron- based nanoparticles can cause ROS synthesis and decrease glutamate uptake, potentially leading to neurodegeneration.

P-227

Protective effect of melatonin supplementation on red blood cell in metabolic syndrome patients

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Metabolic syndrome (MS) is now regarded as a major risk factor for cardiovascular disease. As prooxidant/antioxidant balance is disturbed in the course of this disorder, it is possible that melatonin may exhibit protective effect against oxidative damage of blood cells.
MS was diagnosed according to International Diabetes Federation definition (2005). The investigated group consisted of patients before and after the melatonin supplementation (5 mg per day for two months) and was compared to control group of healthy individuals on normal diet.
Our experiments show that erythrocytes from patient group exhibit significantly higher TBARS and total cholesterol levels whereas the protein thiol concentration, Na^+K^+ATPase and glutathione peroxidase activities were decreased in comparison to those of healthy volunteers.
After two month melatonin supplementation, TBARS and cholesterol concentrations significantly decreased, whereas the Na^+K^+ATPase, catalase and glutathione peroxidase activities increased. The glutathione concentration was also higher.
These results show that melatonin supplementation has a protective effect on erythrocytes of MS patients.

Abstracts
– Stem cells –

P-228

Embryonic-derived NS cells show a maturation-dependent pattern of voltage-gated Na^+ currents

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Changes in the gating properties of Na^+ channels were studied in ES-derived neural stem (NS) cells during *in vitro* neuronal differentiation with the use of the whole-cell and cell-attached variants of patch-clamp technique. NS cells represent a novel stem cell population remaining stable and highly neurogenic over multiple passages.Voltage-clamp recordings during neuronal differentiation of NS cells indicated significant changes in the key properties of Na^+ currents. A voltage-gated and tetrodotoxine-sensitive Na^+ current,absent under self-renewal conditions, was first recorded following application of differentiative agents. Current density increased with time of exposure to differentiating conditions. Whole-cell and single channel analysis revealed that the observed increase in current density was due at least in part to changes in steady-state activation and inactivation properties. Namely, half activation potential shifted from -34 mV to -45 mV, while half-inactivation potential shifted from -94 mV to –78 mV. Furthermore, a contribution to the increase in Na^+ current density could also be given by an enhancement in channel expression, as suggested by an augmentation in the number of single channels per patch area, with increasing neuronal differentiation. Interestingly, those changes in Na^+ channel activity well correlate with the capability of NS cells to generate action potentials during *in vitro* neuronal differentiation.

O-229

Spontaneously proliferating and differentiating cells in the culture of new born rat cardiomyocytes

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A subpopulation of small cells (volume 141±6 μm^3) have been found in the neonatal cardiac myocytes culture whereas the same parameter of the remaining cells was 819±68 μm^3 on the 1st day. In contrast to main part of hypetrofied cardiomyocytes, the small cells were able to proliferate, form colonies and differentiate spontaneously into cardiac myocytes in the culture. On the 8th day there were slight, slow (2-3 beats/min), arrhythmic contractions in the centre of colonies. Pulsing cells were not united by common contraction and had individual beating profile. On the 11-12th days, the colonies displayed comprehensive contractile activity with the pulsation rate 25-28 beats/min and reached 46 beats/min on the 23^{d}-25th day and 58 beats/min on the 26-30th days in the culture. It has been shown that receptors of the surface membrane and sarcoplasmic reticulum of colony cells gradually mature. By estimating the Ca^{2+} transition under the specific agonists action: acetylcholine, KCl and caffeine, we have detected the activity of basic structural and functional elements of excitation–contraction coupling in contractile cells inside colonies. The small cells ability to proliferate and differentiate under the influence of near mature cardiomyocytes allows us to put forward a hypothesis, that they belong to a category of resident stem cells.

O-230

The plant hormone abscisic acid stimulates the proliferation of human hemopoietic progenitors through the second messenger cyclic ADP-ribose

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Abscisic acid (ABA) is a hormone involved in pivotal physiological functions in higher plants. Recently, ABA was demonstrated to be produced by human granulocytes, β pancreatic cells and mesenchymal stem cells (MSC) and to stimulate cell-specific functions. Here we show that ABA expands human hemopoietic progenitors *in vitro*, through a cADPR-mediated increase of the intracellular calcium concentration. Incubation of $CD34^+$ cells with micromolar ABA also induces transcriptional effects, which include NF-κB nuclear translocation and transcription of cytokines encoding genes. Stimulated human MSC produce and release ABA at concentrations sufficient to exert growth-stimulatory effects on co-cultured $CD34^+$ cells, as demonstrated by the inhibition of colony growth in the presence of an anti-ABA monoclonal antibody. These results provide a remarkable example of conservation of a stress-hormone from plants to humans and identify ABA as a new hemopoietic growth factor involved in the cross-talk between HP and MSC.

O-231

Expression of uncoupling proteins (UCPs) during neuronal differentiation and brain development

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UCP4 is a member of the mitochondrial anion transporter family and one of the three UCPs (UCP2, UCP4 and UCP5) associated with the nervous system. In our previous work we have shown that UCP4 appears in brain at embryonic day 14 (E14). We hypothesized that the UCP4 expression can be related to neuronal differentiation. To prove this idea we now investigated protein and mRNA levels in two different systems: (i) mouse embryonic stem cells of line D3 (mESC) and (ii) brains from pre- and postnatal mice. UCP4 was not present in undifferentiated mESC. During differentiation of mESC in neurons the expression of neuronal marker MAP2 and UCP4 started at the same time - as early as on day 7 in culture - and were increasing simultaneously. (ii) The comparative analysis of gene transcripts prepared from whole embryos, brains and different brain regions (neocortex, hippocampus, cerebellum) demonstrated that levels of UCP4 mRNA were increasing from E8 till E19, reached an expression peak between E19 and postnatal day 0 (P0) and remained constant in adult animals. In contrast, expression of UCP5 was increasing permanently until birth, whereas UCP2 expression was invariant in time. Our results suggest that UCP4 contributes to specific neuronal functions.

Abstracts
– Stem cells –

O-232

Therapeutic plasticity of adult stem cells for neurological diseases

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Stem cells are considered as a possible source of cells for tissue repair. In this scenario damaged tissues will be reconstructed by newly formed cells with the result of a recovery of lost functions. Thus, the rationale for utilizing stem cells for the treatment of neurological diseases such as multiple sclerosis (MS) stemmed from the idea that they differentiate in neural cells regenerating the damaged tissues at the basis of irreversibile disability. However, while multipotential embryonic stem cells may provide in the future an optimal source of cells competent for myelin and axons repair in MS, their use is still far from being exploitable in a clinical setting. Moreover, it is widely accepted that adult stem cells may *in vitro* transdifferentiate in neural cells but recent experimental data have challenged the biological importance of this event *in vivo*. Nevertheless, several experimental studies have provided new evidences supporting the use of adult stem cells derived form different human tissues for the treatment of MS. In the cases of mesenchymal stem cells and neural stem cells current experimental data support an unexpected therapeutic plasticity mediated by diverse paracrine mechanisms including neuroprotection, induction of local neurogenesis and modulation of the immune response. Therefore, current experimental and clinical studies support the use of stem cells for the treatment of neurological diseases such as MS.

P-233

Regulation of self-renewal in Cancer Stem Cells

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We are characterizing the biological differences between normal and transformed SCs. SCs are defined by their ability to generate more SCs, termed self-renewal, and to produce cells that differentiate (*asymmetric cell division).* SCs, however, possess the ability to expand in number (i.e. during development, in adulthood after injury/disease); this increase is not accounted by asymmetric divisions, in which only one daughter cell maintains SC identity. Recent findings in invertebrates indicate that SCs can also generate daughter cells destined to acquire the same fate (*symmetric cell division*). On the other hand, SC quiescence is critical to maintain tissue homeostasis after injury. Here we show increased symmetric divisions of CSCs in breast tumors (due to inactivation of the p53 tumor suppressor) and dependency of leukemia development on quiescent leukemia SCs (due to transcriptional up-regulation of the cell cycle inhibitor p21 by leukemia-associated fusion proteins). We suggest that SC asymmetric divisions function as a mechanism of tumor suppression, that SC quiescence is critical to the maintenance of the transformed clone and that symmetric divisions of SCs permit their geometric expansion.

Abstracts
– Solar energy conversion and photosynthesis –

P-234

ms-Delay Fluorescence (ms-DF) as an Indicator of Stress Factors Action on Photosystem II

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Stress factors such as heavy metals, strong light and low temperature were investigated by measurement of transient pictures of ms-DF in intact plants, isolated chloroplasts and pure PSII particles. The heavy metals action on PSII was investigated on wheat seedlings. The targets for toxic action of Al, Mn and Co ions were found to be a Q_A-Q_B acceptor side of PSII. The damage site for Cd^{2+} may be partners for recombination with $P680^+$ - depend on of medium pH - either Y_Z or $CaMn_4$-cluster. Investigated ions (Mn^{2+}, Al^{3+}, Cd^{2+} and Co^{2+}) have lead to reduction of chlorophyll-protein complexes pigment fund and Cd^{2+} and Co^{2+} destroyed also an apoproteins, especially of PSII. The photoinhibition of PSII was investigated in intact leaves of barley and maize seedlings at low and normal temperature. A very sharp reduction of intensity of ms-DF second fast component, possibly $Y_Z{}^*$P680ÿ$Q_A{}^-$ radiative recombination of maize seedlings was observed even after short illumination by strong light at 4°C. pH dependence photoinhibition of chloroplasts and pure PSII particles have shown that strong light damaged of $CaMn_4$-cluster in great degree than Y_Z .

P-235

ms-Delayed fluorescence – a probe for Photosystem II donor side *in vitro* photoinhibition

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It is well known that fast component of induction curves of millisecond delayed chlorophyll fluorescence (ms-DF) originates via radiative recombination of reaction center with product on the donor side of PSII. In view of our previous data *(J. Photochemistry and Photobiology B: Biology 86, 2007)* it was shown that partners for radiative recombination of $P_{680}Q_A{}^-$ localized as a hole on the donor side of PSII. Depending on pH this hole might be on the $CaMn_4$-cluster or on Y_Z and their recombination with $P_{680}Q_A{}^-$ can be monitored by the ms-DF fast component. For analysis of site of damages, photoinhibition of PSII particles from spinach at different pH was monitored by ms-DF. During photoinhibition of PSII (4000 μmol photons m^{-2} s^{-1}) the fast component of ms-DF was shown to be more stable at pH 5.5 and ratio of the fast component to the steady-state level of ms-DF was approximately constant, while at pH 8.5 the fast component essentially decreased, the steady-state level increased and ratio of these components rapidly went down. It is possible concluded that strong light damaged of recombination reaction with $CaMn_4$-cluster [($P_{680}Q_A{}^-$)-($S_{i+1}{}^\circ$)] in great degree than recombination reaction with Y_Z [($P_{680}Q_A{}^-$)-($Y_Z{}^\circ$)]. In the presence PpBQ – artificial electron acceptor – the protection of PSII from photoinhibition has been observed only at acidic condition.

O-236

Photochemically controlled molecular devices and machines

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The macroscopic concepts of a device and a machine can be extended to the molecular level. Molecular-level devices and machines operate via electronic and/or nuclear rearrangements and, like macroscopic devices and machines, they need energy to operate and signals to communicate with the operator. The extension of the concepts of a device and a machine to the molecular level is of interest not only for basic research, but also for the growth of nanoscience and the development of nanotechnology.
If a molecular device or machine has to work by inputs of chemical energy, it will need addition of fresh reactants ("fuel") at any step of its working cycle, with the concomitant formation of waste products that compromise the operation. Currently there is an increasing interest in the use of light to power molecular devices and machines.
The lecture will illustrate examples of recent achievements [1,2,3], which include molecular wires, switches, plug-socket systems, extension cables, antennas, and light powered nanomotors.

[1] V. Balzani, A. Credi, M. Venturi: "*Molecular Devices and Machines. Concepts and Perspectives for the Nanoworld*", Wiley-VCH, **2008**.
[2] V. Balzani, A. Credi, M. Venturi, *Nanotoday*, **2007**, *2*, 18.
[3] R. Ballardini, et Al. *Adv. Funct. Mat.*, **2007**, *17*, 740.

O-237

Unravelling the structure of the water splitting site of photosynthesis and implications for mechanism of catalysis

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Photosystem II (PSII) is a multi-subunit membrane protein complex which catalyses the oxidation of water to molecular oxygen and reducing equivalents. The reaction occurs at a catalytic centre composed of 4 Mn ions and a Ca ion, is thermodynamically demanding and generates highly oxidised species. Unavoidable side reactions cause detrimental effects on the protein environment leading to the rapid turnover of the reaction centre D1 protein. To understand the mechanisms of water oxidation and D1 turnover structural information is required. Initially the positioning of various protein subunits and their transmembrane helices were determined by electron microscopy. More recently a refined structure of the cyanobacterial PSII unit has been elucidated by X-ray crystallography giving details of specific environments of the redox active cofactors. The implications of these structural studies will be discussed in relation to the unique facets of PSII function, particularly the water splitting reaction. Importantly this new knowledge is providing a blue print for the design of photochemical catalysts which can mimic the photosynthetic water splitting reaction and thus give hope that new technologies will emerge to provide humankind with a sustainable energy supply.

Abstracts
– Solar energy conversion and photosynthesis –

P-238

Probing the dark cycle to reveal regulatory networks controlling photosynthetic efficiency

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The food, fibre and fuel needs of an ever-increasing population are one of the challenges facing current society. Higher plant photosynthetic efficiency is ∼1% whereas the theoretical maximum is thought to be ∼6-8% giving potential for great improvements. It is not however clear where in the photosynthetic process the additional losses are occurring.
We are adopting a systems biology approach to reveal the regulatory networks that control photosynthetic efficiency, the challenge being to make *quantitative* measurements of the input parameters to models of the network of photosynthetic reactions, and also to identify missing physical parameters and processes. The aim being to understand how they interact with other key physiological pathways and what impact they hold over photosynthetic efficiency.
We report initial results in this poster from experiments using conventional and novel proteomic methods. E.g. an optical technique based on a non-linear 2DIR method is being developed for proteomic and metabolomic analysis. Unlike many conventional proteomic methods, which provide information on the *relative* levels of various proteins and metabolites, the viability of the 2DIR method as a *quantitative*, sensitive and high throughput proteomics platform has recently been demonstrated. In collaboration with Klug this project will employ 2DIR as a both a proteomic and metabolomic tool.

O-239

Mechanisms of light harvesting and excess energy dissipation in plants and algae

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In oxygenic photosynthetic organisms, reaction centre complexes catalyze electron transport from water to CO2 using light energy absorbed by tetrapyrrole pigments bound to so called “antenna proteins”. A major challenge in performing this type of photosynthesis consists on the difficulty of operating “one electron” transport through a multi-step pathway in the chloroplast environment which is the source of oxygen of biosphere. In fact, synthesis of ROS, mainly singlet oxygen and superoxide anion and consequent photoinhibition is an intrinsically unavoidable consequence of photosynthesis that must be prevented and/or controlled in order to avoid photodestruction and death. Mechanisms involved include chlorophyll (Chl) triplet quenching, ROS scavenging and controlled heat dissipation of excess Chl singlet excited states. The latter process has been studied for over 40 years with little success in elucidating its mechanism. Reverse genomics and ultrafast-spectroscopy led to the proposal that the transient formation of carotenoid radical cations, followed by charge recombination might be the underlying mechanism of energy dissipation while three proteins belonging to the LHC superfamily could be the site hosting the reaction.

P-240

The A2 low energy monomeric chlorophyll of CP29

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To investigate the role of low energy spectral forms in energy transfer among chlorophyll protein complexes, we characterized one of the redmost chlorophylls of PSII antenna complexes, the A2 of CP29. Pigment stoichiometry of the wild type (CP29wt) and mutant lacking the A2 binding residue (CP29a2) reconstituted complexes confirmed the loss of a chlorophyll *a*. To exclude the possibility of a marked excitonic interaction of the A2 with other chlorophylls, we quantified the decrease in dipole strength after mutagenesis as a function of the frequency and performed a second derivative analysis of the difference absorption spectrum CP29wt *minus* CP29a2. These data, along with circular dichroism spectra of both complexes, support the idea that the A2 is a monomer in CP29, in disagreement with recent suggestion of involvement in an excitonic dimer (Mozzo et al., BBA 2008). Chl*a* A2 reorganization energy and inhomogeneous broadening were then determined through a thermal broadening analysis in the 80-285 K temperature range. These parameters allowed us to calculate the Förster Overlap Integral of the homogeneously broadened A2 bandshape, a fundamental factor for energy transfer rate estimations between chromophores. A comparison with FOI of chlorophyll in solution suggests that chl*a* A2 may connect CP29 complex to PSII *core* favoring energy transfer from CP29 towards other inner antenna low energy chlorophylls.

P-241

Heterologous expression of [FeFe]-hydrogenase from Chlamydomonas reinhardtii

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Hydrogenases are considered as potential energy sources. In particular, the ability of the green alga *C. reinhardtii* to reduce protons to hydrogen gas upon illumination by means of a [FeFe]-hydrogenase is a phenomenon of great scientific interest, as it would need only light and water to generate energy. However, the catalytic activity is strongly inhibited by the O_2 produced during photosynthesis; furthermore, the protein is expressed at very low levels, only in conditions of strict anaerobiosis. This mutually exclusive nature of O_2 and H_2 photoproduction represents a crucial problem in the development of H_2 bio-production. The study of the structure-function relationship of [FeFe] hydrogenases, which would help to clarify the molecular mechanisms underlying both H_2 production and O_2 sensitivity, requires the characterization of purified native and modified proteins, which can be obtained by site-directed mutagenesis. We expressed the algal hydrogenase in the cyanobacterium *Synechocystis sp.* PCC 6803, which holds a bidirectional [NiFe]-hydrogenase with a well known maturation system (1). We obtained two constructs to stably transform *Synechocystis*, enabling it to express the *C. reinhardtii* hydrogenase in an active form. This suggests that the [NiFe]-hydrogenase maturation pathway is able to drive the biosynthesis of functional [FeFe] enzymes. These data open new perspectives about the indispensable presence of HydE, HydF and HydG auxiliary proteins (2,3) to obtain a correctly folded [FeFe]-hydrogenase.

Abstracts

– Solar energy conversion and photosynthesis –

P-242

Identification of the sites of Chlorophyll triplet quenching in relation to the structure of LHC-II from higher plants. Evidence from EPR spectroscopy

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The Chl a molecules involved in the triplet-triplet energy transfer to the central luteins in trimeric LHC-II are identified by time-resolved and pulse EPR techniques. The concept of spin conservation during triplet-triplet energy transfer is exploited. The sites with the highest probability to form triplet states, which are quenched by the central luteins, are Chl603 and Chl612. Unquenched Chl triplet states are also produced by photo-excitation in the LHC-II complex. Putative sites of these triplet states are Chl614, Chl611, Chla604 and Chl613, since they do not contribute to the formation of the observed carotenoid triplet states.

P-243

H2 production mediation by proton-motive force or proton-ATPase in Rrodobacter sphaeroides

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Rhodobacter sphaeroides strain A-10 isolated from mineral springs in Armenia produces H_2 with high rate in anaerobic conditions under light. In our previous work the inhibition of H_2 production by *N,N'*-dicyclohexylcarbodiimide (DCCD), the F_0F_1-ATPase inhibitor, was shown, and DCCD-inhibited ATPase activity was determined. Therefore it is possible to admit a role of this ATPase in H_2 production by *R. sphaeroides*; otherwise DCCD inhibition of hydrogenase is not ruled out. In order to examine the mediatory role of proton-motive force (PMF) or proton ATPase in this process transmembrane electrical potential ($\Delta\Psi$) and ΔpH are determined and the ATPase activity is studied in *R. sphaeroides* grown under light. PMF was determined under anaerobic conditions in the dark. At pH 7.5 the $\Delta\Psi$ was of –94 mV and the reversed ΔpH was +30 mV, resulting in the PMF of –64 mV. But $\Delta\Psi$ was not affected by DCCD. Moreover, adenine nucleotide phosphates (ANP) content is essential for cell functioning and may result with ATPase activity. The percentage of ATP was calculated from the total quantity of ANP in whole cells, it was 46.6 %. A relatively high concentration of ADP (~35 %) and AMP (~18 %) and accordingly low energetic charge were noted; these might be indicative for the ATPase activity, a further study is required. Relationship between H_2 production and ATPase activity by *Rh. sphaeroides* is suggested; possible mechanisms are discussed.

O-244

Investigation of [NiFe]- and [FeFe]-hydrogenases: basis for a biotechnological hydrogen production

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Hydrogen is considered the fuel of the future if produced from sun light-driven water splitting. A hydrogen economy based on genetically modified organisms, immobilized enzymes or biomimetic synthetic catalysts requires a profound knowledge of the structure and function of the respective enzymes in Nature.[1] Light-induced water splitting is performed by a tetranuclear manganese cluster located in photosystem (PS) II of oxygenic photosynthesis. The protons generated by PS II can be converted to molecular hydrogen by the enzyme hydrogenase, which is for example found in green algae and cyanobacteria.

[NiFe]- and [FeFe]-hydrogenases are the two main classes of this enzyme. They contain bridged binuclear transition metal cores, which are tuned by a special ligand environment to efficiently convert protons to hydrogen – or *vice versa* – via a heterolytic mechanism.[2] The various intermediate states in the catalytic cycle are studied by EPR, FTIR and electrochemical techniques. The activation of the enzyme, the inhibition by CO, the sensitivity to molecular oxygen as well as substrate binding and product formation have been investigated. This led to proposals for the catalytic cycles of the [NiFe]- and the [FeFe]-hydrogenases.[1,2]

1. W. Lubitz, E.J. Reijerse, J. Messinger, *Energy Environ. Sci.* **2008**, *1*, 15
2. W. Lubitz, E.J. Reijerse, M. van Gastel, *Chem. Rev.* **2007**, *107*, 4331

O-245

Structural bases for the pH-dependent activation of violaxanthin de-epoxidase

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Violaxanthin de-epoxidase (VDE) is the enzyme responsible for zeaxanthin (Z) production. The synthesis of this carotenoid in plants exposed to high light conditions is an important photoprotection mechanism, enhancing excess energy dissipation and reactive oxygen species scavenging. The inactive enzyme is normally soluble but, upon activation by low pH, it binds to the thylakoids membrane, where its substrate is found.

We present here the first structural data on this enzyme obtained at both acidic and neutral pH. At neutral pH, VDE is monomeric with its active site occluded in the lipocalin barrel. Upon acidification, the barrel opens up resulting in a functional dimerization of the enzyme. The channel linking the two active sites of the dimer can harbour the entire carotenoid substrate and thus allow the parallel de-epoxidation of the two violaxanthin β-ionone rings, making VDE an elegant example of the adaptation of an asymmetric enzyme to its symmetric substrate.

Structural data opened the possibility to investigate deeper this enzyme and further work allowed the identification of its active site, the protein domains responsible of its membrane association as well as key residues involved in the pH dependent conformational change.

Abstracts
– Solar energy conversion and photosynthesis –

P-246

Photosynthetic apparatus response under heat stress

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The mechanisms of PSII thermoinactivation and adaptation under high temperature impact and participation of hydrogen peroxide at these processes were studied. The twice suppression of oxygen evolving activity of thylakoids with simultaneous decrease in D1 protein content and the release of extrinsic 33 kDa polypeptide from WOC after 15-20 min heating at 40°C were registered. Using inhibitor analysis it was shown that thermoinduced degradation of D1 protein after 20 min heating occurred by proteases. The participation of FtsH protease in thermoinduced D1 protein degradation was observed. Level of transcription of *psbA* gene in chloroplast was raised after 20 min heating and was decreased through 1 h. The content of hydrogen peroxide was increased three times after 20 min of heating and was decreased to normal level through 1 h and was raised after 2.5 h again. It is interesting that level of peroxidation lipids products was increased after 2.5 h heating only. Received data indicated that hydrogen peroxide is signal molecular at the photosynthetic apparatus under heat stress. During heating the inactivation of WOC and D1 protein is occurred. As result the H_2O_2 is generated. Hydrogen peroxide as signal molecule activates transcription of *psbA*. Turnover of PSII is occurred. More long heating induces degradation of proteins and lipids and H_2O_2 represents as the destructive agent.

P-247

Fluorescence lifetime spectrum of the plant photosystem II core complex

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The photosystem II kinetic model (diffusion or trap-limited) is still much debated. There is discussion about whether energy transfer from the core antenna (CP47 and CP43) to the reaction center complex (D1-D2-cyt *b*559) is rate-limiting (transfer to trap-limited). This study investigates this problem in isolated core particles by exploiting the different optical properties of the core antenna and the reaction center complex near 680 nm, due to P680 and an isoenergetic pheophytin. This was used as a marker feature for the reaction center complex. If the transfer to the trap-limited model were correct, assuming excited-state thermalization, the specific reaction center fluorescence decay lifetime should be shorter near 680 nm, where there is reaction center complex specificity, than at the other emission wavelengths. Such a selective reaction center feature was not observed in fluorescence decay measurements. At the
experimental resolution used here, we conclude that the trap-limited energy transfer to the reaction center could, at the most, be 20% limiting. Thus, the transfer to the trap-limited model is not supported.

Abstracts
– Glycobiophysics –

O-248

Solvent occupancy analysis in ligand structure prediction: the case of Galectin-1

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Formation of protein ligand complexes is a fundamental phenomenum in biochemistry. During the process, significant solvent reorganization is produced along the contact surface. Using MD simulations in explicit solvent combined with statistical mechanics analysis, thermodynamic properties of water molecules around proteins can be computed and analyzed in a comparative view. Based on this idea, we developed a set of analysis tools to link solvation with ligand binding in a key carbohydrate binding protein, human galectin-1 (hGal-1). Specifically, we defined water sites (WS) in terms of the thermodynamic properties of water molecules strongly bound to protein surfaces. We then succesfully extended the analysis of the role of the surface associated water molecules in the ligand binding and recognition process to many other carbohydrate binding proteins.
Our results show that the probability of finding water molecules inside the WS, $p(v)$, with respect to the bulk density is directly correlated to the likeliness of finding an oxhydril group of the ligand in the protein-ligand complex. This information can be used to predict possible complex structures when unavailable, and suggest addition of OH-containing functional groups to displace water from high $p(v)$ WS to enhance drug, specially glycomimetic-drugs, protein affinity and/or specificity.

O-249

Thermal aggregation of glycated Bovine Serum Albumin

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Aggregation and glycation processes of proteins are of peculiar interest for several scientific fields. Serum albumins are widely studied proteins for their ability to self-assemble in aggregates and also to undergo to non enzymatic glycosylation in cases of diabetes. In this work we report a study on thermal aggregation of glycated bovine serum albumin (BSA) prepared with different concentrations of glucose at pH 7.4. Increasing concentration of sugar modulates the effect of different glycation levels on the protein aggregation. Fluorescence spectroscopy, FTIR absorption, static and dynamic light scattering are used to follow the time evolution of the aggregation process and of protein conformational changes. Conformational changes of secondary and tertiary structures are measured by FTIR absorption; the kinetics of Amide I, Amide II and Amide II' bands are monitored. The kinetics of tryptophans fluorescence give complementary information on the tertiary structure changes and on the polarity modification of the fluorophores environment. The aggregates growth is studied by dynamic light scattering measurements and Rayleigh scattering peak. The results show that the partial unfolding of the protein is not affected by the glycation, while the presence of increasing amounts of glycated molecules progressively inhibits the aggregates formation.

O-250

Interaction between frutalin with biomembrane models and its correlation with cell adhesion property

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Frutalin is a homotetrameric lectin D-Galactose (D-Gal) binding that activates natural killer cells *in vitro* and leukocyte migration *in vivo*, being a potent lymphocyte stimulator. In this study we investigated the interaction of frutalin with different phospho/glyco-lipids using Langmuir monolayers as biomembrane mimetic system. The results attest the specificity of the protein for the carbohydrate D-Gal when attached to the biomembrane model. The adsorption kinetics for frutalin to the mixed monolayers containing glycolipids showed that the interaction depends on the presence of charged groups and on the position of the D-Gal on the polar head of the glycolipid. Using Brewster angle microscopy (BAM), we investigated the morphology of the interface for the binary mixtures containing GalCer, where small domains were formed at high lipid packing, suggesting that frutalin can induce the formation of like-lipid rafts domains *in vitro*. The results obtained with the membrane models were associated with those from fibroblast adhesion induction. The cell adhesion promoted by frutalin is in accordance with the results observed in Langmuir monolayers, which probes the specificity of the interaction between the lectin and D-Gal on cell-membrane surfaces. Based on these results, frutalin can be considered as a promise biotechnological tool to actuate in tissue engineering regeneration. Supported by FAPESP, CNPq and CAPES

O-251

Perception of Nod Factors in the Legume-Rhizobia symbiosis: in silico studies on Medicago truncatula

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The mechanism of symbiosis between legume plants and Rhizobial bacteria is a relevant topic of interest since it is at the basis of the nitrogen fixation process. This mechanism is strictly related to the production of lipochitooligosaccharides, Nod Factors (NF), which allow the bacterial invasion into the legume host roots. In *Medicago truncatula*, the recognition of NF from the symbiont *Sinorhizobium meliloti* requires the *NFP* gene, able to encode a LysM-motif receptor-like kinase. The extracellular part of this protein is characterized by three LysM motifs: only one of them seems to be involved in the NF recognition. Herein, we report an *in silico* investigation of different structural aspects of this process. First of all, the conformational behaviors of a new generation of NF analogues were elucidated by MDs simulation. Then, a homology model of the LysM2 domain from *M. truncatula* was proposed and compared with a model of LysM domain from *Pisum sativum*: docking calculations of natural NF identified a common binding site in which the carbohydrate portion is the main responsible of the binding. Both studies provide support for the idea that the carbohydrate part of NF plays a key role in the interaction with LysM domains, while the lipid moiety modulates ligand specificity probably interacting with a potential second receptor.

Abstracts
– Glycobiophysics –

O-252

Glyconanoparticles: Nano-biomaterials for application in biotechnology and biomedicine

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To study and intervene in carbohydrate interactions our laboratory has developed a chemical strategy (Glyconanotechnology) to produce sugar functionalized gold nanoclusters with multivalent carbohydrate display (glyconanoparticles, GNPs). By combining these tools with biophysical and analytic surface techniques (ITC, AFM, TEM, SPR) we have demonstrated and evaluated Ca^{2+}-mediated carbohydrate-carbohydrate interactions involved in cell adhesion processes. The GNP system complements other currently available multivalent systems incorporating carbohydrates and presents some advantages as: 1) exceptionally small core size; 2) high stability in water and physiological buffers; and 3) multivalency and multifunctionality with control over ligands number. Specific GNPs have been applied to the inhibition of melanoma metastasis in mice and as inhibitors of HIV-*trans* infection. Magnetic GNPs have also been used in cellular labelling and imaging as probes for MRI.
In this lecture, the Glyconanotechnology strategy will be presented and some application will be highlighted.

Abstracts

– Condensed colloidal phase in biology: from proteins crystals to amyloid fibrils –

P-253

Temperature-induced aggregation of the monoclonal IgG1 antibody Rituximab is governed by coagulation

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It is crucial for pharmaceutical protein formulations to have low aggregate content. Using the monoclonal IgG1 antibody Rituximab as a model system, we have studied the mechanisms by which antibodies aggregate at physiological pH when incubated at 52–65°C. Light scattering showed two coupled stages: An initial fast stage followed by several hours of exponential growth of the scattered intensity. Data analysis showed the fast formation of a large species, which subsequently increased in size. The aggregate number density had a maximum suggesting that aggregates increase in size by coagulation. The analysis also predicted the actual underlying increase in aggregate mass to be linear and reach saturation. This was confirmed by size-exclusion chromatography of incubated samples. In an Arrhenius plot the activation energy of the first stage was similar to the unfolding energy of the CH2 domain, suggesting a pivotal role of this domain in the aggregation process. CD and fluorescence showed only minor structural changes in the temperature interval studied. We conclude that coagulation is the main mechanism driving Rituximab aggregation and that aggregation is due to small structural changes.

P-254

Dynamic Light Scattering aggregation studies of Aβ (1-40) peptide

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Heparan sulfate (HS) proteoglycans play a role in formation of amyloid plaques by facilitating formation of amyloid aggregates. Heparins and low-molecular weight heparins which mimic HS sequences could interact with amyloid peptides putatively involved in neurodegenerative processes. The aim of this work is to study the influence of chain length and structure of heparin oligosaccharides on the aggregation of amyloid. To this purpose, the interaction with amyloid peptide $A\beta_{1-40}$ with a number of heparin/heparin oligosaccharides had been investigated using dynamic light scattering (DLS) which permits to determine the size and the stability of molecular aggregates in solution. As indexes of aggregation in solution, two parameters were chosen, i.e., the area under the autocorrelation function (AF) and the average hydrodynamic ratio (Rh). We evaluated the aggregation kinetic of different preparations of Aβ (1-40) and the pH of solubilization. A clear indication was obtained for the trend of aggregation of the peptide $A\beta_{1-40}$ alone and in the presence of oligosaccharides. The size of the aggregate in the presence of the tetrasaccharide is definitely lower than for the peptide alone. The size of the aggregates increases with increasing size of the oligosaccharides. In fact, the octasaccharide promotes further aggregation.

O-255

Modification by Dopamine adducts links α-synuclein to oxidative stress in Parkinson disease

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Oxidative stress has been proposed to be involved in the pathogenesis of Parkinson disease (PD). A plausible source of oxidative stress in nigral dopaminergic neurons is the redox reactions that specifically involve dopamine (DA) and produce various toxic molecules, i.e., free radicals and quinone species. α-Synuclein (αSyn), a small protein found in Lewy bodies characteristic of PD, is also thought to be involved in the pathogenesis of PD. To investigate the possibility of a synergistic role of oxidative stress and αSyn in PD, we analyzed the modulation of DA toxicity by αSyn overexpression in dopaminergic human neuroblastoma cells. Our results indicate that the increased expression of αSyn enhances the cellular toxicity induced by the accumulation of intracellular DA. We then correlated our results with the structural modifications induced by the oxidation products of DA on αSyn by studying two potential pathways for the oxidative chemistry associated with DA. The first one is the auto-oxidation reaction which leads to the concomitant formation of radical and quinone species. The second is the enzymatic oxidation, mediated by tyrosinase, which leads only to the accumulation of quinones. Our data suggest a link between DA and αSyn in the progression of PD and provide novel insights on both the mechanisms involved in the oxidative chemistry and the aggregation properties of αSyn.

O-256

Expanded ataxin-1 induces membrane mechanical instability: evidences from model systems and cells

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Spinocerebellar ataxia type 1 is a neurodegenerative disorder involving the expansion of a polyglutamine stretch beyond a threshold in the protein ataxin-1, with intracellular deposition of amyloid aggregates. Ataxin-1 variants with polyglutamine stretches of pathological length are not associated to disease when the stretch is interrupted by histidines.
Mounting evidence suggests that ataxin-1 aggregates interact with the nuclear membrane. To get insight into the mechanisms leading to neurological disorders, we studied model lipid membranes containing an expanded pathological form of ataxin-1 or expanded normal forms interrupted by histidines. Electrical measurements on planar bilayers showed the occurrence of current steps, much larger for the pathological form, indicating the formation of pore-like structures. Atomic force microscopy measurements showed that the longer the polyglutamine tract, the smaller was the force required to penetrate the bilayer with the AFM tip. The smallest penetration force, and then the strongest membrane destabilization, was observed for membranes containing the expanded pathological form. Experiments on COS1 cells provided evidence that pathological protein aggregates damage the nuclear membrane eventually causing cell autophagy.

Abstracts

– Condensed colloidal phase in biology: from proteins crystals to amyloid fibrils –

O-257

Conformational dynamics and oligomerization pathways of the ovine prion protein

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Prion diseases are deadly neurodegenerative diseases affecting human and mammalian species. According to the ‘protein-only’ hypothesis, the key event in the pathogenesis is the conversion of the α-helix-rich monomer (PrP_c) into a polymeric β-sheet-rich pathogenic conformer (PrP_{sc}). We have used a combination of biophysical techniques with Molecular Dynamics simulations (MD) to elucidate the molecular mechanisms of PrP_c unfolding and polymerization. Under well established conditions, three β-sheet-rich soluble oligomers were generated from the partial unfolding of the monomer, which were found to form in parallel. To obtain a deeper insight into the molecular events, double-cystein mutants were designed, thus ‘locking’ different regions of PrP. Single mutations were also performed, which affected dramatically and selectively the PrP oligomerization pathway. Furthermore, we have now identified the minimal region that leads to the same oligomerization profile as the full-length PrP, namely, H2H3. The existence of at least three distinct oligomerization pathways and the effect of single mutations reveal the conformational diversity of PrP and a possible relationship with prion strain phenomena. The identification of domains involved in the conversion process may lead to a better understanding of the effect of mutations or gene polymorphism on the evolution of prion pathology.

P-258

Analysis of physico-chemical parameters affecting collagen I liquid crystal organizations

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The collagen I is the major structural protein of the body and it is found organized in a hierarchical manner in the extra-cellular matrix of several tissues (bone, tendon, cornea and sclera, etc..). It is responsible for their specific architecture.
It is already known that collagen I is capable of forming cholesteric liquid crystalline phases that once stabilized by a pH increase, lead to collagen matrices that mimic the organization of the organic matrix found in bone [1]. In the present work, we analyze physico-chemical ways to modulate long range organizations obtained in this liquid state. We study the effect of collagen concentration, pH (2.6 / 3.6) and acids (hydrochloride acid / acetic acid) over the liquid crystalline order. In a original approach, correlation of collagen endofluorescence and SHG signals has enabled us to quantify cholesteric pitch and phase transition as a function of collagen concentration within a gradient.
Cholesteric pitch have proved to be very responsive to physico-chemical conditions. Indeed, the results allowed us to quantify a I / cholesteric transition as a subsequent transition from cholesteric to a phase hexagonal or colummar.
[1] Besseau & G.Guille (1995) J. Mol Biol. 25: 197-202

P-259

A mutation in APP gene with dominant-negative effect on amyloidogenesis: a light scattering study

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We studied the effect of a single-point mutation (A673V) on the aggregative properties of Alzheimer’s peptides Aβ1−40. By laser light scattering it was possible to follow the early stages of aggregation of $A\beta 1-40_{wt}$ and $A\beta 1-40_{mut}$ (within 48 hours after sample preparation), when intermediates in the aggregation pathway, rather than the mature insoluble fibrils, are formed. Results show that both the kinetics and the extent of aggregation are strongly enhanced by the mutation. On the reverse, coincubation of mutated and wild-type peptides slows down the aggregation process and results in Aβ aggregates that are much more unstable against dilution. It is evident that the interaction between mutant and wild-type Aβ1−40 interferes with nucleation or nucleation-dependent polymerization, hindering amyloidogenesis. These results account for the highly amyloidogenic effect of the mutation in vitro, if alone, reversely reduced by the mutated-wild type mix. Also a clinical case exists that constitutes the in-vivo evidence of these two opposing effects. This finding may offer grounds for the development of therapeutic strategies, based on modified Aβ peptides or peptido-mimetic compounds, for the potential treatment of Alzheimer’s disease.

P-260

Solid-state NMR structural studies of Alzheimer’s disease amyloid β(1-42) in lipid bilayers

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Amyloid-β peptides are believed to cause loss of nerve cell function in individuals suffering from Alzheimer’s disease, where evidence suggests that interaction with the cell membrane correlates strongly with cytotoxicity. Previous studies report a range of different but plausible structures, which depend on the molecular environment of the peptide. This sensitivity to sample preparation suggests that the structure relevant to disease is found in lipid bilayer membranes. While model membrane vesicles are too large to be studied by conventional solution NMR, solid-state NMR is one of the few technologies available to study such systems. We present recent measurements which suggest a novel peptide structure in a lipid bilayer environment: (i) rotational-echo double-resonance (REDOR) distance measurements between selectively enriched ^{13}C-carbonyl and ^{15}N-amide positions constrain dihedral angles of intervening residues and suggest that at least part of the Aβ(1-42) peptide folds into a β-sheet like conformation, in contrast to the helical and coiled structures in previous reports; and (ii) double quantum filtered DRAWS measurements indicate that the extended strand does not assemble into an in-register parallel sheet as reported for amyloid fibrils.

Abstracts

– Condensed colloidal phase in biology: from proteins crystals to amyloid fibrils –

P-261

Capturing the initial events of In-cubo crystallization of membrane proteins

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Lipid based methods for the crystallization of membrane proteins including *in-cubo* crystallization are becoming popular in recent times. However, a complete understanding of the basic principles behind these methodologies is still elusive.
The crystallization of membrane proteins in the lipid cubic phases involves following major steps: removal of membrane proteins from the native membrane using detergent-like molecules, followed by their insertion and equilibration into a lipid bilayer. The crystallization itself commences with precipitant induced osmotic dehydration which in turn stimulates the nucleation that subsequently leads to a crystal growth.
Using time-resolved X-ray scattering (SAXS) and ultraviolet spectroscopy we have been able to gain new insights into the mechanism behind protein insertion into these complex three dimensional lipid structures including information on the timescales of protein folding relative to crystal growth and the effect of protein insertion on the morphology of the surrounding lipid matrix.

P-262

Amyloid Gels: Formation and mechanical properties of insulin fibrillar networks

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The formation of insulin fibrils is characterized by an initial apparent lag-phase, related to the formation of oligomers, protofibrils and aggregation nuclei. Afterwards, the aggregation proceeds via fibril elongation, thickening and/or flocculation, and eventual gelation. Here, we focus on the formation of such a gel, made of insulin amyloid fibrils, upon incubation at high temperature and low pH. By light scattering and rheological techniques, we monitor the development of the structural, dynamical and mechanical properties of fibrillar aggregates, up to the dynamic arrest of the sample and to the appearance of a non-ergodic behaviour, which marks the onset of gelation. Our experiments were able to reveal the structural details hidden in the apparent lag-phase, displaying the slow fibril nucleation and elongation. This initial stage is followed by the known exponential growth of structures of different sizes. These two kinetic stages of structural growth are mirrored by the kinetics of the viscoelastic properties and, in particular, by the growth of the elastic modulus. Our results show that the appearance of a noteworthy elastic network, is associated with the initial fibril nucleation and elongation more than with the formation of large structures, which causes the eventual gelation.

P-263

AFM studies of artificial amyloid fibrils and filaments

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Amyloid fibrils are beta-sheet-rich superstructures of peptides or proteins. Although these aggregates have first been found in connection with protein-misfolding diseases, such as Alzheimer's or Parkinson's Disease, there is evidence that the ability to form fibrils is a generic property of any polypeptide rather than a result of specific, disease-related amino-acid sequences. Fibrils can easily be formed in vitro from non-disease-related proteins and even from synthetic peptides. These artificial fibrils can thus serve as model systems to investigate the biophysical properties of amyloid fibrils.
The system presented here is built from the artificial peptide $TTR_{105-115}$, which contains 11 amino-acids and which forms well-defined nanorods of ca 10 nm diameter. We present Atomic Force Microscopy (AFM) results of their inner morphology by chemically dissecting the rods into their constituent filaments without completely breaking up the aggregates. In contrast to the stiff rods, their constituent filaments of ca 1-2 nm diameter were much more flexible. This shows that the particular way filaments are arranged around each other can massively change the mechanical rigidity of the resulting structures in amyloid fibrils, which could be one cause of the different strains in amyloid diseases.

O-264

Investigating toxic protein nanoclusters and their interactions with living cells

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Amyloid protein aggregation is responsible for many neurodegenerative diseases, such as Alzheimer's and Parkinson's. It appears that quasi-stable small soluble aggregates are the key to amyloid toxicity. Though amyloid aggregation appears to be a nucleation mediated process, simple nucleation theory is unable to explain the stability of these intermediate species. We investigate this issue with Fluorescence Correlation Spectroscopy in solution and on cell membranes. Our initial data, varying the pH and the ionic strength of the solution, show that a charged colloid model can explain the overall stability of these species. In addition, this understanding suggests possible ways of modulating the stability of these aggregates in solution. Some of these strategies successfully decimate the stable aggregate population, and also reduce the toxicity of amyloid beta to cultured neurons.

Abstracts

– Condensed colloidal phase in biology: from proteins crystals to amyloid fibrils –

P-265

The initial stage of proteins aggregation leading to amyloid fibrils: a SAXS study

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Under some conditions, a protein converts from its soluble form into highly ordered aggregates called amyloid fibrils, which are associated with many human diseases. In order to tackle the prevention and treatment of these diseases, we need to understand the mechanism of the pathological aggregation of proteins. In vitro amyloid fibril formation is preceded by the formation of metastable non fibrillar forms, which are responsible for cytotoxicity underlying neurodegeneration. The molecular mechanisms leading proteins into prefibrillar aggregates are still unclear. We present a SAXS study performed at ID02 beamline of ESRF on the apomyoglobin mutant W7FW14F, which at physiological pH, firstly aggregates in prefibrillar forms that are cytotoxic and then forms amyloid fibrils. The first stages of W7FW14F oligomerization are induced by a pH jump. Data show that big changes in W7FW14F in solution happen in less than 100 ms. Singular Value Decomposition (SVD) of the data yields a set of functions, from which all the scattering curves can be reproduced. The major result of our study is the determination of the presence of different oligomers in each step of the process. Hence, time-resolved SAXS experiments together with the estimation of different oligomers via SVD method, can be a new and useful approach to investigate the first stages of amyloidogenesis.

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Ability to undergo amyloid aggregation is affected by protein size

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Short polypeptide chains, typically between 28 and 253 residues, are generally involved with the conversion from the native state into insoluble fibrillar aggregates. The low prevalence of large proteins is disproportionate with their high occurrence in the human proteome. In order to explore the propensity of large proteins to form amyloid-like fibrils, the 486-residue hexokinase-B from *Saccharomyces cerevisiae* (YHKB) has been induced to aggregate under two separate conditions, at low pH in the presence of salts and at pH 5.5 in the presence of trifluoroethanol. Such conditions are among the most promising to form amyloid-like fibrils by normally globular proteins. Under both conditions YHKB aggregates very rapidly into species with significant β-sheet structure, as detected by circular dichroism, and a weak Thioflavin T and Congo red binding. Atomic force microscopy revealed globular aggregates eventually clustering into large amorphous aggregates at low pH, while in the presence of trifluoroethanol ribbon-like structures with distinct morphology from typical amyloid fibrils were observed. They had irregular width, no twist, and were connected by thinner fibril segments perpendicular to them. In general, YHKB aggregates displayed an unusual softness, as they were very easily perturbed by the AFM tip. These results suggest that inability to form amyloid fibrils may prevent large proteins from being associated with protein deposition diseases.

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TTR amyloid protofilament assembly revealed by AFM imaging and single molecule force spectroscopy

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In this work we used AFM to follow the amyloidogenesis pathway of transthyretin (TTR) to form protofilaments. Single-molecule force spectroscopy (SMFS) of native TTR and protofilaments were also compared in order to evaluate dynamic and structural differences. We observed that this pathway proceeds through the formation of transient amorphous aggregates, followed by the occurrence of annular oligomers. Although implicated in cytoxicity, the role of such oligomers within the amyloidogenesis pathway is poorly understood. We show that the annular species display a tendency to stack, forming tubular-like structures that precede the formation of protofilaments. The protofilament height and pitch resemble those of TTR amyloid reported in previous structural studies. Upon solvent exchange, we also observed protofilament disassembly that revealed structures reminiscent of the initial TTR annular oligomers. SMFS of protofilaments showed a time-dependent increase in the length of the manipulated structures, suggesting that associations between monomers stabilize with time. Force spectra of native TTR and protofilaments contained transitions spaced by ~4 nm, indicative of sequential unfolding of individual β-strands. Based on these results a model of TTR protofilament assembly is proposed.

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Fibrillogenesis of hen egg-white lysozyme at acid pH

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Several proteins can form amyloid fibrils under given environmental or thermodynamic conditions that affect their native conformation.
Lysozyme forms amyloid fibrils upon incubation of solutions at acid pH and at about 65°C for a few days.
Differential Scanning Calorimetry experiments confirm that lysozyme is mainly unfolded above 60°C at pH 2. We monitored the growth of aggregate size by Dynamic Light Scattering for some days at different temperatures. Our results show that the fibrillogenesis is characterized by an initial apparent lag phase and a subsequent growth with quadratic dependence upon time of the scattering intensity. This behaviour recalls a simple kinetic model of nucleation and elongation, with nuclei in equilibrium with monomers. At the end of the incubation at high temperature, we collected Atomic Force Microscopy images which show fibrils with a diameter of a few tens of nanometers and a length of a few microns, characterized by a periodicity along the elongation axis. Interestingly, the fibrils morphology exhibits no branching or thickening. This is consistent with the non-exponential growth observed in light scattering experiments.
In order to elicit the role of repulsive electrostatic interaction in protein unfolding and self-assembly we extended our study at different pH.

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Fluorescence microscopy studies of IAPP fibrillation at model and cellular membranes

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Type 2 diabetes mellitus (T2DM) is characterized by islet amyloid deposition and beta cell death, the main culprit being a small 37 a.a. peptide hormone, islet amyloid polypeptide (IAPP), which forms fibrils under pathological conditions. We studied the interaction of IAPP with giant (GUVs) and large (LUVs) unilamellar vesicles as well as with INS-1E cells. By using confocal / two-photon excitation fluorescence microscopy and GUVs, we tested the influence of charge (DOPC:DOPG 7:3) and model raft systems, displaying liquid-ordered (l_o) / liquid-disordered (l_d) phase coexistence (such as DOPC:DPPC:cholesterol 1:2:1), respectively, on the kinetics of IAPP fibril formation. A preferential partitioning into the l_d phase was observed and fibrils grew along with lipid uptake. Fluorescence spectroscopy leakage experiments with carboxyfluorescein-filled LUVs and the corresponding ThT kinetics of IAPP fibril formation were carried out as well. Moreover, using the WST-1 reduction assay and fluorescence microscopy, we could show that the red wine compound resveratrol is a potent inhibitor of IAPP fibrillation and its cellular toxicity on INS-1E cells, these findings highlighting the potential role of resveratrol in future clinical applications, i.e., in the treatment of T2DM.

O-270

Protein condensation diseases - a colloid physicists viewpoint

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A broad class of diseases, such as cataract, Alzheimer's disease and sickle-cell disease, involve protein association phenomena as an essential aspect. The basic element common to all members of this class of molecular condensation diseases is the subtle interplay between protein interactions that produces condensation into dense, frequently insoluble mesoscopic phases. Among this class of diseases, cataract is particularly important as the world's leading cause of blindness. This disease is most often the consequence of an uncontrolled aggregation (or phase separation) of the proteins in the eye lens that results in a loss of its transparency.
The high concentration protein mixtures present in the eye lens are normally stable and produce a high refractive index that aids the eye in adaptive focusing of light. Moreover, the proteins themselves exhibit a rich variety of repulsive interactions, attractive interactions, sizes, phase transitions and self-association. These mixtures also exhibit the pathological aggregation and opacification of the cataract disease that has inspired their study. In my presentation I will illustrate how we can use a combination of small-angle neutron and X-ray scattering experiments combined with molecular dynamics computer simulations to identify, measure and model the molecular interactions and emergent optical and viscoelastic properties and the phase behavior of the relevant, complex cytoplasmic mixtures.

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Temperature and metal ions induced aggregation of human serum albumin

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An increasing number of experimental studies is demonstrating that the propensity of forming amyloid fibrils is not limited to proteins related to neurodegenerative diseases but it is a more general phenomenon. We present data on human serum albumin (HSA) aggregation induced by a combination of thermal and metal ions effects investigated by optical density, fluorescence and electron paramagnetic resonance (EPR).
The turbidity experiments as a function of temperature show that the HSA aggregation starts at about 70 °C which is higher than the denaturation temperature ($\sim$ 60 °C). In the presence of copper and zinc metal ions the onset temperature for protein aggregation is markedly reduced as the metal:protein molar ratio is increased from 1:1 to 10:1. Moreover, the copper is more effective in inducing protein aggregation compared to zinc ion.
The HSA aggregation analyzed by ThT fluorescence at different incubation temperatures lacks a lag phase and the kinetic traces can be fitted by double exponential functions. The ThT fluorescence increase evidences the formation of protein aggregates with fibrillar features. EPR experiments for the Cu(II)-HSA complex show the binding of Cu(II) in the protein native state in a square planar coordination with 4 equatorial N atoms, and is not influenced by the heat treatment of the protein. The overall results suggest that HSA aggregation is compatible with a downhill process that does not require the formation of an aggregation nucleus.

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Study of structure/toxicity relationship of amyloids by infrared spectroscopy

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Amyloid diseases (Alzheimer, Parkinson, type II diabetes, prion) correlate with protein aggregation. All the proteins associated with these pathologies aggregate into amyloid fibrils with a common ß-cross structure. According to many *in vitro* studies, the toxicity of various amyloids seems to be linked to some intermediates formed during the aggregation pathway and to their interaction with membranes. Our aim is to understand the link between structure and toxicity of amyloids by studying their interaction with model membranes. For this, we use as an amyloid model, the Prion-Forming Domain PFD218-289 (Wild Type WT) of Het-s, a prion protein of the fungal *P. anserina.* When expressed in yeast, WT is not toxic whereas one of these mutants, M8 is toxic. *In vitro*, M8 forms very unusual short amyloid fibers contrary to WT which polymerizes as long fibers. Furthermore, ATR spectra have shown that M8 is essentially assembled into mixed parallel and anti-parallel ß-sheets whereas WT displays a predominant parallel organization. We are currently studying the interaction between WT or M8 with lipid Langmuir film by Polarized Modulation-InfraRed Reflexion Absorption Spectroscopy (PM-IRRAS). This technique allows determining the secondary structure of proteins and their orientation in the membrane. The study of interactions with different charged phospholipids is following.

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Studying alpha-synuclein aggregation by fluorescence polarization based kinetics

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Alpha-synuclein (syn) is linked to Parkinson's disease (PD) by two evidences: the accumulation of amyloid fibrils of the protein and the autosomal dominant forms of the disease (A53T, A30P and E46K mutants). Protein oligomers seem to be the most toxic species, causing dopaminergic neuronal death, probably disrupting cell membrane (Volles & Lansbury, 2002). The aggregation of syn follows a nucleation dependent mechanism, i. e., aggregation is favoured only after the formation of an oligomer composed of a critical number of monomers (Wood et al., 1999). As the constitution of the nucleus is a rare event, early aggregation stages are difficult to study. To explore the early stages of the aggregation process a fluorescence polarization (FP) based method (Luk et al., 2007) was applied to study syn oligomerization. FP depends on the size of the fluorescent molecule, therefore it is suitable for the detection of oligomers formation. We measured aggregation kinetic properties under several different conditions. A 100 to 1 mixture of syn and Oregon Green labelled syn was used to analyze the aggregation behaviour of wild type (wt) and PD mutants. The wt protein shows the fastest aggregation rate. This aggregation process is slowed down by the presence of the chaperone 14-3-3eta in wt syn samples. Finally, dimers formed by a disulfide bond at the N-terminus or C-terminus of syn, when tested for their aggregation behaviour, show a different propensity to aggregate.

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Sol-gel transition in DMSO/lysozyme/water mixtures

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A remarkably high viscosity has been induced in aqueous solutions of lysozyme by the addition of certain structurally related organic solvents, such as tetramethylurea (TMU), dimethylsulfoxide (DMSO), dimethylformamide (DMF), and hexamethylphosphortriamide. DMSO-induced gelation is observed in samples fulfilling the two following requirements: (1.) lysozyme concentration in excess of 5 mM, and (2.) volume fractions of DMSO exceeding 0.7. Based on spectroscopic data, the whole process was characterized as consisting of two mutually independent stages. The first involves an extensive transition of the polypeptide backbone, from a predominantly helical to increased random coiled and beta-sheet structures, with the occurrence of non-orthodox protein secondary structures at regions above the solvent critical point. The second stage consists of short-lived interchain contacts leading to an entanglement of the macromolecular system as a whole.
Here we present a set of scattering and microrheology experiments that investigate both the structural and dynamic properties of the gels under various experimental conditions.

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Deceleration/acceleration of Pi release with ramp stretch/release protocols in skinned rabbit fibres

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In vivo, skeletal muscle actively shortens and also resists lengthening, for example when landing after a jump. We study the energy during shortening and during lengthening by measuring the rate of Pi release which results from ATP hydrolysis.
We show here the rate of Pi releases in an in vitro protocol that involves muscle stretched followed by muscle shortening. It is hypothesised that the magnitude of deceleration of Pi release in the stretch phase is less than the acceleration during the release due the energy input of the motor that is used to apply the length changes. Therefore the end point of Pi release is similar to isometric conditions. Pi release responses to a ramped stretch (5%) followed 100 ms later by a symmetrical release at low velocities (0.5 L_0/s^{-1}) were measured in permeabilised fibre bundles of rabbit psoas at 20°C. Laser diffraction and high speed video were also recorded to confirm length change. We show that the rate of Pi release drops during the stretch phase, returns to isometric levels during the length hold phase, and finally accelerates during the ramped release. Tracking of sarcomere length change using video analysis demonstrated that laser diffraction is unreliable at times during stretches due to lack of uniformity of the sarcomere spacing.

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Monitoring the interaction of single G-proteins with rhodopsin disk membranes upon light-activation

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Heterotrimeric G-proteins interact with their G-protein coupled receptors (GPCRs) via key binding elements comprising the C-terminal segment of the α-subunit and the two lipid anchors at the α− and γ−subunit. Direct information about diffusion and interaction of GPCRs and their G-proteins is mandatory, as these properties will affect the timing of events in the complex signal transduction cascade. In the case of the photoreceptor rhodopsin, receptor packing in the membrane and the related diffusion coefficients are discussed controversially (1, 2). By using single particle tracking we show that the encounters of rhodopsin with the fluorescently labeled C-terminus of the α-subunit as well as with the Holo-G-protein transducin change upon rhodopsin light-activation. Our results indicate confined areas of interaction for the C-terminal segment of the α-subunit with inactive rhodopsin disk membranes and less restricted diffusion of the receptor-bound C-terminal segment after light-activation. This suggests dynamic short-range order in rhodopsin packing and specific structures for efficient interaction (3).

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O-277

Biosensing applications of micro/nano structured silicon

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Several topics related to porous silicon (PS) biosensing properties were carefully considered in this study. PS allows the increasing of the immobilised biomolecule number on its surface and the creation of stable covalent bonds due to its controllable chemistry. Beside this, PS is suitable for electrical (conductance, impedance), electrochemical and optical amplification of the detected signal. The experimental results on the fabrication of the PS microstructures such as: (i) protein immobilization and detection using microarray technique; (ii) DNA biomolecule detection by impedance or by fluorescence spectroscopy; (iii) very sensitive SERS biosensors (RAMAN signal of 11-mercaptoundecanoic acid); (iv) sensitive element for neurons in NutMix culture are in detail presented. Various characterisation techniques have been used, optical and scanning electron microscopy (SEM), X-ray diffraction, Raman, laser fluorescence and impedance spectroscopy for investigation molecule attachment on the Au/PS structures. We have demonstrated that different morphologies of PS as-prepared or coated with gold nanoparticles have an important role in biomolecule/cell detection, due to its large internal surface combined with specific optical properties, being in the same time sensing element/support for immobilization of sensing biomolecules as well as transducer for biochemical interactions.

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Distribution of pigments in ciliates by multidimensional confocal fluorescence microscopy

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The colored ciliates *Blepharisma japonicum* and *Fabrea salina* show photomotile responses triggered by endogenous pigments of the hypericin family. *B. japonicum* exists in two forms: the wild one contains red blepharismin; the other one, generated by irradiating the cells with dim visible light, blue blepharismin. *B. japonicum* shows step-up photophobic response, whereas *F. salina*, that contains fabrein, shows step-down photophobic responses and positive phototaxis.
We showed by confocal microscopy that the pigments are localized not only in pigment granules, but also in the cilia. This fact implies that the structure of the pigment in the cilia differs from that in the cell body, (pigment granules are too big to fit in the axoneme) and suggests that ciliary pigments might play a decisive role in photoreception.
Fluorescence Lifetime Imaging Microscopy (FLIM) shows that there is a spatial distribution of lifetimes, which are shorter for the pigment in the cilia. This might indicate a functional role for these pigments. Furthermore, lifetimes for *F. salina* are always longer than those of *Blepharisma*. In order to further characterize the structure of ciliary pigments by means of spectroscopic methods, we have also performed spatially resolved static and time-resolved fluorescence anisotropy measurements.

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– Photosensory biophysics –

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Light perception by phototropin studied with time-resolved infrared spectroscopy

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The blue light receptor phototropin regulates the growth of plants towards the light. It contains two light-, oxygen-, or voltage-sensitive (LOV) domains and a kinase domain. LOV domains bind noncovalently flavin mononucleotide (FMN) as chromophore. Upon illumination, the triplet excited state of flavin reacts within few microseconds with a nearby cysteine under formation of a photoadduct, which represents the signaling state. In response to adduct formation, a Jα helix adjacent to the LOV2 domain dissociates and allows for autophosphorylation by the kinase domain. The mechanism of the photoreaction and the signaling pathway from FMN to the Jα helix are still unclear. We have investigated the LOV2 domain of *Arabidopsis* phototropin 2 by microsecond FT-infrared spectroscopy. The difference spectrum recorded at 2 μs provides evidence that the flavin is unprotonated in the triplet excited state. Therefore, a previously proposed ionic mechanism of bond formation is disfavored. Changes in secondary structure were detected concomitant with adduct formation that relax with a time constant of 120 μs. This early adduct intermediate has not been previously characterized. The final adduct state is formed in milliseconds by further alterations in secondary structure. These findings raise the question of whether the early or late adduct intermediate propagate the signal to the Jα helix.

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Calcium transport and phototransduction in isolated rod outer segments

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Ca^{2+} concentration in photoreceptor rod outer segment (OS) strongly affects the generator potential kinetics and light adaptation. Light stimuli may produce voltage changes exceeding 40 mV: since the OS Ca^{2+} extrusion is entirely controlled by the Na^+:Ca^{2+},K^+ exchanger (NCKX), it is important to assess how the NCKX ion transport is affected by voltage and intracellular factors. The NKCX regulation was investigated in whole-cell recorded OS, using ionic conditions that activated maximally forward and reverse exchange. In all species examined of amphibia and reptilia, the forward (reverse) exchange current increased about linearly for negative (positive) voltages and exhibited outward (inward) rectification for positive (negative) voltages. Since hyperpolarization increases Ca^{2+} extrusion rate, the recovery of the dark level of Ca^{2+} (and of the generator potential) after light stimuli results accelerated. Mg-ATP doubled the size of forward and reverse exchange current without modifying their voltage dependence, indicating that Mg-ATP regulates the number of active exchanger sites and/or the NCKX turnover number. Ca^{2+} jumps achieved via photolysis of caged-Ca^{2+} produced current transients, possibly originating from electrogenic partial reactions. No monovalent cation substituted for Na^+ at the NCKX binding sites, but Rb^+ substituted for K^+, while Sr^{2+}, Ba^{2+}, Mg^{2+} substituted for Ca^{2+} with an apparent permeability ratio of 0.78, 0.20, <0.05:1, respectively.

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Gold colloids-fluorophore complexes for protein detection assay

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Noble metal nanoparticles (NP) are endowed with peculiar optical properties related to the surface plasmon resonances (SPR). The interaction of surface plasmons of gold nanoparticles with fluorophores a few nanometers away from the surface modifies their brightness and excited-state lifetime, and this effect can be exploited to obtain nanodevices for protein-protein recognition. We studied different types of constructs based on gold NPs on which derivatives of fluorescein were bound. The interaction of this fluorophore with the gold surface plasmon resonances, mainly occurring through quenching, affects its excited-state lifetime, that is measured by fluorescence burst analysis in standard solutions. The binding of proteins to the gold NPs through antigen-antibody recognition further modifies the dye excited-state lifetime. This change can therefore be used to measure the protein concentration. Streptavidin-functionalized gold NPs of size 5-10 nm are used to bind biotin-fluorescein and biotin-antibodies for specific proteins. We have first tested the constructs for bovine serum albumine (BSA) detection. The data reported here indicate that one can measure the concentration of BSA in solution with an apparent limit of detection of 5±2 pM. We have then extended the study to the antitumor protein P53 - P53 antibody interaction in standard solution and directly in cellular extracts.

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Microbial rhodopsins: receptors, channels, and pumps from a single design

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The microbial rhodopsin family is comprised of ∼5000 homologous proteins containing 7 transmembrane helices forming a pocket for the chromophore retinal. Most are light-driven ion pumps ("transport rhodopsins") and others are photosensory receptors ("sensory rhodopsins"). Phylogenetic analysis indicates frequent lateral gene transfer of proton pumps among prokaryotic and unicellular eukaryotic species, followed by coupling of the pump's mechanism to the cell's existing signal transduction machinery to create photosensors. This evolutionary path is strongly supported by our studies of sensory rhodopsins in various organisms which demonstrate remarkably diverse signaling mechanisms with diverse transducer partners. The best studied are the phototaxis receptors in haloarchaeal prokaryotes (SRI and SRII) and in eukaryotic algae (channelrhodopsins). SRI and SRII transmit signals by protein-protein interaction to control a phosphorylation cascade that modulates motility. Channelrhodopsins are light-gated cation channels that depolarize the membrane mediating calcium ion influx into the flagellar axoneme. Crystallography and molecular biophysics have begun to clarify how modifications of the same architecture enable the rhodopsins to carry out their distinctly different molecular functions. Interconversions of their functions by mutation reveal the elegant simplicity by which evolution uses existing genes to create proteins with novel functions.

Abstracts
– Single molecule biophysics –

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Fluorescent voltammetry of single redox proteins

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We report a detection method for the redox state of proteins which combines FRET-based fluorescence/confocal microscopy on dye-labeled protein with cyclic voltammetry. By using this combined method, electron transfer properties can be revealed from protein to electrode or from redox enzyme to substrate. We applied the fluorescent detection to azurin, a blue copper protein from the bacterium Ps. aeruginosa, fluorescently labeled on the N-terminus for monitoring the redox state of the protein. The dye fluorescence is quenched by energy transfer to the copper in oxidized, but not in reduced azurin.
Fluorescence results demonstrated that Cy5-labeled wt-azurin switched in fluorescence intensity by up to 85% by varying the applied potential. Labeled zinc-azurin was used as a control sample and did not show any fluorescence switching.
For single molecule studies wt-azurin was labeled with ATTO 655 dye and a mixed SAM was used, with 8-Hydroxy-1-octanethiol as a blocking agent to prevent non-specific binding of protein on the surface, and 1,10 decanedithiol for the specific covalent binding to the protein. Preliminary data show that single-molecule fluorescence switching with the potential is indeed possible.

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Effects of pH on transport properties and ionic selectivity of amphotericin B-induced channels

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Amphotericin B (AmB) is an antifungal antibiotic which, despite the severe side effects, is still used for the treatment of systemic fungal infections. In this study we investigated the influence of pH upon the selectivity and the transport properties of AmB channels inserted in reconstituted, ergosterol-containing zwitterionic lipid membranes. Our electrophysiology experiments carried out on single and multiple AmB channels prove that at pH=2.8 these channels are anion selective, whereas at neutral and alkaline pH's (pH=7 and pH=11) they become cation selective. We attribute this to the pH-dependent ionization state of the carboxyl and amino groups present at the mouth of AmB molecules. Surprisingly, our data reveal that the single-molecule ionic conductance of AmB channels varies in a non-monotonic fashion with pH changes, which we attribute to the pH-dependent variation of the surface and dipole membrane potential. We demonstrate that when added only from one side of the membrane, in symmetrical salt solutions across the membrane and low pH values, AmB channels display a strong rectifying behavior, and their insertion is strongly favored when positive potentials are present on the side of their addition.

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Light scattering study of DNA over a wide chain length range: comparison with wormlike model

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This work reports light scattering measurements on DNA in aqueous solutions (100 mM NaCl ,1mM EDTA and 10 mM Tris-HCl buffer, pH 7.8) over a wide range of molecular weights (10^2-10^5 base pairs) and shows that, in the above standard solvent, shorter chains ($< 10^4$ base pairs) behave as a "wormlike chain" and their diffusion coefficient as obtained by dynamic light scattering measurements, confirm the prediction of standard wormlike model, whilst longer chains ($>10^4$ base pairs) behave in a different manner. Dynamic and static light scattering and SEM analysis indicate that DNA molecules 10^5 base pairs long, condense into compact structures in our solvent condition. Calculations done using a wormlike model are also presented and discussed in comparison both to our experimental data and to other data reported in the literature.

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The psychostimulant amphetamine increased NO generation measured by EPR as well as amino acid release in the rat brain

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Nitric oxide (NO) is a novel messenger that modulates many functions of the nervous system. The involvement of NO in brain damage was shown mainly by indirect evidence and the data are controversial. The short half-life of NO makes its direct detection difficult. We measured NO generation using EPR spectroscopy based on determination of the amount of paramagnetic mononitrosyl-iron complexes. The aim was to elucidate whether psychostimulant drug amphetamine (AMPH) modulates formation of NO and lipid peroxidation (LPO) products as well as the neurotransmitter release in rat brain. The output of glutamate, aspartate, GABA and acetylcholine (ACH) was monitored in striatum by microdialysis with HPLC detection. AMPH produced 2-fold elevation of NO generation and LPO formation in brain areas. While AMPH increased the aspartate, GABA and ACH release, the glutamate output was not affected. Pretreatment with the neuronal NOS inhibitor was highly effective in abating the rise of NO and neurotransmitter levels but failed to influence the LPO intensity elicited by AMPH. The findings suggest that activation of NO synthesis is a potent factor in the AMPH-induced neurotransmitter release.

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– Single molecule biophysics –

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CC/PBSA: Prediction of mutational effects on protein-protein binding affinity and protein stability

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Modifying the stability or the binding behavior of the involved proteins by mutation can influence the activity of cellular processes. For an efficient identification of possible mutation-sites a fast calculation of the free energy of proteins is crucial.
Here we developed a fast and reliable method (CC/PBSA) [1] for the prediction of the change in stability of proteins and binding affinity of protein-protein complexes upon mutation. The energy function of CC/PBSA is based on gas phase energies, solvation free energies and entropic contributions. The protein flexibility is taken into account by generating random conformations based on geometrical constraints only applying the CONCOORD[2] program.
We applied CC/PBSA on the TEM1-BLIP complex, which is important in bacterial antibiotic resistance. The results of single- and double-point alanine scanning are used to detect hot spots, cooperative effects, and the corresponding energy distribution.
CC/PBSA is freely accessible on our web-server: `http://ccpbsa.bioinformatik.uni-saarland.de`

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[2] de Groot B. L. et al., **Proteins** 29 (1997), *240-251*.

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Unwinding the DNA helix in force clamp condition

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We use a dual-laser optical-tweezers (DLOT, Smith et al., *Science*, 1996) to define the highly cooperative conformational transition in the molecule of DNA, where the natural B-DNA has converted into a new overstretched conformation called S-DNA (Bensimon et al., *Phys. Rev. Lett.*, 1995; Cluzel et al., *Science*, 1996). Single molecules of double stranded λ-phage DNA (in a solution with 150 mM NaCl, 10 mM tris-HCl, 1 mM EDTA, pH 8.0. and 27 °C) are stretched either in length clamp or in force clamp mode. When the DNA molecule is stretched in length clamp mode with a ramp lengthening, it shows the previously described highly cooperative overstretching transition at ∼60 pN, attributed to unwinding from the B-form to the 1.7 times longer S-form. Stretching the molecule in force clamp mode with a staircase of force steps at 5 s intervals (step size 1-2 pN, rise time 1-2 ms) shows, for any given clamped force F in the region of the overstretching transition, different amounts of DNA elongation (ΔL) with exponential time courses. The analysis of the elongation rates allows to recover all the necessary parameters for an effective two-state model able to reproduce the out-of-equilibrium properties of the system. The results imply an unwinding cooperativity of 27 bps. This value is significantly lower than that obtained assuming force independent rate constants. Supported by MiUR, Ente Cassa di Risparmio di Firenze and ITB-CNR (Milano).

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Nanoscopic and spectroscopic investigation of p53-based complexes at single molecule level

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p53 is a transcription factor that plays a widely recognized role in preventing cancer development in response to DNA damage. The tumor suppressor activity of p53 involves the formation of several complexes whose detection and study, at single molecule level, could be extremely relevant to understand, in detail, the mechanisms governing the cancer defense processes, as well as to develop ultrasensitive biosensors. p53-based complexes, are investigated at molecular level, by combining Atomic Force Microscopy (AFM), Atomic Force Spectroscopy (AFS) and Surface Enhanced Raman Spectroscopy (SERS) with the support of computational docking. Our attention is mainly devoted to study complexes between p53 and the electron transfer azurin which has been demonstrated to interact with p53, by promoting its stabilization. A possible competition between azurin and the cellular oncogene mdm2 is also investigated.

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Is there a specific erythrocyte membrane receptor for fibrinogen? An atomic force microscopy approach

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Fibrinogen (Fg) contributes to erythrocyte (RBC) hyperaggregation by an increase in. RBC-Fb protein binding considered to be non-specific. Glycoprotein $\alpha_{IIb}\beta_3$ is a specific integrin receptor for fibrinogen on platelets. We showed that there is a single molecule interaction between Fg and an unknown receptor on RBC membrane, with a lower affinity when compared with platelet binding. We evaluated if RBC-Fg binding is through an integrin-like receptor or not. Interactions between Fg and platelet/RBC receptors were studied by force spectroscopy. Force curves were performed between Fg-functionalized atomic force microscope tips and RBC or platelets. To evaluate if the Fg-RBC binding is calcium-dependent, similar studies were performed in the presence of Ca^{2+} or EDTA. We also carried out studies in the presence of a $\alpha_{IIb}\beta_3$ inhibitor and of methyl-ß-cyclodextrin (to disrupt lipid rafts by cholesterol depletion). RBC-Fg single forces were of 300-380 pN and of 400-480 pN for platelet-Fg binding in presence of calcium. A significant decrease of the platelets-Fg force-rupture was obtained in the presence of EDTA or $\alpha_{IIb}\beta_3$ inhibitor. Significant lower Fg-RBC force value was obtained in the presence of MβCD, but not in the presence of EDTA. Conclusion: Fg-RBC binding seems not to be calcium-dependent but the existence of cholesterol on RBC membrane is important.

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Helixlike pili is a prerequisite of uropathogenic *E. coli* to adhere to host and withstand urine flow

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Many infection processes start with primary adhesion of pathogenic bacteria to host cells. The Gram-negative uropathogenic *Escherichia coli* (UPEC) bacteria, invades the urinary tract region and cause in some cases severe infections, pyelonephritis, if they can withstand the rinsing action of urine and ascend to the kidney, via the bladder and ureters. To mediate adhesion, UPEC express quaternary surface organelles that are assembled from $\sim 10^3$ identical subunits into a helix-like coil, with a single adhesin located at the tip. It is believed that the single adhesin mediate attachment to host cells while the helix-like structures act as shock absorbers to dampen the irregularly shear forces induced by urine flow. To unravel the biomechanical properties of such quaternary structures, in particular in terms of their force-elongation and kinetic behavior, Force-Measuring Optical Tweezers (FMOT) have been used. A plethora of different types of pili have been identified in the literature and we show, using FMOT, that those dissimilarities might reflect the host environment. For example, we have found differences among pili expressed at diverse environment inside the urinary tract, which imply that pili presumably have evolved to resist specific forces under *in vivo* conditions. It is thus worth striving for understanding bacterial adhesion in order to figure out alternative to the over-abundance of antibiotics worldwide.

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Optical and electrophysiological detection of single phages across a lipid membrane

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We present an investigation study of the ejection of single T5 bacteriophages. In vivo studies of DNA ejections from the bacteriophage capsid show that the T5 genome is introduced in the bacterial host in two steps. First 8% of the genome is ejected then after a pause of a few minutes the rest is internalized. Bulk in vitro studies showed that various mutants of T5 eject their genome in solution following a single or a multistep process. By immobilizing single bacteriophages on a surface and following their ejection by fluorescence microscopy we showed that in all cases the ejection occurs in one step, but some mutants seem to have a subpopulation for which the triggering signal of the ejection is transmitted more slowly to the capsid entrance. We then reconstituted the phage receptor FhuA into giant liposomes and followed the ejection of the DNA into the liposome by fluorescence. Finally we incorporated FhuA in a suspended bilayer and followed the infection of the phages through the bilayer both by fluorescence labeling and electrophysiological measurements. We will discuss the influence of the cross membrane potential on the ejection speed of the DNA.

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Biofunctional micropatterned surfaces to study the spatio-temporal organisation of LFA-1

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Lymphocyte function associated antigen-1 (LFA-1) adhesion depends on receptor occupancy and lateral organization on the cell membrane. However, the signals and mechanisms which dynamically reorganize LFA-1 into high avidity clusters are still a subject of many studies. To obtain deeper insight on the mechanisms that control and regulate LFA-1 clustering, patterned surfaces of immobilized LFA-1 ligand areas were fabricated using microcontact printing.
The diffusion of LFA-1 expressed by monocytes stretched over patterned surfaces was followed in time using single molecule TIRF microscopy. Single LFA-1 nanocluster trajectories on individual cells showed an increase of immobile LFA-1 fraction and a slow-down of diffusing LFA-1 on the ligand areas compared to the non-ligand areas. Moreover, single-cluster intensity analysis indicated a reorganization of LFA-1 nanoclusters in microclusters upon ligand binding. Finally, single particle motion analysis of LFA-1 trajectories in close neighborhood to the ligand areas showed no assisted diffusion of LFA-1 towards the adhesive regions, consistent with random ligand-encountering and binding. We are currently investigating the effect of cell membrane organizers to regulate the spatio-temporal organization of LFA-1.
R. Diez-Ahedo et al, Small, in press.

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Direct observation of twisting steps during Rad51 polymerization on DNA

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The human recombinase hRad51 is a key protein for the maintenance of genome integrity and for cancer development. This protein plays a central role is the DNA strand exchange occurring during homologous recombination. Here we report the polymerization and depolymerization of hRad51 on duplex DNA observed with a new generation of magnetic tweezers, allowing the measurement of DNA twist with a resolution of 5° in real time. At odds with earlier claims, we show that, after initial deposition of a multimeric nucleus, nucleoprotein filament growth occurs by addition of single proteins, involving DNA twisting steps of 65±5°. Simple numerical simulations support that this mechanism is an efficient way to minimize nucleoprotein filament defects. This behavior, consisting of different stoichiometry for nucleation and growth phases, may be instrumental in vivo. Fast growth would permit efficient continuation of strand exchange by Rad51 alone while the limited nucleation would require additional proteins such as Rad52, thus keeping this initiation step under the strict control of regulatory pathways. Besides, our results combined with earlier structural information, suggest that DNA is somewhat less extended (4.5 versus 5.1 Å per bp) and more untwisted (18.2 versus 15 ° per bp) by hRad51 than by RecA, and confirm a stoichiometry of 3-4 bp per protein in the hRad51-dsDNA nucleoprotein filament.

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Elucidation of the mechanism of the lambda bacteriophage epigenetic switch

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The lambda bacteriophage epigenetic switch determines the growth lifestyle of the virus after infection of its host (*E. coli*). It is now clear that the switch consists of a ∼2.3 kbp-long DNA loop mediated by the lambda repressor protein. Using tethered particle microscopy (TPM), magnetic tweezers and AFM, our laboratory has novel, direct evidence of loop formation and breakdown by the repressor, the first characterization of the thermodynamics and kinetics of the looping reaction and its dependence on repressor non-specific binding and DNA supercoiling. These *in vitro* data provide insight into the different possible nucleoprotein complexes and into the lambda repressor-mediated looping mechanism which leads to predictions for that *in vivo*. The significance of this work consists not only of the new insight into a paradigmatic epigenetic switch that governs lysogeny vs. lysis, but also the detailed mechanics of regulatory DNA loops mediated by proteins bound to multipartite operators and capable of different levels of oligomerization.

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Single Particle Tracking: towards understanding the influence of the probe using a model system

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Two well-known methods for measuring dynamical properties in lipid membranes are Fluorescence Recovery After Photobleaching (FRAP) and Single Particle Tracking (SPT). FRAP probes the average dynamical properties of a population of fluorescently labeled molecules whereas SPT obtains these properties from observations of the trajectories of individual molecules tagged with a colloidal particle (the "bead"). When results of FRAP are compared with those of SPT, FRAP yields significantly higher diffusion constants than SPT. To understand the origin of this difference, we have developed and tested a model system to evaluate the influence of the bead on the dynamics of a diffusing molecule. We use a dsDNA tether to attach a bead to a supported lipid bilayer (SLB). The DNA tether is modified at one end by cholesterol for anchoring to and diffusion in the membrane, and at the other end by biotin for tagging with a bead. With this system, we can vary several parameters: distance between SLB and bead, size and nature of the bead, lipid composition of the SLB, external force. We can also model the Brownian motion of the bead and the hydrodynamic flow around it. We will present results using $\phi = 200$ nm neutravidin-coated latex particles attached to an eggPC SLB via a 15 bp to 90 bp DNA tether.

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Simulations of single-molecule FRET experiments on ribozymes

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RNAzymes are important biological molecules and that can catalyze the cleavage of their own nucleotide chains. This precess is called *self splicing* [1]. There are several methods to to study kinetic properties of ribozymes at the single-molecule level. In this work we focus on FRET (Förster Resonance Energy Transfer) of single, immobilized molecules. Single-molecule fluorescence spectroscopy gives an insight into the behavior of individual molecules rather than the average behavior of an ensemble of molecules, which makes it possible to observe the kinetic heterogeneity.
We simulated FRET trajectories for single immobilized ribozymes. In our computer simulations we consider two- and three-state kinetic models motivated by actual experiments, published in [2] and [3]. We fitted the histograms of *on* and *off* times to recover the kinetic parameters of the models. We investigated the bias and standard deviation of the recovered parameters when one or two thresholds are applied to FRET trajectories between adjacent states. We found that two thresholds give better parameter estimates than one-threshold analysis.

References:
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2. S.Liu, G.Bokinsky, N.G.Walter, X.Zhuang, *PNAS*, 2007, **104**, 12634
3. M.A. Ditzler, E.A. Alem'n, N.G. Walter, *Biopolymers*, 2007, **87**, 302

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Liposomes as scaffold for controlling the environment of single enzymes New insights in old premises

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Single molecules studies have probed protein conformational fluctuations and monitored the oscillating activity of enzymes that remained masked in ensemble measurements. Various statistical models have attempted to connect the conformational motions to the fluctuating activity of enzymes and suggested that they adopt inter-converting conformations each one of them exhibiting different catalytic activity. The main drawback of these studies is the non specific interactions that may bias the observed catalytic behavior.
We have developed new innovating tools to study the behavior of single enzymes. As a first step we utilized liposomes as scaffold to confine enzymes while keeping them under physiological conditions. Keeping them in their native conformation allowed us to monitor the inherent properties of their behavior. As a second step we developed a new model that accurately connects enzyme activity behavior to conformational motions. Using this approach we accurately predicted the behavior of single lipases in a highly controlled environment. We modulated the enzyme's conformational mobility and activity by systematically varying its accessibility to liposomes. As we anticipated, that resulted in altering the time the enzyme spends on active conformations.Our results provide new insights on interpreting the behavior of enzymes.

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– Single molecule biophysics –

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Single molecule force spectroscopy and recognition imaging

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In single molecule force spectroscopy, interaction forces of ligand-containing tips with receptors on probe surfaces are quantified. Here he attachment of human rhino virus 2 (HRV2) to the cell surface, the first step in infection, was characterized. Sequential binding of multiple receptors was evident from recordings of characteristic quantized force spectra. This suggests that multiple receptors bound to the virus in a timely manner. Unbinding forces required to detach the virus from the cell membrane increased within a time frame of several 100 ms. The number of receptors involved in virus binding was determined and estimates for on-rate, off-rate, and equilibrium binding constant were obtained. Furthermore, we show that accurate free energy values of membrane protein unfolding can be obtained from single molecule force measurements. By applying a statistical theorem developed by Jarzynski, we derived equilibrium unfolding free energies of from unfolding force data acquired at different force loading-rates and temperatures. Finally, we present a method for the localization of specific binding sites and epitopes with nm positional accuracy. A magnetically driven AFM tip containing a ligand covalently bound via a tether molecule is oscillated at a few nm amplitude, during scanning along the surface. In this way, topography and recognition images on membranes and cell surfaces were obtained simultaneously.

P-300

Magnetic contrast neutron reflectivity delivers significant improvements in resolution for membrane structure analysis

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Neutron reflection (NR), with its high resolution and use of contrast variation, is a unique tool to gain information on the orientation and structure of lipid bilayers in the z-axis perpendicular to the surface.
To improve the use of NR we have used a highly oriented and stable layer of membrane proteins and lipids made possible by self-assembly upon a gold surface. This also provides a model of the bacterial outer membrane. As the dimensions of the layer can be predicted with accuracy, the system provides a molecular ruler for improvements in methodology and the complexity of the layer structure adds significantly to the modelling challenge. Early improvements in resolution were obtained through sample preparation and gold smoothness. Further improvement however required clearer discrimination between similar models. This was achieved using the new approach of magnetic contrast variation which uses a magnetic layer to provide two different scattering length densities for oppositely polarised neutrons. During this data collection the sample is unchanged. We show that this approach delivers significant improvements in data analysis and resolution of the protein, lipid and solvent structures. The method will provide a new level of understanding of membrane structure and dynamics.

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Single centrosome manipulation reveals its electric charge and associated dynamic structure

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We demonstrate laser manipulation of individual early *Drosophila* embryo centrosomes in between two microelectrodes to reveal that it is a net negatively-charged organelle with a very low isoelectric region (3.1 ± 0.1). From this single-organelle electrophoresis, we infer an effective charge smaller or of the order of 10^3 electrons, which corresponds to a surface-charge density significantly smaller than that of microtubules. By investigating the centrosome hydrodynamic behavior, we show that its charge has a remarkable influence over its own structure. Specifically, we find that the electric field which drains to the centrosome expands its structure to a physiological size a 60% larger than previous electron microscopy determinations, a self-effect which modulates its structural behavior via environmental pH. This methodology further proves useful to study the action of different environmental conditions, such as the presence of Ca^{2+}, over the thermally-induced dynamic structure of the centrosome.

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A single-molecule mechanical assay to study DNA replication coupled to DNA unwinding

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Mechanical force at the molecular level is involved in the action of many enzymes. For example, the Phi29 DNA polymerase mechanically unwinds the DNA helix as it moves processively along the DNA replicating one strand of the DNA molecule. Using Optical Tweezers we have developed a single molecule mechanical assay to elucidate the physical mechanism of DNA unwinding by the Phi29 DNA polymerase as the protein replicates the DNA. A single DNA hairpin is hold between an optical trap and a mobile surface. As a single polymerase works on the DNA hairpin, its replication and unwinding reactions can be measured in real time by measuring the change in extension in the DNA polymer, revealing the fluctuations of its rate in response to the DNA sequence. Moreover, by gradually pulling on the opposite strands of the DNA hairpin we can promote the controlled mechanical unwinding of the DNA helix and determine the effect of increasing unwinding forces on the polymerization and unwinding rates. The effect of force and DNA sequence on the activities of the wild type and an unwinding-deficient polymerase mutant will allow us to determine the inner workings of this molecular motor.

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Control of the translocation of single DNA molecules through alpha-hemolysin nanopores

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The analysis of single nucleic acid molecules by electrophoretic threading through nanopores is under intense investigation as a rapid, low cost platform for DNA sequencing. Biological nanopores such as staphylococcal alpha-hemolysin (HL) have an added advantage over solid-state nanopores because they can be modified by genetic engineering with atomistic precision. Although we showed that all four DNA bases can be identified in an immobilized ssDNA molecule (1), the translocation of free DNA is too quick to observe single bases. Here we show that by a small increase of the net internal charge of the HL nanopore (e.g. by introducing a ring of 7 arginine residues), we have augmented the frequency of DNA translocation events through the pore and dramatically lowered the voltage threshold required for DNA translocation (2). By further increasing the net positive charge of the transmembrane barrel region of the pore (e.g. by introducing 49 extra positive charges) we have also reduced the speed at which DNA translocate the pore by more than two orders of magnitude.
These experiments provide a means of controlling DNA translocation with protein nanopores, which might be translated to solid-state nanopores by using chemical surface modification.

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(2) Maglia, G.; *et al. PNAS USA* **2008**, *105*, 19720-5.

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Force measurement study of the interaction between 23S rRNA and the ribosomal protein L20

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Single molecule force measurements enable to probe the complex structure and folding dynamics of RNA molecules and furthermore to investigate the numerous interactions with proteins that affect RNA folding in vivo. We use a double optical tweezers setup to study a region of the ribosomal RNA 23S from E.Coli and its interaction with the essential ribosomal protein L20. The apparatus permits us to probe the dynamics of the RNA structure with milisecond time-resolution. First, we pull the RNA and show that it mechanically unfolds in several reproducible steps. We use these results in combination with structure prediction tools to evaluate the possible structures of the RNA fragment in vitro. The most probable structure exhibits a unique binding site for L20. Then, force measurements are done on the RNA in presence of L20. We show that the protein specifically binds the RNA and stabilizes it. The binding site is recognized with a resolution of a few bases. Finally, two bases are mutated on the RNA fragment. In presence of these mutations, in vivo and in vitro binding of L20 to 23S RNA is abolished. The single molecule approach gives an explanation of this result: the force measurements show that the mutated RNA folds differently from the natural one and that it does not bind L20.

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Population shift effects of Adenosine kinase upon inhibitors binding

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Large scale domain motions are structural rearrangements often adopted by enzymes to achieve their full functionality. Understanding the mechanism responsible for such movements by means of computational approaches is of great interest especially in rational drug design, since induced fit or population shift effects are closely related aspects of the problem. Unfortunately, the simulation time required to sample these events by conventional techniques such as plain molecular dynamics, is unfeasible. Here, the domain motion required by Adenosine kinase (AK) to achieve its (pre-)catalytic conformation was studied using well-tempered metadynamics [Barducci, A. et al. *Phys Rev Lett.* (2008), **100**: 020603] with path collective variables (PCVs) [Branduardi, D. et al. *J Chem Phys.* (2007), **126**: 054103]. First, a low energy path for the *apo* form of the enzyme was obtained, and the potential of mean force along the PCVs was reconstructed. Then, the large scale movement was simulated in the presence of two inhibitors known to bind to the enzyme in different conformational states. The adopted approach was proven to be successful both to understand the mechanistic features of the AK domain motion and to provide a picture of the population shift effects upon ligand binding.

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Inferring maps of forces inside cell membrane microdomains

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Mapping of the forces acting on biomolecules in cell membranes has spurred the development of effective labels, e.g. organic fluorophores and nanoparticles, to track trajectories of single biomolecules [1]. Standard methods use particular statistical observables, namely the mean square displacement (MSD), to extract cues on the underlying dynamics. Yet, MSD is not an appropriate tool to access force fields and becomes easily a biased estimator in the presence of positioning noise. Here, we introduce **general inference methods** [2] to fully exploit information hidden in the experimental trajectories, providing sharp estimates for the forces and the diffusion coefficients within membrane microdomains. Rapid and reliable convergence of the inference scheme is demonstrated on trajectories generated numerically. The inference method is then applied to **infer forces and potentials** acting on the receptor of the ε-toxin labelled by lanthanide-ion nanoparticles. Results show a constant diffusivity inside a complex force field confining the receptor inside a specific domain. Our scheme is applicable to any labelled biomolecule, and results presented here show its general relevance to the issue of membrane compartmentation and protein motion.

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Abstracts
– Single molecule biophysics –

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Unzipping DNA with a nanopore

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The use of proteinaceous or artificial nanometer size pores has become a promising approach for sensing biomolecules at the single molecule level. It was shown that alpha-hemolysin, a toxin from *staphylococcus aureus*, can be employed to sense the translocation of DNA strands through a lipid bilayer.
The pore dimension allows the electrophoretically driven passage of single stranded DNA whereas double stranded structures need to open prior their translocation.
We study the unzipping process of the double stranded part of DNA both experimentally and theoretically. We show that in a first approximation the duplex opens progressively in a sequence dependent manner. The experimental results can be accounted for by modeling the unzipping process as a free diffusion of the unzipping fork in the energy landscape defined by the sequence of the basepaires. We then discuss the effect of the pore/DNA interaction on the translocation process. We further characterize the effects of the pore geometry, showing that the pre-confinement of the DNA in the pore vestibule is essential to the unzipping process.
We eventually discuss the possibility to use this nanopore approach to sequentially probe RNA secondary structures.

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A mathematical model of neurotransmission at the input stage of the cerebellum

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The granule cell (GC) of the cerebellum has some peculiar properties compared to other cells of the vertebrate brain. The GC has a small soma (diam=6 microm) and just a few (2 to 6) excitatory and inhibitory inputs (E&I). The E&I GC synaptic inputs are formed inside the cerebellar glomerulus, which favors neurotransmitter diffusion between neighboring sites (1) protracting postsynaptic receptor activation and (2) causing cross-tall between E&I synapses through presynaptic receptors. Neurotransmitter release probability (p) can be regulated by long-term plasticity (LTP and LTD at E synapses) and by $GABA_B$ and mGlu presynaptic receptors (both at E&I synapses). The p change in turn modifies short-term plasticity, affecting the first response in a train much more than the followings. To gain insight into the role that p changes might have in computations performed at the cerebellum input stage, we have built detailed biophysical models of the E&I synapses. The model has allowed to investigate glomerular processing of high frequency inputs reaching the cerebellum. Optimal synaptic transmission through the MFs inputs resulted when GOs inhibited synchronously the GCs. The role of feed-forward inhibition onto synaptic transmission is under investigation.

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Velocity, processivity and individual steps of single myosin V molecules in live cells

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We report the tracking of single myosin V molecules in their natural environment, the cell. Myosin V molecules, labeled with Quantum Dots, are introduced into the cytoplasm of living HeLa cells and their motion is recorded at the single molecule level with high spatial and temporal resolution. We perform intracellular measurements of key parameters of this molecular transporter: velocity, processivity, step size and dwell time. Our experiments bridge the gap between in vitro single molecule assays and the indirect measurements of the motor features deduced from the tracking of organelles in live cells.

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Monte Carlo simulations of single-molecule pulling experiments

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The atomic force microscope (AFM) is a tool for imaging, measuring and manipulating matter at the nanoscale. Single-molecule pulling experiments give information on the thermodynamics and kinetics of biomolecules.
The purpose if this work was to develop software to simulate single-molecule pulling experiments and to analyze single-molecule pulling data. The long term objective is to asses the accuracy and precision of the parameters recovered from single-molecule pulling data.We carried out simulations for two different models [1,2,3], using a wide range of loading rates. From the simulated force-extension curves we extracted the kinetic parameters and compared them with the values used for simulations. The kinetic parameters were the intrinsic rate coefficient (k_0 Kramers rate), the location of transition state (x) and the free energy of activation (ΔG). We have found that the loading rates have a small effect on the recovery of the free energy of activation, but have a significant effect on the recovery of the Kramers rate.
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Abstracts
– Single molecule biophysics –

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Uracil-DNA glycosylase (UNG) interacting with mismatched DNA – a single-molecule approach

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Genetic information coded in DNA provides complete instructions to cells regarding metabolism and proper functioning. A number of endogenous and exogenous sources can damage the genomic DNA. Unrepaired DNA damage can give rise to mutations and may cause cell death. Cells have evolved different mechanisms to repair damaged DNA and to protect genetic integrity. Uracil in DNA occurs as a result of incorporation of dUMP in the place of dTMP during replication and deamination of cytocine, resulting in U:A and U:G base pairs, respectively. Uracil-DNA glycosylase (UNG) is a DNA repair protein that searches and removes uracil from DNA. UNG removes mismatched base by flipping it out from the base stack into the active site. The exact mechanism by which UNG searches DNA for uracil is unknown. Therefore, in our study we use Atomic Force Microscopy (AFM) to investigate the interaction between single UNG molecules with DNA carrying the U:A or U:G mismatches. Furthermore, we study the complexation of DNA and UNG protein. AFM images of UNG bound to DNA reveal the structural changes at the level of single complex, e.g. DNA kinking that may occur upon binding of UNG to DNA. Imaging of DNA-protein complexes can provide a a new level of understanding of UNG-DNA interaction.

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In-vitro assembly of Polyoma VP1

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One essential element of a virus is its protein shell, the viral capsid, which encloses the viral genome. The murine Polyomavirus is a non-enveloped DNA tumor virus with an icosahedral T=7d structure. Besides the knowledge of the structure, it is of utter importance to understand the process of viral assembly [1]. The assembly reaction of Polyoma VP1 does not show the typical sigmoidal kinetics in light scattering experiments. The apparent kinetics is of fourth order, which appears rather unrealistic. In order to gain knowledge on capsid composition during assembly beyond ensemble average, we apply methods of single molecule fluorescence, namely fluorescence correlation spectroscopy (FCS), fluorescence-intensity-distribution-analysis (FIDA), and single-particle-imaging (SPI).
The molecular brightness and the diffusion time reported by FCS are in agreement with the results from light scattering. FIDA as well as SPI, moreover, point to a polymerization of subunits to complete capsids along the assembly pathway without pronounced intermediates.
Mixing experiments show that already early in the course of the assembly reaction no exchange of pentamers between capsids occurs, and that the effect of Breathing [2] can be excluded for Polyoma VP1.

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O-313

The rate of topoisomerase II activity correlates with persistence length of DNA

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Type II topoisomerases catalyze the transection of one double helical segment by another to modify the topological state of DNA. Reversible 5' linkages to the phosphate-sugar backbones are established on each strand of the "gate" segment to create an opening through which the enzyme drives the "transfer" segment. A recent crystal structure shows that the "gate" segment bends approximately 150° upon binding to the enzyme. Bending a stiff polymer like DNA requires considerable energy and could represent the rate limiting step in the catalytic (topological) cycle. Using modified deoxyribonucleotides in PCR reactions, more rigid DNA fragments have been produced and used as substrates for topoisomerase II-mediated relaxation of plectonemes introduced in single molecules using magnetic tweezers. Before relaxation the persistence lengths were measured by fitting force extension data with the worm-like chain model. Topoisomerase II was found to release mechanically introduced supercoils more slowly in stiffer DNA suggesting that DNA bending might be a rate limiting step in topoisomerase II activity.

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Single-molecule pair interactions of porcine submaxillary mucin with soybean agglutinin

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We report on the interaction forces in the range 100 -- 400 pN determined between pairs of the lectin soybean agglutinin (SBA) and a modified porcine submaxillary mucin (Tn-PSM) using a single-molecule approach. Lectins are carbohydrate-binding proteins with biological activities related to i.e. cellular recognition, adhesion, growth and metastasis. Here, dynamic force spectroscopy is used to investigate pairs of SBA and Tn-PSM with the aim to understand the mechanisms of binding and cross-linking of multivalent lectins. The unbinding force increased from 103 pN to 402 pN with increasing force loading rate and the lifetime of the complex in the absence of applied force was 1.3 – 1.9 s. Published kinetic parameters describing the rate of dissociation of other sugar lectin interactions are in the range 3.3 x 10^{-3} – 2.5 x 10^{-3} s. The long lifetime of the SBA – TnPSM complex is compatible with a previously proposed "bind and jump" mechanism. This mechanism has also been suggested for lectins binding to multivalent carbohydrates and globular glycoprotein. The bind and jump mechanism is also similar to that observed for binding of proteins to DNA, and suggest a common conserved binding mechanism of ligands to the two biopolymers and possibly between ligands and all biopolymers, as recently suggested.

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Direct observation of rotation of F1-ATPase from Saccharomyces cerevisiae with mgi mutations

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Mitochondrial Genome Instability (*mgi*) mutations allow yeast to survive the loss of mitochondrial DNA. A number of these mutations occur in the genes encoding the F_1 portion of the ATP Synthase, and have been shown to uncouple ATP Synthase (*Wang et al.* 2007). The mutations cluster around the collar region of F_1 where the alpha, beta and gamma subunits interact and are thus likely to affect F_1 rotation. Single molecule studies of the thermophilic *Bacillus* PS3 F_1-ATPase have revealed kinetic and structural information that cannot be discerned using other methods, including the presence of 40 and 80 degree physical substeps (*Yasuda et al.* 2001) and the order and kinetics of chemical substeps. We are interested in using single molecule techniques to observe the effects of *mgi* mutations on enzyme kinetics and torque production in F_1 from the yeast *Saccharomyces cerevisiae*. Using a high speed imaging camera, we have captured the rotation of wild-type and mutant forms of yeast F_1-ATPase. Rotation data for the wild-type and preliminary data for some *mgi* strains will be presented. We show for the first time that at saturating ATP, wild-type yeast F_1 rotates approximately four times faster than the thermophilic F_1. Kinetic and substepping behaviour in yeast appears to be similar to that observed in bacterial F_1.

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Single molecule AFM force spectroscopy and steered molecular dynamics of contactin-4 protein

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Contactin-4 (CNTN4) is an axonal cell adhesion protein that contains six IgC2 and four FnIII domains and is responsible for creating neural outgrowths. Recent research shows that mutation of CNTN4 gene may be responsible for autism, while its absence in transgenic mice results in lack of smell sense. We use a homemade AFM single molecule puller and steered molecular dynamics simulation to test its elastic properties through force spectroscopy. Stretching experiments show unfolding of FnIII domains while IgC2 domains stay coiled as they are stabilized by disulfide bonds. Unfolding of Contactin-4 molecule appears to play role of buffer that helps to protect neural contacts from damage when neural cells are subjected to shock or tumor.

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Counting fluorescent molecules by photon-antibunching

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Information on the stoichiometry of labelled biomolecules is highly desirable. A method called photo-antibunching has successfully been used to determine the number of fluorescence emitters in multichromophoric systems. A statistical model to estimate the number of fluorescent molecules in a confocal observation volume is established based on photon-antibunching in Time-Correlated Single-Photon Counting (TCSPC) scheme with 4 Avalanche Photon Diodes (APDs) for detection of individual photons. Numerical simulations based on realistic experimental conditions show that the model is able to estimate the number of molecules with moderate errors. Experiments on immobilized double-strand DNA oligonucleotides with photon stabilizing agents show promising results even when the number of molecules is $\sim$ 20. The proposed method allows us to monitor labelled single molecules and in the near future we plan to implement it in complex biological systems (cell extracts/live cells).

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Single-molecule Anatomy by Atomic Force Microscopy and Recognition Imaging

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Atomic Force Microscopy (AFM) has been a useful device to visualize cellular and molecular structures at a single-molecule resolution. Especially, AFM imaging under the TREC^{TM} (Topography and RECognition) mode (recognition imaging) can map a specific protein of interest within an AFM image. In this study, we employed an antibody-coupled AFM cantilever in recognition imaging, and dissected the structural/functional domains of α Actinin-4, an actin binding protein that cross-bridges actin filaments and anchors it to Integrin via tailin-vinculin-α actinin adaptor-interaction. A use of monoclonal anti-α actinin-4 antibody enabled us to map its epitope in the amino-terminal actin-binding domain within a single molecule AFM image of α Actinin-4.

Abstracts
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Confined toxin receptor motion imaged with lanthanide doped labels and analyzed by inference

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Luminescent lanthanide-ion doped nanoparticles (NPs) are attractive single-biomolecule labels. They are synthesized directly in water, are highly photostable, and display narrow emission *without intermittency*. We coupled $Y_{0.6}Eu_{0.4}VO_4$ NPs to ε-toxins produced by *Clostridium perfringens*, which bind to a specific receptor on MDCK cells. Single-molecule tracking experiments using these labels produce long (5 min) uninterrupted trajectories with temporal resolution down to 20 ms or localization precision down to 15 nm. We found that the toxin receptor exhibits confined motion in cell membrane microdomains. To analyze the receptor trajectories, we used a novel approach based on an inference method [1]. This method fully exploits the information of the ensemble of the trajectory, in contrast to the usual mean square displacement analysis. Applying both techniques to recorded trajectories, we highlight the difference in extracted parameters. From the shape of the confining potential, obtained by mapping the forces inside the domain, we can deduce information about the mechanism of confinement. In combination with experiments on cholesterol depletion and cytoskeleton destruction, this technique will shed light into the nature of the membrane micropatterning.

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Determination of physical properties of an elastomeric peptide by AFM stretching experiments

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By stretching a polymer in solution using single molecule techniques it is possible to infer about its physical properties. AFM stretching experiments allow for a full characterization of the elasto-mechanical properties of the sample under study. In the presented work, single molecule AFM force spectroscopy experiments were used to determine mechanical properties of a peptide obtained from Exon 28 (Ex28) of the human elastin gene. Elastin is a protein with important mechanical properties and, in particular, it shows quasi ideal elastic behavior associated to the presence of many hydrophobic unstructured domains (such as Ex28) into the protein structure. Ex28 coded polymer was used as a starting point to obtain bio-materials with specialized elasto-mechanical functions. In particular, a mutated polypeptide based on the EX28 sequence was synthesisized with the aim of obtaining a new polymer with the same mechanical and physical properties of the native molecule but with increased aggregation properties, induced by a cross-linking reaction. AFM stretching experiments were used to verify the mechanical properties of the engineered proteins at a single molecule level. The obtained results allowed not only to address this question, but also to give some insight into the first aggregation steps of the polymer towards the formation of reticulated structures.

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Biophysical characterization of a DNA G-quadruplex formed in the promoter of human MEF2D gene

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G-quadruplex is believed to be involved in many crucial biological processes, such as the gene regulation and the maintenance of human telomere. MEF2D, a member of MEF2 (myocyte enhancer factor-2) family of transcription factors, regulates the response of heart to cardiac stress signals. We found that a G-rich sequence starting at -145bp of MEF2D promoter can form a very stable intramolecular G-quadruplex. Here we present a detailed biophysical characterization of this quadruplex. We also found that this quadruplex is more stable than its duplex form under physiological conditions by CD melting and single molecular FRET. We observed subpopulations in smFRET measurements possibly due to different conformations of the quadruplex. We characterized its unfolding process by monitoring the change in FRET when it hybridizes to its C-rich complimentary strand. Finally, we proposed several possible structures of this quadruplex based on the smFRET and fluorescence DMS footprinting results.

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Abstracts
– Statistical, soft matter and biological physics –

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Effect on platelet behaviour by changes in membrane elasticity and cytoskeletal structure

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Platelet arrest on von Willebrand factor (VWF) occurs transiently via the platelet receptor GPIb. Depending on the combination of shear stress and surface density of VWF binding sites the platelets either only adhere, pull tethers or generate microparticles. These processes are influenced by the properties of the platelet membrane and the cytoskeleton. Under shear flow conditions these platelet characteristics were examined with reflection interference contrast microscopy and a viscosimeter. In addition we quantified platelet adhesion energy and tether pulling forces. Disrupting platelet F-actin had several effects. The tethers were shorter, the membrane contact area was larger, the receptor αIIbβ3 density of the microparticles was depleted and no platelet spreading occurred. Breaking up the microtubular system had different implications. The number of severed tethers tripled, the contact area was smaller, microparticle formation was increased and the area of spread platelets was reduced. However changes in the membrane elasticity had no effect on the platelets. Our results suggest that platelet attachment and adhesion is not only determined by the platelet adhesion receptors and their cytoplasmic linker proteins, but that cytoskeletal structures have also a crucial influence on how platelets interact with thrombogenic surfaces.

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Intrinsic gravity versus metabolically inert infrastructure and basal metabolic rate in living mass

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'Self gravitation bio' is an emerging concept in biophysics. Intrinsic or 'self' gravitational force might exert when mass increases beyond critical level in biological particle pyramid. (http://en.wikipedia.org/wiki/Biomechanics_of_intrinsic_gravity) 'Metabolically inert infrastructure' (MII) consists of total body mass (body water, dissolved substances, mineral and organic deposits) and serves as storage of nutrients, transport and distribution of these materials. To act independently as living body, MII is suggested also to provide structural support to the organism with density-gradient buoyant force against intrinsic and extrinsic gravitational attraction for the biological mass. It is shown that 'amniotic fluid', 'isotonic saline to ailing patient', 'growth factors', 'cultural medium' and other 'medium matrices' act as counter-gravity mechanism for micro to macro living organisms under center-of-biomass reference frame.
Controversy over relationship between BMR (basal metabolic rate), RMR (resting metabolic rate), PAL (physical activity level), on one hand, and mass of the living organism, on the other, (as described in Rubner's 2/3 law, Kleiber's 3/4 law that continued to be contested by many) can be favorably resolved substituting the concept of self gravitation bio, considering 'mass' as synonym of gravitational force under center-of-biomass reference frame. Bioenergetics would be an unequal but opposite entity of self-gravity reinforced by extrinsic gravity.

O-324

Towards mechanistical understanding of adsorption: combining technologies in situ and in real time

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A growing number of researchers in different surface related fields present evidence from more than one analytical technique when detailing their findings. Thus a logic and useful development is to combine technologies for simultaneous measurements on a single sensor surface. To develop a biosensor platform such as an assay fast and accurately, mechanistical understanding of why the platform works is essential. Quartz Crystal Microbalance-with Dissipation (QCM-D) technology and instrumentation provides an open platform and enable easy and precise quantification of mass, thickness and viscoelastic properties of surface bound molecules. These parameters provide both a reference tool in assay development, but also detecting and identifying your target molecules as the combination of mass and material property info in many cases provide a unique response for a specific molecule. By combining QCM-D with other technologies, a range of useful info is put within easy reach of the researcher.
Recent advances will be presented where simultaneous real time and *in situ* measurements using QCM-D together with electrochemistry, ellipsometry and fluorescence microscopy enables manipulation of films as well as quantification of the variation of water content as a result of conformational changes in immobilized molecules. Examples will include new data from the formation of protein films, polyelectrolyte multilayers and polymer brushes.

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Pacing associated tachyarrhythmias in patients with implantable cardioverter defibrillator

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Background: Implantable cardioverter defibrillators (ICDs) are save-life device for patients at risk of sudden cardiac death, and help cardiac patients to avoid slow beat-rate by means of anti-bradichardia pacing (ABP) feature. However, recent literature evidenced the presence of ventricular tachyarrhythmias (VTs) immediately following ABP, leading to the hypothesis that ICD devices might be pro-arrhythmic.
Aim: Study differences between pacing-associated tachyarrhythmias (PAT) and other spontaneous VTs.
Signals and methods: 165 spontaneous VT episodes are retrieved from 37 patients with ICD. VTs are examined and PAT episodes, classified. Characterization of VTs is based on the analysis of 20 seconds immediately preceding VT-onset quantified by: heart cycle (HC-PreVT), prevalence of premature ventricular contraction (PVCprev) and prevalence of paced beats (Pprev). Significant differences are evaluated by Student-T or Chi-square tests with $p<0.05$.
Results: 20 VT episodes from 8 patients are PAT episodes (12%). Cardiac activity preceding PATs vs. non-PATs differs: PAT episodes have higher HC-PreVT ($p<0.01$), greater Pprev ($p<0.001$), and greater PVCprev ($p<0.001$). Analysis also shows that PAT episode often occur when the paced beat immediately follows a premature contraction.
Conclusion: The study indicates that new ICD generations should avoid ABP after premature ventricular contraction.

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Watching hemoglobin at work inside intact red blood cells by Time-Resolved Wide Angle X-Ray Scattering

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Our efforts in recent years have been to study in great detail the way hemoglobin works in confined geometries [1-3]. To this aim we have contributed to the development of a new experimental technique, time-resolved wide-angle X-ray scattering (TR-WAXS), that enables one to watch proteins at work in solution [4,5]. A very recent and challenging application of this technique is the study of hemoglobin functioning inside intact red blood cells (RBC). Our preliminary results show that, by using laser pulses of about 150 ns, it is possible to photolyse hemoglobin inside RBCs obtaining about 40% - 60% photolysis. Good structural signals from hemoglobin are obtained, with limited radiation damage to the cells: this opens the possibility of studying the conformational transitions of hemoglobin in its “natural” environment. Preliminary results concerning the timing of the R->T quaternary transition inside the RBC and comparison with the behaviour in dilute solution will be discussed.

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Microscopic origin of the very-low energy vibrational dynamics in proteins

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Important functions of biological processes require large atomic rearrangements and conformational fluctuations. For proteins, atoms are mostly displaced along the soft directions identified by the delocalized, low frequency vibrations. The study of very low energy vibrational spectrum of proteins is therefore of particular interest. As a step toward understanding their functional role, we have investigated the low frequency vibrational motions ($0.03 \div 10$ meV) in different proteins by performing inelastic neutron scattering and low temperature ($1.5 \div 30$ K) specific heat measurements. For the first time for biological systems, the well-known Boson peak found in neutron scattering spectra at ~ 3 meV is put in relationship with a clear anomaly of the measured specific heat located at around 7 K. This departure from the Debye-like behavior further expands the analogy of proteins with glassy systems. Quite interestingly, in the very low sub-meV energy range and below $\sim$3K, we observe an additional anomalous behaviour, which could be ascribed to the existence of two-level-systems states. Increasing the hydration degree, the low energy vibrational region is drastically altered, revealing that the addition or removal of the hydrogen bond network around the protein deeply modifies the interatomic forces, affecting the character of the vibrational modes.

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Dynamics of a minimal neural model consisting of an astrocyte, a neuron and an interneuron

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In the nervous system of vertebrates there are more glial cells than neurons: from 10 to 50 times, depending on the animal type. Glial cells are not able to generate action potentials but, nevertheless they play an important role for the functioning of the different brain’s area. The astrocytes are the more abundant cells of the macroglia and through their processes they modulate synaptic activity. In this contribution a biophysical neural network model consisting of a pyramidal neuron, an interneuron and the astrocyte is studied. The corresponding dynamical properties are mainly investigated by using numerical simulations. The results show that the presence of the ATP and of the interneuron strongly impacts the neural activity. Moreover, it is shown that the fluxes of calcium through the cellular membrane strongly affect the modulation of the neural activity arising from the astrocyte.

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Target site search strategy of gene regulatory proteins

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Gene expression is orchestrated by a host of regulatory proteins that coordinate the transcription of DNA to RNA. Regulatory proteins function by locating specific binding sequences of DNA and binding to these sequences to form the transcription initiation complex. In many instances, these regulatory proteins only have several hundred copies that must efficiently locate target sequences on the genome-length DNA strand. The non-specific binding of regulatory proteins to random sequences of DNA is believed to permit the protein to slide along the DNA in a stochastic manner. Periodically, a thermal kick or an interaction with another bound protein will disengage the regulatory protein from the DNA surface, leading to three-dimensional diffusion. Eventually, the protein will reattach to the DNA at some new location that is dictated by both the diffusivity of the protein and the DNA configuration. Cycling through these random events constitutes a search strategy for the target site. We build a reaction-diffusion theory of this search process in order to predict the optimal strategy for target site localization. The statistical behavior of the DNA strand acts as a necessary input into the theory, and we consider several governing behaviors for the DNA strand. We explore the impact of DNA configuration and protein occlusion on target site localization in order to predict how protein expression will vary under different experimental conditions.

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Maximum entropy production principle (MEPP) in generalized Michaelis-Menten kinetics

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MEPP is a physical principle, widely used for quantitative explanation of non equilibrium phenomena in physics, chemistry and biology [1-3]. Here, we applied MEPP to study two and three state reversible Michaelis-Menten kinetic schemes of enzymatic reactions. By applying constraints as a diffusional limit for kinetic constants, constant free energy differences between enzymatic states and constant thermodynamic force, we calculated Shannon information entropy and entropy production of the entire reaction system as a function of forward rate constants. We found maxima in the net steady-state metabolic flux, total entropy production and Shannon entropy for equal values of forward rate constants. Moreover, for these values an analytical expression derived gives a relation between substrate and product concentrations at which enzymes operate in the optimal way. In conclusion, we demonstrated that MEPP is an appropriate selection principle for evolutionary optimization of enzymes.

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P-331

A model coupling vibrational and rotational motion for the DNA molecule

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A largely used mechanical model for vibrational motion of DNA has been proposed by Peyrard and Bishop (PB). In this model, DNA is represented by a pair of harmonic chains coupled by a nonlinear potential. The most frequently used nonlinear potential for this purpose is the Morse potential. Some extensions of the original model are proposed considering, for example, the helicoidal structure of DNA. An important objective for this kind of model is to understand the phenomenon of thermal denaturation and, through this, get some knowledge about other processes such as the genetic transcription and drug intercalation. It is possible to obtain from this type of model interesting properties, such as the average stretching between base pairs as a function of the temperature using the transfer integral operator. Dynamical properties of this model were also explored and several phenomenological quantities are studied, such as energy localization. In this work, we extend the original PB model by introducing rotational motions for the nucleotides. In this way, both the vibrational and rotational motion for each nucleotide are considered. The stretch of the base pairs is given by the Morse potential in the same way as in the original PB model. However, the coupling between the two kinds of motion, rotation and vibration, is obtained through a nonlinear combination of them in the Morse potential.

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Modeling of fibrin gels using confocal microscopy, light scattering and turbidimetry

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Fibrin gels are biological networks playing a fundamental role in blood coagulation. Confocal microscopy of fibrin gels shows a collection of straight fibers, not uniformly distributed in space, connected together at low-order (3-4) branching points. Although each fiber is quite straight (mass fractal dimension D_m=1), for the overall system D_m>1. Based on the confocal images, we generated three-dimensional (3D) synthetic gels made of cylindrical sticks of diameter d, joined together at randomly distributed nodal points. The resulting 3D network is no more random but ordered on length scales of the order the average fiber length, and exhibits a fractal morphology with $D_m \sim 1.2$-1.7. The gel structure is analyzed by means of its 3D correlation function $g_{3D}(\mathbf{r})=\langle n(\mathbf{x})\, n(\mathbf{x}+\mathbf{r})\rangle_{\mathbf{x}}$, where $n(\mathbf{x})$ is the gel local density. Since the gel is isotropic, g_{3D} depends on the modulus $r=|\mathbf{r}|$ and can be obtained by averaging 2D correlation functions evaluated at different heights of the gel volume. From this analysis we recover the fiber diameter d (FWHM of g_{3D}), the fractal dimension D_m (power-law decay of g_{3D}) and the gel mesh-size ξ (minimum in g_{3D}). Furthermore, the 3D-Fourier transform of $g_{3D}(r)$ gives the gel power spectrum I(q), which compares quite well with elastic light and multi-wavelength turbidimetry data taken on real gels.

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Biological physics in Physical Review

M. C. Foster
Physical Review Editorial Office, Ridge, New York, U.S.A.

Physical Review Letters and Physical Review E invite submission of your best work in biological physics. Submissions must present significant new results in physics; topics may range from the microscopic to the macroscopic. We will provide information on the different types of articles published in the journals, on authors and referees, and on the review process. For publication in Physical Review Letters, the work should be important and of broad interest. For Rapid Communications in Physical Review E, the work should be important for the field. Biological physics papers published in Physical Review are indexed in MEDLINE. In an effort to bring important research to the attention of a wider community, the Physical Review journals have recently begun highlighting important work with Viewpoints in the online publication Physics. All Physical Review journals may be accessed through the website http://publish.aps.org/.

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Control of DNA replication by anomalous reaction-diffusion kinetics

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DNA replication requires two distinct processes: the initiation of pre-licensed replication origins and the propagation of replication forks away from the fired origins. Experiments indicate that these origins are triggered over the whole genome at a rate *I(t)* (the number of initiations per unreplicated length per time) that increases throughout most of the synthesis (S) phase, before rapidly decreasing to zero at the end of the replication process.
We propose a simple model for the control of DNA replication in which the rate of initiation of replication origins is controlled by the interaction with a population of rate-limiting proteins. We find the time set by reaction-diffusion kinetics for such proteins to find, bind to, and trigger a potential origin. The replication itself is modeled using a formalism resembling that used to study the kinetics of first-order phase transitions.
Analyzing data from *Xenopus* frog embryos, we find that the initiation rate is reaction limited until nearly the end of replication, when it becomes diffusion limited. Initiation of origins and hence *I(t)* is suppressed when the diffusion-limited search time dominates. We find that, in order to fit the experimental data, the interaction between DNA and the rate-limiting protein must be subdiffusive. We also find that using a constant nuclear import of the limiting proteins leads to a more accurate description of the experimental data.

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Probing the molecular mechanism of antibiotics diffusion through the OmpF channel

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In Gram-negative bacteria, the outer membrane porin-F (OmpF) is the preferred entry point of antibiotics. Bacteria can resist to antibiotics by altering the expression and the structures of OmpF. A key feature in the structure of OmpF is the presence of a constriction zone, characterized by both spatial and electrostatics restrictions. To study the process of antibiotics translocation at a molecular scale, we performed molecular dynamic simulations accelerated with the metadynamics algorithm. We studied the diffusion of antibiotics with different structural and chemical properties through OmpF wild-type and variants. The free energy surface suggests faster translocation for the cephalosporins compared to the penicillins antibiotics, and also for OmpF mutants compared to the wild type. Further, the conservation of favored orientation and affinity sites of antibiotics inside the OmpF channel reveal which specific interactions govern translocation.
The calculated energy barriers and rate determining interactions for translocations compared well with the electrophysiology measurements and liposome swelling assays from our collaborators. This study demonstrates how theory and experiments can be combined to reveal the structural determinants and mechanism of OmpF permeation. This will benefit to the design of antibiotics with improved transport properties.

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Assessment of novel pore lining motif in a viral channel forming protein, a computational approach

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Viral genomes encode for a series of membrane proteins which are embedded or attached to the lipid membrane of the host, or penetrate them during the very first step of viral invasion. We are focussing especially to those of the former which are known to assemble and from channels or pores for small ions or substrates. With these channel forming proteins the virus manipulates the host cell interior for the benefit of its replication. Strategies are developed to answer the question about the assembly process of these proteins based on the 'two stage model' and the substrate flux.
Only few experimental data are available to answer these questions, consequently we stress computational methods to derive the adequate answers. The methods applied are *ab inito* molecular dynamics (MD) simulations based on density functional theory (DFT) and conventional MD simulations. Potential routes for assembly of the three transmembrane domains of 3a protein from SARS-CoV will be outlined and compared with computational data from other viral membrane proteins. With a novel pore lining motif suggested for 3a, the dynamics of ions at selected positions within the putative pore will be assessed.

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Attractive interactions between like-charged lipid surfaces induced by charged nano-particles

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Attractive interaction between like-charged lipid surfaces mediated by spherical nanoparticles with spatially distributed charge is theoretically described by using functional density theory and MC simulations. The spatial distribution of charge within a single nanoparticle is considered by two effective charges at a finite distance. The minimization of the free energy is performed to obtain the equilibrium configuration of the system. Both, the rigorous solution of the variational problem and the MC simulations show that orientational ordering of nanoparticles subject to the gradient of the electric field gives rise to an attractive interaction between charged lipid surfaces for high enough charge densities of the interacting surfaces and large enough separations between charges within the nanoparticle. The attraction is explained by orientational ordering of dimeric charges in the electric field which lowers free energy.
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Translocation through nanopores: Ion conductance, selectivity and facilitated diffusion

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To control the passage across the bacterial cell wall nature created a large number of "nano"- channels which may act as selective gates for water soluble molecules. Here we focus on porins from *E. coli* which control permeation through interactions with the channel surface. Comparing single channel temperature dependent conductance measurements with all atom modeling allow conclusions on the mode of permeation. For example, surprisingly modeling OmpF-conductance revealed not only a good agreement with the experiment over a broad range of temperature but also the selectivity for ions. The primary task of porins is to provide facilitated diffusion. We investigated facilitated diffusion of maltooligosaccharide or antibiotics. Time resolved conductance measurements allows conclusion on the flux and molecular modeling identifies the limiting interactions with the surface, reveal potential barriers and pathways. Exploiting the selectivity of natural or bioengineered channels has promising applications for detecting molecules, characterizing molecular interaction, sequencing DNA, protein folding etc.

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Charge transfer in DNA: Role of base pairing

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Charge migration along DNA helices may be biologically important because extended electronic states could play a role in the processes of sensing of DNA damage and/or DNA repair via long-range charge transfer. We measured conductivity and other physical characteristics of several models of natural and diversely damaged molecules of DNA. DNA polymers were mimicked by various sequences of 32-nucleotide-long double helices with fully Watson-Crick (WC) paired bases, with several central bases mismatched, and also with chemical modifications that included removal of bases from a few central nucleotides (abasic DNA), and neutralization of phosphate charges by their derivatization. The model DNAs were investigated by scanning tunneling microscopy, time-resolved THz spectroscopy, Raman spectroscopy, circular dichroism, and modeled by molecular dynamics simulations. DNA has the highest conductivity in its biologically most relevant double helical form with WC base pairs and negatively charged phosphates equilibrated with counterions. Mismatches and all chemical modifications always lower the conductivity. The mechanism of charge transfer is consistent with electron or hole hopping between parallel stacked bases. These observations and data showing that the natural DNA has also the most regular double helical form suggest that the continuous base stacking is critical for charge transfer.

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Determination of root-mean-square pairwise RMSD from experimental B-factors

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Root-mean-square deviation (RMSD) is a measure used to give information on the global structure of macromolecules. For example, pairwise RMSD (pRMSD) is used to assess similarity of the lowest energy NMR structures or for clustering large ensembles of structures. On the other hand, to obtain information on the local structure of a macromolecule and its dynamics, root-mean-square fluctuation (RMSF) is often used. RMSF can be calculated from MD simulations, but also from experimental X-ray B-factors. Since pRMSD and RMSF report on different features, it is interesting to ask what the relationship between them is. First, we provide a mathematical derivation showing that, given a set of conservative assumptions, the $\langle \mathrm{pRMSD} \rangle_{RMS}$ is directly related to the $\langle \mathrm{RMSF} \rangle_{RMS}$ and, consequently, experimental B-factors. Second, we demonstrate this on structures taken from distributed-computing MD simulations of the native and unfolded state of villin headpiece. Both our analytical and computational results suggest a strong correlation: $\langle \mathrm{RMSF} \rangle_{RMS} = [(S-1)/2S]^{1/2} \langle \mathrm{pRMSD} \rangle_{RMS}$, where S is the number of compared structures. Furthermore, if $\langle \mathrm{pRMSD} \rangle_{RMS}$ is defined as a generalized radius of gyration in the space of 3D structures and using RMSD as a measure of distance, the following identity holds: $\langle \mathrm{RMSF} \rangle_{RMS} = \langle \mathrm{pRMSD} \rangle_{RMS}$. Our results provide a basis for determining the level of structural diversity of molecular ensembles, as captured by $\langle \mathrm{pRMSD} \rangle_{RMS}$, directly from experiment.

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Inference of structure of biochemical networks from time-series of reactant concentrations

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We propose a new method for inferring the structure of a biochemical network from the time-series of the reactant species. It consists of two parts: the first is the quantification of the correlation between the time-series profiles. Correlation in time series data can be used to reveal dependencies between variables and to infer the graph of connectivity among species. The second part consists in the elimination from the connectivity graph of the relationships that have a non-null correlation coefficient, but that are not biochemically plausible. The cutting of false correlations from the graph is performed through the estimation of the parameters ("calibration") of the network. To calibrate the network for detecting null dynamics correlations, we developed the software tool KInfer (Knowledge Inference). Based on a new probabilistic model of the variations in reaction concentrations, KInfer infers the values of the kinetic rate constants, their confidence regions and the level of noise in the input data by maximizing the likelihood to obtain the observed variations given the network model. The a priori knowledge required as input is minimal, as it consists only in the time-series of reactant concentrations. The minimal a priori knowledge and the probabilistic formulation of the calibration method make the accuracy of the predictions strong against experimental, biological and stochastic noise, and allows to use it to cut the null-dynamics edges of the connectivity graph.

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Nuclear centering in fission yeast mediated by kinesin-8 motor proteins

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In the fission yeast *Schizosaccharomyces pombe*, the nucleus is positioned at the cell center. Since the nucleus determines the cell division site, keeping the nucleus at the center is crucial for ensuring symmetrical cell division (*1*). Microtubules push against the cell ends and exert force on the nucleus (*2*), but how the cell regulates these forces in order to center the nucleus remains unknown. Here we tackle this problem by using a combination of live cell imaging, cell manipulations by optical tweezers, and a theoretical model. We show that microtubule pushing forces can center the nucleus because of a larger number of contacts between the microtubules and the proximal cell end than the distal one. Moreover, kinesin-8 motors (Klp5/6) increase the rate of microtubule catastrophe (transition from growth to shrinkage) in a microtubule length- and contact-dependent manner. Thus, the motor behavior results in a longer contact between a microtubule and the proximal than the distal cell end. Taken together, our experimental and theoretical results provide a novel centering mechanism, where kinesin-8 motors increase the efficiency of nuclear centering.

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Electron transfer and protein dynamics in photosynthetic reaction centers embedded in sugar glasses

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The coupling between electron transfer and protein dynamics has been compared in reaction centers (RC) from the wild type (wt) and the carotenoid-less mutant R26, by combining Brownian dynamics simulations and the kinetic analysis of charge recombination. Upon incorporation of the RC into a progressively dehydrated trehalose matrix the electron transfer between the primary photoreduced quinone and the photoxidized donor accelerates progressively and becomes broadly distributed. This behaviour reflects the hindrance of protein relaxation following charge separation and the inhibition of interconversion between conformational substates. In extensively dehydrated matrices the recombination kinetics is two-times faster and three-times more distributed in the wt RC, indicating a larger inhibition of the internal protein dynamics. In line with this findings Brownian dynamics simulations reveal a larger rigidity of the carotenoid-containing structure, in which a cluster of residues close to the quinone acceptors is stiffened as compared to the R26 RC. The *in silico* and experimental results indicate that the introduction of an internal void in the RC structure has long-range effects on the protein dynamics and that the coupling between the glassy matrix and the RC interior depends markedly on the local mechanical properties of the protein.

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Anomalous dynamical properties of protein hydration water in the picosecond timescale

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The single-particle and collective dynamical properties of protein hydration water have been studied by neutron scattering experiments in a wide temperature and hydration range. An unprecedented accuracy has been achieved thanks to the availability of a large amount of fully deuterated protein powder and the use of the high-flux spectrometers IN5 and BRISP. The protein under investigation was the Maltose Binding Protein (MBP), which is a well-known and widely studied model of biosensor systems.
We found that the low-temperature single particle dynamics of MBP hydration water shows clear features that can be traced back to amorphous systems. More in detail, its vibrational density of states is simply described as the superposition of the contributions of low-density and high-density amorphous ice. The quasielastic signal, which appears at the higher temperatures, can be excellently described with a fractional power law which may put in relationship with the peculiarities of fractal systems. Quite strikingly, there is a strong similarity, on both the qualitative and quantitative point of view, with the behaviour of hydrated proteins.
The collective dynamics of protein hydration water is characterised by the presence of two modes, whose dispersion curves are reminiscent of those of bulk water. However, the relevant damping factors suggest a strong similarity with glassy systems.

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Behavior of alpha-chymotrypsin in macromolecular crowding situation

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Traditionally, studies of diffusion-controlled reaction of biological macromolecules have been made in diluted solutions. However, the high concentration of macromolecules in intracellular environments results into non-specific interactions (macromolecular crowding), which have a great importance on the kinetics and thermodynamics of possible reactions that occur in these systems. In the literature there are studies concerning Monte Carlo (MC) simulations, giving results that are satisfactory agreement with experimental data, showing, for example, that the protein diffusion in cell cytoplasm is reduced considerably. In addition, there are MC studies about enzymatic reaction, which predict a temporal dependency of the velocity constant in macromolecular crowding. In this work, we try to compare the predicted behavior by MC simulation with the results obtained from the study of the diffusion and reaction of a model protein (alpha-chymotrypsin) using spectroscopic techniques in highly confined media in order to study experimentally the temporal dependence of its diffusion and reaction coefficients.

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Pathways of proteins and RNA mechanical unfolding: an Ising-like model

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Many features of protein folding have been shown to be described by an Ising-like model (one-dimensional, with long-range, multispin interactions) whose equilibrium thermodynamics is exactly solvable [1-3].
We have generalized such a model to the problem of mechanical unfolding. The equilibrium thermodynamics is still exactly solvable, and the characteristic kinetic responses found in force ramp and force clamp experiments are well reproduced [4,5].
Unfolding and refolding pathways and intermediates can also be studied, again with good agreement with experiments [6].
Applications to various proteins and RNA fragments will be discussed.

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Insulin protective effects against cell degeneration induced by A-beta amyloid

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Diabetes and Alzheimer's disease are connected in a way that still is not completely known. Diabetes has been implicated as a risk factor for developing Alzheimer's disease. Some diabetes drugs appear to decrease the cognitive decline associated with Alzheimer's disease.
It has been recently demonstrated that extracellular injection of insulin is able to protect neurons against A-beta amyloid cell death. One of the proposed theories to explain such an effect is that the hematic glucose levels affect the metabolism of the hippocampus, a part of the brain (associated with memory, emotion and motor skills), which is strongly damaged in Alzheimer's patients.
The aim of this study is to investigate the effect of insulin on the A-beta induced degeneration and oxidative stress on the neuroblastoma LAN5 cell line. In particular, the present study looks into the role of insulin in the inhibition of A-beta specific degenerative apoptotic pathways. Preliminary results indicate that insulin dissolved in culture medium in its hexameric form (as tested by absolute scale light scattering) is able to reduce neurodegeneration induced by A-beta amyloid in a dose dependent manner. The link between diabetes and Alzheimer's disease may provide new targets for future Alzheimer's treatments. Moreover, due to the increased incidence of diabetes in western countries, a deeper understanding of such a link is relevant in order to control the escalation in the number of people dealing with dementia.

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Imaging of brain activation using core techniques as fMRI, PET and synchrotron radiation in parallel

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Functional magnetic resonance imaging (fMRI), magnetic resonance spectroscopy (MRS), positron emission tomography (PET) and synchrotron x-ray emission imaging form a highly complementary set of imaging core technologies for studying brain function and energy metabolism. Owing to differences in the information each conveys and the temporal and spatial scales on which they measure labeled substances, a single working hypothesis can be tested from different angles to provide a cross-validated, consistent and coherent explanation. The CIBM is a unique research facility in Europe for advancing our understanding of biomedical processes in health and disease. The housing of a 7-Tesla human magnet, 9.4 and 14.1-Tesla animal magnets, an animal PET imaging facility and fully-equipped neurochemistry and RF laboratories has enabled us to develop and perform well-targeted experiments around one research theme. In parallel, a long-term collaborative project using synchrotron x-ray transmission and emission imaging at Elettra enables us to combine these core technologies towards understanding brain function in vitro and in vivo, from tissue to cells. A brief presentation of these methods will be followed by their application in studying the visual system from man to mouse.

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Lipid loss in giant unilamellar vesicles induced by an electric field

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Electropermeabilization is a commonly used physical method which can induce a transient permeabilization of the cell membrane allowing the entry of therapeutic molecules into the cell and is thus of great interest in the fields of cancer treatment and gene therapy [1].
However, very little is known about the mechanisms occurring at molecular level. There is clearly some microscopic reorganization of the membrane which is responsible for this change in its transverse transport properties. Rather than studying the change of these transport properties, we adopt a simple strategy based on the use of giant unilamellar vesicles and videomicroscopy, as described below.
We apply a series of permeabilizing electric pulses to the liposomes, and we observe a size decrease down to a critical radius beyond which their size no longer changes. This decrease in size points to the fact that during the physical processes leading to electropermeabilization, lipids are lost from the vesicles. Our results published in [2] suggest different possible modes for lipid loss, which can be small vesicles, pores, or tubules formation.

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A new theoretical spectrum for the shape fluctuations of giant unilamellar vesicles: coexistence of bending and hybrid curvature/compression modes

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We study thermal undulations of giant unilamellar vesicles (GUVs) of lipids by flickering spectroscopy. Getting values for the mechanical parameters of lipid bilayers requires the experimental fluctuation spectra to be scrutinized in view of the classical Helfrich's theory. Pure bending modes are revealed unable in predicting the large fluctuations systematically found at high wavevectors. Hybrid curvature-dilational modes have been invoked as a more efficient mode of motion in producing high curvatures. A bimodal spectrum of the thermal undulations has been theoretically developed for the shell-like topology. From this new description, two important consequences emerge a priori, the dependence of the fluctuation dynamics on either vesicle size and on bending/compression parameters. For POPC and DOPC vesicles containing cholesterol the experimental fluctuation spectra are well described by the new spectrum. Reconciliation between experiments and theory is achieved when this bimodal spectrum is considered. The new theory opens enormous possibilities for better exploring membrane mechanics in GUV models.

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Peptide dimer motifs in the phospholipid environment – structure, interaction and molecular design

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Based on recently reported homodimeric peptide pores with a D,L-alternating configuration a novel double helical hairpin-motif of a membrane active gramicidin A analog was designed.[1, 2] The CD spectroscopic analyses of the peptide-lipid complexes revealed the structural preservation and elucidated the importance of a zwitterionic interaction of the peptide termini.[3] The peptide design was enhanced regarding the versatile functionalization with analytical probes as well as molecular recognition moieties like peptide nucleic acids (PNAs) to observe the effects of aggregation and specific organization within model lipid membranes even at high peptide-to-lipid ratios.[3] X-ray reflectivity on lipid bilayer stacks in combination with heavy atom labeling and spectroscopic studies of vesicle systems provides information about the peptide structure and interaction in the native fluid state of the membrane system.[2, 3] For this, the Fmoc-diiodo-allylglycine building block was created to serve as a novel iodine label pinpointing at a certain position with respect to the membrane normal.[3]// [1] Alexopoulos et al., *Acta Cryst. D60* **2004**, 1971-1980. [2] Küsel et al., *ChemPhysChem* **2007**, 8, 2336-2343. [3] Schneggenburger et al., *ChemPhysChem*, submitted.

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Self assembly of patchy particles and DNA-functionalized dendrimers

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I will report numerical results on the phase behavior of very simple models of patchy particles with the aim of understanding the interplay between phase separation and self-assembly and how the fraction of surface allowing for attractive interactions controls the collective behavior of the system. The case of Janus particles, particles characterized by a surface divided evenly into two areas of different chemical composition, will be discussed.
I will also discuss the self-assembly of a simple model for four single strands of DNA tethered to a central core, and show that the model exhibits a rich phase diagram that includes at least four thermodynamically distinct amorphous phases (polyamorphism) in a one-component system. The dense phases consist of a hierarchy of interpenetrating networks, reminiscent of a woven cloth.
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Water domains investigated by delayed luminescence

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The key role of water in living systems has been widely studied in Literature, along with its anomalies, consequence of the extensive three-dimensional hydrogen bonding of water molecules. Moreover protein-water interactions take place at protein surface where cell water has been recognized to behave differently from bulky water. The two-states theory of water assumes that water is a mixture of microdomains of different structure and density, the low-density water (LDW) and the high-density water (HDW) domains, and ions partition selectively into LDW or HDW domains. The idea developed in this work was to explore the ordered water structure by measuring Delayed Luminescence (DL) from salt aqueous solutions in which water structuring is anticipated. It appeared that DL signal from salt solutions is significantly relevant when prevalence of LDW domains is foreseen, with a decay time probability distribution function characterized by a broad maximum in the microsecond range. The obtained results support the ability of DL to reveal the different properties of LDW and HDW domains induced by salt molecules. Moreover, the results reveal the existence of clusters, whose characteristics strongly depend on the specific ion effects, of surprisingly long lifetimes not observed till now. This could give new insight into biological water properties.

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Water dynamics in cells

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Water is essential to life and a major scientific interest lies in a detailed understanding of how it interacts with biological macromolecules in cells. We studied water dynamics in whole cells with neutron scattering [1,2,3]. The cellular environment is extremely crowded with macromolecules and water molecules are permanently in close contact to biological interfaces. We measured water dynamics in *E. coli* and human Red Blood Cells with neutron scattering [2,3]. The data revealed two populations of water in the cells: a major fraction which has dynamical properties similar to those of bulk water (relaxation times ∼ps) and a minor fraction in the order of ∼10% which is interpreted as bound hydration water with significantly slower dynamics (relaxation times >49 ps).

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P-355

The effect of Dextran 70 concentration on red blood cell deposit formation

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Red blood cell (RBC) deposit formation was examined by means of an optical method. Blood was obtained from healthy donors and measurements were performed at initial hematocrit 40%. The intensity of scattered light was measured during sedimentation of RBCs suspended in saline - Dextran 70 solutions at different polymer concentrations (2 - 6 g/dl). The changes in the intensity of the scattered light manifest RBC aggregate formation, their sedimentation and the process of deposit formation. The deposit formation curve was determined. It is shown that the concentration of dextran affects the deposit formation. An empirical model has been used to describe the experimental data. The parameters of the deposit formation curve as a function of dextran concentration are analyzed.

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Multi-joint analysis of locomotion in the first neonatal rats flown in space

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The first mammalian neonatal animals in space were the rats flown on the Space Shuttle Endeavor during a 9-day mission, STS-72. The development of locomotion in weightlessness was evaluated using two litters of neonatal rats, launched at postnatal days 7 and 15. Age- and cage-matched animals were used as ground controls. Free walking was videotaped from the landing day.

Although preliminary analysis of walking showed differences in both hindlimb and forelimb joint angles and a hyperextension of the hindlimbs was apparent, the numerical values reached the significance level only for the ankle angle measured at specific moments of the step cycle: foot contact, maximum loading with weight, foot lift and maximum flexion during swing.

We report here on the behavior of all the joints during the whole step cycle, by computing the integral of these angles over the step cycle. The results were affected by the differences in the walking speed (the young animals walked faster than the controls), so we scaled the integrals by the step cycle duration. We found that, besides the ankle, the knee was also more extended throughout the whole step cycle in both groups of animals. Moreover, all the joints (including the toe and the hip) were affected in the same way (hyperextended), since the differences were still significant when we added together these angles. The animals recovered slowly, with significant differences remaining after 14 days of readaptation.

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Biosensor technology for platelet function testing

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Under normal conditions, platelets circulate in the vascular system having very low interaction with each other and with other cells. The platelets become activated when the biological system is disturbed, for instance by vascular damage in which blood gets in contact with collagen. Upon activation, different types of receptors/molecules are exposed on the cell membrane to support adhesion, spreading and aggregation of the platelets onto the damaged vessel. The measurement of altered platelet function is particularly important in cardiovascular diseases such as thrombosis.

We are investigating biosensor technologies for the detection of functional properties of platelets. An important study is the specific and non-specific stimulation of platelets in a biosensor cartridge. We will present experimental results on biosensor platelet activation using the platelet-specific membrane markers P-selectin and GP1b.

Abstracts
– Statistical, soft matter and biological physics –

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Single cell adhesion on micro-patterned substrates

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Cell adhesion and motility are processes involved in fundamental biological phenomena. They imply multimolecular scaffolds as anchorage points and actin cytoskeleton filaments to build internal stress and eventually crawl onto the substrate. These processes, very dynamic by nature are out of equilibrium. We study cell adhesion on micro-patterned substrates where the introduction of a finite distance between the possible anchorage points of the cell modifies drastically the organization of the cytoskeleton and the anchorage point's distribution. Because statistical quantification shows that some shapes are more likely than other, we believe they represent particular organizations of the system which should minimize the energy dissipation. We checked this hypothesis by using the Cellular Potts Model. Shapes obtained by simulation are in excellent qualitative agreement with experimental shapes. They depend on phenomenological parameters such as interaction between cells and the extra cellular matrix, a line tension and an elastic modulus. The aim of this work is to link model parameters to physico-chemical properties of cells and to establish phenomenological relations between relevant biochemical regulators controlling the cytoskeleton organization.

P-360

Hydrogen dynamics in bio-mimetic membrane, and the effect of pore forming peptides

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In this contribution we report on investigation of model membrane dynamics by means of Quasi Elastic and Inelastic Incoherent Neutron scattering and on the effect of membrane inserted pore forming peptide Gramicidin.
Model membrane are realized by highly oriented, hydrated phospholipid bilayer stacks of DMPC (1,2-dimyristoyl-sn-glycero-3-phoshatidylcholine) hydrated with D2O in excess of solvent condition.
The bilayer were supported on mica substrates and prepared at different concentrations of Gramicidin, a 15-residue oligopeptide showing antimicrobial activity by forming pores on the membrane surfaces which allow water and small ions to permeate across the membrane.
Incoherent QENS and INS spectra, measured on IN5 and IN13 spectrometer at ILL, allows to obtaining information on the mean dynamics of the Hydrogen atoms in the system. Moreover, by proper orientations of the membrane plane respect to the scattering wave vector Q, we were able to derive information on in plane and out of plane motions of the phospholipids.

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The using of media products for the creating attracted bioenergetic brain rhythmus advertisement

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The technical efficiency of bioenergetical influence of advertisement is present with assistance of consciousness of memory at revision and hearing of advertisement on TV, radio stations, mobile phones, at supermarkets and other places. In a basis of useful model it is put a task to improve the method of creating the attracted advertisement, in which the creation of bioenergetical influence by the oscillation of not less 2 electromagnetic fields or video-images is introduced with their creating as the base on the spatial or flat structure formative macro matrix or matrixes with repeatable structure with the brain α-rhythm frequency of extreme attention and δ-rhythm frequency of meditation.
The point of the patent on the device is in the bioenergetical influence increases in addition to the information influence by advertisement of pictures and audio oscillations the bioenergetical influence increases at the consciousness contribution of human memory at the moment of watching or hearing of television, radio stations, working in the Internet, music in the supermarkets, banks, clubs, metro and other places.

Abstracts
– Biomolecular self-assembly –

P-361

Multi-method studies of the membrane spanning domains of proteins

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Due to the immense medical importance of proteins which span the membrane of cells, detailed molecular structural information of these systems is essential. Practical difficulties in employing high-resolution structural elucidation techniques have resulted in a relative paucity of fully resolved membrane protein structures. Therefore a variety of lower-resolution techniques are used to determine structural information of the transmembrane (TM) domains of proteins. One example of such a membrane protein is ErbB-2, a receptor tyrosine kinase responsible for triggering cell division and which is prone to a mutation in its transmembrane domain resulting in permanent activation and oncogenic effects. We have predicted an interface for the mutated TM domain dimer using site-specific infrared spectroscopy containing a repeating sequence of Ile, Val and Leu [1]. Applying the in vivo TOXCAT assay to the TM domain sequence and to specific mutants of it, confirms this proposed interface whilst another proposed interface is discounted. Current studies are focussing on the effect of the TM mutation to the activation of the ErbB-2 receptor and to any possible change in this interface.

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P-362

Glycosyl acridines reducing lysozyme amyloid aggregation

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Numerous diseases have been linked to a common pathogenic process called amyloidosis, whereby proteins or peptide clump together to form amyloid aggregates in the body. An attractive strategy to develop therapies for these diseases seems to be reduction of polypeptide aggregation. We have tested several acridine derivatives characterized by various glycosyl groups for their potential to affect the lysozyme amyloid aggregation *in vitro*. The ability of glycosyl acridines to interfere with lysozyme aggregation was investigated by ThT assay. We found that structure of acridine side chain is factor affecting their anti-aggregation activity significantly. For the most effective compounds the values of IC50 and DC50 were obtained. The reduction of protein aggregation was confirmed by AFM. To investigate influence of the glycosyl acridines on the cell processes we examined effect of compounds on cell viability. We performed glycosyl acridines characterization by high anti-aggregation activity and low toxicity suggesting their possible application for therapeutical purpose. (This work was supported by project of ESF 26220120021 and by Slovak Academy of Sciences in frame of CEX NANOFLUID and VEGA grants 7055, 0056 and 0038 and EU-STRP 0032652 BIODOT.).

P-363

Effect of magnetic nanoparticles on lysozyme amyloid assemblies *in vitro*

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Peptide amyloid aggregation is a hallmark of amyloid diseases including Azheimer's disease or type II diabetes. Recent works have addressed the potential of nanoparticles to affect amyloid aggregation. The experimental data are very controversial suggesting that particle characteristics markedly influence the final effect of nanoparticles on the amyloid aggregation (initiation, acceleration or inhibition of amyloid aggregation). We investigate the ability of electrostatically stabilized magnetic nanoparticles of Fe_3O_4 to affect the amyloid aggregation of lysozyme, as a prototype amyloidogenic protein. We have used a combination of spectroscopic (ThT fluorescence) and local microscopic techniques (AFM). We found, that the ability of magnetic nanoparticles to inhibit formation of amyloid aggregates or destroy pre-formed amyloids exhibit concentration-dependence. The values of inhibition IC50 and depolymerization DC50 were determined suggesting that nanoparticles interfere with lysozyme aggregation at stoichiometric concentrations. The observed features make magnetic nanoparticles of potential interest as a therapeutical agent against amyloid diseases. (This work was supported by project of ESF 26220120021 and by Slovak Academy of Sciences in frame of CEX NANOFLUID, VEGA grants 7055, 0056 and 0038 and EU-STRP 0032652 BIODOT.).

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Comparative analysis of structural and functional properties of Aβ-amyloid aggregated species

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Alzheimer disease (AD) is characterized by cerebral extracellular deposits of β-amyloid (Aβ) fibrils. Aβ aggregation is a multi-step process involving the formation of various conformational species including soluble intermediate species (i.e. Aβ oligomers), protofibrils and fibrils. Such aggregates may have various effects on neuronal and glial function and differentially contribute to AD neurodegeneration. Aim of this study was to investigate the structural properties of distinct Aβ aggregated species and dissect out their effects on neuronal viability. Recombinant Aβ42 and Aβ40 peptides were aggregated *in vitro* in conditions differing by ionic strength, temperature and pH and were analyzed by gel electrophoresis, thioflavin T binding assay and atomic force microscopy (AFM). AFM analysis was performed using both hydrophilic and hydrophobic substrates, to analyze the full spectrum of structural species. Stable low molecular weight oligomers were obtained when Aβ42 was incubated at 37°C for 5 days in low salt concentration buffer. Doughnut-shaped conformational species were detected by AFM alongside globular aggregates (1.6-5.2 nm height range). Acidic pH promoted aggregation of Aβ42 into Thioflavin-positive fibrils and protofibrils. Protofibrils appeared as beaded chains having a mean height of 3.65±1.49 nm. Effects of Aβ on viability of mouse hypppocampal neurons were assessed and correlated with their conformational features.

Abstracts
– Biomolecular self-assembly –

P-365

ORF 8a of the SARS Coronavirus forms an ion channel: experiments and molecular dynamics simulations

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Many genomes of viruses encode small membrane spanning proteins which are proposed to modify membrane permeability for ions and small molecules. These channel or pore forming proteins are getting into the focus for antiviral therapy since they are essential for some of the viruses. One of the general themes of the mechanism of function of the proteins is to self-assemble to form the functional form.
We present a study on the open reading frame (ORF) 8a membrane protein encoded in structural region of Human Severe Acute Respiratory Syndrome Coronavirus (SARS-CoVs). The full length ORF8a protein is 39 residues long and contains a single transmembrane (TM) domain. Full length protein is synthesized using solid phase peptide synthesis and reconstituted into artificial lipid bilayers forms cation-selective ion channels. The bilayer recordings show cation selection channel activity with a major conductance level of around 8.5 pS also at elevated temperatures (38.5°C). In silico studies with a 22 amino acid TM domain are done to assess conformational space of the monomeric ORF8a helix. With this monomeric helix homooligomeric helical bundle models are built and embedded in a fully hydrated 1-palmitoyl-2-oleoyl-sn-glycerol-3 – phosphatidylcholine (POPC) bilayer. Results of both experimental channel recordings and computational modeling show SARS ORF8a to act as a channel forming protein.

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Assembly and bundling of the bacterial Z-ring protein FtsZ

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FtsZ, the ancestral homolog of eukaryotic tubulins, is a GTPase that assembles into a cytokinetic ring structure (Z ring) essential for cell division in prokaryotic cells. The Z ring also recruits other proteins (e.g. ZapA, YgfE, ZipA) to the division site, where they participate in formation of the septum that separates the two daughter cells. We have studied FtsZ polymerization and its dynamic behaviour in real time by right angle light scattering. Similar to tubulin, FtsZ polymerizes into dynamic protofilaments in the presence of GTP; polymer assembly is accompanied by GTP hydrolysis. The kinetics of inorganic phosphate (P_i) released from the GTP hydrolysis have been studied as well, employing a fast and sensitive colourimetric assay. At pH 6.5, approximate 90% of the P_i was released into the media within 20 minutes of GTP addition. The effects of GTP, pH, K^+, and Mg^{2+} were studied in both cases, and the results were used to build up a model for the mechanism of fibre assembly and disassembly. YgfE, a FtsZ accessory protein, is identified as a functional ZapA orthologue. Finally, we have studied the YgfE bundling to FtsZ polymers. It strongly promotes FtsZ bundling and is an inhibitor of the GTPase activity.

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The effect of &beta-amyloid peptide on polymer cushioned membranes

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Beta-amyloid (Aβ) is a peptide implicated in the neurodegenerative process characteristic of the Alzheimer's disease (AD). To clarify its mechanism of action it is crucial to elucidate the interaction of Aβ with the neural membrane. In previous work we demonstrated the capability of Aβ to penetrate and perturb stacked lipid bilayers. In this study we considered polymer cushioned lipid bilayers as a model for neural membranes. The polymer cushion is aimed to preserve the membrane natural fluidity; it is obtained depositing charged polyelectrolites layer-by-layer; the lipid membrane is built on the top of the polymer film by fusion of unilamellar vesicles. The floating membranes were kept always in contact to the subphase. The kinetics of adsorption of the lipid double layer at the polymer/water interface was monitored by neutron reflectivity; different experimental conditions to obtain the best surface coverage were exploited. After administration of Aβ to the subphase the lipid membrane still adhered to the polymer cushion, but its structure was modified by the interaction with Aβ. Neutron reflectivity showed a change of the scattering density profile in the direction perpendicular to the membrane plane, suggesting a penetration of Aβ inside the double layer. A change in the surface morphology was detected by AFM imaging; AFM film-rupture experiments showed that Aβ weakens the lipid packing.

O-368

Physical mechanisms controlling DNA ejection from tailed bacteriophages

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Bacteriophages are complex molecular assemblies which multiplication relies on bacteria infection. The process starts with the binding of the phage on its specific host receptor and the injection of its genome into the host cytoplasm. Our work aims to determine the physical mechanisms and forces driving the DNA transfer from the phage capsid. The *in vitro* DNA ejection has been analyzed by using light scattering and gel electrophoresis measurements for three phages (T5, SPP1 and Lambda) belonging to the same family (*Syphoviridae*). Our results reveal two forces contributing to drive the DNA transfer: the first one is originated from the pressurization due to the strong confinement of DNA into the capsid; the second one comes from a pulling mechanism originated by the presence of condensed DNA outside the capsid. These two contributions were characterized in *in vitro* conditions but they likely play a role in the *in vivo* transfer. The ejection kinetics was also analysed and the characteristic time of the mechanism was studied as a function of the temperature. It appears to follow an Arrhenius law, allowing the determination of the activation energy that governs the transfer. The energy values are close for the different phages, suggesting that the mechanism regulating the ejection is common for a given phage family. Below these general features, our studies also reveal differences between the three phages.

Abstracts
– Biomolecular self-assembly –

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Equilibrium/non-equilibrium transitions in macromolecule interactions

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Usually, so called 'relaxation phenomena' occur on a fast time-scale and 'P-jump' or 'T-jump' techniques are required to follow such events lasting (much) less than 100 ms. We report that, during stability studies of proteins or nucleic acids, such relaxation events can be observed on astonishing long time-scales. We first performed 'melting studies' with nucleic-acid duplexes by using linear variations of temperature (T) with time (t). We observed that even very low rates dT/dt could lead to a frozen state for temperature values below a sharp temperature range, and relaxation to equilibrium beyond that range. This allows defining a 'relaxation temperature' T_r separating the two regimes. Numerical simulations very accurately described the related hysteresis phenomenon observed upon a heating-cooling cycle, which is the hallmark of departure from equilibrium. Analogous observations were made with protein oligomers submitted to either a variable pressure, or variable concentration in denaturant. Importantly, a single theoretical frame predicts that the critical relaxation value X_r (X standing indifferently for temperature, pressure or denaturant concentration) depends on Ln(dX/dt). One may ask whether some thermosensor RNAs known for switching on or off genetic expression by 'feeling' a temperature variation, might also 'feel' dT/dt. If true, the exact switching temperature would depend on dT/dt and faster temperature changes would increase T_r.

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Amyloid beta peptide fibril formation modulated by phospholipid membranes

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Disease-causing amyloid fibril formation can be modulated by many factors including interactions with biological lipid membranes. An increasing amount of evidence suggests that the process of fibril formation *in vivo* and the mechanism of toxicity both involve membrane interactions. Alzheimer's is probably the most well-known amyloid disease and the associated amyloid beta peptide originates from the membrane incorporated amyloid precursor protein (APP). We use recombinant Abeta M1-40 and Abeta M1-42 produced in *Escherichia coli*, which allows us to perform large scale kinetics assays with good statistics where the amyloid formation process is followed in means of thioflavin T fluorescence. The lipid membranes are introduced in the system as large unilamellar vesicles composed of DOPC, DPPC and Sphingomyelin, with and without incorporation of cholesterol. We find that the phase behanviour of the membrane in the vesicles has a large effect on the lag time of the amyloid formation process for both Abeta M1-40 and M1-42. All membranes increase the lagtime to some degree but DPPC has the largest effect. By comparing different phases we can conclude that the translational diffusion in the membrane seems to be more important than the acyl chain ordering. Furthermore, electrostatics, concentration dependence and membrane addition at different time points in the amyloid formation process have been investigated.

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Assembly of bacteriophage T5 capsid: Structural and dynamic characterization

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The *Syphoviridae* coliphage T5 is a well-suited model to study the assembly of large viral capsids. Biochemical and biophysical approaches were used to reconstitute *in vitro* the assembly pathway of its capsid. The T5 structure was recently solved from cryo-EM and image reconstruction. Its icosahedral capsid (T = 13) is built from the major head protein (pb8, 775 copies) forming both the pentons and hexons and from the portal protein (pb7, 12 copies) located at one vertex. Its assembly proceeds by steps. pb8 and pb7 first assemble into a precursor structure called prohead I, which is converted to prohead II by proteolysis of pb8 and pb7 by a head maturation protease. Packaging of the 121 kbp dsDNA is then driven through the portal pore by a molecular motor, the terminase. This promotes expansion of prohead II leading to the mature capsid. The different assembly steps and the conformational changes accompanying capsid maturation were characterized using Proheads I either self-assembled from the overproduced and purified capsid proteins or isolated from a phage mutant. These precursor capsid structures were analysed by Small Angle X-ray Scattering. The 3D structure of prohead II and of the expanded capsid were solved from cryo-EM. Our data show that the assembly process of a large icosahedral capsid can be efficiently reconstituted *in vitro*.

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Alp14 and Dis1 as catalysts of microtubule dynamics in S. pombe

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Microtubules are involved in many vital processes. Their rigid structure can resist high forces while their intrinsic ability to switch stochastically between growth and shrinkage phases allows them dynamically to reorganise. In cells, a sizeable network of microtubule binding proteins control and regulate microtubule dynamics. Alp14 and Dis1 are members of the Dis1/ XMAP215 family that are major players in S.pombe. The deletion phenotypes of Alp14 and Dis1 are similar, but nonetheless distinct. Both are involved in the formation of spindles but Alp14 is also involved in the maintenance of cytosolic microtubules in interphase. The restrictive temperatures of Alp14-deletion and Dis1-deletion mutants are different. Alp14 interacts with Alp7, a potential member of the TACC protein family. I am working to reconstitute Alp14/Dis1-dependent microtubule dynamics in vitro, using purified S. pombe tubulin. Both Alp14 and Dis1 express well in insect cells and can be readily purified. Preliminary data show that both proteins bind tubulin at low salt concentrations and that both influence the dynamics of pig brain microtubules. My goal is a complete functional analysis of Alp14 and Dis1, individually and in combination, to test candidate molecular mechanisms for Alp14/Dis1-catalysis of S. pombe microtubule dynamics.

Abstracts
– Biomolecular self-assembly –

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Tethered bilayer lipid membranes: an interesting model membrane platform

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Model membranes mimic the essential function of a natural membrane. However, the complexity is reduced in order to allow the study of fundamental processes.
Tethered membranes consist in principle of a lipid bilayer that is covalently linked to a solid support through a spacer group. This architecture allows the characterization of the membrane itself as well as of incorporated membrane proteins using surface analytical techniques. We have established a versatile system of various anchor lipids, which allow membrane formation on different surfaces. The architectures have been characterized by surface Plasmon techniques, neutron reflectivity and electrochemical methods. The membranes are electrically insulating and allow for the functional incorporation of ion channel proteins. Polymerizable lipids allow to pattern the membrane and to study lateral diffusion processes.
Furthermore, the membranes can be used as a sensing platform, where embedded membrane proteins act as actual sensing units.

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Complexes between DNA and PEGylated chitosans: physicochemical properties and transfection efficacy

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Materials used as gene delivery vehicles must be able to condense DNA into small sizes to facilitate transport and crossing various barriers. One of the polycations investigated for DNA compaction is chitosan, which has the advantage of being safe and biodegradable. As a step towards reducing the aggregation behaviour of DNA-chitosan complexes, chitosans were modified by grafting PEG-chains on the backbone. It is known that the transfection efficacy depends on the chitosan chain length. Additionally, the degree of pegylation might influence the condensation process. Here a systematic biophysical study of pegylated chitosans and how the interplay between chitosan chain length and degree of pegylation affect the compaction of DNA in terms of particle size and structure, stability in PBS and when exposed to serum, and transfection efficacy is presented. Three different chain lengths of chitosans are employed, and for each sample three pegylation degrees are investigated and the properties of the DNA-PEGchitosan complexes compared to complexes formed when employing the original, chitosan for DNA compaction. It is found pegylation of chitosans can be used to increase both the stability of the DNA-chitosan complexes when exposed to serum as well as increase their transfection efficacy in HEK293 cells.

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The role of proline isomerisation in the aggregation process and fibril formation of alpha-synuclein

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Alpha-synuclein (α-syn) plays a central role in Parkinson's disease. The aggregation of this protein, which contains five proline residues (P108,P117,P120,P128,P138), is accelerated in vitro by FK506 binding proteins (FKBPs), a family of enzymes with a peptidyl-prolyl *cis-trans* isomerase activity (PPIase). FKBPs catalyze the cis-trans conformational change of proline, often a rate limiting step in protein folding. To elucidate the role of the proline residues in aggregation, we constructed a mutant P(108,117,120,128,138)A α-syn . The kinetics of the aggregation of the mutant were studied with turbidity and Thioflavin T fluorescence (ThT). Turbidity measurements show the formation of early, ThT negative aggregates which is as fast for both WT and mutant. Fibril formation however is faster for the proline-deficient mutant. We also studied the effect of FKBP12 on the aggregation of the mutant. Although WT α-syn early aggregate formation is accelerated by the addition of 1 μM FKBP12, this effect disappears in the mutant. Addition of (1pM-1μM) FKBP12 accelerates the fiber formation of WT α-syn, which is abolished in the mutant.
We can conclude that α-syn fiber formation is accelerated for the proline-deficient mutant, which suggests a role for the proline residues in fiber formation. Furthermore all accelerating effects of FKBP12 are abolished in the mutant which suggests that the PPIase activity of FKBP12 is responsible for the accelerating effect on the aggregation of WT α-syn.

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Structural characterization of prion peptide PrP82-146 in form of oligomers and fibrils

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The major component of amyloid plaques in the Gerstmann-Sträussler-Scheinker disease is a prion peptide fragment from 81-82 to 144-153 residues. Here, we present a structural study of PrP82-146 in form of oligomers and fibrils by Fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM). After incubation at 37°C, the unfolded peptide was found to aggregate into oligomers characterized by intermolecular β-sheet infrared bands and by a wide distribution of oligomer volumes. After a lag phase, a conformational rearrangement of oligomers into fibrils, with a parallel orientation of the cross β-sheet structures, was observed. By AFM, different morphologies were also detected for fibrils that displayed high heterogeneity in their twisting periodicity and a complex hierarchical assembly. In addition, we also studied thermal and random aggregation. The PrP82-146 peptide was found to undergo several aggregation pathways, whose end products display different structural properties and intermolecular interactions. These findings underline the high plasticity of the prion peptide, a peculiar feature of prion proteins to overcome species barriers *(Natalello et al. J.Mol.Biol. 2008;381:1349-1361).*

Abstracts
– Biomolecular self-assembly –

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Polyglutamine aggregation and neurodegeneration

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Polyglutamine (polyQ) diseases are rare but dominant misfolding diseases linked to neurodegeneration. They are caused by the expansion of CAG codon repeats, which encode polyQ tracts in the corresponding gene products. Aggregation of polyQ proteins is thought to be triggered by polyQ expansion but be strongly modulated by the protein context. In the attempt of understanding the molecular bases of polyQ diseases, we are studying the structures, interactomes and aggregation properties of selected polyQ proteins.
Here, we present recent work on ataxin-3, taken as a representative example of the whole family. Ataxin-3 is a ubiquitin specific cysteine protease, involved in the ubiquitin-proteasome pathway and known to bind poly-ubiquitin chains of four or more subunits. The enzymatic site resides in the N-terminal josephin domain of ataxin-3. We have characterized, using different biophysical techniques, the structure in solution and the aggregation properties of josephin both in isolation and in a ubiquitin complex. We demonstrate that interaction with ubiquitin strongly modulates the aggregation properties of ataxin-3 and suggest the importance of protein-protein interactions in preventing aggregation. Our study also provides new insights into the molecular mechanisms which determine ataxin-3 specificity for poly-ubiquitin chains of the correct length and cross-linking.

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Lactalbumin - Lysozyme interaction as evidenced by fluorescence measurements

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Understanding protein–protein interactions and assemblies to control the hierarchical building of well-ordered supramolecular structures is highly relevant to new tailor-made biomaterials. We previously evidenced that contrary to native calcium-loaded α-lactalbumin (holo α-LA), calcium-depleted form (apo α-LA) has the ability to self-assemble with lysozyme (LYS) to form different supramolecular structures in temperature-dependent manner. In the present work, the events occurring at molecular scale were explored using fluorescence techniques. Fluorescence anisotropy and fluorescence lifetime measurements provide a powerful and sensitive mean to measure intermolecular interactions. We showed that LYS interacts with both apo α-LA and holo α-LA to form oligomers, assumed to be heterodimers, at 10°C and 45°C. The dissociation constants for dimerization, found to be in the μM range, were sensitive to the ionic strength. Correlation time calculations suggest that formed heterodimers holo α-LA/LYS and apo α−LA/LYS differed in their shape and/or conformation. Such conformation differences could explain why holo α-LA/LYS complexes are trapped as heterodimers while the apo α-LA/LYS complexes have the ability to further self-assemble into previously reported various supramolecular structures.

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Protein adsorption on hydrophobic surfaces: unfolding vs reassembly

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Upon interaction with hydrophobic surfaces, proteins show a tendency to expose regions that are normally buried in the hydrophobic core. Unfolding is generally perceived as an undesired process in studies aimed to anchor functional proteins at surfaces. Upon an upset of perspective the fine control of the unfolding/re-assembly process could be regarded as a strategy to build up molecular nanostructures for the development of organic-inorganic assemblies. We show that molecular layers patterned at the nanoscale, with long-range order properties extending over the microscopic scale, can be obtained upon adsorption of proteins onto the hydrophobic and ordered surface of pyrolytic graphite. Upon adsorption, proteins lose their native folding and polypeptide chains re-assemble on the surface in a layered fashion, forming a molecular bilayer. The first layer, in contact with the substrate, and the second molecular layer show corduroy-like nanopatterns of different periodicity, with a relative orientation between the first and second layer patterns of 30°. Surface-induced protein unfolding and polypeptide chain reassembly according to a layered ordered structure is a rather general phenomenon since it is observed for different proteins irrespectively of their specific structural properties. The possibility of using these ordered molecular structures as templates for the subsequent patterned deposition of supramolecular aggregates will be discussed.

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DNAs or like-charged polyelectrolytes attraction and assembly

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The electrostatic free energy (fe) of two parallel rigid like-charged polyelectrolytes (PEs) is given as a function of the separation distance.[1] For high linear charge density, z, the fe shows a minimum due to the increasing of the counterion condensation and condensation volume as the two PEs approach. The interaction fe is governed by a critical linear charge density, z_c, inversely proportional to the counterion valence. For highly charged PEs ($z > z_c$, like DNA), the PEs attract the stronger the smaller is the counterion valence, because the fe is dominated by the entropic term due to condensation of counterions in a volume displaying a maximum at short distances. For weakly charged PEs ($z < z_c/2$) the PEs remain undercritical in the whole separation range and therefore repel. For moderately charged PEs ($z_c/2 < z < z_c$), the infinitely separated PEs are undercritical but become supercritical as they approach a critical distance and charge condensation and condensation volume expansion start: in these circumstances the PEs may attract if the counterion valence is sufficiently large.
In the case of many DNA rods, hexagonal clusters may be formed. [1]Pietronave, S.; Arcesi, L.; D'Arrigo, C.; Perico, A. J. Phys. Chem. B **2008**, 112, 15991-15998

Abstracts
– Biomolecular self-assembly –

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Stability of inverted hexagonal mesoscopic phase affected by phospholipid anisotropy

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The role of phospholipid anisotropy in the stability of inverted hexagonal phase was considered. The equilibrium configuration of the system was determined by the minimum of the free energy involving the contribution of the isotropic and deviatoric bending and the interstitial energy of phospholipid monolayers. The shape and local interactions of a single lipid molecule were taken into account. The minimization with respect to the configuration of the lipid layers was performed by the Monte Carlo simulated annealing method. At high enough temperature the lipid molecules attain a shape exhibiting higher intrinsic mean and deviatoric curvatures which fits better into the inverted hexagonal phase than into the lamellar phase. For the mathematical model the advanced geometry with non-spherical cross-section of inverted hexagonal phase was calculated, resulting in lower energy in non-spherical cross-section. Theoretical results are in a good agreement with the small angle X-ray scattering experimental data.

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Adsorption of Yeast Cytochrome c on conductive substrates investigated by spectroscopic ellipsometry

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We present the results of a Spectroscopic Ellipsometry (SE) study of the adsorption process of Yeast Cytochrome c (YCC) on gold and graphite substrates, according to methods already applied to study the growth dynamics of organosulphur SAMS on gold [1]. SE investigation was carried out both in situ, at room temperature during protein deposition, and ex situ. On gold, SE data demonstrate the formation of an about 3-4 nm thick layer, consistent with the formation of a dense monolayer of YCC molecules, confirmed by AFM inspection. Both in situ and ex situ measurements were characterized by well defined spectral features related to the Soret band. Analysis of the fine position of this feature allowed to obtain information on the oxidation state of the iron ion of the heme group. SE data suggest that proteins have preserved their native structure. A completely different adsorption mechanism was observed on highly oriented pyrolytic graphite (HOPG)[2]. Ex-situ SE data on YCC/HOPG, supported by AFM observations, indicate the formation of an ultrathin molecular layer (~0.7nm) related to complete protein unfolding at the hydrophobic surface.

[1] M. Prato et al. J. Phys. Chem. C, 2008 ,112
[2] Svaldo-Lanero et al., Soft Matter 2008, 4

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Observing protein aggregates on surfaces

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Protein aggregation is an important topic of current protein research as it is associated with several human diseases including Alzheimer's disease, Parkinson's disease, and Type II diabetes. Although protein aggregation mechanisms and conditions have been comprehensively investigated, studies on the formation and the fate of protein aggregates in contact with solid interfaces are scarce.
We have comprehensively investigated the structure of protein assemblies that form spontaneously upon protein adsorption on solid interfaces using a surface sensitive fluorescence imaging technique based on super critical angle fluorescence (SAF) detection. Combining this technique with FRET we not only succeeded to detect protein aggregates deposited on surfaces but also to characterize their behavior in real time, i.e., their emergence, growth, or spreading. The model protein BSA, for instance, was found to exhibit a certain tendency for aggregation in the buffer solution. These protein clusters can deposit onto solid surfaces and spread resulting in a large, flat structure after some time.[1]
A different possibility how protein aggregates emerge on surfaces consists of a direct deposition of protein monomers to pre existing aggregates. Such a growth of protein aggregates on the surface has been observed in a model system using the protein α-synuclein, which is tightly associated with the Parkinson's syndrome.
Ref.: [1] M. Rabe et al., *Soft Matter*, 2009, **5**, 1039-1047.

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In vivo determination of structure and distributions of supra-molecular associations

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Förster Resonant Energy Transfer (FRET) from an optically excited to a non-excited molecule has been widely used to probe molecular interactions in living cells. Changes in the molecular makeup of a cellular region occurring during the acquisition of fluorescence images place tight constraints on the FRET technology and data analysis, which could not be addressed satisfactorily until recently. We will describe a method for imaging protein complex distributions in living cells with sub-cellular spatial resolution, which relies on a spectrally resolved two-photon microscope (Raicu et al, 2009, *Nature Photon.* **3**: 107-113) and a simple theory of FRET in oligomeric complexes (V. Raicu, 2007, *J. Biol. Phys.* **33:**109–127). Then, we will overview recent results on the determination of the supra-molecular structure and distributions in living cells of oligomeric complexes of some G protein-coupled receptors.

Abstracts
– Biomolecular self-assembly –

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A multi-resolution approach to the structure and function of integrin αIIbβ3

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Integrins are heterodimeric transmembrane receptors involved in mechanical anchoring and two-way signaling. Each α and β subunit has a modular structure with a large extracellular portion, a single transmembrane region, and a cytoplasmic domain. Integrins activation mechanism is regulated by controversial conformational changes: while crystallography revealed similar bent shapes for resting and primed extracellular region constructs, ligand binding-induced large structural rearrangements in smaller constructs suggested extension, "opening" and tails separation. In a multi-resolution approach, we used experimental and computed hydrodynamics to discriminate among αIIbβ3 integrin models built on X-ray, NMR, and EM data. In contrast with X-ray data and 3D EM maps, an extension is needed to match the hydrodynamics of full-length, solubilized αIIbβ3; an electron tomography-based model fares better. Consistent with that, and with our averaged 2D EM images, a conformational change in the head region (β3 hybrid domain swingout) coupled to a simple transmembrane helices shift matches priming agents-induced frictional changes in full-length αIIbβ3. Our multi-resolution study thus suggests that in integrins extension and immediate tail separation are uncoupled from head domain rearrangements following activation.

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Supported Biomimetic Membrane designed for the investigation of a bacterial toxin translocation

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Supported Biomimetic Membranes (SBM) were developed for protein-membrane interactions studies. Phospholipid vesicles were chemically linked onto amine grafted gold or glass surfaces; after an osmotic choc and liposomes fusion a continuous membrane bilayer was formed. The anchoring phospholipid molecule (DSPE-PEG-NHS) incorporated into the vesicles allowed the formation of a water-filled compartment between the surface and the bilayer. This first SBM model was used to monitor the membranes binding properties (dependent of calcium) of the adenylate cyclase toxin (CyaA) from *Bordetella pertussis*. The SBM model was improved in order to study the translocation of the catalytic domain of CyaA across the bilayer. Naturally, the CyaA catalytic domain, when it reaches the target cell cytosol, associates with intracellular calmodulin (CaM) an activator of the adenylate cyclase activity of CyaA. To mimic this biological phenomenon, CaM was first immobilized on the surface (gold or glass) and in a second step membrane construction was performed over the CaM layer. The formation of the biomimetic membrane onto the CaM layer was monitored by SPR while membrane fluidity and continuity were analysed by fluorescence. Our results demonstrated the potentialities of SBM for the study of protein insertion into and translocation across biological membranes.

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Can peptaibols used as elicitors ?

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Peptaibols are peculiar peptides produced by fungi associated to plants. They are composed by 4 to 20 amminoacidic residues and exhibit antibiotic and antifungal properties. Due to their amphypatic nature, they can form ion channels in biological membranes. By making use of experimental models of biological membranes (biomimetic membranes) currently employed in the Laboratory of Bioelectrochemistry, and models of plant membranes (corn seed root), that are used in the International Laboratory of Plant Neurobiology (LINV), we characterized synthetic peptides such as Trichogin GAIV and its shorter homologues (4 and 8 residues). We studied these peptaibols in a dioleoylphosphatidylcholine monolayer supported by Hg using different electrochemical techniques (AC,VC,EIS). The experimental technique employed in the LINV (Clark microelectrode coupled to MIFE® system) allows to measure oxygen flux in the solution contacting plant cell membranes, after treatment with different peptide concentrations. Preliminary results might indicate that short peptides can influence the whole metabolism of the plant and can therefore be used as "elicitors" in order to induce an acquired systemic resistance.

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Natural pigments induce perturbation of beta-amyloid peptide self assembly

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For a long while the conventional view has been that Alzheimer Disease is brought about by the beta-amyloid fibrils found in the senile plaques, but more recently it has been suggested that the main neurotoxic species would be the soluble oligomeric species, apparently prone to interact with cell structures and macromolecules potentially inducing neuronal dysfunction. Peptide-peptide interactions resulting in self-assembly phenomena of beta-amyloid yielding fibrils can be modulated and influenced by small organic molecules that might also be effective therapeutic tools to ideally target both oligomeric and fibrillar species. In this perspective, polycyclic aromatic molecules are of special interest because they might disrupt the molecular architectures precursors of beta-amyloid fibrils by means of weak, non-covalent aromatic interactions, like stacking interactions.
We have performed an *in vitro* spectroscopic study (light scattering, circular dichroism, FTIR and fluorescence) of the effects on beta-amyloid fibrillogenesis of the natural pigment hypericin extracted from *Hypericum perforatum*. Our results show that, thanks to its structural characteristics and peculiar spectroscopic features, hypericin can be easily used to *in vitro* monitor the appearance of initial aggregation states of beta-amyloid peptides and, more importantly, that hypericin can interfere with the early stages of polymerization process, playing the role of an aggregation inhibitor.

Abstracts
– Biomolecular self-assembly –

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Self-assembly of NADPH:protochlorophyllide oxidoreductase macrodomains *in vivo* and *in vitro*

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Chlorophyll biosynthesis is light-dependent in angiosperms because the reduction of protochlorophyllide (Pchlide) into chlorophyllide is driven by a photoenzyme, NADPH:Pchlide oxidoreductase (POR). The unique properties of POR are due to its ability to assemble into dimers and oligomers within the prolamellar body (PLB) membranes of etioplasts studied mainly in leaves of dark-grown seedlings under laboratory conditions. We extended these studies to plant organs developed in the nature: cabbage heads, leaf primordia inside buds, pericarp-covered regions of sunflower cotyledons, potato tubers and seedlings germinating under the soil. In electron microscopic and fluorescence spectroscopic studies we found in many of these organs poorly developed PLBs in which POR was mainly in monomer state. As a consequence, the chlorophyll accumulation was slow and photo-oxidation processes occurred at illumination. *In vitro* we artificially induced the aggregation of POR monomers into oligomers in glycerol and sucrose containing buffers. This resulted in the increase of the photoreduction rate at the expense of photo-oxidation. These results underline the importance of the self-assembly of POR and the PLBs in chloroplast development and chlorophyll synthesis in nature.

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Self-assembly of phenylalanine oligopeptides: insights from experimental and computational studies

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The diphenylalanine peptide (FF) forms well ordered nanotubes and its derivatives form nano-assemblies of various morphologies with promising material applications. We demonstrate for the first time by electron and laser microscopy and FTIR spectroscopy that the related, triphenylalanine peptide (FFF) assembles into rather planar nanostructures, rich in β-sheet. In addition, we conduct 0.4-μs replica exchange M.D. simulations of aqueous FF and FFF solutions in implicit solvent. The peptides coalesce into aggregates and participate frequently in open or ring-like linear networks, as well as elementary and network-containing structures with β-sheet characteristics. Polar and nonpolar interactions, as well as the surrounding aggregate medium contribute to the network stabilities. Within a network, consecutive peptides are linked by head-to-tail interactions; the aromatic sidechains of neighbor peptides assume approximately "T-shaped" orientations. These features are observed in FF crystals and could characterize early formations, or stabilize the mature nanostructures. The FFF aggregates acquire higher stability and peptide-network propensity compared to the FF aggregates due to energetic contributions[1,2].

1. Tamamis et al. Biophys. J. in press, 2009.
2. Tamamis et al. NIC Series 40, 393-396, 2008.

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Bacterial protein self-assembly on surfaces of well-defined chemistry

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S-layers are one of the most common cell envelope components of prokaryotic organisms and represent the simplest biological membrane developed during evolution. These (glyco)proteins, which can self-assemble into 2-D crystalline nanostructures on lipid films, liposomes, and polymers, play already an important role in nanobiotechnology.

In this work, we present new findings concerning the recrystallization of bacterial proteins on substrates with defined chemistry.

Manipulation of the protein-sample interaction was carried out by changing the relative height of OH and CH_3 terminated moieties in self-assembled monolayers (SAMs). We have found that differences in chain length lead to: i) protein bilayer-protein monolayer transition, ii) preferential protein side adsorption, and iii) increase of the crystal lattice parameters.

Further manipulation of the protein-sample interaction was achieved by using silane chemistry. We will show that sample hidrophobicity speeds up recrystallization kinetics and reduces the crystalline domain size (and layer compliance). The protein-adsorbed mass per unit area on these substrates is reported for the first time.

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Stabilizing effects of α_{s1}-casein, a natively unfolded protein, on the aggregation of biomolecules

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α_{s1}-casein is one of the four types of caseins, a group of calcium phosphate-binding proteins that, in the form of micellar aggregates, makes up the largest protein component of bovine milk. The structure of α_{s1}-casein is that of a triblock polymer with a hydrophilic tract interposed between two hydrophobic blocks. Due to the lack of a compact folded conformation, this protein can be classified as one of the intrinsically disordered (or natively unfolded) proteins. This class of proteins is known to exert a stabilizing activity on biomolecules through specific interaction with hydrophobic surfaces that partially unfolded molecules may expose to the solvent. Here we present results on α_{s1}-casein effects on the thermally induced aggregation of Gluthathione S-transferase, a ligand-binding anzyme, and 1-40 β-amyloid peptide involved in Alzheimer's disease. By means of Light Scattering and Circular Dichroism experiments, we attempt to reveal the molecular details of α_{s1}-biomolecules interaction.

Abstracts

– Biomolecular self-assembly –

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Oligomerization of Concanavalin A in live cells detected by fluctuation analysis

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We report an experimental study on ConcanavalinA (ConA) aggregation in live cells. In vitro, close to physiological temperature, ConA readily forms fibrils involving secondary structure changes leading to β-aggregate structures. The effect of ConA on cell cultures and formation of protein aggregates were measured by confocal fluorescence microscopy. In particular, we monitored protein aggregation in live cells by means N&B analysis, Cross-N&B and RICS. N&B showed the aggregation kinetic and the progressive formation of ConA oligomers at cell surface. This suggests that, at cell membrane where local concentration is higher, nucleation sites for aggregation are provided. In parallel, the morphology of the cells changes indicating the progressive cell compaction and death. Aggregation and binding of small aggregates to the cell surface were assessed by RICS: it is possible to distinguish regions where small aggregates are diffusing and regions where they are bound to the cell. Oligomers formation may stimulate non-specific cellular responses due to the exposure of reactive regions of protein structure and of progressive formation of cross−β structures. Moreover, aggregates stoichiometry was measured during the kinetic by Cross-Variance N&B.

Abstracts

– Structure-function relationship in channels, pumps and exchangers –

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Reconstitution of fluorescent voltage gated ion channels in giant unilamellar vesicles

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The physics of membrane/channel and channel/channel interactions is difficult to investigate in cells where it is nearly impossible to modify relevant parameters to deduce physics laws. To overcome these difficulties we built a model system in which voltage gated ion channels were reconstituted in Giant Unilamellar Vesicles (GUVs) for the first time.
As a first step, we successfully expressed KvAP (a voltage gated potassium channel) in E-coli. The channel was purified, fluorescently labelled and reconstituted in small liposomes. Its functionality was checked with electrophysiology via fusion of these liposomes into Black Lipid Membranes (BLM).
As a second step, GUVs were formed from these small proteoliposomes using electroformation in a buffer containing 100 mM KCl salt. The proper incorporation of proteins into GUVs was controlled using confocal microscopy. Functional proteins were detected using the patch clamp technique.
With our protocol, we are thus able to prepare GUVs containing functional voltage-gated ion channels.
One goal is now to study the effect of channel activity on its spatial distribution in these GUVs.

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Listeriolysin pore forming ability in planar lipid membranes at different pH

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Listeriolysin O (LLO) is a cholesterol-dependent cytolysin secreted by the intracellular pathogen *Listeria monocytogenes*. Its main task is to enable escape of bacteria from the phagosomal vacuole into the cytoplasm. LLO exhibits optimal cytolytic activity at low pH but it is still able to bind membranes at physiological or even slightly basic pH values in a cholesterol-dependent fashion. High cholesterol concentrations in the membrane restore the low activity of LLO at high pH values.
Based on this broad pH activity we investigated the electrophysiological properties of pores formed by LLO at room temperature and at different pHs using Planar Lipid Bilayer technique.
LLO is able to form pores both at pH 5.5 and 7.5 with a similar permeabilizing ability and similar heterogeneous conductances in the range of picosiemes to nanosiemens. Cholesterol content directly correlates with LLO activity but it does not change the pore characteristics.
Collectively, our results demonstrate that LLO activity at physiological pH cannot be neglected and that its action at sites distal to cell entry may have important physiological consequences for Listeria pathogenesis.

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Cell hydration as a universal messenger for environmental signal transduction in cell

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The review of our data on the effects of physical and chemical weak signals on physicochemical properties of water, cell volume, activity and the number of membrane proteins (receptors, ionic channels and enzymes, Na^+/K^+ pump and Na^+/Ca^{2+} exchanger), intracellular signal systems in norm and pathology (cancer and nerve disorders) would be presented. Light microscopic, cell voltage- and patch-clamp, isotope, standard biochemical and genetic engineering methods were used. Weak signal–induced effects on cell functional activity (intracellular enzymes activity, the number of functionally active membrane proteins) are realized by changing the physicochemical properties of extra- and intracellular aqua medium. The latter induces the modulation of Na^+/K^+ pump-induced cell hydration, which serves as a primary mechanism through which the autoregulation of pump and regulation of membrane excitability and chemosensitivity are realized. By genetic engineering method in oocytes it was shown that the correlation between Na^+/K^+ pump and Na^+/Ca^{2+} exchanger, which is realized through intracellular messenger systems, plays a crucial role in weak signals transduction in cells and determination of aging-induced increase of cell pathology.

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The two conductive pathways of P2X7 purinergic receptor: different modulation and selectivity

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The $P2X_7$ purinoceptor ($P2X_7R$) is an ATP-gated cation channel that is able to activate a cell permeabilizing pore. $P2X_7R$ cytosolic C-terminal tail is thought to modulate this function. This study was aimed to characterise the biophysical properties of $P2X_7R$ compared to those of the variant lacking the entire C-terminus tail ($trP2X_7R$) by measuring whole-cell currents and intracellular Ca^{2+} variations. A mathematical model is used to describe the experimental results. In $P2X_7R$ expressing HEK-293 cells, the potent agonist 3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate (BzATP) -evoked ionic currents depending on concentration and frequency of agonist applications. The currents were strongly inhibited by extracellular Mg^{2+} in a non-competitive way. By contrast, in $trP2X_7R$ cells, only high BzATP concentrations elicited small currents not affected by Mg^{2+}. Interestingly, BzATP-induced Ca^{2+} influx was present both in $P2X_7R$ and in $trP2X_7R$ cells, albeit in the latter the intracellular Ca^{2+} elevation was smaller. Importantly, in $trP2X_7R$ the intracellular Ca^{2+} rise maintained a competitive mechanism of Mg^{2+} inhibition similar to that observed in $P2X_7R$. The experimental data and the modelling findings support the tenet of a functional structure of $P2X_7R$ possessing two distinct conductive pathways.

Abstracts
– Structure-function relationship in channels, pumps and exchangers –

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"Social" domain organization and dynamics of nicotinic acetylcholine receptor at the cell membrane

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A combination of experimental techniques (patch-clamp, confocal FRAP and FCS, single-particle tracking, high-resolution fluorescence microscopy) has been used to analyze the supramolecular organization of the acetylcholine receptor (AChR), the dynamics of the receptor at the cell surface, and the kinetics of receptor internalization. Changes in cholesterol (Chol) content affected muscle and neuronal-type AChR organization and dynamics at the cell surface. Chol depletion produced gain-of-function of single-channel dwell time. Submicron-sized (∼250 nm) domains, stable over a period of hours at the cell membrane, could be resolved into AChR "nano-clusters" with a peak size distribution of ∼55 nm by STED microscopy. Chol depletion reduced the number of nanoclusters, increasing their size, and changed their supramolecular "social" organization on larger scales (0.5-3.5 microns). FRAP, FCS and SPT experiments provided information on the dynamics of AChR nanoclusters, disclosing the dependence of their mobility on Chol content and cortical cytoskeleton. Chol content at the plasmalemma may thus modulate cell-surface organization and dynamics of receptor domains, and fine-tune receptor channel function to temporarily compensate for acute AChR losses from the cell surface.

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As opposed to the phospholamban pentamer, sarcolipin forms anion-selective channels in biomembranes

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Sarcolipin (SLN) and the phospholamban pentamer (PLN) are two membrane proteins that inhibit Ca-ATPase of the sarcoplasmic reticulum at low concentrations. In contrast to PLN, SLN stimulates maximal Ca^{2+} uptake rates. SLN and PLN were incorporated in a bilayer lipid membrane (tBLM) tethered to a mercury electrode through a hydrophilic spacer. Electrochemical impedance spectroscopy measurements show that SLN forms channels permeable to chloride ion, weakly permeable to phosphate ion and impermeable to inorganic cations such as Na^+ and K^+. A relationship between this property of SLN and its regulatory function on Ca-ATPase of sarcoplasmic reticulum is proposed. ATP increases the permeability of a tBLM incorporating SLN to phosphate ion by associating to SLN with an association constant of 0.1 μM. An explanation for this behavior is provided. SLN can be identified with the „P_i transporter" described by A.G. Lee et al. Conversely, both electrochemical impedance spectroscopy measurements and molecular dynamics simulations provide strong evidence that the pore of the pentameric form of PLN does not act as a chloride channel.

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Electron transfer during O_H to E_H transition of the aberrant *ba₃* oxidase from *Thermus thermophilus*

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Cytochrome ba_3 from *Thermus thermophilus* belongs to the large family of structurally related heme-copper oxidases. It accepts electrons from cytochrome c_{552} at the P-side of the membrane and uses them to reduce oxygen to water. The energy released in this reaction is used for proton pumping across the membrane to form of an electrochemical proton gradient, used by the cell for formation of ATP. In this work we followed the kinetics of single-electron injection into the oxidized nonrelaxed state ($\mathbf{O_H}\rightarrow\mathbf{E_H}$) of cytochrome ba_3 by time-resolved optical spectroscopy. Two main phases of electron transfer were resolved. The first ($\tau\sim17$ μs) includes oxidation of Cu_A and simultaneous reduction of both low and high spin hemes. The second ($\tau\sim420$ μs) reflects reoxidation of both hemes by Cu_B. This is in significant contrast to the $\mathbf{O_H}\rightarrow\mathbf{E_H}$ transition of aa_3-type oxidases, where the fastest phase is due to transient reduction of the low-spin heme *a* only. On the other hand, the single-electron reduction of the relaxed **O** state in ba_3 oxidase consisted of only rapid electron transfer from Cu_A to heme *b*, which is similar to that in aa_3 oxidase. This indicates a functional difference between the relaxed **O** and the pulsed $\mathbf{O_H}$ states of cytochrome ba_3.

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RyR activation in cultured SHR cardiomyocytes at the end of the prehypertensive period

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The rate of $[Ca^{2+}]_i$ elevation after the ryanodine receptor (RyR) activation by 4-chloro-m-cresol (4-CmC) and L-type Ca^{2+} channels (DHPR) activation by Bay K8644 was studied in cultured (5 days) cardiomyocytes of spontaneously hypertensive (SHR) and normotensive rats (WKY, Wistar) during 6 weeks of postnatal development. The differences in DHPRs and RyRs activities began to be evident after 3 weeks age when CICR formation has finished and became more expressed at the end of prehypertensive period (6 weeks). In response to 4-CmC (2 mM), a drastic increase in the rate of $[Ca^{2+}]_i$ accumulation (2.85±0.8 times) in SHR myocytes was registered after 20 days age versus a decrease in the rates of Ca^{2+} efflux from the sarcoplasmic reticulum of Wistar and WKY rat cardiomyocytes. BayK (80 μM) also induced more sharp $[Ca^{2+}]_i$ elevation in SHR myocytes (3.35±0.25 times) as compared with Wistar (2.32±0.16 times) and WKY (1.22±0.09 times) ones of the same age. Our results argue that in SHR and WKY cardiomyocytes, as opposed to normotensive Wistar rats, gradual growth of DHPR activity is observed, which follows in parallel with CICR formation in the excitation–contraction coupling during early postnatal ontogenesis, and drastic activation of RyR2 in SHR myocytes after the process termination.

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Modified valinomycins as sensors in model membranes

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The cyclododecadepsipeptide valinomycin is composed of two amino acids (L-Valine and DValine) and two hydroxyl acids (D-α-Hydroxy-isovaleric acid and L-Lactic acid). They form a 36membered ring of alternating amino and hydroxyl acids. In the cyclic structure, the polar groups are oriented towards the central cavity, whereas the rest of the molecule is relatively nonpolar. This enables the complexation of ions and valinomycin acts as a selective ion transporter (K^+) in lipid membranes.
When one of the amino acids is exchanged, e.g one L-Valine by an L-Lysine, selected functionality can be engineered into the depsipeptide while maintaining its ion conducting properties.
We induced several modifications into valinomycin, i.e. a Biotin binding unit or a Ferrocene group to induce an electrochemical active center. The ion conducting properties of the modified ion carriers have been probed in planar lipid bilayers as well as in solid supported membranes.

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Putative potassium channels in Synechocystis sp. PCC 6803

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We are interested in the identification and characterization of potassium channels in the cyanobacterium *Synechocystis sp.* PCC 6803, an organism which is considered the ancestor of plant chloroplasts. A bioinformatic screening of *Synechocystis* proteome identified, among others, two proteins on which we focused our attention. The first one (SynCaK) displays sequence homology to MthK, a Ca^{2+}-dependent potassium channel from *M. thermoautotrophicum*. The second one (SynK) is predicted to contain six transmembrane regions and the typical selectivity filter of all potassium channels. Our goal is to understand their roles in the physiology of cyanobacteria. We cloned their coding sequences in fusion with GFP and the hybrid proteins were expressed in Chinese hamster ovary cells. We evaluated the presence of both proteins in plasma membrane by fluorescence microscopy and then we proceeded to their functional characterization using patch clamp technique.This analysis will allow us to gain information about channel activity, regulation and pharmacology. We also plan to evaluate the importance of SynCaK and SynK channels in photosynthesis. To test the hypothesis that they could be involved in regulating this process, we will produce deletion and site-specific mutants in *Synechocystis*. Finally we would also identify the homologous of these channels in the higher plant *A. thaliana* and obtain some information about their localization and function.

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Open channel structure of MscL from FRET microscopy and simulation

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Mechanosensitive channels open in response to membrane bilayer deformations occurring in physiological processes such as touch, hearing and osmoregulation. Here, we have determined the likely structure of the open state of the mechanosensitive channel of large conductance from *E. coli* (MscL) in a natural environment using a combination of patch-clamp studies, FRET spectroscopy, EPR data, molecular and Brownian dynamics simulation. Structural rearrangements of the protein are measured while controlling the state of the pore by modifying lipid bilayer morphology. FRET efficiency changes can be related to distance changes using a Monte Carlo analysis program in conjunction with detailed orientational analysis. These measurements are used as restraints in all atom molecular simulations in order to determine the likely structure of the open state, whose probable conductance is derived from Brownian dynamics simulations.
Transition to the open state occurs via large rearrangements throughout the protein that create a wide pore nearly 30 Å in diameter. Both transmembrane helices are found to line part of the pore. The N terminal helix is found to lie along the face of the membrane where it can act to sense membrane tension and directly transfer this to the pore lining helices. The method of coupling spectroscopic data with simulations is likely to be of great value for studying conformational changes in a range of membrane proteins.

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Pirolid protects MCF-7 cancer cells against oxidative stress induced by doxorubicin and paclitaxel

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Doxorubicin (DOX) and paclitaxel (PTX) are anticancer drugs commonly used in chemotherapy of breast cancer therapy, however, the use of these drugs is limited by the risk of developing heart failure. Generation of reactive oxygen species contributes to the cardiotoxicity of doxorubicin. Nitroxides are low molecular weight, stable free radicals reacting with ROS and they present antioxidant properties.
The aim of this study was to analyze the effects of Pirolid (PD) on the oxidative stress induced by DOX and taxane in MCF-7 breast cancer cell line.
Results from MTT test revealed that PTX is more cytotoxic towards MCF-7 cells than DOX. The IC_{50} was 0.3μM and 3μM, respectively. PD alone does not influence cell viability. PD in combination with both drugs did not change viability of cells.
Both drugs increased the level of carbonyl groups in cells. The highest level of peroxide was observed in cells incubated with DOX (approx. 3-fold). Nitroxide alone did not influence the level of peroxide in the whole range of concentrations. Combination of PD with DOX and PTX reduced the level of carbonyls depending on its concentration.
PD did not affected on the level of peroxide in cells suspension. DOX and PTX increased (2-fold) the level of carbonyls. PD decreased the level of peroxides in cells treated with DOX and PTX. The lowest concentration of peroxide was observed at 50μM of PD. These results show that PD protect MCF-7 cells against oxidative stress induced by drugs.

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Two gating modalities in the pore of the miniature K^+ channel Kcv

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Kcv is a viral protein that forms functional K^+ channel in heterologous systems. Because of its miniature size (94 amino acids) we use Kcv as a model system to study and manipulate basic properties of the K^+ channel pore. By analysing single-channel recordings we highlighted two voltage-dependent modalities of gating in Kcv: a slow and a fast gating. The presence of a slow gating is revealed by the very low (in the order of 1-3%) mean open probability. Slow gating is not related to the presence of a bundle crossing, as shown by accessibility of the cavity to MTS reagents. Channel opening might involve the transient formation of salt bridges between residues at the N and C termini of the channel, as suggested by mutational experiments inspired by molecular dynamics simulations of Kcv. Fast gating, analyzed by beta distributions, is responsible for the negative slope conductance in the single-channel I/V curve at extreme potentials and can be explained by depletion-aggravated instability of the filter region.

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Azurin at Au/SAM junctions: pressure effects on the short-range and long-range electron transfer

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We exploited the Au-deposited self-assembled monolayers of the type: $[-S-(CH_2)_n-CH_3]$ (where $n = 4$, 9 and 15) with hydrophobically adsorbed redox protein – azurin to verify intrinsic electron transfer mechanisms according to the charge-transfer theory. The enthalpies and volumes of activation were determined through the variation of temperature (2–50 ^oC) and pressure (5–150 MPa) and experimental values were compared with those expected on the theoretical grounds. For the case of $n = 4$ the activation enthalpy definitely contains a large contribution originated from frictional-like dynamics of protein, and the activation volume has a small positive value. For $n = 15$ the value of activation enthalpy directly matches 1/4 of that for the reorganization energy, and the activation volume attains substantially negative value. For $n = 9$ we observed the intermediary performance. The whole kinetic pattern is consistent with the smooth changeover between adiabatic and nonadiabatic mechanisms of electron transfer.

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Atypical mechanism of conduction in potassium channels

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Potassium channels can conduct K^+ ions with rates of up to $\sim 10^8$ ions per second at physiological conditions, and they are selective to these species by a factor of 10^4 over Na^+ ions. Ion conduction has been proposed to involve transitions between two main states, with two or three K^+ ions occupying the selectivity filter separated by an intervening water molecule. The largest free energy barrier of such a process was reported to be of the order of 2-3kcal mol^{-1}. Here, we present an alternative mechanism for conduction of K^+ in K^+ channels where site vacancies are involved, and we propose that coexistence of several ion permeation mechanisms is energetically possible. Conduction can be described as a more anarchic phenomenon than previously characterized by the concerted translocations of K^+-water-K^+.

P-409

Effect of phloretin on the properties of single channels formed by alpha-hemolysin

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The role of the membrane dipole potential (φ_d) is of a particular interest due to a powerful impact of this potential on the membrane permeability and lipid-protein interactions. Channel forming activity of gramicidin A, alamethicin, syringomycin E, HPA3 peptide, and OmpF porin are influenced by φ_d. We have studied the effect of the membrane dipole modifier, phloretin, on the properties of single channels formed by a wild-type alpha-hemolysin in planar lipid bilayers. The single channel of a ~1000 pS conductance exhibits transitions into a number of low-conductance states as the transmembrane voltage exceeds ~130 mV (regardless of the voltage polarity). The phloretin addition to the bathing solutions (20 μM) (after the hemolysin channel was formed in the membrane) shifts dramatically the channel voltage-dependence. Transitions to the low-conductance states are observed at ~30 mV. The effect of the phloretin addition was not observed in the case when it was introduced into the bathing solution before alpha-toxin. Since phloretin reduces φ_d, the data may report on the influence of the electric potential profile on the energy of the low-conductance state of the alpha-hemolysin channel. The alternative explanation of this effect consists in a specific interaction between the phloretin and toxin channel. The work is supported in part by RFBR (# 09-04-00883), SS (# 1135.2008.4), and the Program of the RAS «Molecular and Cell Biology».

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The effects of telmisartan on electrical responses of left ventricular papillary muscle in rats with streptozotocin(STZ)-induced diabetes mellitus

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Experimental studies have shown that the AT1-receptor antagonists telmisartan (Tel) has a PPAR-activating property, but there does not appear to be a class effect. To test telmisartan's importance, we investigated its effect on electrical activities (EA) in diabetic (D) rats. The purpose of this study was to investigate the effects of the Tel on EA of diabetic papillary muscle (DPM) with STZ-induced. In this study, we used four groups: (1) nondiabetic control (NDC) group (C), (2) Tel–treated NDC group (C+Tel), (3) diabetic group (D), and (4) Tel-treated D group (D+Tel). Diabetes was induced by a single i.v injection of STZ. In the study, membrane potential (MP) and action potential (AP) recorded after the establishment of diabetes. 1) MP was decreased significantly in both Tel-treated C and D rats (from -71,4±0,7 to -64,7±0,8 mV and from -69,9±0,6 to -63,8±0,7 mV). 2) AP unchanged in D group, whereas C+Tel and D+Tel groups showed increase in AP compared with C and D groups. 3) Repolarization time was prolonged in diabetic rats. 4) In C+Tel and D+Tel groups depolarization rate values increased significantly. On the other hand, in D group repolarization rate values descreased increased significantly compared to baseline values in Tel solution. As a result, our data suggest that the beneficial effects of Tel-treatment on the EA of the DPM appear to be due to the diminished K^+ currents.

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Coupling of water and glucose fluxes in epithelial cells expressing sodium-glucose cotransporter

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The mechanism of water transport through epithelia is still under debate. In the present work we tested the hypothesis of isosmolal water transport using MDCK cells stably expressing the human sodium-glucose cotransporter (hSGLT1). Tagging hSGLT1 with EGFP enabled determination of its abundance in the plasma membrane by fluorescence correlation spectroscopy (FCS). By monitoring tiny shifts in the concentration of water-soluble dyes in the vicinity of epithelia, FCS also allowed assessment of the water fluxes through confluent cell monolayers grown on permeable supports. Fitting the set of differential equations for the osmotic drift and for the back diffusion to the experimentally determined dye distribution permitted calculation of water flow both in the presence and in the absence of an osmotic gradient. From the calorimetric measurements of glucose transported across the cell monolayer, the water:glucose stoichiometry was derived. Dividing the increment in osmotic water flux due to hSGLT1 expression by the number of hSGLT1 copies in the plasma membrane resulted in a single transporter water permeability p_f of 4.6 x 10^{-14} cm^3/sec. Thus, p_f is close to the single channel water permeability of aquaporin-1. Consequently, even small osmolyte concentration differences between the cytoplasm and the basolateral buffer solution are sufficient to drive a substantial water flux.

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Electrostatic interactions in the active site of Complex I from Escherichia coli

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Complex I, the first member of the respiratory chain, serves as a proton pump catalyzing transfer of two electrons from NADH to ubiquinone coupled with the translocation of four protons across the membrane. So far the mechanism of energy transduction by Complex I is unknown.
The NADH-binding cavity of Complex I has a very prominent feature – the presence of two invariant amino acid residues, glutamate and tyrosine, that are exposed to the solvent and located in the vicinity of the FMN, the primary electron acceptor in the enzyme. It was suggested that they might be involved in the binding of NADH through interaction with its nicotinamide moiety. In this work we assessed the function of corresponding Glu95 from the NuoF subunit of *E. coli* Complex I by mutation for glutamine.
We showed that the negative charge of glutamate in the catalytic site is needed for the electrostatic repulsion of negatively charged phosphates of nucleotides. This process facilitates release of the product NAD^+ and, as a result, accelerates turnover of Complex I. We also found that glutamate, as one of the four negatively charged amino acid residues surrounding the isoalloxazine ring of the FMN at a distance of 4-6 Å, has a share of 40 mV of the overall 130 mV depression of the midpoint potential of this redox cofactor.

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Mutations in the actuator of ACA8, a Ca-ATPase of A. thaliana, generate partially deregulated pumps

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ACA8 is a type 2B CaATPase with a regulatory N-terminus whose autoinhibitory action can be suppressed by binding of calmodulin (CaM). ACA8 N-terminus is able to bind a region of the small cytoplasmic loop connecting transmembrane domains 2 and 3. To define the role of this interaction in autoinhibition we have analysed a number of single point mutants produced by mutagenesis of ACA8 E252-N345 sequence. Mutation to Ala of any of 6 acidic residues (E252, D273, D291, D303, E302, D332) originates an enzyme with normal activity in the presence of CaM, but less CaM-stimulated. These results highlight the relevance of a negative charge of the surface area of the small cytoplasmic loop in ACA8 autoinhibition. The most deregulated mutant is D291A ACA8, which is less activated also by controlled proteolysis or by acidic phospholipids; moreover, the phenotype of the D291A mutant is stronger than that of D291N ACA8 suggesting a more direct involvement of this residue in autoinhibition. Of the other mutants (I284A, N286A, P289A, P322A, V344A, N345A), only P322A ACA8 has a basal activity higher than that of the WT. These results provide the first evidence that the small cytoplasmic loop of a type 2B CaATPase plays a role in the attainment of the autoinhibited state.

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Biomechanical analysis of capillary leukocyte trafficking by microfluidic investigation

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Circulating leukocyte sequestration in pulmonary capillaries is arguably the initiating event of lung injury in Acute Respiratory Distress Syndrome (ARDS) [1]. We present a microfluidic investigation of the roles of actin organization and myosin II activity during the different stages of leukocyte trafficking through narrow capillaries using specific drugs. The deformation rate during entry reveals that cell stiffness depends strongly on F-actin organization and hardly on myosin II activity, supporting microfilament role in leukocyte sequestration. In the transit stage, cell friction is influenced by stiffness, demonstrating that the actin network is not completely broken after a forced entry into a capillary. Conversely, membrane unfolding was independent of leukocyte stiffness. The surface area of sequestered leukocytes increased by up to 160% in absence of myosin II activity, showing the major role of molecular motors on microvilli wrinkling and zipping. Finally, cell shape relaxation was largely independent of both actin organization and myosin II activity, whereas a deformed state was required for normal trafficking through capillary segments [2].

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Heteromerization properties of voltage dependent potassium channels

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Voltage-gated potassium channels are either homomeric or heteromeric tetramers composed of four α-subunits. In order to bring a contribution to the comprehension of channel heteromerization we have been investigating the properties of two plant voltage-gated K^+-channels by using electrophysiological and fluorescence techniques. Experiments were focussed on KDC1 and KDC2, coexpressed in Xenopus laevis oocytes. KDC1, the first potassium channel cloned from Daucus carota, belongs to the subfamily of α-modulatory silent channels as it doesn't form functional homomeric channels by itself. On the contrary KDC1 forms functional heteromeric channels when coexpressed with homologous subunits. KDC2, the last K^+ channel cloned from D. carota, belongs to the KAT1 family and shares an overall identity of 63% with KAT1. To correlate KDC1 functional properties with its localization in oocytes, KDC1 and/or KDC2 subunits were labelled with GFP and their properties investigated by confocal microscopy and voltage-clamp. We found that the KDC1-EGFP fusion protein is not targeted to the plasma membrane unless it is coexpressed with KDC2. Moreover electrophysiological experiments demonstrated that the heteromeric KDC1-KDC2 channel has altered selectivity and activation properties with respect to homomeric KDC2 channel.

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Role of Calcium ions in Nickel potentiation of NMDA currents

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NMDA receptors are glutamate-gated channels distributed throughout the brain in the excitatory synapses and are critical for the nervous system function. They are assembled from two types of subunits, the essential NR1 and at least one NR2(A,B,C,D). Nickel (Ni) modulates the current flowing through NMDA receptors in a different way depending on the NR2 subunit present. We have recently identified several domains of the channel involved in Ni interaction, but many aspects of this modulation remain elusive. In this work we intended to determine the role of calcium (Ca) ions in the potentiation induced by Ni on the current through NR1/NR2B recombinant NMDA receptors. When Ni was applied in the presence of the physiological concentration of Ca (1.8 mM), a voltage-independent potentiation of the current was observed with a Kp of 2.5 μM. This effect was progressively reduced by decreasing Ca concentrations and it was no more detectable with 0.18 mM Ca or in the presence of barium (Ba, 0.3 mM). In this last case the effect of Ni on NR1/NR2B receptors was mainly inhibitory (Ki(-60mV)=156μM). Therefore a physiological concentration of Ca is necessary to induce Ni amplification of the current. Many data in the literature indicate a correlation between Ca ion entrance through the channel, NMDA current facilitation and cytoskeleton; however, in our experiments, the application of the actin perturbing agent cytochalasin-D did not produce major modifications in Ni effect.

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Proton transfer in cytochrome c oxidase studied by time-resolved FTIR spectroscopy

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Cytochrome *c* oxidase is a terminal complex (C*c*O, Complex IV) of a respiratory chain that is located in an internal membrane of mitochondria or plasma membrane of bacteria. C*c*O is an electron transfer enzyme that reduces O_2 and uses the redox energy of the O_2 reduction for the proton translocation across the membrane. The electron- and proton-transfer generate a transmembrane electrochemical gradient ($\Delta\mu H^+$) that is used for ATP synthesis and all other kinds of work for the cell needs. The proton translocation mechanism of C*c*O requires 'channels' for the H^+ uptake and expulsion within the enzyme. The proton transfer occurs on a time-scale of micro-to-milliseconds.
In order to study the proton transfer in C*c*O, a flow-flash approach based on a time-resolved FTIR spectroscopy was developed and applied. Our FTIR flow-flash approach (the measurement of the reaction of C*c*O with O_2) allows to reach a time resolution up to tens milliseconds. With this approach and site-specific mutants of C*c*O where the catalysis is slowed down, separate steps of the proton transfer were studied. The results showed that a unique cross-linked Tyr-280 (in a combination with a time-resolved visible spectroscopy and electrometry) serves as a proton donor for the dioxygen bond cleavage during the O_2 reduction by C*c*O. Furthermore, the protolytic transitions of Glu-278 – a key amino acid in the proton transfer mechanism in C*c*O - were shown for the first time.

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Determination of calcium currents in cation channels using a novel fluorescence/patch-clamp approach

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The patch-clamp technique combined with fura-2 fluorescence detection is suitable to investigate calcium fluxes. We used the excised patch configuration and focused the photomultiplier to the tip of the recording pipette where the fluorescent dye was present (FLuoresence combined with Excised Patch = FLEP). This configuration has several advantages, i.e. absence of delay in loading the fluorophore, of interference by endogenous calcium buffers and of photobleaching. Here we present an application for the determination of fractional calcium currents (Pf) in a plant non-selective cation channel, showing that Pf can be modulated by cytosolic calcium and potassium. FLEP is very efficient for measuring small calcium currents (<1 pA) of sufficiently long duration; fluorescence signals are amplified by integration in time, as the calcium/fura-2 complex accumulates at the tip of the recording pipette and diffuses slowly. We propose this technique not only for the study of calcium transport pathways, but also for other transporters of divalent cations as nickel and manganese known to quench fluorescence thus reducing both 340 and 380 nm components. Moreover, using the appropriate fluorophore the technique may be extended to further ion species, e.g. BCECF for investigating proton transport pathways. Ref: Gradogna A, Scholz-Starke J, Gutla PV, Carpaneto A (2009) The Plant Journal, 58:175-182

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Effects of flavonoid Naringenin on non-selective cation channels isolated from plant vacuoles

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The Slow Vacuolar (SV) channels are ubiquitous in all tissues of higher plants. The SV channel is a non-selective cation channel permeable to both monovalent and divalent cations. SV currents recorded in a typical patch-clamp experiment require unphysiologically high cytosolic and low vacuolar calcium concentrations for full activation. We aim at looking for endogenous plant substances which might be able to modify or shift the voltage activation threshold of this channel towards more physiological conditions. Flavonoid Naringenin [Nar] is present in all plant species where it plays a central role in the flavonoid biosynthetic pathway. Nar is stored in the vacuoles in glycosylated form called Naringin. When Nar was added to cytosolic bath solution, we recorded a dose-dependent reversible decrease in SV channel activity. When we investigated the effect of Nar on the voltage dependence of the channel, we observed that the activation threshold of the SV channel is shifted towards more positive voltages. Our group has evidences that approximately 10% of the total SV current at high (e.g.> 50 mV) positive voltages is mediated by calcium. Therefore, in order to verify whether Nar affects both potassium and calcium conductance, we performed experiments by combining the patch clamp technique with fluorescence measurements using the fluorophore fura-2: both SV currents and calcium signals were abolished by 1 mM [Nar].

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Beyond steady-state protein dynamics

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To study protein dynamics beyond steady-state experiments we have developed a novel laser-pump:neutron-probe experiment which allows us to monitor temporal changes in protein dynamics during a working cycle of a protein. Protein dynamics has been extensively studied, but so far, the correlation of internal protein dynamics with the function of proteins was investigated only indirectly in steady-state experiments by variation of external parameters by variation of external parameters like temperature or hydration. The method comprises of an in-situ optical activation of a protein and a time-dependent sampling of the dymamic response using quasi-elastic neutron scattering. With the membrane protein bacteriorhodopsin, a light driven proton pump, we can demonstrate for the first time temporary alterations in the protein dynamics after triggering the working cycle. This observation is a direct proof for the functional significance of protein structural flexibility, in connection with the large-scale conformational changes in the protein structure occurring during the operation of a "molecular machine".

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O-421

NF676, is a cell permeable and specific inhibitor of the calcium release channel ryanodine receptor

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Excitation-contraction coupling in skeletal and cardiac muscle is tightly regulated by the calcium release channel of the sarcoplasmic reticulum, the ryanodine receptor (RyR). We could previously show that suramin is a potent activator of the RyR via the calmodulin binding site. Calmodulin shows dualistic action, i.e. activation or inhibition of the ryanodine receptor, depending on the absence or presence of Ca^{2+}. Screening of suramin analogues identified NF676 as a use-dependent inhibitor of the skeletal muscle RyR (RyR1). Here we show that NF676 inhibits high affinity [^{3}H]ryanodine binding and single channel recordings of the purified RyR1. NF676 induced a reduction of open probabilities in a concentration dependent manner, with no effect on current amplitude and unitary conductance. Importantly, NF676 triggers flickering episodes of channel openings and closings before the RyR1 is frozen in a complete non-conducting state, which is fully reactivated by the RyR agonist ATP. Moreover, zwitterionic behaviour of NF676 facilitates plasma membrane permeation, which prevented caffeine induced Ca^{2+} transients in skeletal muscle cells and cardiomyocytes. Conversely, IP_3 mediated Ca^{2+} signals were not altered by NF676. *This work was supported by Herzfelder'sche Familienstiftung and FWF.*

Abstracts

– Structure-function relationship in channels, pumps and exchangers –

O-422

Electron transfer patterns and inherent mechanisms for cytochrome c and azurin at Au/SAM junctions

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Bioelectrochemical devices composed of Au electrodes coated by self-assembled monolayers (SAMs) of different composition and thickness are ideal systems to probe ET patterns and mechanisms for redox proteins. Representative proteins, cytochrome *c* and azurin were studied by using the combinations of four different strategies including the variation of SAM thickness ($[-S-(CH_2)_n-\omega]$, with n running over the range 3 to 20, throughout), solution viscosity (varied by adding of the viscose additive – glucose), temperature (0 to 50 ^{o}C) and hydrostatic pressure (up to 150 MPa), aiming the identification of different intrinsic ET patterns and interplay between them in the framework of generalized charge-transfer theory. We demonstrated the full adiabatic (frictional) control for the case of thinner SAMs, the intermediate (mixed) regime, and the complete changeover to the nonadiabatic mechanism (long-range tunneling) for the case of thick SAMs owing to the variation of electronic coupling, in a nice agreement with theoretical predictions.

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Mitochondria regulate Ca^{2+} influx and determine patterns of ER Ca^{2+} refilling in acinar cel

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Store-operated Ca^{2+} entry (SOCE) is mediated by activation of SOC-channels of plasma membrane following the emptying of endoplasmic reticulum (ER) Ca^{2+} stores. The SOCE is required for calcium signaling, secretion of neurotransmitters and proteins, but the mechanisms of natural SOCE regulation are not well understood. We utilized several imaging methods to measure Ca^{2+} signals in cytoplasm ($[Ca^{2+}]_{cyt}$), inside the ER ($[Ca^{2+}]_{ER}$) and Mit ($[Ca^{2+}]_{Myt}$). In the absence of external Ca^{2+}, emptying the ER with acetylcholine leaded to the activation of SOCE that resulted in generation of $[Ca^{2+}]_{cyt}$ signals following readmission of external Ca^{2+}. The rate and amplitude of SOCE were significantly lower when Mit had been depolarized with FCCP or CGP 37157. The patch-clamp experiments have directly shown that Mit are capable to capture Ca^{2+} entering through SOCC, which are characterized by generation of $[Ca^{2+}]_{Mit}$ transients, observed both in EGTA- and BAPTA-buffering inside solutions. These data suggest that Ca^{2+} sequestration by Mit is associated with the formation of microdomains and prevents Ca^{2+}-dependent SOCC inactivation. We also found that inhibition of Mit under prolonged cell stimulation resulted in complete inhibition of SOCE as well as decrease and deceleration of ER refilling. Thus, Mit regulate calcium recycling and maintain the SOCE controlling dynamic interplay between SOCE and sustained ER refilling under prolonged stimulation.

P-424

Antibiotic affinity to bacterial porins – Conductance measurements and biological relevance

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Influx of antibiotics into the periplasm of gram-negative bacteria is facilitated by porins that form channels in the outer membrane. We propose that certain natural antibiotics have been optimized by co-evolution to take advantage of the charge distribution in non-specific porins to achieve binding and thereby facilitating their uptake in bacteria. We investigate the permeation pathways of antibiotics into bacteria by reconstitution of a single porin into an artificial lipid bilayer and measuring the binding of antibiotic molecules through the time-resolved modulation of a small ion current. We have been able to characterize facilitated translocation of several antibiotics through *Escherichia coli* and *Enterobacter aerogenes* porins. Noise analysis of ion currents through a porin in the presence of effective antibiotics revealed binding kinetics at a single molecule level. We report for the first time temperature dependent antibiotic translocation that revealed complete energy profile. Combining these results with microbiological assays and molecular dynamics simulations, we conclude the molecular mechanism of antibiotic permeation. Our approach may contribute to the rational design of new antibiotics against clinical bacterial strains for the most efficient delivery to target sites.

P-425

Monitoring of the electrostatic surface potential on the Na^+/K^+-ATPase cytoplasmic headpiece

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We introduced an original method for the monitoring of the changes in the electrostatic surface potential, using the quenching of the intrinsic tryptophan fluorescence by acrylamide or iodide. This approach opens new way to understanding the dynamic processes within the proteins. Our experiments revealed that the conformation of the Na^+/K^+-ATPase large cytoplasmic loop (C45) in the presence of the ATP (without magnesium) substantially differed from the conformation in the presence of Mg^{2+} or MgATP or in the absence of any ligand not only in the sense of geometry but also in the sense of the electrostatic surface potential. Moreover, our data indicate that the effect of the ligand binding is not restricted only to the close environment of the binding site and that the information is in fact transmitted also to the distal parts of the molecule. This property could be important for the communication between the cytoplasmic headpiece and the cation binding sites located within the transmembrane domain.

Abstracts
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P-426

Structural and dynamical properties of OmpF and OmpC porins: a case study on β-lactamase inhibitors

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The striking presence of outer membrane (OM) in gram-negative bacteria of E.coli represents a strong barrier for any molecule to penetrate inside bacteria. In particular for β-lactam antibiotics, which have their target located inside the bacteria; the first step towards reaching inner part of bacterium is the cellular uptake. Ubiquitous presence of porins (such as for instance OmpF, OmpC) in the OM, function as a channel facilitating the transport of molecules (such as, for instance antibiotics) across the OM. Bacteria can exhibit resistant towards antibiotics by: (i) decreasing their uptake by under-expressing the porins or/and (ii) production of inactivating enzymes such as ß-lactamases. To combat the latter mechanism ß-lactamase inhibitors (such as, sulbactam for instance) are prescribed together with the antibiotics. Like the antibiotics, the inhibitors must penetrate the OM, the main path being through porins. It is thus evident the biological relevance of investigating the mechanisms by which porins can regulate entry/exit across the OM. To achieve this goal, molecular dynamics simulations were performed to explore the structure and dynamics of pores formed by OmpF and OmpC porin. From the analysis of data obtained from our simulations, we identified the key residues buried behind the L3 loop, which may play be crucial for porins to exert their biological role. As a case study, we report results about the diffusion of sulbactam through the two porins.

P-427

Anionic lipids reveal molecular determinants involved in single and coupled gating in KcsA

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Channel clustering and coupled channel gating modulate the activity of KcsA (Molina et al, 2006). The occurrence of a similar behavior in other channels points out to clustering and coupled gating as a potentially important drug target to modulate channel activity.
We have identified molecular determinants involved in single and coupled channel gating in KcsA. First, we detected that clustering and coupled gating of KcsA is modulated by anionic lipid. Also, a model for the interaction between two KcsA open channels was built. The docking predicts intermolecular sites which includes the non-annular lipid binding site. This explains how an excess of anionic lipid disrupts interactions between channels, destabilizing clustering and coupled gating in KcsA. In addition, the docking model reveals molecular determinants involved in single and coupled channel gating. This interaction involves W87, which affects the neighbouring channel through specific interactions in the extracellular mouth stabilizing the selectivity filter in an open conformation. The coupled gating is also explained since this mechanism affects the opposite channel in a mutual manner. Finally, mutants KcsA E71A and KcsA W87A disrupt the coupled gating of KcsA, thus, supporting the model. We think that this coupled gating phenomenon could correspond to the second gate previously detected by fluorescence methods (Blunck, et al, 2006).
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O-428

Local anaesthetic binding to Shaker channels: Role of aromatic residues

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Local anaesthetic, antiepileptic and antiarrhythmic drugs acting on Nav and hERG channels have been assumed to bind to aromatic residues in the internal vestibule; to F1764 and Y1771 in Nav (Ragsdale et al., 1996) and to Y652 and F656 in hERG (Mitcheson et al., 2000). Despite a lack of such residues in Kv channels, local anaesthetics, antiepileptic and antiarrhythmics bind to Kv channels with a considerable affinity. To explore the role of aromatic residues for the binding we investigated the effect of bupivacaine, benzocaine, phenytoin and quinidine on Shaker channels mutated to residues corresponding to the most C-terminal of the two aromatic residues in the S6 segment of the Nav and the hERG channels, (V473Y and P474F respectively). The channels were expressed in *Xenopus* oocytes and the currents measured with the two-electrode voltage-clamp technique. The results suggest that aromatic residues do not increase the binding affinity of the studied compounds to Kv channels. Rather, the affinity decreases (as reflected in typical K_d values for bupivacaine on V473F, P474F and wildtype channels, being 550, 740 and 300 μM, respectively). Thus, aromatic residues seem not to be necessary for high-affinity binding of the studied compounds to Kv channels. How this relates to their suggested roles in the Nav and hERG channels, remains to be evaluated.

P-429

Mito. bov. ADP/ATP carrier in detergent is predominantly monomeric but also forms multimeric species

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ADP/ATP carriers (AACs) are major and essential constituents of the inner mitochondrial membrane. They drive the import of ADP and the export of newly synthesized ATP. They were described as functional dimers from the 1980s until the structures of the AAC shed doubt on this consensus. We aimed to ascertain the published biophysical data claiming that AACs are dimers and to characterize the oligomeric state of the protein before crystallization. Analytical ultracentrifugation sedimentation velocity experiments clearly show that the bovine AAC is a monomer in 3-laurylamido-N,N2 dimethylpropylaminoxide (LAPAO), whereas in Triton X-100 and reduced Triton X-100, higher molecular mass species can also be identified. Neutron scattering data for monomeric bovine AAC in LAPAO does not give definite conclusions on the association state, because the large amount of detergent and lipids is imperfectly matched by contrast methods. We discuss a possible way to integrate previously published biochemical evidence in favor of assemblies, the lack of well-defined multimers that we observe, and the information from the high-resolution structures, considering supramolecular organizations of AACs within the mitochondrial membrane.

Abstracts

– Structure-function relationship in channels, pumps and exchangers –

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Modulation of the voltage-gated sodium channel Nav 1.5 by rCssII, a toxin from the scorpion Centruroides suffusus

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The main cardiac voltage-gating sodium channel, Na_v 1.5, generates the fast depolarization of the cardiac action potential and plays a key role in cardiac conduction. Its importance for normal cardiac function has been exemplified by the description of numerous naturally occurring genetic variants of the gene SCN5A, which encodes Na_v 1.5, that are linked to various cardiac deseases. Subsequently, studies of this channel localization have led to its identification in immature and denervated skeletal muscle and in the brain neurons.
In our effort to identify high affinity ligands for this channel, we have investigated the effects of the recombinant CssII (rCssII), a four disulfide-bridged scorpion toxin isolated from the venom of the scorpion *Centruroides suffusus*. Human cardiac sodium channel α subunit SCN5a was expressed in CHO cells and macroscopic Na^+ currents were recorded with patch-clamp technique in whole cell configuration. The electrophysiological experiments have highlighted a strong affinity for the channel at low nanomolar concentration. Compared with control conditions, rCssII toxin affects in reversible way the kinetics of activation and inactivation and marked decrease the peak Na^+ influx.
Research supported by International Collaboration between CNR and Mexican CONACyT

P-431

Role of ions and lipids on the conformation, stability and function of the potassium channel KcsA

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The K^+ channel KcsA is an integral membrane protein from *S.lividans*, used as a model system for studies on ion channels and oligomeric membrane proteins. Its atomic structure has been solved by X-ray diffraction, which shows an assembly of four identical subunits around a central aqueous pore, including the so-called selectivity filter. This channel is able to permeate K^+ at high flux rate and it is blocked by Na^+ (physiological blocker). Fluorescence, circular dichroism and Fourier transform infrared experiments carried out in our laboratory demonstrated that K^+ and Na^+ are able to bind to KcsA in a competitive manner. This binding is indeed associated with channel conformational changes, which seem to be related to the permeation and blockade processes. To further investigate this phenomenon we carried out a detailed study of chemical and thermal denaturation of wild-type and mutant KcsA channels. These two types of experiments were in agreement and indicate that both cations are able to stabilize the channel through conformational changes, being K^+ the more efficient one, even more when lipids are present. Particularly, mutant channels with a structurally altered selectivity filter show that ion and lipid-induced global conformational changes are intimately associated to the conformation of this selectivity filter (conductive and non-conductive forms).

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Molecular dynamics simulations of mechano-gating in the E-coli mechanosensitive channel MscL

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The bacterial mechanosensitive channel of large conductance MscL is constituted of homopentamer of a subunit with two transmembrane inner and outer α-helices, and its 3D structure of the closed state has been resolved. The major issue of MscL is to understand the gating mechanism driven by tension in the membrane. Although several models for the opening process have been proposed with Molecular Dynamics (MD) simulations, as they do not include MscL-lipid interactions, it remains unclear which amino acids sense membrane tension and how the sensed force induces channel opening. We performed MD simulations for the mechano-gating of MscL embedded in the lipid bilayer. Upon tension in the bilayer, Phe78 in the outer helix was dragged by lipids, leading to a tilting of the helices. Among amino acids in the outer helix facing the bilayer, Phe78 at the water-lipid interface showed the strongest interaction with lipids, thus may work as a major tension sensor. Neighboring inner helices cross each other in the inner leaflet, forming the most constricted part of the pore. As tension increases, the crossings move toward the cytoplasm associated with an expansion of the constricted part. During the movement, a hydrophobic water block environment around the constricted part was broken followed by water penetration and permeation.

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Low free energy barrier for ion permeation through double-helical Gramicidin

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The pentadecapeptide gramicidin forms a cation-specific ion channel in membrane environment. The two main conformations are the head-to-head helical dimer (HD) known as the channel conformation and the intertwined double helical form (DH) often referred to as non-channel conformation. In this comparative study [1], the energetics of single potassium ion permeation by means of the potential of mean force (PMF) for both gramicidin conformations embedded in a DMPC bilayer has been addressed by molecular dynamics simulations.
A significantly decreased free energy barrier by ∼25 kJ/mol for potassium ion passage through DH as compared to HD is reported. Favorable electrostatic side chain-cation interactions in HD are overcompensated by phospholipid-cation interactions in DH. The latter are coupled to an increased accessibility of the channel entrance in DH due to distributed tryptophans along the channel axis. This result underscores the importance of the lipid environment of this channel not only for the equilibrium between the different conformations but also for their function as cation channels.

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Abstracts

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Aquaporins as cell volume regulators: how membrane tension affects water permeability

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Under extreme conditions many cells control their volume responding to osmotic challenges by unloading or loading solutes to recover their original volume. A faster volume regulatory role triggered by membrane tension has been disclosed for aquaporins in kidney proximal tubule cells, where AQP1 is the main water channel, using isolated brush border membranes. In conventional osmotic studies in animal cells it is common to disregard internal hydrostatic pressures because they are insignificant compared to osmotic forces. However, by using low osmolarity buffers in small radii vesicles, we detected a rise on the internal pressure that creates surface tension and causes membrane stress, with a negative outcome on aquaporin water permeability. These findings suggested a mechanism for volume regulation in kidney proximal tubule epithelia where massive solute and fluid transport occurs.
To further explore aquaporin regulation by membrane tension, yeast cells were used as a model that could bare surface tension some orders of magnitude higher than animal cells due to the existence of a cell wall. The effect of increasing levels of membrane tension on yeast water channel activity was evaluated. An impairment of aquaporin activity correlated with the increase of membrane tension corroborates the volume regulatory role of aquaporin in different cells.

O-435

Interaction of Bax with a mitochondrial potassium channel is crucial for its action in apoptosis

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Voltage-gated potassium channel Kv1.3, the major plasma membrane channel in lymphocytes (Chandy et al, 2004 *Trends Pharm. Sci.,* 25, 280), has been located also to the inner mitochondrial membrane. Lymphocytes genetically deficient for Kv1.3 or transfected with siRNA suppressing Kv1.3-expression resisted apoptosis induced by several stimuli, while retransfection of Kv1.3 restored death. Pro-apoptotic Bax directly interacted with and functionally inhibited Kv1.3. Incubation of isolated mitochondria with recombinant Bax or channel inhibiting toxins triggered hyperpolarization, formation of reactive oxygen species, release of cytochrome c and depolarization. Mutation of Bax at K128, which corresponds to a conserved lysine in Kv1.3-inhibiting toxins, abrogated its effects on Kv1.3 and mitochondria (Szabò et al, 2008 *PNAS* 105, 14861). Likewise, a single point mutation turned Bcl-xL pro-apoptoptic. To test the function of the mutant Bax *in vivo*, $Bax^{-/-}$ $Bak^{-/-}$ mouse embryonic fibroblasts (DKO MEFs) were transfected with either wild type Bax or Bax(K128E). Staurosporine-induced apoptosis was defective in Bax(K128E)-transfected cells, indicating that Bax mediates apoptosis in lymphocytes at least in part via interaction with mitochondrial Kv1.3.

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Biochemical and functional characterization of chloroplast-located plant glutamate receptors

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In *Arabidopsis thaliana* there are twenty genes, grouped into three subfamilies, encoding for homologues of animal ionotropic glutamate receptors (iGLRs). Each protein displays a pore-forming loop, flanked by two conserved helices (plus a third C-terminal helix), a glutamate-binding domain and an N-terminal region. Through pharmacological and/or genetic approaches, many physiological functions have been attributed to plant GluRs, such as the regulation of cytosolic calcium, photomorphogenesis, water balance and carbon/nitrogen sensing and assimilation. According to the endosymbiotic theory, the cyanobacteria are considered to represent the precursors of the present chloroplasts. The first prokaryotic glutamate receptor (GluR0) was identified in the cyanobacterium *Synechocystis*. The putative products of the Atglr3.4 and Atglr3.5 genes display a possible targeting sequence for chloroplast location and show a high degree of homology with GluR0. Using specific antibodies and confocal microscopy, we have localized the two members of the AtGLR subgroup 3, GLR3.4 and GLR3.5 (splicing variant), to the chloroplast in *Arabidopsis* and to the inner envelope membrane in spinach. Electrophysiological experiments indicate the presence of an activity which is compatible with that of glutamate receptors. Furthermore, oxygen evolution measurements suggest that chloroplast-located glutamate receptors may play a role in the regulation of photosynthesis.

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The extrusion mechanism of substrates in RND family efflux pumps: a molecular dynamics study

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The RND transporters of the AcrAB-TolC (E.Coli) and MexAB-OprM (P.Aeruginosa) systems are able to export structurally and chemically different substrates outside bacteria through the membrane, being responsible of multidrug resistance. On the basis of crystallographic information, an extrusion process conceived as a three-cyclic peristaltic pumping has been proposed, but further microscopically well-funded investigations are needed to understand the mechanism. Using different computational methods like adaptive bias force (ABF) and targeted molecular dynamics (TMD), we have investigated the mechanism of substrate uptake and pumping at a molecular level. With the first method we have investigated the passage of antibiotics from the periplasm into the internal pore of the pump, while TMD has been used to assess the effect of conformational changes on the extrusion of drugs (which have been located into one of the proposed binding pockets). Comparison between the active pumps AcrB and MexB (which show different resistance patterns despite their homology) provide insights into the microscopic details of their functioning.

Abstracts

– Structure-function relationship in channels, pumps and exchangers –

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Deletion of the C-terminus destabilizes phosphorylated Na/K pump state containing 3Na ions

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The Na/K pump's extended C-terminus (compared to the SERCA Ca pump's) links 3 transmembrane helices, and its truncation lowers cytoplasmic Na affinity for forming the occluded E1P(Na_3) state. Here we test the effects of C-terminal truncations on interactions with external Na. We deleted the last 2 (YY) or 5 (KESYY) residues in *Xenopus* $\alpha 1\beta 3$ pumps made ouabain resistant by mutations Q120R-N131D (RD) or C113Y (C-Y), and then used two-microelectrode voltage-clamp recording in *Xenopus* oocytes to measure pump currents as 10 mM ouabain-sensitive currents while endogenous Na/K pumps were silenced with 1 μM ouabain. Inhibition by external Na of steady outward pump current ($[K]_o$=15 mM) at large negative voltages was somewhat weaker in both RD and C-Y pumps than in WT pumps, but was severely impaired in all C-terminal truncated pumps. Consistent with this, the voltage dependence of transient charge movements under Na/Na exchange conditions ($[K]_o$=0 mM) was strongly shifted to more negative potentials in the truncated pumps relative to the parent RD or C-Y pumps, shifts comparable to those seen in WT pumps on decreasing $[Na]_o$ several-fold. Together, the results suggest that these C-terminal deletions lower the apparent affinity for external Na ions to bind and become occluded in the Na/K pump. The C-terminus therefore provides contacts important for stabilizing the occluded E1P(Na_3) conformation, regardless of the route of Na ion entry into the binding pocket.[NIH HL36783]

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Deuterium isotope effects on fast gating of the chloride channel ClC-0

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Gating of the *Torpedo* Cl^- channel ClC-0 is modulated by intracellular and extracellular pH, but the mechanism responsible for this regulation has remained so far elusive. Using inside-out patch clamp measurements we studied the dependence of the fast gate on pH_{int} and $[Cl^-]_{int}$. Only the closing rate, but not the opening rate showed a strong dependence on these intracellular factors. Using mutagenesis we excluded several candidate residues as mediators of the pH_{int} dependence. We propose a model in which a proton generated by the dissociation of an intrapore water molecule protonates E166 leading to channel opening. Deuterium isotope effects confirm that proton transfer is rate limiting for gate opening and that channel closure depends mostly on $[OH^-]$. The model is in natural agreement with the finding that only the closing rate constant, but not the opening rate constant, depends on pH_{int} and $[Cl^-]_{int}$.

Abstracts
– Biological motility and molecular motors –

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In situ measurements of the molecular motor of muscle with nanometer-microsecond resolution

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In a contracting muscle, arrays of the dimeric motor protein myosin II pull the actin filament towards the centre of the sarcomere during cyclical ATP driven interactions. When the external load is smaller than the array force, the sarcomere works as a motor, converting metabolic energy into mechanical work; when the external load is larger than the array force, the sarcomere acts as a brake resisting the load with reduced metabolic cost. To investigate the molecular basis of the work production and the braking action of muscle, we use sarcomere-level mechanics and X-ray interferometry in intact single cells from frog skeletal muscle. During isometric contraction, each motor bears a force of about 6 pN. During shortening against high and moderate loads, the number of myosin motors attached to actin reduces in proportion to the external load while the force per attached motor is maintained similar to the isometric value (Piazzesi et al., *Cell* 131, 784-95, 2007). Rapid stretches of 1-5 nm between each overlapping set of myosin and actin filaments in a muscle sarcomere cause the stiffness of the array of myosin motors to increase up to twice the isometric value within 2 ms (Brunello et al., *PNAS USA* 104, 20114-19, 2007), indicating that the high resistance of active muscle to stretch is due to recruitment of the second motor domain of the myosin molecules with the first domain already attached to actin. Supported by MiUR and Ente CRF (Italy), NIH (USA), MRC (UK), EMBL, ESRF.

P-441

The positive role of noise on the transport efficiency of Na, K ATPase

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Na, K ATPase is a molecular motor which is able to transport ions through cell membranes, even against the transmembrane ion concentration gradient. While in vivo this nanoscale soft machine consumes ATP, it may be driven by external fluctuating electric fields, no matter they are periodic or random. Theoretically, the motor conformations can be described by a conformation vector V(t) governed by a multi-dimensional kinetic equation. Given an oscillating electric field with a slight fluctuation, the Boltzmann distributions of these conformations will change with time. The instantaneous transported ion flux is a functional of the quasi-cyclic trajectory V(t) of this non-autonomous dynamical system. Various interesting dynamical properties of this ion pump, including stochastic resonance, can be studied theoretically, some of which have good agreement with recent experimental findings.

O-442

Neck linker docking coordinates the kinetics of kinesin´s heads

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Conventional kinesin is a two-headed motor protein, which is able to walk along microtubules processively by hydrolyzing ATP. Its neck linkers, which connect the two motor domains and can undergo a docking/undocking transition, are widely believed to play the key role in the coordination of the chemical cycles of the two motor domains and, consequently, in force production and directional stepping. Although many experiments, often complemented with partial kinetic modeling of specific pathways, support this idea, the ultimate test of the viability of this hypothesis requires the construction of a complete kinetic model. Considering the two neck linkers as entropic springs that are allowed to dock to their head domains and incorporating only the few most relevant kinetic and structural properties of the individual heads, we have developed the first detailed, thermodynamically consistent model of kinesin that can (i) explain the cooperation of the heads during walking and (ii) reproduce much of the available experimental data (speed, dwell time distribution, randomness, processivity, hydrolysis rate, etc.) under a wide range of conditions (nucleotide concentrations, loading force, neck linker length and composition, etc.). Besides revealing the mechanism by which kinesin operates, our model also makes it possible to look into the experimentally inaccessible details of the mechanochemical cycle and predict how certain changes in the protein affect its motion.

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FLIM reveals that the myosin ATPase microenvironment “senses” force

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Muscle contraction is driven by molecular motors that adapt their energy utilization according to the demands made on them. We test the hypothesis that rate constants controlling the biochemical steps involved in ATP hydrolysis by myosin ATPase are affected by the force of the muscle. Here we use Fluorescence Lifetime Imaging Microscopy (FLIM) of a fluorescently labelled ATP analogue to investigate changes in the environment of the myosin ATPase, caused by different loads applied to skeletal muscle. Single muscle fibres were subjected to cycles of stretches and releases in the presence of rigor solution and 10 μM of coumarin-labelled ATP. FLIM acquisition was synchronised with stretch/release cycles and force measurements, which allow us to investigate the effect of strain on the lifetime of the labelled ATP bound to the actomyosin complex. Characterization of the fluorescence decay by a bi-exponential function resolved the time constant of two populations, namely, free fluorophore ($\tau_1 = 0.47 \pm 0.03$ ns; mean $\pm$ S.D.) and fluorescent nucleotide bound to the actomyosin complex ($\tau_2 = 2.21 \pm 0.06$ ns at low strain). These experiments showed that while the time constant of the free fluorophore did not change with force, the time constant of the fluorescent nucleotide bound to actomyosin showed a linear dependence with the force applied to the muscle of 0.43 ± 0.05 ps/kPa.

Abstracts
– Biological motility and molecular motors –

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Muscle contraction: Pitfalls in the determination of the contractile response

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The contractile response of an active muscle depends on the load. The load is a force /cross-section. There are three fundamental dimensions: the mass, m; the space, l; and the time, t. From these three dimensions are built up all the physical dimensions. As an example the acceleration, a, is given by, $a=l.t^{-2}$. Once the direction and versus are settled the modulus fully defines the physical effect of the acceleration. What about the force? The force, F, is given by, F=m.a. At variance with the acceleration, once the direction and versus are settled, the modulus does not define the physical and the biological effects of the force: the same force is generated by an infinite number of mass-acceleration couples that display different physical and biological effects. The same occurs with the load. Thus defining the load that opposes the contractile force does not define the contractile system. In the studies on muscle contraction the acceleration of the load is not considered nor it is provided a way to extract this information. Thus these systems are poorly defined from the physical as well as from the biological point of view. Models of muscle contraction that consider explicitly both the mass and the acceleration of the load show that, at the same load, the decrease of the acceleration of the load significantly delays the pre-steady state of the contraction and decreases the stiffness of the active fibre.

O-445

Investigating cross-bridge interactions in muscle fibres using Fluorescence Life Time Imaging

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Actomyosin cross-bridges formed when the globular heads of myosins bind to actin filaments are the molecular engines that drive muscle contraction, fuelled by ATP hydrolysis. Critical to this process is the change in shape of the cross-bridge and the change in the interactions with actin, in response to force applied to the muscle, and to the status of the nucleotide in the binding pocket. Although molecular detail is known from X-ray crystallography and biochemistry, understanding of the interplay between cross-bridge shape and chemical state requires studies in muscle fibres generating force. We use Fluorescence Life Time Imaging Microscopy (FLIM) as a probe of the cross-bridge environment.With a fluorescent analogue of ATP[1], fluorescence life-time (FLT) changes when the cross-bridge binds to actin. Now, we show preliminary experiments on the effect of force on FLT. The Essential Light Chain of myosin (ELC) is a ~20 kDa peptide that wraps around a 9nm-long α-helix of the myosin cross-bridge known as the lever arm which tilts during force generation. Using a recombinant ELC, labelled with a fluorophore at a strategic Cys, we replace the native ELC and introduce the fluorescent ELC in muscle fibres. Preliminary experiments demonstrate that the ELC fluorophore also is sensitive to force applied to the muscle fibre. In addition, Förster Resonance Energy transfer occurs between the nucleotide and ELC fluorophores, opening the way for studying structural changes in cross-bridges during force generation by FRET.

P-446

Protein and mRNA expression levels of titin in myocardium of hibernating ground squirrels and SHR

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Titin is a giant protein of vertebrate skeletal and cardiac muscles. Cardiac titin is expressed in two main isoforms: short N2B (~3000 kDa) and long N2BA (~3400 kDa). We have studied changes of titin isoform composition in myocardium of hibernating ground squirrels and spontaneously hypertensive rats (SHR). Using electrophoresis we have revealed considerable decrease (by 2-3 times) in the content of titin relative to myosin heavy chains in SHR heart as compared with that for normotensive rats. Surprisingly that the data of qRT-PCR showed the increase in mRNA content of N2BA and N2B-isoforms in hypertrophic heart more than 2 times in comparison with norm. We suppose that such a result is an effect of depressed translation of mRNA-titin in pathology. We have observed the decrease (by 1,5 times) of total titin amount in heart of hibernating animals in comparison with that for summer active animals. However N2BA/N2B ratio in the heart upon hibernation was increased by 2 times. Similar trend was not revealed for the mRNA level of corresponding isoforms, although we have showed the decrease of mRNA of both titin isoforms in heart of hibernating ground squirrels as compared to their content of summer animals. The decrease in total mRNA level may be explained by repressed transcription or mRNA degradation in the cell during hibernation. These discrepancies in protein and mRNA levels may be considered as the post-transcriptional regulation of titin isoforms expression.

P-447

Hinge-1: a flexible domain in kinesin-1 that facilitates motor cooperativity

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Kinesin-1 is a molecular motor that moves cellular cargo along microtubules. Its functional mechanism is well understood for individual motors. However, the way that many kinesin-1 motor proteins bound to the same cargo move together is not. We addressed the structural basis for this phenomenon using video microscopy of single microtubule-bound full-length motors and various spectroscopy methods were employed to study synthetic peptides derived from Hinge-1 region.
These Peptides show an unexpected profile of secondary structure forming propensities. Video microscopy of single microtubule-bound full-length motors reveal the sporadic occurrence of high compliance states alternating with longer-lived, low compliance states. The deletion of Hinge-1abolishes transitions to the high compliance state. From the results we hypothesize that strain accumulated during multiple kinesin motility populates the high compliance state by unfolding helical secondary structure in the central Hinge 1 domain flanked by unordered regions, thereby preventing the motors from interfering with each other in multiple motor situations.

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– Biological motility and molecular motors –

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The time course of inorganic phosphate release in permeabilized cardiac trabeculae of the rat

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The rate of P_i release was determined in permeabilized rat trabeculae. Contraction was elicited at 20°C by laser-flash photolysis of NPE-caged ATP, and time-resolved P_i release was monitored using MDCC-PBP, a coumarin-labelled phosphate binding protein, which increases its fluorescence intensity five-fold upon P_i binding. The ATPase rate during the first turnover of the total crossbridges (assuming 100 μM myosin heads) was $23s^{-1}$. The rate decreased to a steady state of $4s^{-1}$ after the eighth turnover (0.5-0.6s after activation). This steady state rate is comparable to published values of 3 - $10s^{-1}$, made ~15s after activation using an NADH-linked enzyme assay of ADP release. The advantage of using MDCC-PBP is that the control of mechanochemical coupling can be examined from the onset of force production and as it progresses toward the steady state. Force production and P_i release were simulated using a seven step scheme. Force was attributed to the states in the sequence A.M.ADP.P_i ↔ A.M.ADP ↔ A.M.*ADP*, with strain sensitivity incorporated into the isomerisation of A.M.ADP. The A.M.ADP.P_i and A.M.*ADP* states populated rapidly as force was increasing. In contrast, the A.M.ADP state accumulated slowly after the force plateau was reached and became the dominant force bearing state at the time of the eighth crossbridge turnover. Experiments are on-going to examine how the distribution of A.M states changes in response to rapid length-changes.

O-449

Cellular tension influences the conformational behavior of FAK during cell migration and spreading

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Mechanical forces are important in the regulation of cellular adhesion and migration. The Focal Adhesion Kinase (FAK) has been suggested to transduce cellular forces and govern cell migration. To obtain more insight in the functioning of FAK, a FRET-based optical biosensor for FAK was designed to relate integrin-mediated conformational changes in its FERM domain to focal adhesion behavior during cell spreading and migration in living cells. Imaging of the kinetics of FERM-based FAK conformational changes in spreading cells revealed two consecutive stages of focal adhesion activation. Heterogeneous FERM conformational responses were observed in individual focal adhesions of adherent motile cells, with the active FERM conformation being enriched in growing and sliding FAs, but not in stable and shrinking focal adhesions. Inhibition of the cellular actomyosin system revealed the involvement of Rho-ROCK rather than MLCK-induced tension signaling in the modulation of the FERM response. Our results place the FERM conformational change of FAK at the interface between integrin and force sensing.

P-450

Pushing on microtubules: dominant spindle centering mechanism in C. elegans embryo?

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Asymmetric cell division, where the content of the two daughter cells - as well as their sizes – differ, is found in many organisms. Strikingly, the spindle, first centered, starts to be displaced out of the center only in late metaphase. In C *elegans* embryo, the spindle rocks and is posteriorly displaced during anaphase by force generators asymmetrically localized on cell cortex. Prior to anaphase onset, the spindle is usually assumed to be centered by the same pulling force. It thus requires the force generators to be carefully repressed to distribute forces symmetrically. On live embryos, we measured positional fluctuations of centrosomes during metaphase with 20 nm accuracy. Fourier analysis shows an extremely accurate centering respect to the number of force generators and microtubules. Furthermore, spectrum is close to a Lorentzian, modeled by a spring and a dashpot, suggesting a spindle centering more likely by pushing on microtubules than pulling. Deviation at high frequencies indicates a subdominant pulling force. RNAi of gpr-1/2, known to control force generation, increases slightly centering accuracy; this result supports the hypothesis of an independent centering mechanism. Conversely, zyg-9(RNAi), a microtubule growing factor, decreases centering accuracy, modeled spring stiffness and damping modulus. Conclusion: first, the spindle centering mechanism is independent of cortical pulling force generator. Second, microtubules pushing is likely to center the spindle.

O-451

Molecular mechanisms underlying force generation in vertebrate neurons

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Polymerization of actin filaments is the main source of motility in lamellipodia and is controlled by many regulatory proteins. The underlying molecular mechanisms are only partially understood and now a determination of the dynamical properties of force generation is needed. Using optical tweezers we measured with millisecond temporal resolution and pN sensitivity the force-velocity (Fv) relationship and the power dissipated by lamellipodia of dorsal root ganglia neurons. When force and velocity are averaged over 3-5 s, Fv relationships can be flat. On a finer time scale, random occurrence of fast growths and sub-second retractions become predominant. Maximal power dissipated by lamellipodia over a silica bead with a diameter of 1 μm is 10^{-16} W. Due to the presence of adhesion forces, beads in close contact with a lamellipodium can seal on its membrane reducing the amplitude of Brownian fluctuations often by more than 10 times. Under these conditions, when lamellipodia grow and push the beads, discrete jumps varying from about 5 to 50 nm are detected. When lamellipodia retract, pulling the beads, no discrete events are observed. Our results on the dynamical properties of force generation are: a) force generation is a probabilistic process; b) underlying biological events have a bandwidth up to at least 10 Hz; c) fast growths of lamellipodia leading edge alternate with local retractions; d) force generation is produced in discrete steps with varying amplitude up to 0.2 pN.

Abstracts
– Biological motility and molecular motors –

P-452

On the physics of muscle contraction

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Whichever energy source is chosen as an engine, its force will decrease with increasing velocity. This is connected with a limited power of any engine. Thus, we state that Hill's formula is a mere sequence of the law of energy conservation.
To derive a mathematical dependence "force-velocity", all the means of consumption of fuel energy should be determined – in our case, the energy of the ATP hydrolyze. Moreover, the conformation energy of the crossbridges attached serves as the force source as well.
We state that part of the energy release transforms into the energy of oscillations of myosin proteins; the other part goes into thermal energy of the sarcoplasmic solution. Interaction of the oscillating myosin system with the sarcoplasmic solution controls the process of force generation by a muscle. It is just this interaction that leads to the temperature dependence of force.
The presentation is devoted to constructing a theory based on these simple considerations.
A discussion and constructive critic is especially wanted.

P-453

Alpha-synuclein and its A30P mutant affects the actin cytoskeleton structure and dynamics

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Alpha-synuclein (Syn) is a soluble protein abundant in the brain, primarily enriched at pre-synapses. Syn overexpression and the expression of its A30P mutant participate in the pathogenesis of Parkinson's disease. Many roles have been proposed for Syn, including the regulation of synaptic vesicle pools and of neurotransmitter release. The actin cytoskeleton regulates many aspects of synaptic function and its dysregulation may be a cause of neurodegeneration.
Working both in cell-free and in vivo conditions we demonstrate that Syn and the A30P mutant have different effects on the actin cytoskeleton dynamics. Our results show that Syn binds actin, and decreases actin polymerization rate probably by monomer sequestration. On the contrary, A30P accelerates actin polymerization in vitro and disrupts the cytoskeleton of intact cells. In particular, during dynamic cytoskeleton remodeling, A30P induces the assembly of discrete actin-rich foci. Actin trapping and the impairment of filaments reassembly lead to inhibition of cell movement and of the re-establishment of cell-cell contacts. In A30P expressing cells cytoskeleton-based processes, such as cell migration and the exo/endocytic traffic are inhibited.
Elucidating the dynamics of Syn interaction with actin may contribute to the understanding of its role in neuronal physiology as well as in neurodegeneration.

P-454

Strain response of myosin essential light chain in permeabilized skeletal muscle fibres

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We applied fluorescence lifetime imaging microscopy (FLIM) to investigate the relation between conformation of myosin head and mechanical force in skeletal muscle fibres. Recombinant myosin essential light chain (ELC) was expressed in *E.coli* and labelled at Cys-178 with coumarin. The labelled ELC was exchanged with native ELC in single permeabilized rabbit *m.psoas* fibres. Fluorescence lifetime was measured using Leica SP5 upright confocal microscope equipped with Becker & Hickl time-correlated single photon counting module and 63x 1.2 NA Leica PlanApo dipping objective, with the two-photon fluorescence excitation at 856 nm by Sapphire pulsed laser. After acquiring FLIM images of muscle fibres in relaxed state, the solution was changed to Ca-free rigor. Further images were acquired in rigor with or without 0.5-1% stretch applied by a motor. Both single and double exponential fluorescence decay analysis showed that the lifetime in rigor was lower compared to relaxed (about 80 ps difference for single exponential fit) and to rigor fibres under strain (about 40 ps). These data suggest a change in the microenvironment of coumarin induced by nucleotide binding and strain. This change is likely to be due to interaction between C-terminal domain of ELC and the N-terminal domain of myosin heavy chain related to the lever arm re-orientation process. Supported by BBSRC.

O-455

Pulling as a factor in forming the heterophasic structure of immunoglobulin proteins

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Structure of proteins of immunoglobulin superfamily: human IgG3 Kuc and muscle protein titin, has been investigated by methods of electron microscopy and diffraction with the use synchrotron radiation. Super elasticity of titin, the protein of immunoglobulin superfamily, is a key parameter that determines the mechanical properties of muscle. However, the structural-physical mechanism of titin elasticity under tension remains poorly understood. Here both tension transduction and high elasticity of titin are explained in terms of crystalline polymer physics. X-ray data suggest a model of titin as a nanoscale, morphological, aperiodical array of rigid Ig- and Fn3-type domains covalently-connected by conformationally variable short loops. The line group symmetry of the model can be defined as $\boldsymbol{S}_M$ with axial translation τ_∞. Homologous domains would have similar stability, but the structure of different domains on stretching is subject to different forces because they have different orientations relative to the axis of the molecule. Under the force influence the structure of any domain can become either rigid or flexible depending on its orientation in the titin strand. Pulling geometry forms an active axial structure from latent isotropic random coil structure of titin strand. We are suddenly faced with nanophase-separated morphology of IgG3 Kuc.
Study was supported by RFBR Grant 07-02-01281.

Abstracts
– Biological motility and molecular motors –

P-456

Insights into endothelial cells mechanotransduction from microfluidics

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The remodeling of blood vessels in response to changes in blood flow is mainly realized by endothelial cells (ECs) that convert mechanical stimuli from flowing blood into changes in cell signaling through a process called mechanotransduction. Many of the biological responses to external forces originate at two types of microscale structures: focal adhesions linking cells to their extracellular matrix and adherens junctions that link adjacent cells. This study aims to elucidate the role of the cytoskeleton, cell-matrix and cell-cell junctions in transducing fluid shear stress into intracellular signals in ECs. By using microcontact printing of proteins, we design substrates with defined adhesive islands in order to control shapes of living cells. This confinement of ECs allows to study the organization and the contractile activity of the cytoskeleton in order to redistribute their intracellular forces in response to externally applied forces. We design microfluidic channels with sizes and geometries close to small blood vessels to apply a physiological range of shear stresses on ECs. Our results indicate that cells deposited on a precisely defined adhesive area inside microchannels and subjected to shear stress reorganize their cytoskeleton, their focal adhesions and adherens junctions in response to blood flow. Drugs interfering with the cytoskeleton are used to underline the role of its different components in the cellular adaptation to the mechanical environment.

O-457

Self-organization of dynein motors generates meiotic nuclear oscillations

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Meiotic nuclear oscillations in the fission yeast *Schizosaccharomyces pombe* are crucial for proper chromosome pairing and recombination. We report a mechanism of these oscillations based on collective behavior of dynein motors linking the cell cortex and dynamic microtubules that extend from the spindle pole body in opposite directions. By combining quantitative live cell imaging and laser ablation with a theoretical description, we show that dynein dynamically redistributes in the cell in response to load forces, resulting in more dynein attached to the leading than to the trailing microtubules. The redistribution of motors introduces an asymmetry of motor forces pulling in opposite directions, leading to the generation of oscillations. Our work provides the first direct *in vivo* observation of self-organized dynamic dynein distributions, which, due to the intrinsic motor properties, generate regular large-scale movements in the cell (*1*).

1. Vogel SK, Pavin N, Maghelli N, Julicher F, Tolic-Norrelykke IM (2009) *PLoS Biol* 7(4): e1000087.

Abstracts
– Ion channels in channelopathies and cancer –

O-458

Biophysical characteristics and clinical potential of ion channel expression in human cancer cells

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Whole-cell patch clamp recordings from a variety of human cancer cells showed that functional voltage-gated sodium channel (VGSC) expression occurred specifically with strongly metastatic cells. In addition, where studied, this was accompanied by down-regulation of outward (mainly potassium) currents. This has led to the CELEX ("cellular excitability") hypothesis of cancer according to which metastatic cell membranes are excitable and this promotes their hyperactivity. Importantly, the VGSC genes expressed are embryonic splice variants, which are normally developmentally regulated, hence the phenomenon is 'oncofetal'. In breast cancer, where the predominant VGSC is Nav1.5 the neonatal and adult forms are significantly different and this is reflected in channel activity whereby the neonatal VGSC has much slower inactivation kinetics. The double-charge change at position 211 is critical for this difference. The slow kinetics results in much greater influx of Na^+ into cells and one consequence of this is activation of protein kinase A. This is a tonic effect and, under steady-state resting conditions, it results in a positive feedback effect promoting post-translational trafficking of VGSC protein to plasma membrane. The unique amino acid sequence of the spliced region has enabled the production of a polyclonal blocking antibody specific to neonatal Nav1.5. It is concluded that VGSCs represent novel biophysical targets for clinical management of metastatic disease.

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The extracellular matrix molecule hyaluronic acid modulates L-type voltage-dependent Ca2+ channels

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We studied the effects of hyaluronic acid (HA), a major extracellular matrix molecule, on activity of L-VDCCs in a heterologous expression system and in hippocampal slices. We recorded currents mediated by a major neuronal subtype of L-VDCCs ($Ca_v1.2C$, β_1b, and $\alpha_2\delta_1$) expressed in CHO cells. A five-minute application of 0.1 mg/ml HA potentiated L-VDCC currents at -20, -10, 0 and +10 mV by approximately 50%. Analysis of Boltzmann curves showed that HA increased maximal conductance rather than other parameters ($V_{0.5}$ or k). Treatment with hyaluronidase removed endogenous HA in murine hippocampal slices and specifically impaired long-term potentiation (LTP) induced at CA3-CA1 synapses by repetitive theta-burst stimulation. Blockade of L-VDCCs reduced LTP in control slices to the levels seen after hyaluronidase treatment. A potentiation of L-VDCCs with Bay K 8644 fully restored LTP after hyaluronidase treatment. Removal of HA reduced Ca^{2+} transients elicited by backpropagating action potentials in individual dendritic shafts and spines of CA1 pyramidal cells, whereas pretreatment with nifedipine fully occluded this effect. Thus, HA potentiates postsynaptic L-VDCCs and by this way influences use-dependent synaptic plasticity.

P-460

Separate allosteric binding sites for extracellular calcium ions and protons in human CLC-K channels

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CLC-Ka and CLC-Kb are highly homologous Cl^- channels expressed in the kidney and the inner ear where they mediate transepithelial chloride transport. Both channels heteromerize with the beta subunit barttin. Mutations in CLC-Kb and barttin genes lead to Bartter's syndrome. We analyzed the modulatory effect of extracellular Ca^{2+} and H^+ on CLC-K channels using the *Xenopus* oocyte expression system. CLC-Ka currents increased with increasing $[Ca^{2+}]_{ext}$ without full saturation for $[Ca^{2+}]_{ext}$ up to 50 mM. However, in the virtual absence of Ca^{2+}, CLC-Ka currents are about 18 % of currents measured in 10 mM $[Ca^{2+}]_{ext}$, demonstrating that Ca^{2+} is not strictly essential for opening. Vice versa, CLC-Ka was blocked by increasing the $[H]^+_{ext}$ with an almost complete block at pH 6. Among various reaction models tested, the model that best fitted all state-steady data predicts an allosteric regulation of channel opening by separate binding sites for Ca^{2+} and H^+. Moreover, the best fit suggests that one Ca^{2+} and two H^+ bind to the channel. Kinetic analysis of current responses upon $[Ca^{2+}]_{ext}$ and pH jumps confirmed the allosteric character of modulation. In support of the presence of two separate binding sites we identified several mutations that selectively altered Ca^{2+} or H^+ sensitivity. Our data represent a first step towards a molecular picture of Ca^{2+} and proton regulation of CLC-K channels and suggest that it is of physiological relevance.

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Gating modifiers of voltage-gated sodium channels as a novel strategy for multiple sclerosis treatment

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Multiple sclerosis (MS) is the main known pathology of myelinating cells. An autoimmune reaction occurs against myelin sheets of neurons, so action potential (AP) propagation along the affected nerve fibers has been destroyed and it causes various disorders. Here, we propose a novel strategy for MS symptoms treatment. We modeled neuron by ORCAD software and simulated the action potential propagation along the axon in normal condition. Our model simulated normal neuronal behavior. Then we destroyed the myelin sheet as it occurs in MS and observed destroyed AP propagation as it was reported in MS disease. We investigated the effect of changes in the voltage-gated sodium channel (VGSC) threshold on the efficiency of AP propagation. The results demonstrated that reduction of VGSC threshold improves the propagation of AP by increasing the amount of sodium flux during AP propagation. Although, some researches have proposed VGSC blocker as MS symptom treatment, our result suggests that the increase of sodium current produced by reduction of VGSC threshold, improves AP propagation and probably cure some MS symptoms. So, we suggest that VGSC gating modifiers can be considered as novel strategy for MS treatment. Surely, this results needs to be confirmed by experimental studies.

Abstracts
– Ion channels in channelopathies and cancer –

O-462

Cardiac effects of anabolic steroids: an electrophysiological approach

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Anabolic androgenic steroids (AAS) have been used by athletes and non athletes for almost five decades in order to improve performance. However, the illicit abuse of high-doses of AAS has been attributed as a main cause of several cardiovascular disorders such as arterial hypertension, lipid profile abnormalities, heart failure, hypertrophic cardiomyopathy, arrhythmia and sudden death. The aim of this study was to investigate QT interval and transient outward potassium current (I_{to}) changes in rats treated with nandrolone decanoate (DECA). Male Wistar rats received weekly 10 mg/Kg of DECA (n=10) or vehicle (CONTROL, n=10). Electrocardiogram was recorded weekly, and QT interval was measured. After 8 weeks hearts were excised and single myocytes were isolated from the ventricles of animals. I_{to} was recorded by means of the whole cell patch clamp technique. QT interval was larger in DECA group from 4th to 8th week ($P<0.01$). Analysis of I_{to} showed a decreased current density ($P<0.01$) in ventricular cardiomyocytes of DECA group, compared to CONTROL group. In conclusion, our results show that alterations on ventricular repolarizaton may constitute an early consequence of the chronic administration of high doses of anabolic steroids in rats, and demonstrated QT prolongation and I_{to} density reduction, which may constitute an important marker of arrhythmia vulnerability and sudden death.

O-463

The role of ion dynamics in zebrafish fin regeneration

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The specific and directional ion transport across cell membranes or tissue layers results in differential accumulation of ions and endogenous electric currents. These phenomena have been shown to be important for vertebrate organs regeneration. However, the specific ion nature of such electric currents remains unknown, as well as the role of cellular ion dynamics during regeneration and the molecular signalling pathway that transduces electric cues into cellular responses. We use zebrafish caudal fin as an adult regeneration model to unveil the specific ion composition of the currents associated with wound healing and regeneration, using a non-invasive Ion-Specific Scanning Microprobe setup. Our data suggests a role for potassium (K^+), calcium (Ca^{2+}) and protons (H^+) at different stages of the regeneration process. K^+ and Ca^{2+} extracellular effluxes have both been detected during the wound healing stage. H^+ efflux is triggered during wound healing and is maintained throughout regeneration. We are validating these data with genetic and pharmacological approaches, as well as advanced ion imaging. Overall, our results suggest ion-driven mechanisms underlie adult tissue regeneration and its comprehension may open way for new therapeutic strategies, both in regenerative and developmental medicine and in cancer therapy.

O-464

Mechanisms of transport coupling in CLC Cl^-/H^+ antiporters involved in human genetic diseases

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CLC proteins form an evolutionary conserved gene-family that comprises 9 members in mammals. Four of the human CLCs are passive plasma membrane Cl^- ion channels. The other 5 CLCs are expressed in intracellular organelles. ClC-4 and ClC-5, mutations of which lead to Dent's disease, are secondary active Cl^-/H^+ antiporters, similar to the bacterial ClC-ec1, and with identical 2 Cl^- : 1 H^+ stoichiometry. Cl^- and H^+ transport activity of the exchanger CLCs depends on two Glut residues. Mutating a 'gating glutamate' (E211 in ClC-5) converts the exchanger into anion conductances. Neutralizing the 'proton glutamate' (E268), but not its replacement by some other titratable groups, abolishes Cl^- and H^+ transport. Noise analysis indicated that ClC-5 switches between silent and transporting states with an apparent unitary conductance of 0.5 pS, indicating a very large transport turnover. NO_3^- uncouples H^+ transport but mutating the highly conserved S168 to P, as found in the plant NO_3^- / H^+ antiporter atClCa, led to coupled NO_3^- : H^+ exchange. CLC proteins are a fascinating example of how a very similar protein architecture can be used to provide either a passive electrodiffusive permeation pathway or a strictly coupled secondary active ion transporter. (Supported by Telethon Italy - grant GGP08064).

Abstracts
– Protein-ligand interactions –

O-465

Ligand migration in human neuroglobin highlights a complex system of interconnected cavities

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The complex system of cavities identified in human neuroglobin (Ngb) has been postulated to be of functional significance to the putative NO dioxygenase activity of the protein. The interconnected hydrophobic cavities may support this catalytic activity by acting as reservoir for reactants and providing preferential pathways assisting product removal from the active site. We thus decided to investigate CO rebinding kinetics to Ngb embedded in silica gels to expose ligand migration processes in the geminate phase.
Encapsulation of the CO complexes of reduced neuroglobin, leads to a slight increase in geminate recombination after nanosecond laser photolysis. Increasing the viscosity of the medium, by soaking the gels in glycerol, completely inhibits escape of the photodissociated ligand to the solvent, and highlights a complex, multiphasic kinetic pattern. This finding can be rationalized by assuming the existence of a discrete set of temporary docking sites, capable of trapping the photodissociated ligand for very long times, up to a few ms after photolysis.

O-466

Sampling the flexibility of PPAR-γ

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A promising approach to consider the flexibility of proteins in docking studies consists in performing multiple rigid docking on a representative set of the receptor conformations. Molecular Dynamic (MD) simulation is one of the best adapted methods for structural sampling, but exploring the conformational diversity of a protein is computationally expensive. We present a protocol for generating a wide range of conformational states of a receptor using restrained MD and a partitioning protocol to select a few representative conformations of the binding site from this MD. A way to speed up efficiently MD calculation is using an implicit model to represent the solute-solvent interactions. We explore a protocol using a distance-dependant permittivity function to represent solvent effect and an ensemble of controlled restraints applied on a subset of specific atoms in order to prevent artefactual structural distortions, but preserving receptor's flexibility. Ten 100ns simulations have been performed using different sets of parameters and compared to a reference 30ns simulation with explicit solvent. To select a representative set of conformations, partitioning (k-means algorithm) was applied on the ensemble of simulated conformations. This methodology was applied to the ligand binding domain of Peroxysome Proliferator-Activated Receptor-γ.

P-467

Molecular examination of motifs that lead to the formation of S-nitrosylated proteins

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Physiologically, a wide range of proteins experience structural and functional modifications after the addition of a nitric oxide (NO) moiety to cysteine thiol. This post-translational modification, known as S-nitrosylation, regulates a large number of cellular processes like vasodilatation, cell signaling and others, and the products of S-nitrosylation can be involved during the development of different human diseases. However, little is known about the mechanism by which different proteins specifically bind the NO moiety to their cysteine. Here we show a bio-statistical analysis of some properties of cysteine that are S-nitrosylated in different proteins, including the pK, electrostatic environment, solvent accessibility of the target cysteine and identity of surrounding amino acids. We also chose model proteins (human thioredoxin and S100 protein) to make specific targeted amino acid substitutions around selected cysteines to alter the properties described above. The reactivity and stability of these mutant proteins towards S-nitrosylation will be examined.

O-468

Kinetic and structural study of a bacterial heme sensor

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Denitrifying bacteria control NO and NO_2 cytosolic levels by regulating the expression of denitrification gene clusters via REDOX signalling of specific transcriptional factors that may act as NO sensors in vivo. A protein belonging to the subclass DNR (dissimilative nitrate respiration regulator) from Pseudomonas aeruginosa has been recently suggested to be a heme containing protein. Very recently the three dimensional structure of the apo-form of DNR (in the absence of heme) has been determined by X-Ray crystallography, whereas the holo-form (in the presence of heme) has not yet been crystallized. We have investigated the heme local structure in solution of ferric, ferrous, CO bound and NO bound holo-DNR by X-ray Aborption Spectroscopy (XAS) and we added a kinetic study of the CO bound form by means of a flash photolysis setup using UV-Visible absorption as a spectroscopic probe. The combination of Fe K-edge XANES fingerprints and kinetic study reveal a heme pocket able to bind exogenous ligands like NO and CO with increased plasticity, thus supporting its role as the cofactor involved in NO sensing activity.

Abstracts
– Protein-ligand interactions –

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Thermodynamic bases of nucleoplasmin-histone complexes recognition by the nuclear transport machinery

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The nuclear transport of the chromatin remodeling (nucleoplasmin) and chromatin building (histones) proteins is mediated by importins. Nucleoplasmin (NP) contains a classical bipartite nuclear localization signal (NLS) that is recognized by importin α, while histones present multiple sequence elements (NLS-like motifs) for nuclear targeting. Besides, ternary importin/NP/histone complexes might represent a putative coimport pathway for nuclear import of linker (H5), nucleosomal core (H2AH2B) histones and their chaperone protein NP, enhancing the histone import efficiency. To better understand NP and histone recognition by the transport machinery we studied the thermodynamics of complex formation of importin α (a truncated form) and importin β with histones and NP, and with NP/histone binary complexes by means of isothermal titration calorimetry. Data show that importins interact with the two histone types and NP, and that importin and histones can simultaneously bind to NP. Analysis of the binding energetics reveals an enthalpy driven formation of high affinity binary and ternary complexes. We demonstrate that different amount of importin molecules can be loaded on NP/binary complexes dependent on the histone type, linker or core, and the amount of the bound histones.

O-470

Computer simulation of ligand binding and reactivity of heme proteins

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We present an investigation of the molecular basis of ligand binding and reactivity of heme proteins using computer simulation. A combination of classical molecular dynamics and hybrid quantum-classical (QM-MM) calculations are applied to explore distal and proximal effects on diatomic ligand binding to the heme. Trends in binding energies and in the kinetic constants are illustrated through a number of selected examples.
An investigation of the interplay between ligand migration and protein dynamics obtained through classical molecular dynamics techniques in combination with advanced sampling tools is also presented to yield information about free energy profiles and possible secondary docking sites. Results for truncated N hemoglobin of Mycobacterium Tuberculosis, presented as an illustrative example, suggest that the truncated hemoglobin N has evolved a dual-path mechanism for selective/distinct migration of O_2 and NO to the heme, to achieve efficient NO detoxification.
Finally, we present also an analysis of the molecular basis of hexacoordination in human neuroglobin, which suggest that the flexibility of the CD plays a key role in determining the ligand binding properties.

P-471

Potent flaviviridae inhibitors: targeted finger domain of RdRp

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Studies of protein-inhibitors interactions are helpful to elucidate the mode of action of ligands and thereby providing clues for rational drug design. BVDV, is an important target of drug discovery activities largely because it is essential for viral replication. In BVDV RdRp no specific NNI binding site has been reported till now. Experimental results have shown that different class of inhibitors (Benzimidazole, Imidazoquinolines and Pyridoxyquinolines), have resistant mutations located in the finger domain of the RdRp. All the reported mutations are spatially very close to each other. Thereby, indicating that binding sites of NNI's may lie in the finger domain for these different class of inhibitors.Herein, we have utilized docking procedure to investigate binding sites, binding modes as well as binding affinity of different class of inhibitors.We then used all atom molecular dynamics (MD) simulations to investigate the stabilizing interaction between inhibitor-receptor pairs. Our MD results are in good agreement with experimental data and provide deep insights into the dynamical features of the high affinity inhibitor-receptor binding. Thus identifying the binding modes of our inhibitors and mechanism leading to inactivity of the enzyme can help us to build a microscopically well-funded picture of the functioning of these enzymes.

P-472

Inhibition of ion pumps by drugs

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Ion pumps are integral membrane proteins devoted to ion transport through a lipid membrane phase. The ion pumps Ca-ATPase and Na,K-ATPase are prominent members of the P-type ATPases family. Due to fundamental physiological roles of these proteins, they are very promising drug targets. BioElectroLab has a wide expertise in the study of ion transport by these proteins [1]. Our attention has been recently focused on the interaction of these enzymes with molecules of potential pharmacological interest.
Frequently, drugs exert an inhibitory action on the transport activity of an ion pump, usually confining it in an inactive conformation. Molecules like thapsigargin and cyclopiazonic acid belong to high (nanoM) affinity inhibitors of the Ca-ATPase, whereas clotrimazole and curcumin are medium (microM) affinity inhibitors of both Ca-ATPase and Na,K-ATPase. For each of these compounds a mechanism of action is proposed. Moreover, recent results concerning Ca-ATPase inhibition by clotrimazole analogues will be shown: the relevance of this type of molecules is due to their potential employment as an alternative to traditional drugs against malaria parasite.
Financial support of Ente Cassa di Risparmio di Firenze and of MIUR (PRIN Project) is gratefully acknowledged.
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Abstracts
– Protein-ligand interactions –

P-473

Patterned functionalization of surfaces for guided transport on molecular motors tracks

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Chelator head groups with multiple nitrilotriacetic acid (NTA) moieties have been very well characterized and successfully utilized as high affinity adapters for functional immobilization of oligohistidine tagged proteins to surfaces. We have recently established a generic method for patterning NTA functionalized surfaces by selective photodestruction via a light induced Fenton reaction. Efficiency of different transition metal ions for catalyzing this reaction was tested. Functionality of the patterned protein was confirmed using the interaction between Interferonα2 and its receptor. Implementation of this technique in a confocal laser scanning microscope allowed us to control surface density of binding sites, providing the possibility to vary the surface concentration of immobilized proteins in a spatially resolved manner. We also applied this approach for exploring guided transport of microtubules by kinesin selectively immobilized onto TrisNTA patterns.

P-474

Unraveling the molecular basis for ligand binding in truncated hemoglobins: the *B. subtilis* case

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Truncated hemoglobins (trHbs) are heme proteins present in bacteria, unicellular eukaryotes, and higher plants. Three phylogenetic groups (N, O, and P) have been identified in trHbs. The crystal structure of truncated hemoglobin O of *B.subtilis*, does not show an evident tunnel/cavity system connecting the protein active site with the solvent, a fact that cannot be easily rationalized considering the very high oxygen association rate. Moreover, resonant Raman results of the CO bound protein, showed that a complex hydrogen bond network exists in the distal cavity, making it difficult to assign unambiguously the residues involved in the stabilization of the bound ligand. For these reasons we performed classical molecular dynamics simulations of the oxy, carboxy and deoxy protein, and computed the free energy profiles associated with ligand migration to the active site. Our results suggest that there is a key residue, GlnE11, that may present an alternate conformation in which a wide ligand migration tunnel is formed, consistently with the kinetic data. The results for the CO and O_2 bound protein show also that GlnE11 is directly involved in the stabilization of the coordinated ligand, playing a similar role as TyrB10, and TrpG8 in other trHbs. Our results not only reconcile the structural data with the kinetic information, but also provide additional insight about the general behaviour of trHbs.

P-475

Light-enforced antigen dissociation from MHC molecules via photochromic switches

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Peptide presentation by major histocompatibility complex (MHC) molecules is crucial for immune responses. Photocontrol of peptide dynamics by means of photo-switchable peptide analogs will provide insights in peptide dynamics and its dependence on MHC polymorphism (1-3). We have designed a hemithioindigo (mHTI) photo-switch bearing peptide using the viral epitope RRRWRRLTV (pLMP2) as a template. Incorporation of mHTI in the peptide backbone should result only in a minor change of the overall peptide structure given the relaxed conformation of the Z-isomer. Indeed, the human MHC molecule HLA-B*2705 can tolerate nonpeptidic elements in pLMP2, as evidenced by normal peptide binding. Using the autofluorescent properties of mHTI we determined the stability of the HLA-B*2705/mHTI-pLMP2 complex. Photoswitching from $Z \rightarrow E$ results in a decrease of HLA-complex stability. Based on computer modeling this decrease is due to a reduced interaction of the peptide C-terminus with HLA-B*2705.

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P-476

Quantitative analysis of Tau-microtubule interaction using FRET

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Since a decade, our laboratory has already studied the interaction of tau variant with tubulin (tub) or microtubules (MTs) and phosphorylation process on this interaction. Indeed, FRET assay was a powerful tool to achieve binding parameters between tau and tub *in vitro*: 1/ with MTs stabilized by fluorescein-coupled taxol (Flutax-2) as donor and rhodamine-labelled tau (rho-tau) as acceptor, and 2/ in living cells by confocal laser scanning microscopy (CLSM) with tau/α-tub fused to eGFP/mCherry, respectively. Results revealed 47 ± 3 % energy transfer efficiency from Flutax-2 to rho-tau and a donor-to-acceptor distance of 54 ± 1 Å. By titration, the dissociation constant of tau was determined to 1.0 ± 0.5 μM. A cleavage procedure of αβ-tub was performed to determine the influence of the C-term tails of αβ-tub on the tau-MT interaction. No difference in distances and binding parameters was observed. CLSM images displayed a heightened concentration of fluorescent tau in patches along MTs. FRET experiments revealed in particular higher efficiencies between GFP-tau to mCherry-α tub proteins in these locations. Overall, our results suggested no involvement of the hypervariable and highly acidic C-term tails of tub in MT/tau binding. A molecular model is proposed in which Flutax-2 is directly accessible to tau molecules. Besides, the modified distribution of fluorescent tau could be implicated in local change of mechanical properties of MTs.

Abstracts
– Protein-ligand interactions –

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Determination of protein-ligand binding thermodynamics by thermal shift assay

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Thermal shift assay determines the effect of ligand binding on the protein thermal denaturation equilibrium. Several models have been derived to describe the general cases of protein-ligand binding. First, the model describing protein stabilization and destabilization by ligand binding to the native and unfolded states. Second, the split of protein melting transitions by tightly binding ligands is presented when the transition of free protein and ligand-bound protein occur separately. Mathematical equations describing the protein unfolding and ligand binding thermodynamic parameters at various temperatures are presented. The protein melting temperature shift can be determined by various techniques such as differential scanning calorimetry, circular dichroism, and intrinsic or extrinsic fluorescence. The advantages of fluorescent techniques are presented. The melting temperature shift caused by ligand binding is dependent on the thermodynamic parameters of protein unfolding and ligand binding, including enthalpy, entropy, and heat capacity, thus allowing determination of binding thermodynamics. Application of the models in the design of Hsp90 chaperone and carbonic anhydrase inhibitors is discussed. Comparison with isothermal titration calorimetry data is presented.

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Studying interactions between COP1 regulatory protein and HY5 transcription factor

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This work addresses important questions of protein-ligand interactions and selective protein recognition. Protein-ligand bindings are crucial for many cellular processes. Dependable methods for predicting binding sites would lead to a better understanding of proteins' selective recognition and would, in turn, help research on fighting diseases. Presented here is a study of interactions between the WD domain found in a regulatory protein COP1 and the motif V-P-E/D-Φ-G (Φ=hydrophobic residue) found in a transcription factor HY5. COP1 and HY5 proteins have opposing roles in developmental regulation. COP1 can repress HY5 by directly binding with it. The repression involves specific interactions between the WD domain and the motif V-P-E/D-Φ-G. Previous experimental research showed that mutations in the motif's V-P pair resulted in a large decrease in HY5 repression. To study effects of similar mutations, residues in the motif V-P-E/D-Φ-G were systematically substituted with other residues. Interactions between the mutated motif and the WD domain were studied. Distributions of binding sites, bond lengths, local shape complementarity, and interaction potentials were modeled for each residue substitution. The study identified binding sites critical for the COP1-HY5 binding. Some HY5 residues in the vicinity of the motif were also found to be important in the binding. The significance of the results for understanding selective protein recognition is discussed.

P-479

Probing bio-molecular bonds with magnetic force for biosensor applications

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We investigate new technologies to be applied in next generation biosensors, which not only measure biomarker concentrations but also probe bio-molecular interactions. The concept is based on the response of ligand-receptor pairs to an applied force or torque[1,2]. We use functionalized magnetic beads and magnetic fields to apply translational and rotational forces on the molecular bonds.
In a model experiment, polystyrene surfaces were coated with anti-biotin and beads were coated with biotin. After incubation, a constant magnetic force was applied to the beads and the number of bound beads was measured as a function of time. This was repeated for a range of forces. The dissociation rate (k_{off}) is determined for each force and k_{off} at zero force is extracted from these data.
Rotational forces were exerted on protein-G coated beads bound to IgG antibodies immobilized on a surface. Rotating fields revealed an oscillating behavior, which can be understood from the balance between the applied magnetic torque and the torque due to the deformation of the biological bond.
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P-480

Modeling the interactions of T-cell receptor with MHCII bound to type II collagen peptide 261-273

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Rheumatoid arthritis (RA) is an autoimmune disorder, leading to pathological damage at the level of joints, associated with the HLA class II allele HLA-DR4. Although etiology of RA is unknown, type II collagen (CII) is a potential antigen candidate and it is believed that T cell responses in collagen-dependent RA are directed towards the immunodominant pathogenic epitope CII(261–273). Despite recent advances in characterization of class II major histocompatibility complex (MHC) and T-cell receptor (TCR) contacts in this epitope, the atomic details of TCR-CII(261-273)-MHC complex are not known. Here, homology modeling and molecular docking studies have been used to derive a three-dimensional model of TCR β chains, obtained from a DR4+ subject, in complex with CII(261-273)/HLA-DR4. The best complex from docking was further refined using molecular dynamics simulations for 20 ns. The proposed model represents a reasonable structural basis for understanding CII(261-273)-MHCII complex recognition by TCR and for rational design of inhibitors targeting TCR-pMHC interface.

Abstracts
– Protein-ligand interactions –

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Serratia marcescens heme acquisition system: heme transport and protein:protein interactions

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Heme transport systems in bacteria are required and might be potential target for antibacterial drugs. The heme acquisition system, Has, exists in pathogenic as well as in opportunistic bacteria but only for the latter one extensive studies have been conducted, constituting as such a model system. The outer membrane receptor HasR, the central component of this system, functions in synergy with a secreted high affinity heme binding protein, the hemophore HasA. HasA extracts heme from host hemoproteins and returns it to HasR. Then, the energy given by a protein complex of the inner membrane is used to allow heme entrance across the bacterial membrane and to eject the empty hemophore from the receptor. Reconstitution of this heme acquisition system in *E coli*, overexpression and purification of its various components have allowed us to obtain sufficient amount of protein to perform NMR and biophysical studies to analyse at the molecular level the different steps of heme acquisition by HasR.

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rBPI21 aggregates and neutralizes negatively charged lipopolysaccharides and biomimetic membranes

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Antimicrobial peptides (AMPs) are short positively charged polypeptides. They are important due to their potential to provide an alternative to conventional therapy against bacterial infections. $rBPI_{21}$ is a 21 kDa peptide based on the N-terminal region of the neutrophil bactericidal/permeability-increasing protein (BPI). It was shown that this AMP possesses bactericidal effects on Gram-negative bacteria and higher affinity for lipopolysaccharide (LPS), neutralizing its effect. The peptide use against meningitis, is in Phase III clinical trials. Here, we demonstrate that $rBPI_{21}$ promotes aggregation of negatively charged large unilamellar vesicles (LUV) and LPS aggregates, by dynamic light scattering, while for zwitterionic phosphatidylcholine (POPC) LUV the size remains unchanged. The aggregation increases with peptide concentration until peptide promotes massive aggregation followed by sample flocculation/precipitation. With the $rBPI_{21}$-lipid interaction there is a progressive change in the zeta-potential of the LUV systems and LPS aggregates. LUV systems composed of phosphatidylglycerol (POPG) and POPC:POPG mixtures have higher zeta-potential variations than POPC LUV. For LPS aggregates, $rBPI_{21}$ neutralizes the surface charge and at higher peptide concentrations overcompensates it. Results demonstrate that the interaction of the peptide $rBPI_{21}$ with LPS aggregates and LUV systems has electrostatic and hydrophobic contributions.

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Study of HSA-Bilirubin interaction by QCM-D and AFM

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Human Serum Albumin (HSA) is the most abundant non-glycosilated plasma protein in the human body. This multifunctional protein has ligand-binding and transport properties, antioxidant functions and enzymatic activity [1]. Bilirubin interacts with albumin in order to be transported from the blood to the liver where it is secreted. The interaction occurs specifically in HSA I-domain of its three domains [2]. In our work, we investigate the interaction between HSA and bilirubin by Quartz Crystal Microbalance with Dissipation (QCM-D) and Atomic Force Microscopy (AFM) [1,3]. Albumin was adsorbed on negatively charged silicon oxide. However, HSA was removed after rinsing with PBS. HSA adsorbed on positively charged polyelectrolyte multilayers leads to a stable layer of surface mass density of 272 ng/cm^2. Cross linked albumin with glutaraldehyde after its adsorption on NH_2-terminated thiols is also stable reaching surface mass density of 296 ng/cm^2. A preliminary bilirubin adsorption results on cross linked HSA substrate show that 200 ng/cm^2 is immobilized. Taking into account that HSA-bilirubin stoichiometry is 1:1, the outcome demonstrates that the 70% of HSA I-domains remain active.

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P-484

Spectroscopic characterization of the complex between azurin and p53 transactivation domain

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Recent reports have shown that the bacterial redox protein azurin can enter into cancer cells and induce apoptosis by stabilizing p53. The formation of a complex between the two proteins has been demonstrated, but little is known about binding features. For the first time, we show here that azurin binds to the N-terminal region of p53 with a dissociation constant in the 5-10 μM range. Trp phosphorescence lifetime measurements revealed conformational changes of azurin induced by the interaction with p53(1-63). Acrylamide quenching of Trp phosphorescence also indicated a significant increase of the overall flexibility of azurin upon binding to p53(1-63). No change of the fluorescence emission of p53(1-63) was detected in the presence of azurin. The latter finding suggests that W23 of p53 is not directly involved in domain binding to azurin, indicating that the binding site is distinct from that of MDM2. The present results may assist the design of novel cancer treatments based on p53 stabilization by azurin.

Abstracts
– Protein-ligand interactions –

P-485

The *Escherichia coli* membrane insertase YidC reversibly binds its substrate Pf3 coat protein

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The membrane insertase YidC of *E. coli* belongs to the Oxa1 family of mitochondria and plays an essential role in facilitating the insertion and assembly of membrane proteins. We have previously shown with detergent-solubilized ($C_{12}PC$) YidC, labelled with ANS, and Pf3 coat that the initial step of the membrane insertion process, the binding of the substrate Pf3 coat to YidC, is reversible [*Biochemistry 47,* 6052-6058 (2008)]. The dissociation constant K_D for that particular system is about 1 μM. In order to obtain data for the native system we used in this study membrane-reconstituted (DOPC and DOPE/DOPG) YidC. The effect of the initial binding was examined *in vitro* by fluorescence quenching of the 11 tryptophan (Trp) residues of YidC which are highly sensitive fluorescent probes for changes of the tertiary structure. Quenching of the Trp fluorescence after titration with a Trp-free Pf3 mutant indicates a change in the YidCs tertiary structure upon binding to its substrate. The binding data show a K_D value in the range of 0.5–1.8 μM. The influence of different environments (lipid membranes, DDM micelles) on the secondary structure of YidC as well as on the YidC large periplasmic domain P1 was investigated by circular dichroism (CD). The CD data show that the secondary structure of YidC changes upon reconstitution into a membranes when compared to the detergent solubilized state. Particularly, the P1 domain is considerably affected by the detergent $C_{12}PC$.

P-486

A simulation approach to Multiple Sclerosis: study of a peptide with a pharmaceutical potential

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Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system, leading to premature death. One of the potential targets of the autoimmune reaction is the myelin protein MOG that has been crystallized in complex with the 8-18C5 MS-autoantibody. The analysis of contacts and buried surface area combined with an alanine scanning computation reveals the key role of MOG fragment 101-108 for the interaction with 8-18C5. A docking simulation shows that the 101-108 fragment, excised from MOG, and kept in crystal-like conformation, is still capable of fitting into the binding pocket of the antibody. We then studied, through Replica Exchange Molecular Dynamics simulations, the structural equilibrium distribution of the free peptide and of a number of analogs stabilized by a disulfide bond. We found that the free peptide yields a significant fraction of crystal-like conformations and the proportion of native-like structures is further increased by the disulfide bridge. When we tried to dock the centroids of the most populated clusters to 8-18C5, we discovered the existence of a docking funnel whose bottom is populated by stable complexes where the peptide occupies the same spatial region as in the crystal. We therefore conclude that the MOG 101-108 fragment may be used to develop a diagnostic assay or a drug for MS.

P-487

A link between hinge-bending domain motions and the temperature dependence of catalysis in IPMDH

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Enzyme function depends on specific conformational motions. Since conformational flexibility strongly depends on temperature, temperature dependent enzyme kinetic studies with measurements related to dynamics can give us some insight at atomic level into these functionally relevant motions. The catalytic efficiency (k_{cat}/K_M) of 3-isopropylmalate dehydrogenase for its substrate (IPM) has unusual temperature dependence, showing a local minimum at 35°C. In search of an explanation, we measured the individual constants k_{cat} and $K_{M,IPM}$ as a function of temperature, and found that the van't Hoff plot of $K_{M,IPM}$ shows a sigmoid-like transition in the 20-40°C temperature range. By means of various measurements including H-D exchange and FRET, we showed that the conformational fluctuations, including hinge-bending domain motions increase more steeply with temperature above 30°C. The thermodynamic parameters of ligand binding determined by ITC as a function of temperature were found to be strongly correlated to the conformational fluctuations of the enzyme. Because the binding of IPM is associated with a hinge-bending domain closure, the more intense hinge-bending fluctuations at higher temperatures increasingly interfere with IPM binding, thereby abruptly increasing its dissociation constant and leading to the observed unusual temperature dependence of the catalytic efficiency.

P-488

Non-specific component of protein-protein interactions

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Protein-protein interactions (PPI) are the central pillar supporting most of biological functional activity on the molecular level. A binding event between two proteins typically consists of two stages: 1) diffusional search of the binding partners for each other, and 2) specific recognition of the compatible binding surfaces followed by the formation of the complex. We focus here on the non-specific component of PPI, which refers to all physico-chemical properties of the binding partners (such as size, charge, isoelectric point, hydrophilicity etc.) that are independent of the exact details of their binding sites, but which could in turn affect their localization or diffusional search for one another. It is known that proteins co-localize due to segregation into different cellular compartments, sequestration via anchor and scaffold proteins or even chemical modifications. We suggest that the non-specific component of PPI determines in part the co-localization and clustering of the binding partners, which then directly in a non-specific fashion influences their interactions. We examine the possibility that such signature might be encoded within the experimental 3D structures of a large set of known mutually interacting proteins. We provide preliminary evidence that this indeed may be the case, and corroborate our findings by using different statistical tests to compare those features of the known interacting partners, and ascertain correlations and commonalities between them.

Abstracts
– Protein-ligand interactions –

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Dissecting the molecular dynamics of cell surface receptors in immune cells using dual-color FCCS

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Dual-color Fluorescence Cross Correlation Spectroscopy (FCCS) has been used to explore the molecular dynamics at immune cell surfaces, with a particular focus towards the regulation mechanisms of natural killer (NK) lymphocytes.
NK cells are critical mediators of anti-viral immunity and protectors against cancer spread. Their activity is governed by a fine-tuned balance between inhibitory and activating receptors, where Ly49A and KIR receptors represents the inhibitory ones. Their ligands are MHC class I receptors.
FCS is a technique based on the analysis of intensity fluctuations of fluorescent molecules excited by a focused laser beam. The technique offers information about molecular dynamics at the single molecular level, in the nanosecond to millisecond range. Dual color FCCS expands FCS by correlating the intensity from two different colors. By labeling two potential interaction partners with dyes emitting at different wavelengths, the amount of interaction can be determined.
Here, we will report on recent FCCS data exploring the interaction between the inhibitory receptors and their ligands, as well as different labeling strategies used to enable these measurements.

P-490

Dissecting the colicin translocon

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Pore-forming colicin N hijacks *E. coli* outer membrane protein OmpF and exploits it as both a receptor and translocator to cross the outer membrane [1]. It is currently a matter of debate if the translocation route is through the OmpF lumen or the interface between OmpF and the lipid bilayer. Recent electron microscopy data from our laboratory suggests the latter route for translocation [2]. The colicin N/OmpF complex in detergent has been studied by SANS to examine the translocation pathway undertaken by colicin N. By using a combination of deuterated OmpF and hydrogenated colicin we have been able to derive a low resolution structure of individual proteins in the binary complex. Low resolution structural studies supplemented by targeted mutagenesis and screening techniques including ITC, potassium efflux assays and AUC have allowed us further our understanding of colicin N translocation.

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P-491

Functional studies of membrane-bound and purified human Hedgehog receptor Patched expressed in yeast

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The Sonic Hedgehog (Shh) signalling pathway plays an important role both in embryonic development and in adult stem cell function. Inappropriate regulation of this pathway is often due to dysfunction between two membrane receptors Patched (Ptc) and Smoothened (Smo), which lead to birth defects, cancer or neurodegenerative diseases. However, little is known about Ptc, the receptor of the Shh protein, and the way Ptc regulates Smo, the receptor responsible for the transduction of the signal. To develop structure-function studies of these receptors, we expressed human Ptc (hPtc) in the yeast *Saccharomyces cerevisiae*. We demonstrated that hPtc expressed in a yeast membrane fraction is able to interact with its purified ligand Shh, indicating that hPtc is produced in yeast in its native conformational state. Using Surface Plasmon Resonance technology, we showed that fluorinated surfactants preserve the ability of hPtc to interact with its ligand after purification. This is the first report on the heterologous expression and the purification of a native and stable conformation of the human receptor Ptc. This work will allow the scale-up of hPtc production enabling its biochemical characterization, allowing the development of new therapeutic approaches against diseases induced by Shh signalling dysfunction.

P-492

Reaction pathway analysis for (de)methylation process of histone tails

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Histone proteins are fundamental to the cells since they are involved in cell regulatory processes, such as chromatin regulation, gene silencing and transcription, cell cycle control, and epigenetics, which are controlled via post-transcriptional modifications of the histone protein tails. These modifications are categorized under four main groups: methylation, acetylation, ubiquitination, and phosphorylation. Among them methylation has been very recently proven to be reversible with the discovery of histone demethylase proteins and this modification type has been extensively studied, because the abnormal methylation rates cause the excess proliferation of the cell, which, in turn, triggers the cancer. Therefore, understanding of the details of reaction mechanisms of histone methylation/demethylation dynamics provide the required knowledge basis for preventing the abnormal methylation rates by designing proper inhibitor (drug) molecules.
In this study, we display possible reaction mechanisms (such as amine oxidation via LSD1) for the demethylation of specific histone tail proteins. We try to explain the role/importance of residues that take place in or near to the reaction pocket for the demethylation reaction. We also carry out MD simulations and reaction path (free energy profile) analysis using Free Energy Perturbation (FEP) method for QM/MM hybrid systems in order to compare different possible reaction pathways.

Abstracts
– Protein-ligand interactions –

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A new ITC assay for measuring high- and low-affinity protein–ligand interactions

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Isothermal titration calorimetry (ITC) is the gold standard for the quantitative characterisation of protein-ligand and protein-protein interactions [1]. However, reliable determination of the dissociation constant (KD) is typically limited to the range 100 μM > KD >1 nM. Nevertheless, interactions characterised by a higher or lower KD can be assessed indirectly, provided that a suitable competitive ligand is available whose KD falls within the directly accessible window [2]. Unfortunately, the established competitive ITC assay requires that the high-affinity ligand be soluble at high concentrations in aqueous buffer containing only minimal amounts of organic solvent. This poses serious problems when studying protein binding of small-molecule ligands taken from compound libraries dissolved in organic solvents, as is usually the case during screening or drug development.
Here we introduce a new ITC competition assay that overcomes this limitation, thus allowing for a precise thermodynamic description of high- and low-affinity protein-ligand interactions involving poorly water-soluble compounds. We discuss the theoretical background of the approach and demonstrate some practical applications using examples of both high-affinity (KD <1 nM) and low-affinity (KD >100 μM) protein-ligand interactions.

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P-494

Kinetic and structural explanation for the low enantioselectivity of human 3-phosphoglycerate kinase

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L-Nucleosides comprise a new class of antiviral and anticancer agents that are converted to pharmacologically active nucleoside triphosphates *in vivo.* The last step of the cascade may be catalyzed by 3-phosphoglycerate kinase (PGK), an enzyme that has low specificity for nucleoside diphosphate: NDP + 1,3-bisphosphoglycerate ↔ NTP + 3-phosphoglycerate. Here we compare the kinetics of formation of the complexes of human PGK with different D- and their mirror images, L-nucleoside diphosphates, and the effect of 3-phosphoglycerate thereon. Two types of experiment were carried out: equilibrium experiments allow the estimation of dissociation constants, and stopped-flow experiments the transient kinetics of the interactions. In addition, by the rapid-quench-flow technique, we compare the kinetics of the phospho-transfer and product release steps with each D- or L-nucleotide. Crystallographic and molecular modelling studies allow defining the structural reasons for the low enantioselectivity of PGK. The aim of this basic work on the mechanism of action of human PGK with non-natural nucleotides is to obtain information for the optimization of therapeutic nucleoside analogues that are poorly phosphorylated by PGK.
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P-495

Flexibility of human estrogen receptors and stability of estrogen receptor-ERE: salt dependence

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Estrogen receptor (ER) is a well characterized member of the nuclear receptor superfamily that modulates the expression of estrogen-responsive target genes in response to estradiol and other natural and synthetic chemicals mimicking the estradiol structure. In human, two ERs, ERα and ERβ, lied on two distinct chromosomes, are known. ER exhibits several functional domains: two conserved domains, a short domain C involved in DNA-binding and a large domain E/F responsible for ligand-binding and hormone-dependent transcription activation, are linked by a hinge domain D; a poorly conserved A/B domain, at the N terminus, mediating interactions with the general transcription machinery, is involved in hormone-independent transcription activation. Upon estrogen binding, ERs can specifically bind to a DNA fragment, called estrogen response element ERE, and activate the transcription. The optimal ERE sequence consists of two six base-pair half-sites, AGGTCA, organized as inverted repeats with a three base-pair spacing.
In this study, we have investigated, by fluorescence methods, the effect of KCl concentrations, on the protein conformational flexibility and the thermodynamic stability of hERs – EREs complexes. We show, here, that electrostatic interactions, inside hERs, contribute to its conformational flexibility and its thermal stability. Moreover, the specific interaction between hERs and EREs is poorly sensitive to changes in ionic strength, in opposite to unspecific complexes.

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Effect of osmolytes on the DHFR activity, structure and dynamics

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Osmolytes are small molecules accumulated by a wide variety of organisms in response to hyperosmotic stress. They contribute to save the cellular integrity and to stabilize the macromolecules from environmental stress.
Dihydrofolate reductase activity is inhibited by several osmolytes. We studied the impact of osmolytes on the DHFR structure and dynamics by various techniques. We observe that substrate (DHF) and cofactor (NADPH) diffusions are quite different in glycerol and betaine despite similar viscosities. We demonstrate that the overall structure is maintained at high osmolyte concentrations while no direct interactions can be detected with the enzyme. The k_{off} of substrate analogues decreases with increasing the osmolyte concentrations. The enzyme dynamics, in various media, has been compared with the DHFR behaviour in water described in the literature.
The osmolyte impact appears only partly conditioned by its viscogenic properties which reduce the molecules diffusion and the k_{off} of the product controlled by the M20 loop of the DHFR. We suggest that the osmolytes decrease M20 loop mobility. Comparing the results obtained with different osmolytes, we offer a better understanding of the osmolyte nature dependence of the DHFR inhibition.

Abstracts
– Protein-ligand interactions –

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Label free detection using deep-UV laser-based fluorescence lifetime imaging microscopy

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Label free detection based on native fluorescence excited at UV region shows great potential for the life sciences. It offers simple, low-cost and fast method for sensitive detection of important biological analytes without modification. In this contribution we present a deep UV fluorescence lifetime imaging microscopy system (DUV-FLIM) based on a picosecond deep UV laser using time-correlated single-photon counting method. The described setup is well-suited for biological applications for ultrasensitive detection.
We have showed single UV dye (BMQ) and single protein (*Ec*β *Gal*) molecules detection using DUV-FLIM. Further, the label free detection of protein interaction between *Ec*β *Gal* and monoclonal anti-*Ec*β *Gal* has been demonstrated by means of steady-state and time-resolved fluorescence spectroscopy. We achieved detection sensitivity for the *Ec*β *Gal*/anti-*Ec*β *Gal* pair down to the nanomolar concentration range. We also extended this method to study the interaction of therapeutic drug porphyrin with BSA protein.
Fluorescence resonance energy transfer between protein and Alexa Fluor 350 has been investigated using DUV-FLIM. The intrinsic fluorescence and fluorescence lifetime changes of donor biotin β-Galactosidase have been measured. Energy transfer efficiency and donor acceptor distance have been obtained. Fluorescence images of acceptor AF 350 due to FRET have been observed when excited at 266 nm.

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A single amino acid replacement reduces Pln149 antimicrobial property and lytic activity on vesicles

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Pln149 is an antimicrobial peptide produced from *Lactobacillus plantarum* NRIC149. Analog Pln149 (Pln149a) and a Ser-derived (Pln149S, Tyr to Ser replacement) were synthesized on solid phase and their interactions with biomembrane model systems and inhibitory property on *S.aureus* and *P.aeruginosa* were investigated by CD, leakage assays, fluorescence spectroscopy, and differential scanning calorimetry. Both peptides share the same unordered structure-like CD spectrum in aqueous solution, but a helicoidal induction in the presence of negative vesicles were observed, however Pln149S showed lower helical content than Pln149a. The Ser-derived peptide presented 30% decrease of its leakage activity in different liposome compositions, a threefold increase in the dissociation constant from the liposomes than Pln149a, and close to 40% reduction for the inhibitory activity against *P. aeruginosa* growth. We can conclude that besides the electrostatic contacts between the amphipathic &alpha-helix, formed by the cationic residues from Pln149 and negatively charged phospholipids, the Pln149-membrane binding is a process driven for the hydrophobic interactions from non-polar residues. In this case, there was a significant contribution of the Tyr residue, which must be allocated in a lipid interface, described as the preferential position to this residue in membrane proteins. Supported: FAPESP

P-499

Stability and aggregation studies of human septin 3 (SEPT3)

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The septins are a conserved family of nucleotide binding proteins firstly identified in *Saccharomyces cerevisae* as proteins required for the completion of the cell cycle. Septins are implicated in several cellular functions such as cytokinesis, vesicle trafficking, exocytosis, cytoeskeletal dynamics, cell polarity and sperm motility. Furthermore, they are associated with Alzheimer's and Parkinson's disease. SEPT3 was identified in rat brain being highly enriched in presynaptic nerve terminals. It colocalizes with synaptophysin and dynamin I and is associated with synaptic vesicle. In this work, human SEPT3 without its N-terminal domain (SEPT3GC) was expressed in *E. coli* and purified by affinity and size exclusion cromatographies. Structural stability studies were performed with recombinant SEPT3GC using circular dichroism spectroscopy (CD), right-angle light scattering, and fluorescence spectroscopy. The SEPT3GC CD spectrum showed a conformational transition from a predominantly α-helical starting structure to one dominated by β-sheet, just above physiological temperatures. The formation of irreversible aggregates, detected by light scattering, and their ability to bind Thioflavin-T suggested that SEPT3 forms amyloid-like structures, as has been previously observed for human septins 2 and 4. Our data suggest that amyloid formation by isolated septins *in vitro* may be a general phenomenon whose physiological relevance needs to be further investigated.
Supported by FAPESP

P-500

A molecular dynamics study of the CFTR nucleotide binding domains interaction

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The cystic fibrosis transmembrane conductance regulator (CFTR), the dysfunctional protein in cystic fibrosis, contains two transmembrane domains, two nucleotide-binding domains (NBDs), and a regulatory domain. Opening of the pore have been linked to the ATP-driven tight dimerisation of NBD.
We have studied the NBD1-NBD2 interactions on wild type and cystic fibrosis-related mutations by steered molecular dynamics simulations (SMD). A fully solvated dimer, including the two bound ATPs, was separated by pulling one monomer with an external, increasing force. Interestingly, the force needed to break the mutated (G511D) dimer is significantly smaller than in the wild type case. The effect of a CFTR potentiator, the genistein, has also been tested by repeating the SMD simulations with the small molecule docked at the interface between the two NBD domains. To test the validity of our results we have repeated the separation process for different simulation lengths and force strengths. The amount of distortion on the pulled NBD domain has also been studied. This work is partially supported by the Italian Cystic Fibrosis Foundation (Prog FFC #2/2008), with the contribution of "Mille bambini a Via Margutta"onlus"Blunotte", "Lega Italiana FC Toscana"

Abstracts
– Protein-ligand interactions –

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Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations

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Antimicrobial peptides (AMPs) have been intensively studied at micro and macroscopic levels for over twenty years. Knowledge from these two domains has, however, contributed little to a comprehensive understanding of AMP action; rather, in vivo AMP performance has been only remotely correlated to biophysical properties. We focus on the assessment of peptide accumulation on bacterial membranes as an example of this separation: AMP-bilayer interactions have been subject to extensive biophysical characterization, but conversion of that information into educated estimates of in vivo membrane-bound AMP concentrations is lacking. This has led to the overlooking of important factors for activity. Using simple partition models we were able to analyze available information on AMP activity and interaction with membranes to show that unexpectedly high membrane-bound peptide concentrations are likely in vivo and may, in some cases, be required for triggering bacterial death.

P-502

Time-resolved thermodynamics of calmodulin studied by photothermal techniques

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An accurate molecular level description of the signaling mechanism in Ca^{2+} transducers necessitates the knowledge of the kinetics and energetics of conformational changes associated with Ca^{2+} binding to various calcium binding proteins. With this in mind, we have developed an approach that combines the laser-induced photolysis of photolabile "caged" Ca^{2+} compound, DM-nitrophen, with the photothermal beam deflection (PBD) technique to determine thermodynamic profiles associated with the ligand binding to calcium chelator, EDTA, and Ca^{2+} sensor, calmodulin. This approach allows us to monitor time profiles of volume and enthalpy changes on the microsecond to millisecond timescale. The initial PBD study of Ca^{2+} photo-relase from Ca^{2+} loaded DM-nitrophen reveals the presence of two phases. The first step takes place within first 20 μs upon and is associated with a volume decrease of -7 mL mol^{-1} and enthalpy change of 66 kcal mol^{-1}. On the longer timescale ($\tau = 200$ μs), the second event with a positive volume change of 7 mL mol^{-1} and enthalpy change of 8 kcal mol^{-1} was detected. On the other hand, Ca^{2+} photorelease in the presence of calmodulin is accompanied with an additional phase with a distinct lifetime and volume and enthalpy changes that reflects the metal binding to calmodulin and concomitant structural changes.

O-503

Archaeal protoglobin 3D-structure: novel ligand diffusion paths and heme-reactivity modulation

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Protoglobin (Pgb) from *Methanosarcina acetivorans* C2A, a strictly anaerobic methanogenic Archaea, is the latest entry in the hemoglobin superfamily. Our previous crystallographic studies on Pgb have shown that protoglobin-specific loops and a N-terminal extension completely bury the heme within the protein matrix (1). Access of O_2, CO, and NO to the heme is granted by protoglobin-specific apolar tunnels reaching the heme distal site from locations at the B/G and B/E helix interfaces. Here we report structural and kinetic data on Pgb mutants engineered to probe the protein structural and kinetic properties. Six crystal structures (Pgb mutants: Δ20 (missing 20 N-ter residues), Y(B10)61→A, Y(B10)61→W, F(B12)63→W, F(G7)145→W, I(G11)149→F) show that the mutations engineered essentially restrict access to ligand_tunnel_1.

(1) Nardini, et al. (2008). *EMBO Rep* **9**, 157-63.

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Spectroscopic studies of ligand and substrate binding to Indoleamine 2,3-dioxygenase

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The monomeric heme-containing indoleamine 2,3-dioxygenase (IDO) catalyses the oxidative cleavage of the indole moiety of L-tryptophan (L-Trp). Enhanced L-Trp degradation contributes to various physiological disorders including depression or failure of the immuno-regulating system. The regulation of IDO by inhibitors is extensively studied. However, the catalytic mechanism of IDO on the molecular level is still unknown.

In addition to L-Trp, a wide range of substrates are degraded including D-tryptophan, melatonine or tryptamine. In contrast, indole or histidine do not function as substrates for IDO. To understand the determinants for substrate specificity, we investigate the interaction of the heme iron, the heme-bound ligand and the substrate.

We use (time-resolved) UV/visible and Fourier transform infrared (FTIR) spectroscopy over a wide temperature range (4 – 300 K) to monitor the binding of diatomic ligands to the heme iron and the binding of different substrates to CO-ligated IDO. Changes in the spectra upon addition of L-Trp are analyzed and compared to those induced by other substrates or inhibitors.

Abstracts
– Protein-ligand interactions –

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Improving protein-ligand docking by means of enhanced sampling methods

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Docking simulations can be seriously hampered by the great difficulty to accurately estimate the ligand-protein binding affinity constant K, which is usually derived via the computation of the binding free energy ΔG. Unfortunately, due to the involved logarithmic relationship, errors of less than 1.5 kcal/mol in the computation of ΔG result in about one order of magnitude inaccuracy on K. This can hinder computational methods from discriminating micromolar from nanomolar compounds. To improve the reliability of docking prediction, we make use of enhanced sampling methods, ranging from steered molecular dynamics[1] to metadynamics[2]. We also test several descriptors, such as the recently developed path collective variables[3], to identify the most suitable reaction coordinates accounting for binding and unbinding processes. Using these approaches we investigate ligand docking and undocking and we attempt to describe at an atomistic level the kinetics of binding, which we intend to exploit for drug design purposes. Here, an example of application in drug design is reported.

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P-506

Interaction of lipidated N-RasGDP and N-RasGTP with a POPC bilayer investigated in silico

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The GTPase Ras p21 which is linked to the membrane via a lipid anchor, is a crucial switch in the cellular signal transduction processes that control cell growth and proliferation. The interaction of lipidated N-Ras with the membrane is not completely understood. There are NMR structure data about the lipidated membrane anchor of N-Ras and H-Ras and studies about membrane interaction of lipidated H-Ras. In order to identify the interaction domains of N-Ras with the membrane, we performed 40ns- MD simulations of lipidated N-RasGDP and N-RasGTP bound to a POPC membrane. The starting structures base on a homology model of the crystalstructures of H-RasGDP (PDB-ID: 1QRA) and H-RasGTP (4Q21). NMR data from Reuther et al. was used for modelling of the membrane-anchor region. The minimum distance of the G-domain to the membrane is about 11Å in the starting structure. During the simulation it attaches stable to the membrane. The identified interaction domains are the β2-β3 loop (D47), the helix α4 (K128 and K 135) and the helix α5. No differences occur in the interaction domains between the GTP and GDP bound state during the simulations in contrast to the results of Gorfe et al. for H-Ras. The secondary structure of the N-Ras G-Domain is stable during the 40ns-MD simulations and does not differ from the secondary one of MD simulations for N-Ras without membrane.

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Analysis of the interaction between ROS and uncoupling proteins in bilayer membranes and in neurons

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The production of reactive oxygen species (ROS) in mitochondria is very sensitive to the proton motive force and can be decreased by mild uncoupling, mediated e.g. by uncoupling proteins (UCP)[1]. In contrast, the activation of uncoupling proteins by ROS as a negative feedback loop is a highly controversial hypothesis. UCP activation in mitochondria by 4-hydroxy-2-nonenal (HNE, aldehydic product of lipid peroxidation) was first demonstrated by Echtay et al.[2]. Here we investigate the ability of HNE to activate and/or to regulate the expression of UCP in two different systems: (i) in lipid membranes reconstituted with recombinant UCP and (ii) in primary neuronal cells. Total bilayer conductance was enhanced in the presence of HNE, but this effect was independent on UCP1 and UCP2. The results concerning the HNE-mediated UCP expression after induction of ROS-production and/or after exogenous addition of HNE are discussed for three brain-associated proteins (UCP2, UCP4, UCP5) in view of their possible functions.

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P-508

Mimetic model of the fusion active conformation of gp41 on solid supported lipid bilayers

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Retrovirus entry into cells occurs through fusion of the lipid bilayers that surround the virus and the lipid bilayer of the host cell. Fusion proteins, present on the surface of the virus membrane play an essential role in the early stage of virus entry. Understanding of the molecular mechanism is important for the design and function of modern fusion inhibitors. In this project we analyze the fusion of active conformation of the envelope gp41 from the human and simian immunodeficiency viruses (HIV and SIV). During the infection process gp41 undergoes a sequence of conformational changes where the N-terminal NHR develop a trimer pre-hairpin intermediate. Afterwards the three CHR fold back towards the central NHR and a six helix bundle is formed. This rearrangement forces the viral and the host membrane into close contact and fusion pores may induce membrane fusion.

This decisive molecular step in retroviral fusion has been modeled by rational design of lipopeptide assemblies that mimic a coiled-coil structure serving as a receptor for potential antagonists. For this purpose, SSLBs were functionalized in an *in situ* coupling reaction with peptides originating from the NHR (N-peptides) of SIV and HIV to monitor the interactions with the specific CHR peptides (C34 and T20). Binding of antagonists to surface confined coiled-coil structures has been quantified by ellipsometry, quartz crystal microbalance and was visualized by atomic force microscopy.

Abstracts
– Protein-ligand interactions –

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Breathing motions of a respiratory protein revealed by molecular dynamics simulations

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The small size of Myoglobin makes it the preferred candidate to investigate the structure-function paradigm. In its interior five docking sites have been identified and for long time these Xenon cavities have been classified as packing defects. Recently, it was shown that they might be involved in ligands migration path. However, some questions regarding its role as oxygen carrier NO scavenger remain yet open as well as the microscopic mechanism regulating these biological functions. In this work we made use of standard MD simulations of solvated myoglobin to characterize internal cavities. Our principal results is that we have found several secondary cavities showing volume and occurrence at least comparable to that of Xenon cavities.
In order to analyze and rationalize internal cavities we applied special cluster-analysis: we classified all cavities with respect to the position, size and occurrence ascribing them to different clusters. This analysis highlights possible intrinsic migration paths for small ligands within the protein matrix controlled by spontaneous fluctuations of the protein itself. Moreover, we identified some key residues playing a fundamental role in controlling internal pathways. Our suggestion that the secondary cavities constitute the preferred path for ligand escape is also supported by explicit metadynamics simulations of ligand escape.

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Force distribution analysis (FDA) of the signal transduction of *E.Coli* chaperone Hsp90 (HtpG)

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We use a new method that detects force distribution in proteins. Based on molecular dynamic simulations, changes in inter-atomic forces are calculated, here caused by different ligands. These changes will then be analyzed to detect a signal transfer through a protein initiated by the binding of a ligand to the protein.
Chaperones are ubiquitous proteins, which help other proteins to fold into a native conformation. They are able to refold misfolded proteins with a great variety of mechanisms. In this project, our group focuses on molecular dynamic simulations of HtpG, an *E. Coli* homolog of the human Hsp90 (heat shock protein 90kDa). The full structure of HtpG was published by Shiau *et al.* in 2006, but the mechanism of how HtpG performs its function is still not understood.
The folding mechanism is an ATP driven reaction cycle, in which the functional entity is a homodimer of HtpG. We separate the cycle in three main states: *apo*, ADP- and ATP-bound state. Conventional molecular dynamics simulations are used to build a stable simulation system and provide structure trajectories and primary information about the behavior of HtpG in its different states of the folding process. Experiments indicate that the molecular movement is ATP driven. We use force distribution analysis to elucidate how ATP effects HtpG conformation and dynamics.

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Mystery unveiled: benzimidazole docks different domains in Flaviviridae

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The virus encoded RdRp has emerged as a prime target in the search for specific HCV and other Flaviviridae antivirals. recently, the determination of the HCV RdRp structure in complex with certain benzimidazoles has been reported, these NNI's bind to the surface of the thumb domain, thereby disrupting its interaction with the finger domain, which is necessary for catalytic activity. On the other hand, we have found that, in BVDV, the mutations conferring resistance to same class of inhibitors lie in the finger domain of RdRp, indicating that the mode of inhibition of Benzimidazole class of compounds is different in both HCV and BVDV RdRp. Herein, we have applied docking approaches (binding orientation), Molecular Dynamics (MD) simulations combined with metadynamics to elucidate the microscopic mechanism of the interactions between the ligand and the receptor in order to identify features barely seen in experiment. The recently designed algorithm overcomes the time scale problem by accelerating properly defined reaction coordinates. It mimics the real dynamics of a ligand staying or leaving the receptor and in doing so reconstructs the free energy surface, which in turn gives an idea of the residence time of the inhibitor in the cavity.Finally we identified the binding modes and different mechanism of inhibition of Benzimidazole class of compounds in RdRp of two closely related RNA viruses.

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Prediction of protein-protein complex structures using Wang-Landau simulations

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Protein-protein interactions are essential in the majority of life processes, so they have keen interest in several knowledge areas. However, experimental data on protein complexes is being produced at a quite low pace in comparison to that of the individual components. Thus, in recent years attention has focused into computational approaches to the protein-protein docking problem. A variety of docking protocols have been recently reported, sharing usually the following strategy: fast rigid-body search of the interacting subunits, followed by scoring and refinement of the interfaces.
Although this kind of strategy has proven some good results in the CAPRI blind test it has two main limitations. On one hand one should include full flexibility on the protein structures. On the other, the evaluation should be made with free energy calculations instead of using a scoring function.
In this work we propose to search the native complex structure in the minimum of the free energy landscape. We use the coarse grained potential UNRES to get the potential energy of the possible conformations. They are generated changing the dihedral angles, the side chain rotamers, and lastly the mutual orientation using a new sampling protocol we have developed (Rotation-Based Uniform Sampling; RotBUS). Finally, the free energy calculations are performed using an OMP parallelization of the Wang-Landau algorithm.

Abstracts
– Protein-ligand interactions –

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Structural and functional modification induced on DJ-1 by dopaminoquinones (DAQ)

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Parkinson's disease (PD) is a multifactorial neurodegenerative condition characterized by the progressive loss of dopaminergic neurons in the substantia nigra and by the presence of intracellular inclusions, composed predominantly of fibrillar alpha-synuclein (aS). Post-mortem studies indicate the presence of oxidative damage in the nigral neurons. Dopamine oxidation, which leads to the formation of highly reactive quinones (DAQ), may account for the specificity neurodegeneration observed in PD. DAQ have many potential protein targets for chemical modifications. Among them, we focused our attention on DJ-1, of which mutated forms have been found in familial cases of PD. A possible function of DJ-1 is its redox-dependent chaperone activity that could prevent aS aggregation and fibril formation.
In the present work, we analyzed the structural and functional modification induced on DJ-1 by DAQ. ^{15}N-HSQC spectra of DJ-1 were recorded in the presence of different amounts of DAQ and chemical shift perturbations were used to identify the DJ-1 residues target of DAQ and their relative reactivity toward DAQ. Aggregation assays were also performed to evaluate the functional effects of the DAQ modifications on the chaperone activity of DJ-1.

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Pseudo-enzymatic dealkylation of alkyltins by biological dithiols

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Organic compounds of tin are among wide spread environmental pollutants. Due to physical and/or chemical actions, poly-substituted alkyltins speciate into less substituted, more toxic species. Tetra- or tri-alkyltins show a marked delay in their toxic action with respect to the corresponding di- and mono-alkylated analogs. It has been hypothesized that the delayed toxicity may results from the progressive dealkylation of alkyltins in more toxic di- and mono-derivatives, which bind and inhibit essential enzymes. It has been proposed that alkyltins preferentially target enzyme sulphydryl groups. Previously, we showed that a nine amino acid linear peptide (I_1LGC**W**CYLR_9) containing a CXC motif is able to bind and dealkylate tri-substituted alkyltin compounds into the corresponding dialkyl derivatives. Here, we investigated the time dependence of the degradation of the most common alkyltin derivatives by this peptide. We monitored the reaction kinetics using the intrinsic fluorescence of the tryptophan residue in position 5 of the peptide. We found that all of the alkyltins analyzed are progressively degraded to dialkyl derivatives, following a pseudo-enzymatic reaction mechanism. The end-point of the reactions was the formation of a covalent complex between the disubstituted alkyltin and the peptide. These data agree with the speciation profiles proposed for poly-substituted alkyltins in the environment and reveal a possible biotic degradation pathway for these toxic compounds.

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Characterization of different recombinant NRP1 proteins and interactions with heparin

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Neuropilin-1 (NRP1) is a mammalian membrane glycoprotein involved in tip sprouting processes like angiogenesis and neurogenesis. It has been shown that the interaction of NRP1with heparin/heparan sulfate is implicated in its enhancement of growth factor signalling, however the mechanism is not yet known. Commercially available extracellular domain of NRP1 is available either as a truncated HIS-tagged human protein (hNRP1) or as the rat protein fused to HIS-tagged human Fc and expressed as a dimer (FcrNRP1). Biochemical properties such as kinetics of heparin binding and structural requirements for sugar binding together with biochemical tests of protein properties were performed in order to characterise these commercial proteins. Fc rNRP1 shown high affinity to heparin (Kd=2.5 nM) and required a minimum of dp10 to effectively compete with Fc rNRP1 binding to immobilised heparin. Competition experiments with various modified sugars show that interaction of Fc rNRP1 with heparin is highly ionic and dependent on the position of sulfate groups along the heparin chain. The hNRP1 did not bind to heparin immobilised via NHS-biotin, though it did bind to some heparin affinity chromatography matrices.

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Direct kinetic evidence that Lys 215 is involved in the phospho-transfer step of human 3-phosphoglycerate kinase

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3-Phosphoglycerate kinase (PGK) is a promising candidate for the activation of nucleotide analogues used in antiviral and anticancer therapies. PGK is a key enzyme in glycolysis; it catalyses the reversible reaction 1,3-bisphosphoglycerate + ADP ↔ 3-phosphoglycerate + ATP. Here we explored the catalytic role in human PGK of the highly conserved Lys 215 that has been proposed to be essential for PGK function, by a transient and equilibrium kinetic study with the active site mutant K215A. By the stopped-flow method we show that the kinetics of substrate binding and the associated protein isomerization steps are fast and identical for the wild-type PGK and mutant K215A. By the use of a chemical sampling method (rapid-quench-flow) under multi and single turnover conditions and in both directions of the reaction, we show that the rate-limiting step with wild type PGK follows product formation, whereas with the mutant it is the phospho-transfer step itself that is rate limiting. These data are supported by SAXS measurements which showed no direct role of Lys 215 in the domain closure of the enzyme i.e. the isomerisation step of the reaction. The results are explained by the structural data of the enzyme.

Abstracts
– Protein-ligand interactions –

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The effect of toxins on the inorganic phosphate release during the actin filament formation

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Actin can be found in monomeric (G-actin) and filamentous (F-actin) form in eukaryote cells under physiological circumstances. The first step of actin polymerisation is the formation of actin nuclei by ATP-binding actin monomers. The next step in is the elongation when monomers are associated to the previously formed nuclei or to the ends of the growing filaments. After the association of monomers the bound ATP is hydrolysed to ADP.P_i, and with first order kinetics the release of inorganic phosphate occures. The rate constant of the release is 0,006 s^{-1}, which is a slower process than the hydrolysis itself (0,02 s^{-1}).*Phalloidin*, a cyclic peptide from *Amanita phalloides* can bind to the filaments and stabilizes their structure. *Jasplakinolide* is another cyclic peptide from marine sponge (*Jaspis johnstoni*) which binds actin filaments. The aim of this study was to investigate whether the binding of toxins to the newly created filaments has an effect on the kinetics of the inorganic phosphate release or not. We used absorption photometry measurements to measure the rate of phosphate release. *Phalloidin* decreased the rate of the release substantially. Although the effect of *Jasplakinolide* was weaker, the results showed that the binding of these toxins to the actin can modify the rate of the release of inorganic phosphate from the filaments. These observations are in agreement with the molecular mechanisms by which these toxins stabilise the actin filamens.

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Selectivity and interaction of cation and anion modulatory sites in kainate receptors

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Ionotropic glutamate receptors mediate excitatory synaptic transmission in the brain and are heavily implicated in memory and learning as well as in numerous neuropathological conditions. One family of iGluRs, the kainate receptors, show unusual sensitivity to changes in external ion species resulting in an apparent requirement for both sodium and chloride ions for activation. Our recent work revealed the location and selectivity of the cation binding sites. Despite this progress, it is still unclear how the cation binding sites confer sodium selectivity and how apparent affinity for chloride is influenced by the presence of cations. We have attempted to address these questions by performing extensive free energy calculations using all-atom molecular dynamics simulations. The rank order of cation binding obtained from relative binding free energy calculations is in agreement with experimental measurements of apparent affinity. These calculations also reveal that the pair of cation binding sites in the dimer interface act independently. Binding free energy calculations performed using a reduced model of the binding site show that cation selectivity can been attributed to both the rigidity and high charge density of the binding sites. Finally, a potential of mean force derived from umbrella sampling simulations indicate that the presence of cations stabilize the anion binding site considerably.

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Functional protein immobilization on glass-type surfaces

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The immobilization of membrane proteins onto solid supports enables protein interactions and conformational changes to be probed by spectroscopic techniques under highly defined conditions. Here, we present a novel method for covalent protein immobilization on glass-type surfaces using a bottom-up approach. In this approach the 4'-phosphopantetheinyl group of coenzyme A (CoA) was transferred to the acyl carrier protein-derived YbbR tag of the target protein by means of the phosphopantetheinyl transferase Sfp. The glass-support was rendered biocompatible by coating it with an ultrathin layer of PEG (polyethylene glycol), followed by functionalization with CoA through maleimide chemistry. Immobilization of YbbR-tagged proteins in presence of Sfp was followed in real time by label-free detection using reflectance interference spectroscopy. The immobilization procedure was thus systematically optimized by means of binding specificity, enzyme activity and functionality of the immobilized protein. This approach was employed for immobilizing the type I interferon IFNα2 in order to probe ligand recognition by ifnar1 and ifnar2 and ligand-induced ternary complex formation. The versatility of this technique was further enhanced by its combination with photopatterning methods. This immobilization technique can provide a beneficial tool for bioanalytical and biophysical applications at the single molecule level.

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Molecular basis of Rabies viral pathogenicity: interaction viral glycoprotein-neuronal domains

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Rabies Virus (RABV) infects neurons exclusively and causes lethal encephalitis. Pathogenic RABV strains favor neuronal survival, whereas non-pathogen strains lead to neuronal apoptosis. The use of recombinant RABV showed: 1/ the G protein determined the induction of the survival or death phenotypes; 2/ the last 4 COOH amino acids of the G protein cytoplasmic domain (CytoG) are critical. These residues form a binding site for PDZ domain (PDZ-BS). One of the 6 amino acid differences between survival and death G-proteins are located in this PDZ-BS. Results of two-hybrid experiments showed that $\text{CytoG}_{survival}$ interacted PDZ domain of Ser/Thr kinases (MAST), while CytoG_{death} interacted also with 3 additional PDZ containing host proteins. To understand the fine structural basis for the specificity of the PDZ-CytoG complexes, we determined the 3D structure and the dynamics of the MAST-CytoG complexes by NMR. The structures, as well as the affinities and constant kinetics, of the MAST2 PDZ complexes with both CytoG are similar. We conclude that difference by one aa in the PDZ-BS of the two strains cannot drastically modify the interaction with MAST2-PDZ, in agreement with the two-hybrid data. Preliminary results suggest that the interaction of CytoG_{death} with one additional cellular partner blurs the pro-survival signals engaging the infected cells through apoptotic trails.

Abstracts
– Fluorescent proteins –

P-521

CFP modifications induced by reactive oxygen species: photophysical and structural studies

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We aim to understand the basis of the photophysical changes in fluorescent proteins (FP) induced by reactive oxygen species (ROS). Indeed, ROS might be involved in photochemistry of FP, leading to their photobleaching or photoconversion. In addition, FP may be used to investigate cellular events like phagocytosis or mitochondrial activity, where ROS are naturally produced. In the latter cases, an accurate analysis of FP's fluorescence signals requires the full knowledge of reactions between ROS and FP. In the future, this work may help in developing either photoresistant or photoswitchable FP and improving their use for imaging under oxidative stress conditions. Using γ-radiolysis as a quantitative source of ROS, we investigated the reactions of OH and O_2^- radicals on the Cyan Fluorescent Protein (CFP) and the modifications of the CFP's photophysical properties by OH radicals were explored in detail (submitted). In order to address the corresponding chemical changes in the protein, we devised a mild proteolysis protocol that for the first time offers a peptide mass fingerprint almost covering the CFP sequence (Alvarez et al. Biochemistry 2009). Then, we achieved the meticulous characterization of the CFP oxidation products by mass spectrometry and proposed a mechanism to account for their formation by pulse radiolysis.

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Simultaneous Intracellular Chloride and pH measurements using GFP-based sensor in LDCV

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Chloride ion participates in many physiological functions including control of neuronal resting potential, charge balance during endosome acidification, and regulation of cell volume. As a consequence dysfunctions in regulating membrane chloride permeability lead to severe diseases including motor disorders, cystic fibrosis and epilepsy.
At present processes regulating intracellular chloride ion concentrations are still widely unexplored mainly as a consequence of limiting methods to quantify chloride fluxes in living cells. In the present work a highly specific, genetically encoded sensor is developed for detecting simultaneously intracellular pH and chloride concentration. The sensor is obtained by fusion of a red fluorescent protein (DsRed-monomer), insensitive to chloride and pH, to a GFP variant containing a specific chloride-binding site (GFP-Chl). DsRed- GFP-Chl binds the chloride ion following a fluorescence static quenching mechanism, which allows measurements of intracellular pH in a chlorideindependent manner.
The sensor has been successfully tested in different living cells, in a pH range 5-8 and chloride concentration up to 200 mM. For the first time, to the best of our knowledge, it allowed to measure the chloride concentration of dense core vesicles in the secretory pathway. Applicability to high-throughput screening, range of validity and accuracy of time-lapse maps will be discussed.

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Optimizing photoactivatable fluorescent proteins for live-cell imaging

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Recently, novel fluorescent proteins (FPs) have been reported which perform spectral changes in response to irradiation with light of a particular wavelength. Reversibly photoactivatable proteins switch between a bright and a dim fluorescent state. This process is accompanied by photoisomerization of the protein chromophore. Irreversible photoactivation results from photochemical processes within the protein, e.g., photolysis of an amino acid side chain, or an extension of the alternating π-electron system of the chromophore by a β-elimination reaction.
FPs have became valuable tools in live-cell imaging because they allow intracellular protein labeling by using them as fusion tag, and photoactivatable FPs are powerful tools for application in novel subdiffraction imaging techniques. However, the available FPs still offer potential for improvement in various ways. They frequently show a tendency to aggregate or oligomerize, incomplete chromophore maturation, fluctuating emission and low photostability. Here, we will present our recent progress towards engineering the 'perfect' FP.

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Structural basis of photobleaching in a Green Fluorescent Protein

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Fluorescent proteins (FPs) are invaluable fluorescent markers in cell biology. However, their use is often limited by photobleaching of the chromophore, notably in single-molecule, time-resolved or super-resolution imaging studies. We will present the crystallographic studies at near atomic resolution of a photo-activatable fluorescent protein IrisFP that has been observed in a transient radical state *en route* to photobleaching. We took advantage of X-rays to populate the radical, which, under illumination with visible light, presumably forms with low probability from the triplet state. The combined X-ray diffraction and *in crystallo* spectroscopic data (from UV-Vis and Raman spectroscopies) reveal that radical formation in IrisFP involves strong but reversible distortion of the chromophore, suggesting a transient loss of pi-conjugation. These results will help unravel the mechanisms of blinking and photobleaching in FPs, which is of importance to rationally design variants of higher photostability.

Abstracts
– Fluorescent proteins –

O-525

Millisecond photo-switching dynamics of E222Q GFP mutants for sensor and imaging applications

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E222Q mutants of the Green Fluorescent Protein are known to possess photo-chromic properties: the anionic emission, primed by a pump 488 nm laser beam, can be switched between two levels of different brightness by irradiation with a blue, ≅ 400 nm, probe laser light. We have studied here the amplitude and the dynamics of the brightness enhancement of the E222Q mutant of GFPMut2. The fluorescence emission increases almost threefold, under saturating probe laser excitation, for pump excitation intensity in the linear regime. Two characteristic activation times, estimated by means of modulated two colour fluorescence correlation spectroscopy, are detected in the 1-30 ms range, independent of solution temperature and viscosity. The brightness enhancement factor and the characteristic activation times depend markedly on the solution pH. These results indicate that this mutant can be used as a high sensitive intracellular marker for local proton concentration and for modulated excitation imaging.

O-526

GFP-based FRET biosensors for neurodegenerative disease mechanisms

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How misfolding and aggregation of proteins constitutes a toxic insult to neurons remains largely unknown. In order to obtain insight into the molecular biology of neurodegenerative disease, we have developed a number of GFP-based biosensors for the detection and quantification of cellular clearing mechanisms for aggregated proteins. The high load on these protein quality control mechanisms, and their failure to meet normal physiological demand ultimately results in a "de-compensation" condition from which the nerve cell cannot recover. Our FRET/FLIM-based bioassays visualize protein ubiquitination and degradation; proteasomal activity; foldase activity using a folding-impaired GFP mutant which gains fluorescence conditional on the upregulation of chaperone activity; chaperone binding to unfolded proteins; and autophagosome formation/lysosomal integrity via the targeted and sensitive FRET-based measurement of pH changes. These sensors are employed in cellular model systems for Parkinson's and Alzheimer's disease, and Amyotrophic Lateral Sclerosis (ALS) to delineate the molecular pathway of cellular demise, and to gain a mechanistic understanding of the toxicity of protein aggregates and the basis for the vulnerability of neurons.

P-527

Photo-switching mechanism of Enhanced Yellow Fluorescent Protein

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Enhanced Yellow Fluorescent Protein (EYFP) is extensively used as a fluorescent marker. Like other Photo Activatable Fluorescence Proteins (PAFP), it exhibits photo-switching properties. However, the mechanism by which fluorescence can be swiched on or off upon light irradiation is not fully understood at the molecular level. The bright to dark conversion involves a protonation step and structural rearrangements of the chromophore, but it is not clear which of these two steps is the triggering event. To answer this question, we carried out photo-switching experiments at cryo-temperatures. Our data suggest that a photo-induced protonation step (probably in the triplet state) is the primary event in the bright to dark conversion. Our results may bear relevance to other PAFPs, such as Iris, Eos, Dronpa, Padron or Kaede.

O-528

A twice brighter cyan fluorescent protein with monoexponential decay by FLIM screening

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Since the cloning of the green fluorescent protein from *Aequoria victoria*, numerous screens have been performed to improve the brightness of this protein, its spectral variants and fluorescent proteins from other species. The improvement is evaluated by comparing fluorescence intensity of individual bacteria or colonies. In this way also expression level, folding and maturation efficiency, and thickness of the bacterial colony contribute. Here we report a screening method that, in addition to fluorescence intensity, quantifies the excited state lifetime of a fluorescent protein, which is independent of intensity or expression level, and provides a direct measure for the quantum efficiency of the fluorescent protein. The novel approach was used to screen a library of cyan fluorescent protein (CFP) variants randomly mutated at position 65, 148 and 224, yielding an improved bright cyan fluorescent protein named, mPoseidon, with a markedly increased fluorescence lifetime and quantum yield, increased photostability and improved fluorescence intensity *in vivo*. It is shown that mPoseidon is the brightest Cyan Fluorescent Protein in mammalian cells. In addition, several lifetime variants were identified that can be used for lifetime unmixing. It is demonstrated that three CFP variants can be separated and their distribution quantified in a single detection channel.

Abstracts
– Fluorescent proteins –

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Determination of tryptophan rotamers based on Molecular dynamics and Dead-end elimination

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The fluorescent properties of tryptophan residues (W) in proteins are highly dependent on their immediate protein environment. However, the multi exponential decay of single W proteins is not completely understood. The most cited hypothesis contributes a multi exponential decay to the existence of several micro conformations (rotamers) of the W residue within the protein matrix. To determine rotamers we apply a method based on Dead-end elimination (DEE) and molecular dynamics simulations (MD). Low energy rotamers are calculated by DEE while dynamics and further refinement is accomplished using MD. The method was applied on several test cases including the protein mutant *Bc*-Csp L66E from *Bacillus caldolyticus,* which contains a solvent exposed W residue. As resolved by X-ray crystallography, this W residue occupies two conformations. Using DEE and MD we were able to retrieve the W conformations found in the X-ray structure. The results demonstrate the ability of the method to obtain valuable W rotamers, both for solvent shielded as exposed residues. The determined conformations were compatible with the findings based on a method using replica exchange simulations.

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The fluorescence lifetime of Green Fluorescent Protein

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Biotechnological design of the Green Fluorescent Protein (GFP) and the discovery of other proteins boosted the development of the life sciences in the past decade. Tracking protein movements and high resolution microscopy are only a few recent applications which were realized by fluorescent protein technology. Among these examples, the switching between two chromophore state is exploited.
Our aim is to establish fluorescent proteins for bioanalytical fluorescence lifetime measurements. Despite the progress in other fields, quantification with GFP still imposes practical problems [1]. In the past, we observed that the UV-light driven decarboxylation of the nearby aminoacids Glu222 distorts the fluorescence lifetime of GFP [2]. We found out that this chemical reaction also occurs under excitation of the anionic chromophore state with a high quantum yield [3]. Recently, we could show by time-resolved spectroscopy that, indeed, the more susceptible state for this kind of photoconversion is the anionic chromophore state [4]. Suppression of this reaction therefore enables the design of autofluorescent proteins which can be used e.g. for the quantification of ions and which are beneficial as donors in FRET applications.
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[2] G. Jung et al., Biophys. J. 88 (2005), 1932.
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P-531

Intrinsic dynamics in ECFP and Cerulean control fluorescence quantum yield

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Enhanced Cyan Fluorescent Protein (ECFP) and its variant Cerulean are genetically-encoded fluorophores widely used as donors in FRET-based cell imaging experiments. To explain the improvement in the fluorescence properties of Cerulean when compared to ECFP, we have determined the X-ray crystallographic structures of these two proteins at physiological pH, and performed molecular dynamics simulations. Both proteins exhibit a structural heterogeneity in the N-terminal half of their seventh strand, which forms a specific set of van der Waals interactions with the chromophore. The critical H148D mutation present in Cerulean induces a modification of these interactions, and allows the chromophore to be more planar and better packed, albeit only intermittently. As a consequence, the probability of non-radiative decay is significantly decreased. Our results highlight the considerable dynamical flexibility that exists in the vicinity of the tryptophan-based chromophore of these engineered fluorescent proteins, and provide insights which should allow the design of mutants with enhanced optical properties.

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Fixation renders FRET measurements on Cerulean-Venus fusion constructs unreliable

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The advent of fluorescent proteins (FP) gave researchers the opportunity to study proteins *in situ*. Fluorescence resonance energy transfer (FRET) benefited from this.
Cell fixation is a commonly used approach when working with microscopy. However, we have found that FRET efficiency (E) in cells transfected with Cerulean and Venus chimeras could not be reproducibly measured after fixation. To evaluate this problem in detail, we measured E of 3 Cerulean-Venus standard constructs by acceptor photobleaching FRET, intensity-based ratiometric FRET and FLIM-FRET. The constructs were produced as standards (Biophys J, 2006, 91, 99) with 43, 38 and 31% E values, comprising donor-acceptor separations of 5, 17 and 32 amino acids, respectively. Transient transfection of the fusion plasmids was performed into HeLa cells and E was measured in live and PFA or methanol fixed cells.
Literature E values were reproduced when measuring live cells. Conversely, cell fixation caused a deviation of E values. Methanol fixed cells showed E between 1-5% for all the constructs. The effect of PFA fixation on both fluorescence intensity and FRET varied vastly among independent experiments regardless of the measurement modality.
Thus, fixation should be avoided due to the effects it has on FP's fluorescence and consequently on FRET efficiency.

Abstracts
– Fluorescent proteins –

P-533

A combined study of the interaction of outer membrane proteins with cephalosporin antibiotics

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Gram-negative bacteria characteristically are protected by an outer membrane that serves as a selective permeation barrier. Most of the β-lactam antibiotics appear to penetrate the outer membrane through these non-specific channels, and it becomes important to understand the possible interactions between β-lactams and the porin.
Fluorescence techniques have been largely used to characterize both the conformation and the dynamic behavior of large biological structures such as membranes and proteins. The fluorescence of the tryptophan residues is quenched in the presence of the different cephalosporin antibiotics. This reaction between the excited state of the fluorophores and the drug can be described as a formation of a non-fluorescent complex. The dependence of the fluorescence intensity upon quencher concentration for static quenching is proportional to the binding constant for complex formation.
Since β-lactam susceptibility is closely related to the presence of these non-specific porins, *Minimum inhibitory concentration* (MIC) by micro-broth dilution in microplate were used to assess the bactericidal activity of cephalosporin antibiotics upon on *Escherichia coli* strain *BL21(DE3)* and a series of *BL21(DE3)* mutated in different outer membrane proteins.
This combined study of the interactions at single molecular level and at *in vivo* level provides new insights for a better understanding of the antibiotic translocation.

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Photochromic green fluorescent protein mutants: chromophore states unveiled by Raman spectroscopy

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The most widespread genetically-encodable fluorescent markers used for studies in living cells and tissues belong to the green fluorescent protein (GFP) family. Reversibly switchable fluorescent proteins (RSFPs) were developed that can undergo repeated transitions between different states, *e.g. bright* and *dark* forms. This property makes RSFPs particularly attractive as active labels in biological systems for selective photolabeling applications or subdiffraction imaging. We shall present pre-resonant Raman results unveiling the photophysical mechanism underlying the observed photochromic behavior. The variation of spectral properties before and after photoconversion of chemically-synthesized isolated chromophores under different protonation and/or isomerization have been analyzed, and compared to results obtained for the case of complete folded proteins comprising the same chromophores. Experimental results have been analyzed within a time-dependent density functional theory, allowing us to assign all relevant vibrational modes. These results make it possible to discriminate between the effect of *cis-trans* isomerization and of diverse protonation states of the chromophore in the photoproducts of these proteins.
S. Luin *et al*, *J. Am. Chem. Soc.* **131**, 96-103 (2009). R. Bizzarri *et al.*, *Anal. Bioanal. Chem.* **393**, 1107-1122 (2009).

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Structural basis of enhanced photoconversion yield in GFP-like protein Dendra2

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Dendra2 is an engineered, monomeric GFP-like protein that belongs to a sub-class of fluorescent proteins undergoing irreversible photoconversion from a green- to a red-emitting state upon exposure to purple-blue light. This process occurs in the neutral state of the chromophore and is known to result from backbone cleavage accompanied by an extension of the delocalized π-electron system. We have measured the X-ray structure of green Dendra2 and performed a comprehensive characterization of the optical absorption and fluorescence properties of the protein in both its green and red forms. The structure, which is very similar to those reported for the closely related proteins EosFP and Kaede, revealed a local structural change next to the chromophore, involving mainly Arg66 and a water molecule. We propose that this structural change explains the blue shift of the absorption and emission bands, as well as the markedly higher pKs of the hydroxyphenyl moiety of the chromophore. The 20-fold enhancement of the neutral species in Dendra2 at physiological pH accounts for the observed higher photoconversion yield of this protein in comparison to EosFP.

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Red-Shifted Fluorescent Protein Variants of eqFP611

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eqFP611 is a red fluorescent protein (RFP) with the chromophore in a co-planar trans orientation, whereas the cis isomer is preferred by most other RFPs. By using X-ray crystallography, we determined the structures of the dimeric variants d1eqFP611 and d2eqFP611 at high resolution (up to 1.1 Å). For d1eqFP611, we had previously seen a red-shifted species upon irradiation with 532-nm light. Concomitant changes in the Raman spectrum were interpreted as evidence of a trans-cis isomerization of the chromophore. Here we have combined X-ray crystallography and site-directed mutagenesis to assess whether we can create a stable fluorescent, red-shifted eqFP611 variant with a cis chromophore. In a first step, we introduced the N143S substitution. This variant, d2RFP630, is highly fluorescent, with the absorption (emission) maximum red-shifted by 24 (19) nm. With an additional S158C mutation, the chromophore is found completely in the cis form. The variant, RFP639, is highly fluorescent, with excitation and emission maxima at 588 and 639 nm. Still further red shifts appear to be in reach.

Abstracts
– Fluorescent proteins –

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Mechanism and applications of photo- and redox-switchable fluorescent proteins

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Photoswitchable fluorescent proteins have significant advantages over conventional fluorescent labels, and in a revolutionary application, now allow cell biologists to exceed the diffraction limit in light microscopy by a factor of ten. Atomic resolution crystal structures and time resolved spectroscopy of both reversible (mTFP0.7) and irreversible (PA-GFP) photoswitchable fluorescent proteins in the light and dark states explain the long term stability of either state, as well as how illumination at the appropriate wavelength causes the molecules to switch between states. The photoswitching mechanisms will be discussed in terms of photochemistry, light induced chromophore isomerization and excited state proton transfer (ESPT). Mutagenesis of ESPT pathways provide insight into the nature of the rate-determining steps in proton transfer and lead to practical applications, such as redox-sensitive GFP biosensors.

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A spectroscopic approach to the study of chromophoric dissolved organic matter (CDOM) in the sea

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Dissolved organic matter (DOM) in the Ocean represents the largest reservoir of reactive carbon on the Earth. It is produce at each level of the marine food web and it represents the food for heterotrophic bacteria, which can use the different pools of DOM (labile, semi-labile, refractory) with a large range of turn-over times (from minutes to millennia). DOM plays a central role in the global carbon cycle and it has a high ecological significance. Chromophoric DOM (CDOM) is the fraction of DOM capable of adsorbing light at UV and visible spectral regions. It determines the underwater light availability in open and coastal regions with important implication on primary production and biological activity. It is photodegraded to CO_2, CO, with a significant impact on the role of the Ocean as source and/or sink of atmospheric CO_2 and to highly reactive compounds, dangerous for marine organisms. In its pool "humic-like" and "protein-like" fluorophores have been individuated. CDOM optical properties (absorption and fluorescence) together with DOC data collected in some key regions of the Mediterranean Sea will be presented and discussed, in order to (i) investigate the powerful of CDOM optical properties to get information on CDOM "quality" (i) asses the role of DOM in carbon export at depth, (iii) underline the main unresolved question on DOM and CDOM dynamics in the Ocean, with particular emphasis to their biological lability.

O-539

Reversibly switchable fluorescent proteins

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Recently reversibly switchable fluorescent proteins (RSFPs) have become a new branch of the green fluorescent protein (GFP) like family. The RSFPs may be reversibly switched between a fluorescent and a non-fluorescent state by irradiation with light of distinct wavelengths. The key process of this switching behaviour is a light induced change of the chromophore between a *cis*- and a *trans*-isomeric state. The proteins are becoming increasingly important for diverse applications like protein tracking, sub-diffraction resolution microscopy and data storage.
Based on the switching mechanism, we created novel RSFPs with unique and improved characteristics. Padron and bsDronpa are two of these new switchable proteins. Padron features a reversed switching mechanism as compared to all other green RSFPs known to date while bsDronpa exhibits a very broad absorption spectrum extending into the UV. Utilizing the characteristics of both proteins, we performed multi label single detection colour microscopy as well as dual colour sub-diffraction microscopy, the latter with a resolution of 20 nm.
Further, we recently introduced the first monomeric RSFP exhibiting red fluorescence: Using a semi rational mutagenesis approach on the non-switchable mCherry, we generated the switchable monomeric protein rsCherryRev. The use of this protein in single molecule switching microscopy allowed us to record time-lapse live-cell images of the endoplasmic reticulum with sub-diffraction resolution.

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Reversed chromophore protonation explains the large Stokes shift of the fluorescent protein mKeima

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Genetically encoded fluorescent probes such as the green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria*, GFP-related proteins from class anthozoa, and their engineered variants are widely used for *in vivo* imaging of cells and tissues. Recently, Kogure et al. have developed Keima, a red fluorescent that exhibits the largest Stokes shift (180 nm) observed to date. When used in combination with e.g. a cyan fluorescent protein, Keima offers the unique opportunity to perform dual color fluorescence cross-correlation spectroscopy using a single laser line to excite both fluorophores. The molecular determinants of the large Stokes shift of mKeima have been characterized structurally by combining X-ray crystallography with *in crystallo* UV-visible absorption, fluorescence and Raman spectroscopy. Our results reveal a pH-dependant "reverse chromophore protonation" of mKeima, driven by the key residue Asp157. Moreover, the chromophore protonation state is shown to be coupled with different chromophore conformations, *cis* conformation at pH 3.8, and mostly *trans* conformation at pH 8.0. These results will help unravel the mechanisms of fluorescence in FPs, which is of importance to rationally design and develop new fluorescent markers.

Abstracts
– Fluorescent proteins –

O-541

STED nanoscopy in living cells using fluorescent proteins

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Stimulated Emission Depletion (STED) nanoscopy is a light microscopic technique offering a resolution far beyond the diffraction limit: The excitation beam is overlapped with a doughnut-shaped, red-shifted STED beam, which switches off the ability of the molecules to fluoresce in the outer region of the excitation spot. Scanning the nanosized focal spot through the sample renders sub-diffraction images with a sub-second frame rate.
We used the yellow fluorescent protein Citrine to image individual structural elements of living mammalian cells: Vimentin and the tubular network, structures of the cytoskeleton, were recorded with a lateral (x,y) resolution < 50 nm inside the living cell, corresponding to a 4-fold improvement over that of a confocal microscope.
Also, time lapse STED imaging of dendritic spines of YFP-positive hippocampal neurons in organotypic slices outperforms confocal microscopy in revealing important structural details.
As an alternative to fluorescent proteins we used a genetically encoded protein tag which can be labelled with modified organic dyes within living samples.
Thus nanoscale imaging of structures in the interior of living cells greatly expands the scope of light microscopy in cell biology.

Abstracts
– Lipid biophysics –

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Microbicide action and lipid selectivity of defensins: comparison between Psd1 and Hnp1 behaviors

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Defensins are small cysteine-rich cationic proteins or peptides found in both vertebrates and invertebrates. They can be active against bacteria, fungi and many enveloped and non-enveloped viruses; thus, being also classified as antimicrobial peptides (AMPs). In the present work a comparative study between *Ps*d1 (a plant defensin with antifungic properties obtained from the garden pea *Pisum sativum*) and Hnp1 (a human neutrophils defensin) was conducted, in order to shed some light on the mechanism of action at the molecular level of these defensins. Large unillamelar vesicles with different lipid compositions were used for this purpose as biomembranes model systems; namely, POPC/cholesterol (characteristic of the outer leaflet of vertebrates cell membranes) and POPC/ergosterol (fungal) mixtures. Changes on the intrinsic fluorescence of the tryptophan residues present in these peptides upon membrane binding/insertion were followed by fluorescence spectroscopy. Experimental results show the affinity of both defensins for mammalian and fungal sterols. The partitioning behavior of *Ps*d1 showed a high selectivity for ergosterol rich membranes, while Hnp1 has preference for cholesterol rich membranes.

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C and N-truncation of lactoferrampin derived peptides: how does it affect antimicrobial action?

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Lactoferrin is a glycoprotein with two globular lobes, with of two domains each. Since its discovery, the research on antimicrobial properties has been extended to peptides derived from this protein. The largest family studied so far is known as lactoferricin B, obtained from the protein by digestion with pepsin. More recently, a new family of antimicrobial peptides derived from Lactoferrin was discovered by Bolcher *et al* and named Lactoferrampin (LFampin). The original sequence of LFampin contained residues 268-284 from the N1 domain of Lactoferrin. We studied 3 peptides derived from LFampin obtained by extension and/or truncation at the C- or N-terminal sides, keeping the essential characteristics, in order to unravel the main structural features responsible for antimicrobial action. The peptides were tested against model membranes. The ability to adopt helical conformation was followed by CD, the perturbation of the membrane phase transition by DSC, the energetics of interaction by Isothermal Titration Calorimetry (ITC), the partition of the peptide to the membrane by TRFS and the importance of charge effects assessed by Zeta Potential measurements. The results are discussed and compared to the antimicrobial and hemolytic activities obtained by Microbiology techniques.

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From pores to micelles - a peptide-membrane interaction study

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Research in the partitioning of peptides into lipid membranes has been intense for several years. Along with scattering techniques and NMR, isothermal titration calorimetry (ITC) has proven to be a powerful tool for thermodynamic characterization of peptide-membrane interactions. We have studied two antimicrobial peptides, mastoparan-X and melittin, and found that these peptides induce a range of structural transitions of POPC and POPC/POPG membrane systems at different peptide-lipid ratios. We have found that ITC can be used to elucidate the threshold where transitions occur, including the threshold for pore formation and micellation. This has been achieved using a thermodynamic model based on Gouy-Chapman theory, which provides the partitioning constant of the peptide-membrane interaction and thereby the concentration of peptide on the membrane surface. The structural changes have furthermore been visualized by cryo-TEM. We have further investigated the pore formation in detail and found that the thermodynamic parameters of pore formation can be fully characterized using a system specific temperature where the enthalpy of peptide partitioning becomes zero. This allows for an exclusive study of the pore formation process.

O-545

Pulling membrane tubes from solid-supported lipid bilayers with atomic force microscopy

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Information on the elastic and dynamic properties of membranes is essential for a thorough understanding of biological processes such as exocytosis, endocytosis, cell division, and pore formation. We use an atomic force microscope to pull on solid-supported lipid bilayers and observe that an energy barrier must be overcome prior to the formation of membrane tubes. We have modified elastic models of lipid bilayer vesicles to describe the free energy of a planar lipid bilayer in adhesive contact with a surface. From this model we are able to extract force-distance curves for the formation of a membrane tether, including the associated energy barrier. The experimental data can thus be understood in a quantitative fashion. This work enables the atomic force microscope as a quantitative instrument for measuring membrane pulling mechanics, and in future work will allow two-dimensional mapping of elastic properties under tension.

Abstracts
– Lipid biophysics –

P-546

Polarity properties of POPC/cholesterol and egg-sphingomyelin/cholesterol bilayers

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Phosphatidylcholines, sphingomyelins and cholesterol are major constituents of biological plasmatic membranes. As we mix this sterol into pure phospholipid bilayers, there are some important changes in the fluidity and polarity gradients, that depend on the chemical composition as well as on other thermodynamic parameters (e.g. temperature, pressure) [D.Arrais, J.Martins (2007) *Biochim. Biophys. Acta*, 1768:2914-2922]. The variation of one or more of these parameters leads us to significant differences in water permeability, bilayer thickness and molecular packing (reducing the conformational freedom of phospholipid acyl chains and the lipid lateral diffusion).Using the *Py* empirical polarity scale, we have studied the polarity properties of pure POPC and egg-sphingomyelin and of specific compositions in POPC/cholesterol and egg-sphingomyelin/cholesterol mixtures. With increasing cholesterol molar proportions, the polarity values for POPC decrease and we can observe a behavior similar as for homogeneous polar solvents. For egg-sphingomyelin, there is an increase in the polarity values in parallel with augmenting cholesterol concentration and the results show no relation to the thermal behavior of homogeneous solvents. *Acknowledgments*: Dalila Arrais is recipient of a Ph.D. grant (SFRH/BD/41607/2007), from Fundação para a Ciência e a Tecnologia, Portugal.

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ATR-FTIR spectroscopy gives new insights into the lipid membranes in excess water

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The attenuated total reflection Fourier transform infrared *(ATR-FTIR) spectroscopy is ideal for highly absorbing samples* such as water suspensions or even bulk water due to the small light penetration depth. It is also suited for experiments on lipid membranes in excess water. For example, we have studied the hydrogen bonding (H-bonding) between cholesterol (CH) and phosphatidylcholine (PC) or sphingomyelin (SM), which could be important for the stabilization of the cholesterol-rich lipid domains. The ATR-FTIR method enabled *comparison of the carbonyl band shape for PC/CH samples in excess* H_2O *or* D_2O, and has confirmed similar behavior for both [1]. Secondly, we were able to analyze the amide II band for SM/CH samples in excess H_2O. The observations confirm the presence of *H-bonds between CH and N-H group in SM* [2]. Another example is the study of the interlamellar water structure, which could influence the water-mediated phenomena in membranes. *H-bonds in interlamellar water* in partially hydrated lipid multibilayers are stronger with respect to bulk water. In contrast, we show by ATR-FTIR spectroscopy that the H-bonds *are weaker in multibilayers in excess water* [3].

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P-548

Single Mixed-Lipid GUV Method Reveals Interaction of Viper venom with Lipid Membranes

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Studies on the interaction of snake venom and organized lipid interfaces have been conducted using a variety of systems, including bilayer lipid membranes (BLMs), small and large unilamellar vesicles. Giant unilamellar vesicles (GUVs) with a mean diameter of 30 μm have a minimum curvature and mimic cell membranes in this respect. GUVs are ideal for studying lipid/lipid and lipid/protein interactions using microscopy techniques with membrane fluorescence probes.

GUVs were formed from the total lipid fraction from bovine brain by the electroformation method, developed by Angelova and Dimitrov (1987). *Vipera lebetina obtusa* venom was added to the sample chamber before the vesicles were formed. The membrane fluorescence probes, ANS and pyrene, were used to assess the state of the membrane and specifically mark the phospholipid domains.

ANS and pyrene allows us to quantify the fluidity changes in the membrane by measuring of the fluorescence intensity. The presence of viper venom in GUVs media reveals a noticable decreasing of membrane fluidity compare the control, while the binding of fluorophores with GUVs modified by venom lead to appearance of channel activity. It was recognized early that the vipers' venom components preferred an organized lipid substrate near the lipid's phase transition and were particularly active against micellar lipids. These studies also emphasize the importance of a membrane surface curvature for its interaction with enzymatic components of venom.

P-549

Preliminary characterization of atomistic computer models of galactolipid bilayers

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The main lipid component of thylakoid membranes are galactolipids, which constitute more than 75% of its lipid composition. The most common types of galactolipids found in thylakoid are monogalactosyldiacylglycerol(MGDG), whose headgroup consists of a single molecule of beta-D-galactose and digalactosyldiacylglycerol(DGDG), whose headgroup consists of two galactose molecules: alpha-D- galactose and beta-D-galactose linked by O-glycosidic bond Majorty of thylakoid galactolipid have gamma-linolenic acid both in the sn-1 and sn-2 position. Atomistic computer models of MGDG and DGDG molecules have been constructed and parametrized in the OPLS-AA force field. The molecules were used to construct three bilayer systems for molecular dynamics (MD) simulations:(1) composed entirely of DGDG molecules,(2) composed entirely of MGDG molecules,(3) composed of a mixture of DGDG and MGDG molecules the ratio 1:2. The systems have been MD simulated using the GROMACS 3.3.1 package. The generated trajectories were analysed to determine a number of structural parameters among them: membrane thickness, average area per lipid, electron density profile accross the bilayer, order parameters, organisation of the bilayer/water interface as well as several dynamic parametrers like diffusion coefficients, lifetimes of conformational states, lifetimes od lipid lipid interactions.

Abstracts
– Lipid biophysics –

P-550

Leaflet asymmetry and lipid phase transitions in liposomes using fluorescence techniques

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Many studies on phase separations of lipids in bilayers and their leaflet asymmetry make use of fluorescence techniques. We used a dithionite assay, steady-state fluorescence anisotropy and fluorescence resonance energy transfer (FRET) for characterization. Dithionite assay was performed to measure the fluorophore distribution in the inner and outer leaflets of symmetric membranes. For low concentrations, the fluorophore is distributed almost homogeneously, whereas for concentrations > 0.5 mol % it accumulates in the outer leaflet. We discuss these effects taking into account the presence of multilamellar liposomes and dynamic effects produced by higher local bending elasticities. The results point out possible artifacts in the use of fluorophores to characterize bilayers under the assumption of their homogeneous distribution. Dithionite relative bilayer permeability is discussed. The results won by FRET and steady-state fluorescence anisotropy regarding lipid phase transitions are in good coincidence to each other and to results reported in the literature. FRET provides complementary information on liquid/liquid phase separations with inhomogeneous distributions of the different fluorophores used (NBD-PE and Lissamine-rhodamine-PE) and additional phase transitions which have not be reported so far.

O-551

Correlation of the lateral structure of lipid bilayers and monolayers using two photon excitation fluorescence microscopy

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Most of the reported fluorescence microscopy applications on Langmuir lipid films are focused in obtaining fluorescence intensity images using particular fluorescence probes. In this type of experiments the probes are generally utilized to obtain “contrast” between membrane regions (lipid domains) displaying dissimilar physical properties. The obtained information largely depends on the partition of the fluorescent probes for the lipid domains and the obtained fluorescence intensity pictures are not providing any information about the local physical properties of the lipid film. Local physical properties of these distinct regions can be determined by exploring fluorescent probe related parameters such lifetime, emission shift, polarization. However these fluorescent probe’s properties are almost unexplored in this type of experiments. This presentation will focus in describing a new experimental setup that includes a specially designed film balance on top of a custom built multiphoton excitation fluorescence microscope. This setup allows obtaining LAURDAN GP images (1) on the lipid film and monitoring the film’s physical properties at different pressures. These measurements were compared with similar measurement done in planar membrane bilayers allowing establishing interesting correlations among these two model systems.
Bagatolli L.A. 2006. “To see or not to see: lateral organization of biological membranes and fluorescence microscopy” Biochim Biophys Acta 1758:1541-1556

P-552

C-Polycystin-2 and its specific interaction partners

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Polycystin-2, encoded by PKD2, is an integral membrane protein with a size of 110 kDa and 968 amino acids. The protein, which is known to be a Ca^{2+} permeable, non selective cation channel, interacts with several proteins such as Polycystin-1, PIGEA14 or PACS-1/-2 etc. The interaction takes place through a proposed coiled-coil domain of Polycystin-2 located at the C-terminus of the protein.If mutation of either PKD2 or PKD1 (gene product of Polycystin-1) occurs, the interaction between the proteins is disturbed leading to increased formation of renal cysts. This *autosomal polycystic kidney disease* (ADPKD) is one of the most common genetic diseases causing renal failure due to the enormous cyst formation. In this study, the interaction of the C-terminus of Polycystin-2 with its postulated specific interaction partners has been investigated in terms of its biological relevance. Binding affinities as well as kinetic parameters of the interaction were determined. In particular, the interaction of C-Polycystin-1 and PIGEA14 immobilized on solid supported membranes with C-Polycystin-2 has been quantified by means of the *quartz crystal microbalance* (QCM) method. A dissociation constant of about 100 nM was obtained. The results are compared with those obtained by *surface plasmon resonance* (SPR) technique using a different immobilization strategy.

P-553

Effect of helix kink on the activity and selectivity of an antimicrobial peptide

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The bactericidal activity of antimicrobial peptides is linked to their ability to perturb the permeability of bacterial cells. They often show α-helical conformation, and many peptides have a kink in the middle of this structure, caused by Pro or Gly. In order to understand the role of the kink we designed various analogues of P5, in which the central Pro residue was moved from its central position, or removed altogether (in analogue P5F). Displacement of the Pro residue caused a decrease of the antimicrobial activity, and an increase in the toxicity against erythrocytes, with the less active and more toxic peptide being P5F. Fluorescence studies suggest that both P5 and P5F bind on the membrane surface. Fluorescence experiments show that the activities of the two analogues correlate with their affinity for different kinds of lipid bilayers: the kinked P5 peptide has a dramatically higher affinity for negatively charged vesicles (mimicking the composition of bacterial membranes) than for neutral liposomes (similar to mammalian cells), while analogue P5F exhibits comparable affinities for both membranes. Therefore, our data suggest that the central Pro-induced kink is involved in selectivity, inhibiting peptide binding to the membranes of eukaryotic cells.

Abstracts
– Lipid biophysics –

P-554

Different action mechanisms of antimicrobial peptides: fluorescence experiments and MD simulations

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Most antimicrobial peptides exert their activity by perturbing the permeability of bacterial membranes, but the molecular details of this process are still debated. Here, we compare fluorescence experiments and molecular dynamics simulations regarding the interaction of two antimicrobial peptides (PMAP-23 and trichogin GA IV) with lipid membranes, showing that their mechanisms of bilayer perturbation are significantly different.
PMAP-23, a cationic peptide member of the cathelicidin family, associates to the membrane close to its surface and parallel to it, and in this arrangement it causes a severe perturbation to the bilayer, both regarding its surface tension and lipid order. On the other hand, trichogin GA IV, a neutral peptide member of the peptaibol family, undergoes a transition from a surface-bound state to an inserted orientation. In the first arrangement it does not cause any strong membrane perturbation, while in the second orientation it may span the bilayer from one side to the other, despite its relatively short length, by causing a significant thinning of the membrane.

P-555

Perturbation of lipid membranes by HIV-1 Tat

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HIV-1 Tat belongs to the accessory proteins of HIV and has regulatory functions. Tat is concentrated in the nucleus and nucleolus of infected cells. The protein is composed of 86 amino acids with a molecular weight of 11 kDa. Tat is a transcriptional activator protein, which stimulates RNA polymerase II-mediated transcription elongation. Therefore, Tat interacts with Cyclin T1 and binds to the TAR RNA element. Tat has different domains. With respect to the interaction with lipid membranes, the most important structural motif is its basic region, including 6 arginine and 2 lysine residues. The peptide derived from this basic region belongs to the cell-penetrating peptides and is able to translocate through lipid membranes. The major aim of this study is to investigate the influence of full length HIV-1 Tat on artificial lipid membranes. As a starting point solid-supported membranes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) immobilized on silicon dioxide were used. The influence of the lipid head groups on the interaction with Tat was analysed by using membranes composed of the negatively charged lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) in a mixture with POPC. Fluorescently labelled Tat was used to localise the protein in the membrane. The impact of Tat on lipid membranes was investigated by fluorescence and atomic force microscopy, showing that it is capable of perturbing lipid membranes.

P-556

Influence of polyphenol extracts from fruits on biological and model membranes

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Biological activity of polyphenol extracts from apple, strawberry and chokeberry was studied. Polyphenols were shown to be good antioxidants and to act as antyhemolytic agents. Both the activities are the result of polyphenols incorporation into biological membranes. To check potential changes they induce in membranes some experiments were performed with the use of erythrocytes, and lipid membranes.
It was found that the extracts studied induced shape transitions of erythrocytes. They were classified according to the Bessis-Brecher scale. Strawberry extract induced mainly discocytes and discoechinocytes. Populations of discocytes, echinocytes and some discoechinocytes were found on applying apple extract, while chokeberry induced mainly the formation of echinocytes and spheroechinocytes. The results evidence that the polyphenols incorporate into the external monolayer of lipid bilayer of the erythrocyte membrane. The results of the fluorimetric experiments showed that all the extracts changed fluidity in the hydrophilic part of RBC membrane. The changes observed were biggest for chokeberry extract and smallest for strawberry one. Incorporation was also followed by changes in electrical resistance of black lipid membranes and in shifting the temperatures of main transition (T_m) and pretransition (T_p) in liposomes.
This work was sponsored by Ministry of Science and Education, scientific project no. N N305 337034.

P-557

Membrane permeabilization and shape transition: role of asymetry and depth of modification

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The photosensitizing properties of three chlorins are compared in solution and when incorporated in dioleoyl-phosphatidylcholine vesicules. In solution, they possess a similar efficacy to generate singlet oxygen and a similar ability to induce the peroxidation. However, the role of the photosensitizer localization within the lipidic bilayer is strongly highlighted, when chlorins are incorporated in liposomes, both by the changes in order of peroxidation efficacy but by the measurements of the photoinduced permeation of the liposomes. The results are discussed in relation with the technology of photochemical internalization, PCI.
Then, using Giant unilamellar vesicles, we asymmetrically oxidize the membranes. We observed different shape transitions, such as budding, typical of membrane curvature modifications. The asymmetry of the shape transitions are in accordance to a lowered effective spontaneous curvature of the leaflet targeted. This effect is interpreted as a decreased preferred area of the targeted leaflet compared to the other, due to the secondary products of oxidation. Permeabilization of GUV by photooxidation is interpret as the opening of a pore above a critical membrane tension due to the budding of vesicles. The effective spontaneous curvature of photosensitized vesicle at lysis was estimated. Additionally photooxidation was shown to be fusogenic.

Abstracts

– Lipid biophysics –

P-558

Investigating the structure of pores formed by antimicrobial peptides using EPR spectroscopy

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Spin label Electron Paramagnetic Resonance (EPR) is a spectroscopical technique effective to study the molecular mobility of membrane components and the membrane–peptide interactions, as the timescale of EPR is optimally matched to the rotational motions of lipids in membranes.
We applied EPR to study the pore-forming mechanism of two antimicrobial peptides (AMP) that create pores of different dimensions when interacting with liposomes. HP(2-20) is derived from the N-terminus of Helicobacter pylori ribosomal protein L1, and HPA3 is an HP(2-20) analogue where Gln and Asp at positions 17 and 19 were substituted by Trp.
We studied the interaction of the two AMP with zwitterionic and negatively charged vesicles, doped with phospholipids spin labelled in the lipid head or at the C5 or C10 positions; the different phospholipids allow us to study the peptide-membrane interaction at different depths relative to the membrane surface. We studied the interaction of the AMP with vesicles following the influence of AMP on label mobility as a function of temperature and membrane depth.
We also prepared spin-labelled DMPC/DHPC bicelles, doped with lanthanide ions (Dy^{3+}/Tm^{3+}) that allow us to macroscopically orient the system using the magnetic field of the EPR spectrometer. We studied the interaction of AMP with the oriented bicellar system monitoring the effect of AMP on the order parameter of the phase.

P-559

The transformation of vesicle and lateral distribution of mobile membrane inclusions

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Membrane inclusions such as membrane embedded peptides or proteins exhibit curvature dependent interaction with the surrounding lipid matrix due to the mismatch between their intrinsic curvature and the local membrane curvature. This interaction causes an inhomogeneous lateral distribution of the inclusions and a corresponding adjustment of the vesicle shape. By taking into consideration that the membrane free energy includes elastic energy of the lipid bilayer and a contribution due to an inclusion-membrane interaction, the configurations of lipid vesicles with mobile inclusions have been studied theoretically. The variational problem to calculate equilibrium vesicle shapes is solved by applying a Ritz method based on an expansion in spherical harmonics. In general, vesicle shapes adjust to the presence of inclusions by increasing regions with favorable curvature and decreasing regions of unfavorable curvature in such a way that the lateral distribution of inclusions becomes inhomogeneous. If the number of inclusions or the inclusion-membrane interaction exceeds a certain value, the prolate shapes become globally stable.

P-560

Pressure jumps – an accessible trigger for biomolecular transformations

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High pressure can be used to induce many structural changes in biological systems: from triggering phase changes in model membranes to causing protein unfolding, in fact any change involving a volume reduction is promoted by pressure. As well as being broadly applicable, pressure changes can be applied very quickly both up and down in contrast to other structure change triggers such as temperature jumps. Fast pressure jumps allow the trigger to be decoupled from structural changes, so with fast structure probe techniques such as time resolved X-ray diffraction, the out-of-equilibrium evolution of these systems can be monitored. Despite great advantages, high pressure remains under-utilised primarily due to its technical difficulty.
In response to this technology vacuum a high pressure user facility based around a pressure jump cell for small and wide angle X-ray diffraction has been commissioned at beamline I22, Diamond Light Source, UK and will be freely available to the user community. The cell is highly robust requiring virtually no user intervention during an experiment and the pressure system is computer controlled with a graphical user interface and is integrated with the beamline. Pressures between 0 and 0.5 GPa are accessible and jumps can be carried out in approximately 5 ms at temperatures from -10 to 120°C. Sample changing has been made simple and fast with a dedicated sample loading port and modular sample holders allow optimisation for a broad range of samples.

P-561

Temperature dependence of the interaction of lipopolysaccharide with polymyxin

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Lipopolysaccharide (LPS) is the main component of the outer membrane of Gram negative bacteria. LPS is also known as endotoxin because of its potency to induce sepsis, a serious source of mortality in many clinical cases. Among LPS-neutralizing agents, polymyxin B (PxB) is considered as the “gold standard” due to its high efficiency of binding and detoxification of endotoxin. In this work, we have further explored the interaction of PxB to LPS from the rough mutant of *Salmonella minnesota* (Re-LPS) both in the gel and in the liquid crystalline phase, using isothermal titration calorimetry (ITC) and fluorescence based techniques. The effect of PxB binding on LPS-membrane integrity was determined with a fluorescence quenching assay treating vesicles of LPS labeled with NBD-PE with dithionite. Thermodynamic parameters associated with the binding process as well as binding stoichiometry were obtained from ITC experiments. Finally, ITC was conducted with *Enterococcus faecalis* and *Salmonella typhimurium*, as representative examples of a Gram negative and a Gram positive bacterium respectively.

Abstracts
– Lipid biophysics –

P-562

Cholesterol displaces ceramide from its tight packing with sphingomyelin in the absence of ld phase

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A set of biophysical approaches have been applied to study the phase behaviour of palmitoylsphingomyelin (pSM)/cholesterol (Chol) model membranes upon palmitoylceramide (pCer) addition. Fluorescence spectroscopy of di-4-ANEPPDHQ-stained pSM/Chol vesicles reveals no segregation of large liquid-ordered (l_o) microdomains. In contrast, the formation of disperse, compositionally homogeneous l_o pSM/Chol (3:1) nanodomains over a pSM gel (L_β) phase is proposed. DSC measurements show that pCer addition to vesicles with coexisting l_o and L_β phases (low [Chol]) induces the formation of large gel-like pSM/pCer microdomains, coexisting with a l_o pSM/Chol phase. The ΔH for the pSM/pCer phase at high pSM/(Chol+pCer) ratio is close to that of the binary mixture in the absence of Chol, supporting immiscibility, but no displacement, between Chol and pCer-rich phases. On the contrary, both confocal microscopy of GUVs and the DSC data coincide in showing that a rise in pCer increases the gel-like phase to a lower extent than in the pure pSM/pCer mixture, revealing some Chol-induced restriction. In the presence of a pure l_o pSM/Chol phase (high [Chol]), pCer addition is unable to induce the formation of large pSM/pCer microdomains. The present data support the role of Chol as the key determinant in controlling its own displacement from l_o domains by ceramide.

P-563

Inhibition of the surface activity of DPPC films by smooth lipopolysaccharide. Effect of SP-A

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Inhaled bacterial lipopolysaccharide (LPS) may incorporate into the lung surfactant monolayer. In this study, the effect of smooth LPS (s-LPS) on the surface activity of lung surfactant was evaluated. To that end we investigated the behavior of DPPC films containing s-LPS, with and without surfactant protein A (SP-A) in the subphase, using epifluorescence microscopy combined with a surface balance. Our data show that s-LPS injected into the subphase incorporated into DPPC films forming mixed DPPC/s-LPS monolayers. Cospread s-LPS fluidized the DPPC monolayer as demonstrated by epifluorescence images and changes in the compressibility modulus of the monolayer as a function of s-LPS molar fraction (X_{s-LPS}). The presence of low amounts of s-LPS in the monolayer promoted early collapse, preventing high surface pressures to be reached. Moreover, s-LPS hampered the re-spreading of DPPC molecules during dynamic compression at s-LPS concentrations as small as $X_{s-LPS} = 0.02$. Such inhibitory effects could not be relieved by repeated compression-expansion cycles or by adding surfactant protein A. However, SP-A facilitated the squeeze-out of s-LPS from DPPC/s-LPS mixed monolayers, suggesting that SP-A is an s-LPS scavenger.

P-564

Expression and reconstitution of Connexin43 in pore-suspending membranes

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The intercellular communication and electronic coupling between adjacent cells of vertebrates are mediated by gap junctions. These proteins are composed of two connexon hemichannels, whereas each connexon consists of six connexin subunits. Each subunit is characterized by two conserved motives: two extracellular loops and four transmembrane α-helices. In this study, we focused on Cx43. To obtain large amounts of this protein, we expressed Cx43 and Cx43+GFP in a rather new expression system: *Dictyostelium discoideum*. In contrast to human tissue cultures, the system allows for high cell densities up to 30 million cells per mL and the cells can be cultivated by fermentation. Cx43+GFP was successfully visualized in *D. discoideum* by confocal laser scanning microscopy, where it was preferentially found in the plasma membrane.After the cells were harvested, plasma membranes were prepared and both proteins (Cx43 and Cx43+GFP) were verified by western blot analysis. The proteins were solubilized by addition of 5% n-octyl-β-D-glucopyranoside and purified by *ion metal chelate affinity chromatography.* The activities of both proteins were confirmed by a cytochrome C assay. After the purification of both proteins, they were reconstituted in μm-sized pore-suspending membranes. In the near future, we plan to determine the mobility of Cx43 and Cx43+GFP in these membranes by *fluorescence recovery after photobleaching.*

O-565

Organization and dynamics of the serotonin-1A receptor in live cells using fluorescence microscopy

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We addressed the organization and dynamics of the human serotonin$_{1A}$ receptor fused to enhanced yellow fluorescent protein (serotonin$_{1A}$R-EYFP) expressed in CHO cells. Serotonin$_{1A}$ receptors are prototypical members of the G-protein coupled receptor superfamily and represent a prime target for therapeutic actions of several anxiolytic and antidepressant drugs. Interestingly, we observed significant retention in fluorescence of serotonin$_{1A}$ receptors upon Triton X-100 treatment of intact cells at low temperature demonstrating their detergent insolubility. We analyzed the role of cholesterol in the plasma membrane organization of the serotonin$_{1A}$ receptor by fluorescence recovery after photobleaching (FRAP) measurements with varying bleach spot sizes. Our results show that lateral diffusion parameters of serotonin$_{1A}$ receptors are altered in cholesterol-depleted cells in a manner that is consistent with dynamic confinement of serotonin$_{1A}$ receptors in the plasma membrane. Interestingly, results from FRAP measurements performed under conditions of mild cytoskeletal destabilization suggest that receptor signaling is correlated with receptor mobility, in agreement with the 'mobile receptor hypothesis'. Our current work is focused on exploring the oligomerization of the receptor using photobleaching anisotropy measurements and indicates the presence of constitutive oligomers of the serotonin$_{1A}$ receptor in live cells.

Abstracts
– Lipid biophysics –

P-566

Phospholipid membranes dynamics: molecular dynamics vs neutron scattering

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Collective dynamics and single-particle dynamics of hydrated multilamellar phospholipid bilayers (1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine, DMPC) have been studied by means of all-atom molecular dynamics simulations. Here we report results of a gel phase bilayer at 283 K and of a liquid crystal phase bilayer at 303 K. Coherent and incoherent dynamic structure factors and meansquare displacements have been calculated from the trajectories for both the in-plane and out-of-plane lipid dynamics. Moreover, the results have been compared to recent quasi-elastic and inelastic neutron scattering data.

P-567

The antimicrobial peptide, melittin causes membrane micellisation and fusion

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Membrane proteins can be challenging samples to work with and as such often require the use of multiple techniques. Here we present an insight into the structure of the antimicrobial peptide melittin in lipid membranes using various techniques. We explore the conformational changes of membrane-bound melittin and its interaction with model membrane lipid systems with a series of spectroscopic methods that can be used in parallel. The techniques used include linear dichroism and FT–IR for orientation information and circular dichroism for conformation information. We use dynamic light scattering for molecular sizing, fluorescence emission for information on peptide environment, thin-layer chromatography for lipid identification, analytical ultracentrifugation to identify oligomerisation state and calorimetry to investigate the thermodynamics. We observe how the physical properties of both the peptide and the membranes affect the insertion kinetics of the peptide in the membrane.

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Cross-linking of phospholipid membranes by calcium-sensitive synaptotagmins

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Synaptotagmins are vesicular proteins implicated in many membrane trafficking events. They are highly conserved in evolution and the mammalian family contains 16 isoforms. We now show that the tandem C2 domains of several calcium-sensitive synaptotagmin isoforms tested, including Drosophila synaptotagmin, rapidly cross-link phospholipid membranes. In contrast to the tandem structure, individual C2 domains failed to trigger membrane cross-linking in several novel assays. Large-scale liposomal aggregation driven by tandem C2 domains in response to calcium was confirmed by the following techniques: turbidity assay, dynamic light-scattering and both confocal and negative stain electron microscopy. High-resolution cryo-electron microscopy revealed that membrane cross-linking by tandem C2 domains results in a constant distance of approximately 9 nm between the apposed membranes. Our findings show the conserved nature of this important property of synaptotagmin, demonstrate the significance of the tandem C2 domain structure and provide a plausible explanation for the accelerating effect of synaptotagmins on membrane fusion.

Ref: Connell et al. J Mol Biol. 2008, 380(1):42-50

P-569

Hydroquinones modifying lipid membrane morphology

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Quinones are important molecules in cells. Flavina, ubiquinone and coenzyme Q, CoQ, are associated with electron transfer. CoQ, having a hydrophobic tail, is soluble in the internal lipid membrane of mitochondria. Their reduction leads to the equivalent hydroquinones.
We synthesized novel alkylthioquinones ATHs, to investigate their interaction with phospholipids. One or more long hydrophobic chains attached to the quinone ring alter their hydrophobicity and electron availability.
We aimed at their influence on the structure of membranes. Different pHs highlights the charge/polarity effect on the interaction and on the morphology of the bilayer. For that, POPE and POPC, lipids with different charges, but identical chains were selected. X-rays diffraction on POPE/2,6BATH, 100/1 at different temperatures and pH, showed cubic phases coexisting with the usual phases formed by simply hydrated POPE, at different pHs.
For investigation of the charge/polarity, we turned to the POPC/2,6BATH system (smaller charge/polarity). Despite decrease in temperature of phase transition and dimensions of the lattice, the structures were the same as in hydrated lipid, illustrating a less significant effect than on the similar lipid POPE.
Another additive, 2,5ATH, mixed with POPE showed the effect of multiple thioalky chains. No morphology change was seen, compared to pure lipid.
Interestingly, despite both additives differ by one thioalkyl chain only, their influence on the POPE matrix is so different.

Abstracts
– Lipid biophysics –

P-570

Deformation of phospholipid vesicles in high magnetic fields studied by 31P solid-state NMR

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Model membranes with the capacity to align in magnetic fields such as bicelles and magnetically sensitive vesicles are of high interest, since their macroscopically alignment allows for the determination of structure, dynamics and topology of molecules within the membrane [1]. We performed ^{31}P solid state NMR on a magnetically sensitive lipid possessing a large positive magnetic anisotropy introduced in form of a biphenyl unit during lipid synthesis in one of its acyl chains (1-tetradecanoyl-2-(4-(4biphenyl)butanoyl)-*sn*-glycero-3-phosphocholine (TBBPC)). The phosphorus lineshapes of TBBPC MLVs studied at various magnetic fields (7.1T, 9.4T, 11.7T, 16.4T), revealed a drastic change in shape upon exposure to fields > 9.4 Tesla: resonances resulting from phospholipid molecules oriented perpendicular to the magnetic field decrease whereas resonances resulting from parallel oriented molecules increase.
This is a sign for magnetically induced vesicle deformation from vesicle to oblate ellipsoid shape. Factors influencing this drastic deformation, such as magnetic anisotropy, membrane elasticity, lipid chain length and field dependency are discussed based on existing theories [2].
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[2] Helfrich (1973) Chem. Phys. Lett. A43, 409-410.

P-571

Does the lysosomal membrane need triglycerides? A spectroscopic study of a simple model membrane

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Lysosomes are intracellular organelles in which proteins and other macromolecules are degraded. Morphological and functional changes in different compartments of the endocytic pathway are connected to several diseases. A crucial step in understanding biogenesis of lysosomes and their role in disease conditions, is to characterise the properties of the lysosomal membrane. By the use of TLC we have found that lysosomes contain non-neglible amounts of triglycerides (TG). To investigate how the presence of TGs could influence the lysosomal membrane, we have investigated the properties of a mixture of POPC and triolein, as a simple model for the lysosomal membrane. We found the system to form two types of POPC-rich membranes. These were determined as co-existing phases based on their spontaneous and stable separation and named heavy and light phase according to their sedimentation behaviour. By using EPR and fluorescence spectroscopy the physical properties, including order, fluidity and water penetration, of these phases were found to differ markedly despite of their almost identical composition. The results suggest that presence of TGs on lysosomal membranes could have a crucial effect in the barrier functions and thus, the integrity of the organelle.

P-572

Acyl conjugation is a method for increasing membrane selectivity of antimicrobial peptides

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Antimicrobial peptides (AMPs) have received increasing interest as the search for new potential antibiotics has become imperative due to increasing bacterial multiresistance. Acylating the natural occurring Polymyxin B (PMB) significantly enhances antibacterial activity towards two representative Gram-negative bacteria E. coli and K. pneumonia compared to the nonacylated PMB. The aim of the presented work was to study various biophysical parameters, such as partitioning coefficients, zeta potentials and effective charges of a selected AMP, mastoparan-X (MPX) and a propanoylated (PAMPX) and octanoylated (OAMPX) analogue, respectively. Fluorescence spectroscopy, isothermal titration calorimetry and ζ-potential measurements were the main techniques used for the measurements. We employed 2 different LUV model systems; a partially charged (POPG/POPC 1:3) and a neutral system (POPC). For the neutral LUVs there was an increase in partitioning with increasing length of the acyl chain, whereas partioning into the partially charged LUVs was governed by a balance of hydrophobic and electrostatic contributions. The selectivity for the partially charged LUVs over the neutral LUVs was in the order PAMPX, MPX and OAMPX. The modeled effective charge for the peptide followed the same trend as the partitioning coefficient for the three peptides for the respective LUV systems.

P-573

Manipulation of liposomes by laser tweezers: a new way to study vesicle docking and fusion

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Optical tweezers allow trapping of particles of different types in a wide range of sizes [1]. Among these, unilamellar vesicles are of interest as they are known to be effective vectors in drug delivery and they are also studied as models for cell membranes.
This work focuses on the interactions between optically confined unilamellar vesicles and their cross-linking by proteins [2]. Synaptotagmins are vesicular proteins implicated in many membrane trafficking events having an accelerating effect on the membrane fusion. Calcium-sensitive synaptotagmins are thought to confer calcium sensitivity to the fusion of secretory vesicles with target membranes [3].
The novel optical assay reported here allowed us to visualize the cross-linking of 600 nm liposomes mediated by synaptotagmin and calcium. The use of the optical tweezers approach to investigate the function of other fusogenic proteins related to exocytosis (i.e., SNARE proteins) is discussed as well.
References:
1. Ashkin, A. *Proc Natl Acad Sci USA* **94**, 4853-4860 (1997)
2. Connel, E. et al. *J Mol Biol* **380**, 42-50 (2008)
3. Jahn, R. et al. *Nat Rev Mol Cell Biol* **7**, 631-43 (2006)

Abstracts
– Lipid biophysics –

P-574

Polylysine adsorption at charged surfaces of lipid membranes and mica

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Adsorption of monolysine and polylysines at the surface of lipid membranes of varied composition was studied by two methods. Both methods - the electrokinetic one, measured the surface potential of liposomes, and the Intramembranouse Field Compensation sensitive to boundary potential of planar bilayer lipid membranes - detected the positive changes of the potentials for membranes with negatively charged component (cardiolipin, CL). Neither monolysine, nor polylysines adsorbed at neutral membranes (phosphatidylcholine, PC). Pentalysine show the difference between these potentials for the membranes composed from CL/PC mixtures. This difference is attributed to dipole part of boundary potential and indicates the changes in lipid packing. Polylysines show high affinity to the membrane and saturation with plateau. These saturation levels correspond to surface charge densities 0.005 and 0.016 C/m^2 for oligomers with 5 and 12 units, and 0.032 C/m^2 for polymers with 130 and 1435 units. These values do not depend on the ionic strength of background electrolyte but proportional to the content of negatively charged components in the lipid bilayer. The polylysine layer at the mica surface was studied by atomic-force microscope (AFM) technique. It was shown that pentalysine molecules cover the surface by the layer of 0.8 nm thickness and polylysines of high molecular weight by the layer up to 4 nm.

P-575

Lipid bilayer interactions of pHLIP, the third transmembrane domain of bacteriorhodopsin

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The third transmembrane helix of bacteriorhodopsin, also known as pHLIP, is a unique model system for studying the interactions of a natural transmembrane domain with lipid membranes: depending on pH, the water-soluble peptide either adsorbs superficially or inserts as a transmembrane helix on addition of lipid vesicles [1]. Published values for the free energies of these processes were based on a stoichiometric model invoking two distinct sets of binding sites [2]. However, discrepancies between data obtained from different experimental techniques and inconsistencies between experimental and expected temperature dependencies suggest that these values should be taken with caution.
We therefore reassessed membrane interactions of pHLIP using titration calorimetry and fluorescence spectroscopy. If electrostatic effects at the membrane surface are taken into account, the data can be described quantitatively by a partition equilibrium, but not by a stoichiometric binding model. The thermodynamics of membrane partitioning differ substantially from those determined previously [2] and draw a different picture of peptide-lipid interactions. Beyond deepening our insights into the first step of the two-stage model of membrane protein folding, this also sheds light on the ability of pHLIP to drag cargo molecules across lipid membranes.
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P-576

Effects of amphotericin on phospholipid and phospholipid-sterol membrane structure

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Small angle neutron scattering (SANS) studies have been performed to study the structural changes induced in membranes of vesicles prepared from phospholipid and mixed phospholipid-sterol mixtures, in the presence of different concentrations of the anti-fungal drug, amphotericin B (AmB).The vesicles, sonicated to a mean size∼100nm, were prepared using dimyristoylphosphatidylcholine(DMPC) or DMPC-cholesterol or DMPC-ergosterol mixtures –with both of the mixed systems involving 30mol% sterol. Analysis of the SANS data show that when the concentration of AmB added is just above the drug's cmc (∼1μM) there is an increase in the membrane thickness of both the DMPC-chol and DMPC-erg vesicles (both cases + 4Å), but the thickness of the pure DMPC vesicle membranes remains the same as in the absence of AmB. When AmB is added at a concentration in excess of its cmc (∼10μM), the mixed-sterol vesicles show the same changes in membrane thickness as observed with the lower AmB concentration, and the pure DMPC vesicles again remain unaffected. On the basis of these studies, therefore, there appears to be no difference in the structural changes induced by the insertion of AmB into the model fungal cell membranes (mimicked by DMPC-erg vesicles and those resulting from its insertion into the model mammalian cell membranes (mimicked by DMPC-chol vesicles).

P-577

Changes in lipid phase induced by synthetic peptides from E1 structural protein of HGV-C virus

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Hepatitis G virus (GBV-C/HGV) is an enveloped viruses belonging to the Flaviviridae family. Clinical findings have suggested that in people co-infected with GBV-C/HGV and human immunodeficiency virus (HIV), delayed progression of AIDS has been observed (1). The mechanism by which this virus may inhibit the progression of AIDS remain to be elucidated. Enveloped viruses acquire their lipid membranes by budding through host cellular membranes (2), in this process; fusion peptides play an important role. Study of the interaction of HGV-C peptides with lipid membranes could lead us useful information about the mechanism that takes place. In this work we present a study on the effects of E1 peptides on the fluidity of and polarizability of model membranes (DMPC and DPPC liposomes) by DSC and fluorescence polarization. Both techniques showed that the presence of the studied sequences in phospholipid mixtures already affected the thermotropic properties of the gel to liquid-crystalline phase transition. Systems were thermodynamically characterized by ITC. (This work was supported from Ministerio de Ciencia y Innovación. Project number: CTQ2006-15396-C02-02/BQU). (1)Tillmann, H. L. *N. Engl. J. Med.* 2001, *345*, 715-724; (2) Moraes, M.L. et al. Colloid and Surfaces B, 2005, 41, 15 – 20

Abstracts
– Lipid biophysics –

P-578

Effect of UVB radiation and organometallic compounds on biomolecules of membranes

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Our earlier studies showed that organometallic compounds (OTC) in the presence of UVC radiation enhanced the degree of phosphatidylcholine (PC) liposome oxidation, whereas quercetin effectively protected the membrane. The present investigation is concerned with the effect of UVB and OTC (chlorides of diphenyl- and dibutyltin, triphenyl- and tributyltin), both in combined action and separately, on oxidation of erythrocyte proteins, PC liposome and albumin. The degree of oxidation of proteins and liposomes induced by OTC and radiation, also in the presence of selected antioxidants (trolox, quercetin) was determined on the basis of changes in the number of sulfhydryl groups, carbonyl groups and malone dialdehyde, respectively. The studies indicate that UVB induces PC liposome and erythrocyte oxidation, whereas in the case of albumin it causes both an increase in the number of C=O groups and free SH groups (most probably, braking sulphonic bridges). OTC compounds interact with membrane biomolecules both as weak pro- and antioxidants, also in combined action (UVB plus OTC). The prooxidative effects are markedly diminished by application of antioxidants. The action of quercetin results from its ability to incorporate into membranes and formation of complexes with OTC (liposome>erythrocyte>albumine). This work was supported by grant N N3364 34.

P-579

Waves on lipid monolayers

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"For the sake of illustration we shall try to provide a physical basis for the equations, but must emphasize that the interpretation given is unlikely to provide a correct picture of the membrane." [Hodgkin&Huxley, 1952]

To explain the occurrence of reversible heat production during nerve pulse propagation it has been suggested by multiple authors [Wilke,Kaufmann,Heimburg] that travelling sound waves and *not* ion channels may offer a better explanation than the Hodgkin&Huxley theory. Therefore, if sound waves are an essential feature of the nerve membrane they should also appear on lipid monolayers where ion conductivity is evidently absent.

Here we demonstrate that sound waves can be excited on a lipid monolayer by using a set of planar electrodes incorporated into the monolayer and driven by an alternating voltage. Not only do our results indicate propagating waves on lipid monolayers in accordance with their thermodynamic predictions, but importantly, no significant attenuation is detected proposing an adiabatic phenomenon. In order to provide evidence that our physical explanation provides a *correct picture* of the membrane, direct detection of the waves was done, whereas a clear transduction of signals was shown. Finally, the impact of toxins, neuropharmaca and anaesthetics needs to be integrated in our physical picture of the nerve membranes, what can easily be done now and could deliver a new approach for understanding their physical mechanism.

P-580

Automated planar patch clamp: Multiple applications for miniaturized devices

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Patch clamp electrophysiology remains the gold-standard for ion channel research because of the information richness of the data produced. Automated planar patch clamp devices, with their higher throughput and high data information content, have made the technique accessible to a wider audience. Nanion Technologies offers two planar patch clamp workstations combining higher throughput with high data quality. The Port-a-Patch records from a single cell at a time and the Patchliner from up to 8 cells simultaneously with high success rates (typically 60-80%).

Both, the Port-a-Patch and the Patchliner are bench top patch clamp rigs which uses a planar borosilicate glass chip for obtaining a giga-seal for electrophysiological recordings. Suction applied from the underside of the chip is used to attract a single cell to the recording site without the need for optical visualization. Both workstations have been successfully used for whole cell, perforated patch, and cell attached recordings as well as for GUV-bilayer recordings. Due to the versatility of Nanion`s products it is possible to study a wide variety of ion channels including NaV, KV, CaV, ligand-gated, hERG or reconstituted proteins such as KscA, Cx26, OmpC. Special features unique to Nanion products, including but not limited to internal solution exchange and temperature control, expand the experimental possibilities.

O-581

Effects of lysolipids on the mechanical stability of lipid membranes

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Lysolipids (LPCs) and fatty acids (FAs) are the natural products formed by the hydrolysis of phospholipids. LPCs and FAs form micelles in solution and thus act as detergents in the presence of lipid membranes. In the present study we investigate the detergent strength of a homologous series of lysolipids (LPCs) on POPC lipid membranes by use of Isothermal Titration Calorimetry (ITC) and Vesicle Fluctuation Analysis (VFA). The membrane partition coefficient (K) and critical micelle concentration (CMC) are determined and found to obey an inverse proportionality relation (CMC x K $\sim$ constant). The partition coefficient and critical micelle concentration are used for the analysis of LPC's effect on the membrane bending rigidity. The dependence of the bending rigidity on the LPC membrane coverage has been analyzed in terms a phenomenological model based on continuum elastic theory which yield information about the curvature inducing properties of the LPC molecule. The results reveal: (I) an increase in the partition coefficient with LPC acyl-chain length and (II) the degree in acyl chain mismatch between LPC and POPC determines the magnitude of the membrane mechanical perturbation per LPC molecule on the membrane.

Abstracts
– Lipid biophysics –

P-582

The kinetics of membrane-peptide folding and orientation

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Understanding interactions of peptides with lipid membranes is essential if we are to be able to design new and better antibiotic peptides. There are different steps in these interactions and following the kinetics of these processes requires that we combine the information from different biophysical techniques. Here we present data on the kinetics of peptide-membrane interactions using circular dichroism to report on conformation and linear dichroism to report on orientation of the peptides in/on membranes. These are combined with other techniques such as dynamic light scattering and fluorescence spectroscopy to elucidate the mechanisms of action of the peptides. One of the most important aspects of membrane-active peptide design is that of specificity. We investigate specificity for different types of membranes by using libraries of lipids with different properties of charge, chain saturation and curvature stress. In this way one can test for effects of e.g. negatively charged head groups found in bacterial membranes or cholesterol found in animal and human membranes. Using these approaches it is proposed that we will be able to modify peptide sequences, test what part of the kinetic processes are affected and subsequently use this information to design new and better antibiotics.

P-583

Membrane fusion assay based on pore-suspending lipid bilayers

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Membrane fusion has attracted significant interest due to its biological relevance. Thus in recent years a great variety of fusion assays has been developed. Since the process of membrane fusion is not yet fully understood, our aim is to develop a new fusion assay based on pore-spanning membranes, which have been proven mechanically stable, highly insulating and rather tension free. The application of these so-called micro-BLMs should allow simultaneous monitoring of lipid mixing, content release as well as electrical readouts. Furthermore both membrane sides can be addressed individually to apply transmembrane potential or fusion modulating compounds. First results show that the micro-BLMs provide the opportunity to investigate lipid bilayer fusion by means of fluorescence microscopy. The formation of pore-spanning membranes is achieved by the painting technique of DPhPC doped with Oregon Green DHPE dissolved in n-decane. The addition of large unilamellar vesicles doped with Texas Red DHPE allows direct observation of single fusion events. Lipid mixing during fusion leads to a decrease of the donor fluorescence (Oregon Green) and an increase in acceptor fluorescence intensity (Texas Red) in the plane of the planar bilayer due to FRET. In future work simultaneous monitoring of lipid-mixing and content release combined with electrical measurements are planned to gain further insight into different intermediate steps of membrane fusion.

P-584

Highly irregular patterns of cholesterol incorporation into lipid bilayers

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Cholesterol incorporation into lipid bilayers, in the form of multilamellar vesicles or extruded large unilamellar vesicles, has been quantitated. To this aim, the cholesterol contents of bilayers prepared from phospholipid:cholesterol mixtures (33-75 mol% cholesterol) have been measured and compared with the original mixture before lipid hydration. There is a great diversity of cases, but under most conditions the actual cholesterol proportion present in the bilayers is much lower than expected. The maximum solubility of Chol in bilayers containing saturated PC or PC with less than four unsaturations is 50-60mol%, while in polyunsaturated PC, e. g. diC18:4, and in sphingomyelin the maximum Chol contents is 23mol%. A quantitative analysis of the vesicles is thus required before any experimental study is undertaken.

O-585

Imaging phospholipase C/ sphingomyelinase activity in vesicles containing coexisting ordered-disorderd and gel-fluid domains

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A phospholipase C/ sphingomyelinase from *Pseudomonas aeruginosa* has been assayed on giant unilamellar vesicles (GUV) consisting of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and cholesterol, at equimolar ratios. The enzyme activity modifies the chemical composition, thus the physical properties of the bilayer, and conversely the latter influence the enzyme activity. Biochemical assays of enzyme activity, together with confocal fluorescence microscopy examination of GUV provide novel information about the system. The original lipid composition in the absence of enzyme gives rise to lateral phase separation of liquid-ordered and liquid-disordered domains in the GUV. The two enzyme end-products, diacylglycerol and ceramide, have opposite effects on the bilayer physical properties, the former abolishes lateral phase separation, while the latter generates a new gel phase. Morphological examination of individual GUV shows that the enzyme binds preferentially the more fluid (or more disordered) domains, and that, in most cases, it causes the fluidification (disordering) of the other domains..

Abstracts
– Lipid biophysics –

O-586

Structural studies of supramolecular assemblies based on new dissymetrical bolaamphiphile molecules

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In this paper we present a study of a new family of bolaamphiphiles. These amphiphiles are unsymmetrical bolalipids having one sugar polar head and the second glycine betaine polar head. They are potentially useful for pharmaceutical or cosmetics applications as vectors for drugs. Therefore it is important to investigate their self-assembled properties. The chemical variations that we introduced in this new family concern the length of the main chain that connects two polar heads as well as the length of the side chain placed on the position 1 of the sugar moiety. Another variation concerns the introduction of a diacetylenic unit into the main chain in order to rigidify it. We have performed the SAXS (Small Angle X-ray Scattering) measurements on the dehydrated compounds as a function of temperature and observed the lamellar liquid crystalline structures. We also measured the SAXS spectra of aqueous solutions of these compounds that have shown lamellar L_α structure in all cases. These measurements are compared with polarised optical microscopy measurements that confirmed our interpretation.

P-587

The interplay between detergent cohesion and peptide adsorption on the structure and dynamics of a Glycophorin A TM-detergent complex

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In spite of the major interest of membrane proteins at functional, genomic and therapeutic scales, their biochemical and structural study remain challenging as they require, solubilization in detergent micelles. The complexity of this task arises from the structural dependence of membrane proteins on their anisotropic environment and in particular by a delicate balance between different physico-chemical properties. In order to study these properties in a small protein detergent complex, we have used molecular dynamics simulations on the transmembrane part of Glycophorin A (GpAtm) solubilized in micelles of the detergent di-hexanoyl-phosphatidylcholine(D6PC). We show that the molecular aggregates organizes to give distinct populations of detergent molecules. Those molecules which loosely interact with the peptide are preferentially involved in highly cohesive inter-detergent interactions that impose a global bilayer structure to the micelle. Interaction profiles of other detergents with GpAtm depend upon the nature of residues along the surface of the peptide. This topology dependence leads to different modes and strength of interactions that ultimately constrain the orientation of the micelles around the peptide. This simple model illustrates how differential detergent selectivity for faces and strong constraints coming from purely environmental features could influence transmembrane helix packing, membrane protein structure and assembly.

P-588

Budding of giant phospholipid vesicles induced by β2-glycoprotein I

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β_2-glycoprotein I (β_2GPI) is classified among amphitropic proteins: in its inactive form it is dissolved in plasma and in its active form it is bound to membrane. In comparison to other amphitropic proteins it exhibits a distinct membrane interaction behavior. A patch of positively charged amino acid residues contacts anionic phospholipids via electrostatic interactions, and a hydrophobic loop anchores the protein into the outer leaflet of the membrane via hydrophobic interactions. The binding constant depends on physical properties of the membrane such as membrane lipid composition, surface potential, lipid packing density and curvature. The binding of an amphitropic protein alters the membrane's spontaneous curvature as well as the difference between the equilibrium areas of membrane leaflets. Theoretical model has been developed to predict vesicles shape transformations realized by the formation of buds. It was shown that the effects are stronger for flaccid vesicles which were therefore chosen for our experimental investigations. Vesicles were composed of 20 % POPS and 80 % POPC, and flaccidity was achieved by an osmotic adjustment. The solution containing β_2GPI was subsequently injected to a chamber with flaccid vesicles by a syringe pump. Observation took place under a phase-contrast microscope. Experiments showed a concentration dependent occurrence of buds. Their number and size were related to the degree of vesicle flaccidness.

P-589

Influence of lipid oxidation on membrane electroporation

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Reversible membrane electroporation, that can be observed as a effect of electric field influence on biological membranes, finds application in cell delivery of biologically active compounds. The membrane susceptibility for creation of stable pores, depends on electric field parameters applied to the membrane as well as on the membrane environment (ionic strength, pH, temperature) and membrane molecular composition. We have already shown that the changes of van der Waals lipid chain interaction effect the process of pore formation. There are known factors which increase or decrease the membrane respond to electric field. Because pores appear in lipid membrane matrix it make sense to study this phenomenon using model planar lipid membranes. As lipids are very receptive on oxidizing factors we decided to study the relation between lipid oxidation and membrane reaction on electric field. With the use of four electrode galvanostat we are examining the influence of factors initiating lipid oxidation (UV, Fe^{2+}) and the efficiency of some antioxidant compounds of lipid protection on electric properties of lipid membrane. The product of lipid oxidation is determined by characteristic reaction with tiobarbituric acid. We want to determine the role of polyunsaturated fatty acids on membrane properties also. Results of our work will be useful in optimization of medicine distribution, aided by electric field, into cells.

Abstracts
– Lipid biophysics –

P-590

Cell Penetrating Peptide interactions with membranes on nanoporous supports

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Cell penetrating peptides (CPP) have been shown to penetrate cellular membranes. They have been of interest for their ability to translocate not only themselves through cellular membranes, but also carry along with them, cargo as large as iron nanoparticles. The exact entry mechanism remains unclear, but has been shown to vary with peptide sequence. Their translocation properties have been demonstrated through different experiments involving vesicles, cells and living animals. We plan on using nano-black lipid membranes (nano-BLM), which span the pores of nanoporous materials as a model system to study the interaction between CPP and lipid membranes. The nanopores can be used as cellular containers whose interior surface can be functionalized with receptors for biotin-streptavidin recognition. We hope that this system will provide greater control over experimental variables, such as the type of CPP and lipid used, as well as provide kinetic data that can be used to evaluate CPP activity and the kinetics of cargo transport.

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Oxidative stress and the membrane dipole potential; modulation with tocopherol

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Tocopherol, a component of vitamin E well know for its antioxidant properties, has recently been thought also to influence the structure of cellular membranes. Tocopherol treatment reduces hyperglycaemia induced oxidative stress and the associated endothelial dysfunction which is a precursor to the vascular complications of diabetes. Tocopherol and insulin interactions are also modified by hyperglycaemia. It is unclear whether these clinically important effects arise from the anti-oxidant and/or structural properties of tocopherol. We have therefore measured the dipole and surface potentials of phosphatidylcholine vesicles containing different amounts of cholesterol, ketocholesterol and tocopherol. We have also investigated the effects of hyperglycaemia, ketocholesterol and tocopherol, alone and in combination, on microdomain formation and interactions of insulin within the membranes of cultured endothelial cells. Both sets of experiments indicate that tocopherol causes significant modifications of the membrane dipole and surface potentials. The physiological significance of these changes will be discussed.

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Insight into the antimicrobial mechanism of a de novo auto-assembling peptide

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A short (14 residues) *de novo* antimicrobial peptide (K4) composed of a cationic polar head and a hydrophobic tail was studied. It exhibits a broad spectrum of antimicrobial activity on bacteria. No haemolytic activity or cytotoxicity on eukaryotic cells are observed at MIC. When bacteria are lysed by the K4, spherical objects are observed on the SEM micrograph. K4 was structurally studied using various membrane mimetic media such as different micelles and small unilamellar vesicles (SUV) of different composition. CD revealed that K4 adopt various structures (random, β-turn, α-helix) in the presence or the absence of detergents or phospholipids. NMR structures confirmed the α-helical structure of K4 hydrophobic tail in presence of SDS. K4 self-assembles at high concentration as observed by SEM and DLS. We suggest that K4 may act as a surfactant building mixed microsomes composed of peptide and lipids. This destabilization mechanism of the bacterial membrane support the "detergent like model" previously described in the literature.

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Formation of membrane tubular structures induced by phase separation in giant vesicles

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Tubular membrane structures (also known as tethers) exist widely in eukaryotes. Abundant work has been done on tube extrusion from cells and model membranes under the application of external forces. We present a novel system allowing tube formation in the absence of external forces. Aqueous solutions of two chemically dissimilar polymers, polyethylene glycol (PEG) and dextran are encapsulated in giant vesicles, a cell-size model system. The exposure of vesicles to hypertonic solutions induces phase separation of the internal aqueous polymer solution. The excess membrane area created by this vesicle deflation, engages in the formation of tubular structures. Membrane tube formation and phase separation are coupled processes. Hydrodynamic flows and changes in the membrane spontaneous curvature during phase separation might be the driving forces for tube formation. The tubes are rather stable: without external perturbation, they can exist for several days. They prefer to be located in the PEG-rich phase at low polymer concentration. At high concentration, they are absorbed at the interface of the liquid phases to lower the surface energy of the system by decreasing the contact area between the liquid phases. The membrane tubes can be retracted back to the vesicle surface by increasing the membrane tension via vesicle aspiration. Membrane tubes, which can form and be retracted easily, might be relevant to lipid storage in cells.

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– Lipid biophysics –

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Mimicking and quantifying cell-cell and cell-virus interactions via colloidal probe microscopy

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Cell-cell and cell-virus interactions are ubiquitous in living organisms. The analysis of the forces acting between two cells or a cell and a virus gives insight into details of these interaction processes such as their stochastics, cooperativity, reversibility, and energy landscape. Using colloidal probe microscopy in conjunction with solid-supported lipid bilayer techniques, we mimic the contact between two membranous compounds displaying sponge glycolipids or viral peptides on their surfaces. After spreading functionalized lipid bilayers on both - a colloidal probe and a silicon wafer surface - the molecular interactions are quantified by means of force-distance curves. By probing the dynamic interaction strength between the viral peptides N36 and C34 we aim for a deeper understanding of the role of these peptides in the complex process of the formation of the prehairpin intermediate as the key step in retroviral fusion. As far as the cellular interaction of marine sponge cells is concerned, we intend to investigate the strength, specificity, and the Ca^{2+} dependency of the self-recognition between sponge glycans. The systems advantages are the flexibility of the membrane composition and the control over the distribution of receptor molecules in the membranes. Since adhesion in biological systems relies heavily on cluster formation within the biomembrane, we plan to mimic the clustering by "printing" interaction domains and comparing the results to homogeneous samples.

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Interaction of pyrenesulfonate with fluid bilayers and fluorescence quenching by 5-DOXYL-PC

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We studied the partition of the anionic amphiphile 1-pyrenesulfonate (PSA) into MLV and LUV (produced by extrusion), composed by zwitterionic POPC and zwitterionic/anionic mixtures with POPS (until 10 mol %). PSA is an anionic amphiphile that mimics several xenobiotics (*e.g.* pharmaceuticals, pesticides) and endogenous substrates that interact with biological membranes. We found increasing K_p values in parallel with relatively low molar proportions of POPS in mixed fluid and flexible bilayers, comparing with the fluid POPC bilayers at 25°C. Paving on the known details about the physical chemical factors that determine the incorporation of PSA into model membranes and location within the bilayer [M. Manuel, J. Martins; *Chem. Phys. Lipids.* 154 (2008) 79-86], we have studied the quenching of PSA by low concentrations of a DOXYL quencher group covalently linked to an acyl phospholipid chain in fluid POPC bilayers. Steady-state fluorescence of PSA decreased with increasing proportions of 5-DOXYL-PC in the bilayers. The results are best described by a kinetic formalism specific for reactions occurring in two-dimensional media [J. Martins *et al*; *J. Chem. Phys.* 120 (2004) 9390-9393], instead of the classical Stern-Volmer formalism. *Acknowledgments*: M. Manuel is recipient of a Ph.D. Grant (SFRH/BD/40671/2007) from Fundação para a Ciência e a Tecnologia, Portugal.

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Interactions between non-steroidal anti-inflammatory drugs and a PC/cholesterol bilayer

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The non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed and used drugs [1]. There are several side effects connected with frequent use of NSAID, mainly gastrointestinal ulcers and bleedings. A plausible molecular mechanism of these effects are direct interactions of NSAIDs with gastric phospholipids [2, 3]. The influence of three well-known non-steroidal anti-inflammatory drugs with a diverse gastric toxicity (aspirin, ketoprofen and piroxicam) and three newly-synthesized xanthone derivatives (belonging to the NSAIDs) on the structure and dynamics of lipid bilayers was studied using small angle X-ray scattering and molecular dynamics simulations. The results showed some correlation between NSAID toxicity and its binding to the lipid polar groups. This binding increases membrane fluidity by reducing its density due to an increased membrane surface area. A reduced lipid packing in the membrane most likely increases gastric mucosa permeability, which can result in a decreased resistance of the gastric mucosa to luminal acid.

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The role of lipid domains and rafts in ethanol induced membrane alterations – an AFM study

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Ethanol induces phospholipid acyl chain interdigitation. While much is known, important issues remain unclear, such as the role of lipid domains. The main purpose of this study was to follow, in real time, the changes induced by ethanol on supported lipid bilayers with nano to microdomains by *in situ* Atomic Force Microscopy. To this goal, a pure lipid in the fluid phase (DOPC), a pure lipid in the gel phase (DPPC), binary phospholipid mixtures with gel/fluid phase coexistence, and cholesterol-containing, raft -forming mixtures (DOPC/DPPC/cholesterol and DOPC/sphingomyelin/cholesterol) were investigated. From the height differences observed upon ethanol addition to pure lipids (DPPC and DOPC), and to DOPC/DPPC mixtures, it is shown that in the binary system the interdigitation of the fluid phase occurs prior to the gel phase. However, for the lipid rafts mixtures the simultaneous interdigitation of both raft and non raft portions of the membrane is observed both in mica and silicon substrates. For all compositions studied, domain formation or rearrangement accompanied by lipid bilayer expansion occurs as a consequence of interdigitation. These results show the ability of ethanol to influence the bilayer properties in different ways according to membrane composition. Ethanol may exert its biological effects by reducing bilayer thickness, and also by changing membrane proteins conformation and lateral distribution, as a consequence of the altered properties of the lipid bilayer.

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HIV-1 fusion inhibitors-blood cells interaction measurements agree with biomembranes models data

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Enfuvirtide (T-20) was the first HIV-1 fusion inhibitor peptide approved for clinical use. T-1249 is an inhibitor still under evaluation. Previous studies, based on tryptophan intrinsic fluorescence, showed that the peptides interact differentially with membrane model systems (LUV) with different lipid compositions. Studies with human blood cells were necessary to further establish the role of membranes on these peptides mode of action. An experimental strategy was applied based on the membrane dipole potential, as measured by the fluorescent probe di-8-ANEPPS. Human erythrocytes and peripheral blood mononuclear cells (PBMC) were successfully labeled. For both systems, a fusion inhibitor concentration-dependent decrease on di-8-ANEPPS fluorescence excitation ratio (a measure of the spectral shift and dipole potential) was observed. The quantitative analysis of these variations indicated that T-1249 has an approximately ten-fold higher affinity towards erythrocyte and PBMC, when compared with enfuvirtide. This is in agreement with the previously known adsorption of T-1249 on cholesterol-rich membrane domains and with its higher partition constants. HIV associates with erythrocytes *in vivo*, which can constitute a route to deliver peptide to the viral membranes (also rich in cholesterol). Lymphocyte membranes can concentrate and accelerate the drug interactions with its molecular target, gp41 in its exposed conformation.

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Thermodynamic and modelling studies of new dicephalic saccharide-derived surfactants

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New N-dodecyl-N,N-bis[(3-D-gluconylamido)propyl]amine and N-dodecyl-N,N-bis[(3-D-lactobionylamido)propyl]amine (C12-DGA and C12-DLA, respectively), represent a biodegradable family of dicephalic surfactants. The molecules are composed of single hydrophobic tail and two hydrophilic aldonamide-type groupings (gluconyl C12-DGA or lactobionyl C12-DLA) linked by the propylene chain at the nitrogen atom. The micellization processes of C12-DGA and C12-DLA were studied by means of ITC. The critical micelle concentrations, the enthalpies (ΔH_m) and the entropies (ΔS_m) of micellization as well as the contributions of the headgroups to the Gibbs free energies ($\Delta G_m{}^0$(hy)) were calculated. QSPR analysis was also used to predict CMC of studied compounds. The interactions of C12-DGA and C12-DLA with model membranes (DPPC and DPPC/chol. bilayers) were studied by means of DSC. Using quantum computations some basic molecular properties were calculated. The conformational space was explored using molecular mechanics. Obtained results were compared with those for analogical compounds with single head groups, e.g. with also synthesised by us N-alkanoyl-N-methyllactitolamines (C_nMeLA) and common sugar-based surfactants C_{12}Gluc and MEGA-10.

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Current fluctuations in biological lipid membranes from human cell lines

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Lipid Membranes can undergo phase transitions at physiological temperatures. Not only do single lipid component membranes show melting behaviour but also biological lipid membranes from eukaryotes as well as from prokaryotes. Performing calorimetric and monolayer studies we are able to detect phase transitions in extracted membranes from different human cell lines (e.g. keratinocytes and melanoma cells). Close to and in the melting transition regime we measured distinct channel like current fluctuations. The opening times of these current fluctuations can be predicted based on the lateral compressibility measured from the monolayer isotherm and agree well with the experimental data [*]. The applied method of extraction excludes the presence of functional proteins in the membrane rendering the lipid bilayer as the source of the observed current fluctuations.

[*] B. Wunderlich, C. Leirer, A-L. Idzko, U.F. Keyser, A. Wixforth, V.M. Myles, T. Heimburg, M.F. Schneider. Phase state dependent current fluctuations in pure lipid membranes, *in press*

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Development of video-rate imaging microscope using laurdan and its applications to lipid raft

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Various biological functions are located on cell membranes, and the biomembranes maintain heterogeneity as micro-domain even in its dynamic structure. Existence of such domain was reported as phase-separated 'rafts' in a cell. And the bio-functions of membrane protein are affected by physical property of its surrounding lipid bilayers. Laurdan is a useful fluorescence dye to monitor membrane fluidity, and we have developed an instrument to image spatial and temporal change in membrane fluidity by use of laurdan. In order to examine role of 'micro-domain' in biomembrane, we applied this imaging instrument to a giant liposome, CHO cells and PC12D cells. Fluorescence microscope was equipped with a home-build dual-view optical unit. Microscopic image of membranes stained with laurdan is separated into an image at 440 nm and an image at 490 nm by the combination of monochromatic filters and dichroic mirrors. The each image is focused on an image plane of a CCD camera side by side. And generalized polarization (G.P.) image is calculated from those images by personal computer according to the definition of G.P. The G.P. imaging at video rate was applied to a giant liposome of composed of DMPC and DMPE in order to observe phase separation. It was also examined that specific interaction of sphingomyelin and cholesterol in living CHO cells and neuritis protrusion from raft region of PC12D cells stimulated by neuron growth factor.

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Curvature of pore-suspending membranes influenced by Shiga Toxin

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Shiga Toxin (STx) from *Shigella dysenteriae* is an AB_5-class bacterial toxin. Infections with STx lead to the *haemolytic-uraemic syndrome*, which is known to be a major cause for renal failure at an early age. The interaction of the homopentameric B-subunit (STxB), which is responsible for binding and intracellular transport of the holotoxin, with its cellular receptor, the glycosphingolipid Gb_3, is the first step for endocytosis of the toxin. One B-subunit can bind up to 15 Gb_3-molecules to form STxB-Gb_3-clusters causing negative curvature of a membrane. The binding of STxB to *giant unilamellar vesicles*, composed of DOPC, cholesterol and Gb_3 induces tubular membrane invaginations, which were also found in experiments with energy-depleted HeLa-cells. In recent studies, protein and lipid reorganization processes after attaching STxB to solid supported membranes, composed of DOPC/sphingomyelin/cholesterol/Gb_3 have been investigated. The compaction of Gb_3-molecules led to an additional STxB-Gb_3-enriched phase, which was also observed in lipid monolayers at the air/water interface. In this study, the influence of STxB on model membranes will be investigated combining the advantages of free-standing lipid membranes with those of SSMs. The impact of STxB on pore-suspending membranes will be followed by *confocal laser scanning microscopy* and *atomic force microscopy*.

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Kinetics of stearic acids transfer between human serum albumin and polymer-grafted membranes

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Nonesterified fatty acids regulate a broad spectrum of metabolic activities and are involved in many physiological, pathological and/or pharmacological processes in living cells. They spontaneously transfer between donors and acceptors such as fatty acid binding proteins and lipid membranes. We focus on protein-lipid dispersions of human serum albumin (HSA) and sterically stabilized liposomes (SSL) composed of DPPC and appropriate amount of PEG:2000-DPPE in which stearic acids (SA) are inserted either in the protein or in the SSL. Exploiting the fact that HSA has a single tryptophan residue and that the intrinsic Trp-fluorescence emission signal is quenced by the presence of SA, the kinetics of SA transport between HSA and PEG:2000-grafted DPPC membranes is studied by means of fluorescence. It is found that the transfer of SA between HSA and SSL is a first-order process and the kinetics of transfer depends on the type of donor and acceptor matrix, on the temperature (i.e., on the physical state of the lipid bilayers), and on the grafting density of the PEG-lipids at the protein/lipid interface. Indeed, in the absence of polymer-lipids, the rate of transfer increases with temperature in both directions of transfer and it is faster for the passage from DPPC bilayers to HSA. The presence of polymer-lipids reduces the rate of transfer both in the mushroom and in the brush regime of the polymer-chains, especially for lipid membranes in the fluid phase.

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Development of Solid State NMR Methods for Determination of Membrane Protein Structure

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Membrane proteins account for a third of all proteins encoded for by the human genome, and play a vital role in a number of cellular processes. Few membrane protein structures have been determined to date in comparison to soluble proteins. This discrepancy is due to experimental difficulties in preparing membrane protein samples for structural analysis. Traditional techniques for studying membrane proteins by X-ray crystallography or solution NMR use detergent solubilised proteins which can differ from their native confirmations. Solid-state NMR allows the study of transmembrane proteins in lipid bilayers, representing a more native like environment in which to obtain biologically relevant structural information. We have been working on the development of reliable methods for reconstitution of transmembrane peptide into liposomes using Glycophorin A as a model TM protein. Using reconstitution methods based on the removal of detergent by bio-beads, we have used Electron Microscopy to screen the ideal conditions for insertion of GpA into liposomes in preparation for MAS NMR experiments. EM has allowed us to identify conditions favourable for insertion of peptide into lipid vesicles and those that result in aggregation. In order to confirm the secondary structure and insertion of GpA into lipid vesicles, techniques such as CD, OCD, FTIR and DLS have been used to provide quantitative information in addition to the visual results from EM.

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The effect of cholesterol on the diffusion of oxidized phospholipids in supported lipid bilayers

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Oxidation of low density lipoprotein (LDL) is known to be a key step in atherogenesis, leading to inflammation, proliferation and apoptosis of cells of the arterial wall. These effects are largely exerted by oxidatively fragmented phospholipids, which are highly exchangeable between cells, tissues and lipoproteins. In particular, PGPC has been identified in minimally modified LDL and has been reported to elicit a wide range of pathophysiological responses in vascular cells, e.g. the activation of apoptotic signaling pathways.
We investigated here the behavior of the fluorescent oxidized PGPE-Alexa647 compared to DHPE-Bodipy in artificial supported lipid bilayers with different cholesterol contents. The two labeled lipids differ in the type of membrane insertion: while DHPE-Bodipy is anchored to the membrane via two fatty acids, PGPE is incorporated with only one fatty acid. The second chain is an acyl fragment in sn-2 position, which represents the oxidation product of an unsaturated acyl chain. With increasing cholesterol content we observed a decrease in the diffusion coefficient for both lipids. Interestingly, the diffusion of the oxidized lipid was reduced in a higher degree compared to that of the non-oxidized lipid. The calculated ratios of the diffusion constants of PGPE-Alexa647 and DHPE-Bodipy suggest a different type of interaction with cholesterol.

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– Lipid biophysics –

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Effect of cholesterol on the structure of phospholipid and non-phospholipid membranes

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Cholesterol plays an important role in regulating the structural properties of phospholipid and non-phospholipid membranes. In this study we have applied *in situ* energy dispersive X-ray diffraction (EDXD) to investigate the effect of cholesterol on the structure of different phospholipid and non-phospholipid oriented membranes. In detail, phosphatidylcholine (PC) bilayers and niosomal membranes, made of a non-ionic surfactant, polysorbate 20 (Tween 20) [1,2], and/or his pH-sensitive derivative cholesteryl hemisuccinate (CHEMS) [3], were used. The latter system is a very promising nanovector for drug delivery applications.

References

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P-607

Label-free imaging of biological membranes using surface imaging techniques

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Surface plasmon resonance (SPR) is a detection technique traditionally used for specific protein detection, which is now exploited routinely as a generic label-free sensor. SPR responds to changes on a metal surface conditioned to sense the binding of analytes. Thus, the composition of the external medium and metal surface will be fundamental to the signal output. Here we use a model phospholipid membrane system to investigate the effect of altered buffer and surface compositions on SPR signals. Comparisons are undertaken between continuous gold surfaces and gold nanoparticles. We demonstrate that surface electrostatics and the salt composition and molarity of a buffer all have significant impacts on SPR output.

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Extensive bilayer perforation coupled with the phase transition region of an anionic phospholipid

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At low ionic strength dimyristoylphosphatidylglycerol (DMPG) exhibits a broad phase transition region characterized by several superimposed calorimetric peaks. Peculiar properties, such as sample transparency, are observed only in the transition region. We use differential scanning calorimetry, turbidity and optical microscopy to study the narrowing of the transition region with the increase of ionic strength. Upon addition of salt, the temperature extension of the transition region is reduced and the number of calorimetric peaks decreases until a single cooperative event is observed in the presence of 500 mM NaCl. The transition region is always coupled with a decrease in turbidity, but a transparent region is detected within the melting process only in the presence of up to 20 mM NaCl. Optical microscopy of giant vesicles shows that bilayers first rupture when the transition region is reached and subsequently lose optical contrast. Fluorescence microscopy reveals a blurry image in the transparent region, suggesting a different lipid self-assembly. Overall sample turbidity can be related to the bilayer optical contrast. Our observations are discussed in terms of the bilayer being perforated along the transition region. In the transparent region the perforation is extensive and the bilayer completely loses the optical contrast. Financial Support: Fapesp.

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Characterization of phosphatidylcholine chlorohydrins and their effects on the erythrocyte

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Hypochlorite generated *in vivo* under pathological conditions is a known oxidant, able to initiate lipid peroxidation process, which affects the stability of biological membranes. The aim of this study was the analysis of the products formed during the reaction of hypochlorite with phosphatidylcholines containing unsuturated fatty acid residues (1-stearoyl-2-oleoyl-, 1-stearoyl-2-linoleoyl-, 1-stearoyl-2-arachidonylphosphatidylcholine) and their effects on the human erythrocytes. Using electrospray mass spectrometry we observed complete conversion of the lipids into chlorohydrins, which resulted in the decrease of the rotational correlation time and rotational motion freedom of liposomes estimated by EPR using spin probes (16- and 5-doxylstearic acid). Unilamellar chlorohydrin liposomes had lower diffusion coefficient for calcein than liposomes made of parent lipids. Flow cytometry demonstrated fast incorporation of uni- and multilamellar chlorohydrin liposomes labeled with NBD-PE into erythrocytes. This effect was accompanied by the formation of the erythrocyte subpopulations of higher volume, decrease of the rate of fluorescein diacetate hydrolysis, estimated by flow cytometry, and increase of affinity and maximal velocity of the membrane enzyme acetylcholinesterase.

Abstracts
– Lipid biophysics –

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Molecular basis of translocation of novel nucleolar-targetting peptides

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Nucleolar-targeting peptides (NrTPs) have been recently designed by structural dissection of crotamine, a toxin from the venom of a South America rattlesnake (Radis-Baptista et al. 2008. J Med Chem 51:7041). At μM concentration, NrTPs penetrate different cell types and exhibit exquisite nucleolar localization. The aim of this work is to decipher the molecular mechanism for the translocation of NrTPs into cells. Quantification of partition into membranes was carried out, based on intrinsic tyrosine fluorescence. The role of the bilayer phase, anionic lipids, reducing agents and peptide concentration on the extent and kinetics of partition were studied. Both NrTP1 and NrTP2 exhibited high partition to POPC (neutral) lipid vesicles ($K_p \approx 3\times10^3$), which was enhanced by the anionic lipid POPG for NrTP1, but not NrTP2. The peptides showed a decrease in partition for POPC:cholesterol (liquid ordered state) or DPPC (gel) membranes. Depending on the lipid composition, the peptides either increased or decreased their quantum yield upon membrane insertion. Quenching experiments with acrylamide showed no peptide aggregation in solution. Once the translocation mechanism is fully understood we will test NrTPs as carriers of relevant cellular cargos, evaluating their potential clinical application in drug delivery or gene therapy among other applications.

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Thermodynamics characterization of isolated lung surfactant assembled as lamellar bodies

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Lungs are a large extension of $\sim$80m^2 of one layer cells, which is structured as compacted sacs called alveoli, where lung function takes place: the gas exchange. Alveolar endotelium is formed by two kinds of cells: Neumocyte type I (NTI), which covers $\sim$97% of the alveolar surface where gas exchange occurs and, NTII where lung surfactant (LS) is produced. LS are a mixture that lay over a water film in the luminal surface of the alveoli and it is thought to decrease the surface tension to avoid alveolar collapse during expiration. LS are made of phospholipids and proteins, which are determinant for the structure of LS under particular conditions of pressure and temperature. LS inside the cell is packed in organelles called lamellar bodies (LB), and is released to the water/air interphase in other conformation. In the present study we isolate LB from pig's lung and study LB thermodynamic characteristics by microcalorimetry in the presence and in the absence of structural proteins. Phase transition profile of LB with and without proteins is basically the same; while LS in the alveolar lumen have a higher transition temperature (Tm). Mayor changes in Tm are observed between LB and LS from alveolar lumen. Although LS lipid composition in and outside the cell is assumed to be the same, the ground for the differences in Tm under these two conditions is unknown.

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Structure and dynamics of closed melting membranes

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We studied the dynamical and structural properties of large unilamellar vesicles ($\approx$ 120 nm LUVs) of phospholipids (DMPC, $DC_{15}PC$ and DMPC : $DC_{15}PC$ = 1 : 1 molar mixture) in the temperature range around the chain-melting transition, $\approx$ 3°C wide, with 0.1°C resolution and 0.01°C accuracy. Small-Angle (SAXS) and Wide-Angle X-ray Scattering (WAXS) measurements show that across the transition the vesicle behaves as an 'evolving membrane', passing through several different states, each of them being characterized by different proportions of coexisting fluid- and gel-chains molecules. Noteworthy, no kinetics has been detected. On the same samples, a unique and very sensitive laser light scattering technique allows to determine the characteristic times of thermally induced shape fluctuations, connected to the elastic properties of the membranes. Results indicate a clear softening of the membranes in correspondence to the chain-melting transition, as indicated by a manifold increase of the corresponding fluctuation characteristic time. Meanwhile the overall size of the vesicle is not sensibly changed. This softening is likely to be due to the presence of structural defects, eventually driving to local morphological modifications.

P-613

The association of respiratory syncytial virus matrix protein with membrane microdomains

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The association of the matrix protein from respiratory syncytical virus with membranes has been characterised by tensiometry, Brewster angle microscopy, and atomic force microscopy following deposition of Langmuir monolayers onto modified silica substrates. Association of the protein with monolayers containing phosphocholines and cholesterol leads to the formation of materials with new properties that differ from those of either of the pure components. The behaviour of the protein in monolayers rich in cholesterol and sphingomyelin exhibits a significant concentration dependence. At low concentrations, the protein exhibits a simple partioning behaviour. At higher concentrations, lipids are extruded from the monolayer below a critical surface area. These findings are discussed in relation to the recently published structure of the protein (PNAS, 2009, 106, 4441-4446), the documented formation of viral filaments during key stages of the infection cycle and the isolation of the protein from detergent-resistant membrane fractions.

Abstracts
– Lipid biophysics –

P-614

Interfacial behavior of the sub-domain R11-15 of dystrophin rod domain under lipid monolayer by ellipsometry and atomic force microscopy

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Dystrophin is a rod-shaped muscular subsarcolemal protein. Its deficiency is one of the root causes of Duchenne muscular dystrophy. Dystrophin rod domain contains 24 homologous repeats, where the sub-domain constituted by the repeats 11 to 15 (R11-15) was reported to bind actin and membrane lipids.
We analyzed the interaction of R11-15 with lipid monolayers. To better understand the assembly mechanism of this protein with lipids, we studied its adsorption behavior at the air-liquid and lipid/liquid interface using ellipsometry, surface pressure, and atomic Force Microscopy (AFM). Using two different mixtures of phospholipids, ellipsometry and pressure surface data show that R11-15 interacts with the lipid monolayers, but is inserted into the monolayer formed by DOPC-DOPS while it lies below the monolayer of DOPC-DOPE. This indicates that R11-15 interacts more strongly with DOPC-DOPS than with DOPC-DOPE. AFM images show that the pressure of lipid monolayer influences the organization of R11-15. When the lipid surface pressure is 30mN/m, R11-15 forms a striking network, indicating a protein-protein interaction in addition to the protein-lipid interaction. This unique behavior of one part of the central domain of dystrophin may explain its key role in muscle cell.

O-615

A 3-D hexagonal inverse micellar lyotropic phase

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A number of complex three-dimensional lyotropic liquid crystal phases are already known, such as the bicontinuous cubic phases, but so far only a single example has been found – a cubic phase of spacegroup Fd3m – of a structure based upon a complex close packing of inverse micelles (1). We now report the discovery (2) of a novel lyotropic liquid crystal phase, of space group, $P6_3/mmc$, whose structure is based upon a hexagonal close packing of identical quasi-spherical inverse micelles. The model membrane system consists of a hydrated mixture of dioleoylphosphatidylcholine, dioleoylglycerol, and cholesterol. This novel phase has a number of unique features which may render it useful for a range of applications. Firstly, it is the only known self-assembled lyotropic phase whose structure consists of a *periodic close packing of identical inverse micelles.* Secondly, it is stable in *excess aqueous solution*, which is very important for potential biological or biomedical applications.

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P-616

Photooxidation and lateral membrane diffusion of dipole molecules

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The photodynamic oxidation of phloridzin, of the styryl dye di-8-Anepps and of unsaturated lipids was monitored "online" by measuring the collapse of the dipole potential which has been introduced to the membrane by these molecules. Their photodamage occurred at different rates when the target molecules and the singlet oxygen generating photosensitizer (phthalocyanin) were adsorbed to the same or to opposite sides of the planar lipid bilayer. The difference in the oxidation rates were attribute to singlet oxygen transport through lipid bilayer and therefore we were able to estimate the permeability of lipid bilayers to singlet oxygen. However, the apparent permeabilities derived from experiments with different targets were differ from each other. Therefore, we tested the hypothesis that the lateral membrane diffusion of target molecules and oxidation product may have biased our analysis. In line with this anticipation we found that the apparent permeability is dependent on the size of the planar bilayer. The development of a new mathematical model, which takes the mobility of all reacting species in the aqueous and lipid environments into account, allowed estimation of the real membrane permeability to singlet oxygen. It appeared to be very close to that of oxygen in the ground state.

P-617

The effect of TRANS unsaturation on molecular organization in a phospholipid bilayer

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The ingestion of *trans* fatty acids (TFA) formed during the partial hydrogenation of vegetable oils has been linked to a detrimental impact on health by an, as yet, unknown mechanism. We synthesized deuterated analogs of 1-elaidoyl-2-stearoylphosphatidylcholine (*t*18:1-18:0PC) that contains a single "unnatural" *trans* double bond and 1-oleoyl-2-stearoylphosphatidylcholine (*c*18:1-18:0PC) that contains a single "natural" *cis* double bond. Solid state ^2H NMR, complemented by molecular dynamics (MD) simulations, was then employed to compare molecular organization in model membranes prepared from these isomeric molecules. Analysis of spectra recorded as a function of temperature reveals a higher chain melting temperature for the *trans* isomer, indicating tighter molecular packing in the gel state. In the liquid crystalline, however, the difference between the *trans* and *cis* isomers is subtle. Order as probed by the perdeuterated $[^2H_{31}]$18:0 *sn*-2 chain, and corroborated by computer simulation, coincides within $<5\%$. Only in the conformation of the double bond is an appreciable difference implied. Thus, our results contradict the conventional view that TFA resemble saturated fatty acids, which is $>20\%$ more ordered.
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Abstracts
– Lipid biophysics –

P-618

The language of shape: biological reactions are dramatically affected by the shape of lipid membrane

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A plethora of biological process are taking place on the surface of lipid membranes. As a rule membranes in vivo are curved in a variety of complex geometries. Here I will present a quantitave study on the influence of membrane curvature on protein-membrane and membrane-membrane interactions. To gain systematic access to a continuum of membrane curvatures we immobilized liposomes on a surface at dilute densities. Using confocal fluorescence microscopy we imaged single liposomes of different size, and therefore different curvature, and monitored their interaction with a binding partner (proteins or other liposomes).
I will discuss unpublished data on two important classes of biomolecular interactions that exhibited dramatic curvature dependence: A) SNARE-mediated docking and fusion B) anchoring of peripheral proteins.
The following references provide partial information on the single-liposome assay:

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P-619

Single channel recording of α-Hemolysin in nanopore-spanning tethered bilayer lipid membranes

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Artificial lipid bilayer membranes mimic biological cell membranes in many aspects and can be used to study functional processes such as ion channeling, signal transducing, transport of nutrients etc. As most of the functions of a cell are accomplished by membrane proteins, research has been ongoing in studying and characterization of membrane proteins embedded in model lipid bilayer membranes. Black lipid membranes (BLMs) were studied for decades but their potential for practical applications is hindered, mainly due to their lack of stability and limited lifespan. Recently, tethered bilayer lipid membranes (tBLMs) proved to have long life span of up to several months without significant changes in membrane resistance and capacitance. In order to combine advantages of BLMs and tBLMs, we have designed a membrane system which is freely spanned across a single nanopore in a silicon nitride membrane. This system not only mimics cell membranes, but also it allows control over the chemical composition of buffer with unlimited ionic reservoirs on both sides of the membrane. This freestanding tBLM maintains structural stability and lifetime of up to 120 hours without significant decrease in its structural integrity and electrical sealing. For the validation of the tBLM formation, we have inserted well known α-HL pores. We are able to control the amount of α-HL pores insertion and measure single channel ion transport across the tBLM by α-HL pores using a capacitor feedback amplifier.

P-620

Interaction of cationic porphyrins with neutral and negatively charged liposomes

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Porphyrin derivatives are used in photodynamic therapy of tumors. The knowledge of the photosensitizer location within the cell is important. The effect of porphyrin derivatives containing cationic side groups was examined on neutral and negatively charged liposomes by ESR. The ESR signal was first examined as a function of temperature and porphyrin concentration in the dark. A significant change related to the appearance of quasi free spin labels was obtained for spin probes at the 12th carbon atom and was more expressed for the asymmetrical derivative. Illuminating the samples the ESR amplitude decreased for all positions of the spin probe but to different extent. The effect was more expressed in case of the symmetrical derivative, especially for label positions 5 and 12. For the asymmetrical derivative the effect changed from moderate to weak from the 5th to the 16th position. This indicates that the asymmetrical derivative is incorporated nearer to the lipid head groups while the symmetrical one may be located deeper in the membrane. Under oxygen-free conditions both derivatives showed weaker but still pronounced effects..

P-621

Studies on antioxidative efficiency of polyphenols extracted from plants

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Studies on polyphenol extracts from *Helichrysum* L., *Fagopyrum* Mill., *Crataegus* L. and *Hypericum* L. were performed in order to find if they may be applied as a natural free radical scavengers protecting biological membranes against peroxidation. Ghosts erythrocytes were used in the experiments. They were suspended in Tris-EDTA solution, pH 7,4, then.irradiated with a bactericidal lamp without (control) or with a proper amount of the extracts studied. The product of lipid peroxidation was malonic dialdehyde (MA). The colour reaction of MA with thiobarbituric acid (TBA) enables to determine the concentration of MA spectrophotometrically. It was found that peroxidation increased with the irradiation time. However, it significantly decreased when concentrations of poliphenols increased. The best antioxidtive property was found for *Hypericum* L. The antioxidative efficiency sequence of the plant extracts studied was the following: Hypericum L. > Crataegus L. > Fagopyrum Mill. > Helichrysum L.
The results obtained indicate that polyphenol extracts exhibit excelent antioxidative properties that make them good free radical scavengers for efficient protection of biological membranes.

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Abstracts
– Lipid biophysics –

O-622

Catalysis in the membrane: Interfacial mechanism of Phospholipase A2

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Phospholipase A2 cleaves the sn-2 acyl chains of membrane phospholipids and performs a range of physiological functions. One of the least well understood aspects of the its mechanism is how its activity is regulated by the interaction with the substrate membrane. We have used neutron reflection to monitor changes in membrane structure during lipid hydrolysis[1,2]. The penetration depth of PLA2 depends on lipid packing and increases during the lag phase of porcine pancreatic PLA2. By using a selectively deuterated lipid substrate, d31-POPC, we determined the relative membrane-water partitioning of the lipid products *in-situ*. The lyso-lipid product partitions into the solution phase, while fatty acid accumulates in the membrane and increases the affinity of PLA2[2]. PLA2 is inhibited at pH 5, which is consistent with protonation of the catalytic histidine. However, irrespective of pH, PLA2 is fully activated by Me-beta-cyclodextrin, which facilitates the release of the lyso-lipid from the enzyme-substrate complex. Me-beta-cyclodextrin does not interact directly with the membrane surface or the substrate lipids, indicating that product release occurs outside the immediate membrane-water interface.

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P-623

MD simulations of the interaction between limonene and its derivatives with lipid bilayers

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Extracts of the plant *Perilla frutescens* have many uses in the Asian kitchen, e.g. as a popular garnish used in sushi. The plant is also employed in eastern traditional medicine to treat a variety of ailments including colds, food allergy and depression. Two of the main constituents of the plant are limonene and perilla aldehyde. By bio-oxidation, these molecules can be converted to perillic alcohol and perillic acid. These cyclic terpenes possess antibacterial and anti-carcinogenic properties.
The modes of action for these compounds are at present not understood, but their remarkably diverse pharmacological properties suggest that they might target the phospholipid matrix of the cellular lipid membrane. Indeed, the cyclic terpenes can bind to, and alter the physiochemical properties of the lipid bilayer of the membrane, the effects of which can cascade down to several essential cellular processes.
Here, we use molecular dynamics (MD) simulations to investigate the effect of limonene and its derivatives on the properties of lipid bilayers including the changes in acyl chain order parameters, bilayer thickness and the area per lipid. MD can afford molecular-scale dynamic information, often not easily accessible from experimental measurements. This information can be used to interpret existing experimental data obtained by e.g. isothermal titration calorimetry, electron paramagnetic spectroscopy and differential scanning calorimetry.

P-624

Interaction of grape and peach defensins with lipids

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Plant defensins are cysteine-rich antimicrobial peptides found in various plant species. They share a common three-dimensional structure, stabilized by eight disulphide-linked cysteines consisting of three antiparallel β–strands and one α–helix. Most plant defensins show antifungal activity with no effect on mammalian and plant cells. Expression of these peptides in plant tissue is induced by pathogen infection. The mechanism of defensin action is based on membrane permeabilization. This occurs through an interaction with high affinity binding sites on fungal membranes, resulting in alteration of membrane potential. The genes encoding for a peach PpDfn1 and a grape VvAMP defensins were expressed in *E. coli* and purified to homogeneity. They were tested for antimicrobial activity against some fungi and showed to have an inhibitory effect on the spore germination. Biophysical analysis showed that defensins were able to interact with artificial membranes. Binding of defensins to membranes was dependent on lipid composition, increasing with the sphingolipids content. Interaction between peptides and sphingolipids could lead to insertion of the defensins into the membrane resulting in its destabilization.

P-625

RNA-lipid interaction at the air-liquid interface

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There is accumulating evidence of substantial amounts of phospholipids in the cell nuclei[1], although the function of these lipids is still not fully understood. It has been shown that the chromatin complex composed of DNA, RNA and proteins also includes phospholipids, and that RNA colocalize with these[2]. Although the RNA-phospholipid interactions may have important implications to biological function, in gene therapy and in medicine, very little work has been dedicated to the characterization of RNA interaction with phospholipids. The objective of this work is to investigate the adsorption behavior of short single stranded 10 bases long RNA ($ssRNA_{10}$) molecules (similar to miRNA) to lipid monolayers at the air-water interface as well as to study how the presence of RNA affect the domain formation in the monolayers using fluorescence microscopy. Monolayer studies have shown adsorption of $ssRNA_{10}$ to monolayers consisting of zwitterionic DPPC as well as to monolayers consisting of cationic DODAB. The adsorption behavior of these very short nucleic acids differ significantly from the adsorption process for longer nucleic acids as for example a 2000 base pairs long ds DNA ($dsDNA_{2000}$) which has been used as a reference system[3]. The presence of $ssRNA_{10}$ significantly changes the compression isotherm of both DPPC and DODAB monolayers.

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Abstracts
– Lipid biophysics –

O-626

Phosphoinositides in nuclear membrane assembly

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Membrane fusion is a universal phenomenon occuring in many cellular functions. During fertilisation, the nuclear envelope (NE) assembles to provide the pronucleus with an active envelope. A cell-free system mimicking the *in vivo* formation has been developed to study molecular mechanisms involved in membrane fusion: NE membrane vesicles (MVs) from sea urchin fertilised egg extracts, fuse at the surface of a demembranated sperm nucleus. This process is initiated at nuclear envelope remnants (NERs) in the presence of ATP and GTP. The MVs can be divided in two main populations: MV1 and MV2. MV2 has a classical lipid composition while MV1 is enriched in phosphoinositides (PIPs: PI, PIP, PIP_2 and PIP_3). NERs have an unusual lipid composition, enriched both in cholesterol and PIPs. Physicochemical properties of the PIPs were investigated as a function of pH and temperature (T) using NMR, SAXS and DLS to map out their phase state. PIPs-water dispersions are observed in lamellar, hexagonal or isotropic phases depending on T and pH. In parallel, model membranes mimicking MV1 and NERs lipid composition were studied by ^{2}H and ^{31}P NMR. MV1 modelling shows a complex behaviour of PIPs on PC membranes: they order or disorder membranes, whereas the order of $PC/PI/PIP/PIP_2$ membrane is lower than that of PC or PC/PI membranes. NERs modelling reveals that PIPs counter balance the well known ordering effect of cholesterol.

Abstracts
– Single molecule fluorescence –

P-627

HIV1 TAT peptides translocate efficiently into giant unilamellar vesicles

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Cell penetrating peptides like the HIV1 TAT peptide have the property to rapidly translocate the cell membranes and the capability to deliver a wide range of cargoes. The mechanism of the membrane translocation is still under investigation and object of considerable controversy.
We applied and single-molecule and confocal laser scanning microscopy (LSM) to study peptide-membrane interactions. Electron-multiplying CCD cameras yield images of single fluorescent molecules with a time resolution in the range of a few milliseconds only, which allows the tracking of fluorescently labelled peptides and lipids at bio-interfaces in real-time with a localization precision of a few nanometers.
We formed giant unilamellar vesicles (GUVs) from different lipid mixtures and examined their interaction with fluorescently labeled TAT peptides. We found that the passive peptide internalization process depends on lipid composition, charge of the lipid bilayer, and the ionic properties of the medium. A translocation of cationic TAT peptides was observed in membranes containing at least 20 mol% of lipids with a phosphatidyl ethanolamine or a high mol fraction of the phosphatidyl serine head group. In salt-free solution TAT efficiently bound to GUVs, however, in a physiological NaCl solution TAT binding was completely abrogated, but the peptides efficiently equilibrated across the GUV membrane.

O-628

High-resolution spatiotemporal organization of the integrin LFA-1

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LFA-1 is a leukocyte-specific integrin involved in different steps of the immune response. On monocytes, LFA-1 plays a key role in the regulation of monocyte-endothelial interaction during rolling, arrest and extravasation into the underlying tissue. *In-vivo* experiments showed that blood borne lymphocytes can 'switch' within seconds from rolling to arrest. Furthermore, TEM observations of pro-active, ligand-independent nanoclusters confirmed that affinity and clustering are complementary processes required in adhesion. Yet, the mechanisms leading to fast-switching remain obscure. In our group, we used a combination of single molecule fluorescence techniques to study the spatiotemporal organization of LFA-1 on monocytes. We performed optical nanoimaging of LFA-1 nanoclusters in relation to membrane rafts with a resolution of 70nm and accuracy of ∼3nm. In quiescent cells, LFA-1 nanoclusters do not associate with membrane rafts and diffuse freely on the membrane. Binding of the integrin to its ligand ICAM-1 induces the formation of microclusters that further associate with rafts and exhibit reduced mobility, consistent with cytoskeleton interactions. Our work highlights the markedly different spatiotemporal organization of LFA-1 that might explain its concerted action to form larger and stable platforms on the cell surface required for rapid and effective cell adhesion.

P-629

Binding of the HIV-1 NCp7 on oligonucleotides at the single-molecule level

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The nucleocapsid protein (NCp7) of the HIV-1 is a small basic protein which plays key functions in the viral life cycle. The activity of NCp7 mainly rely on its potent RNA- and DNA-chaperone activities that direct the rearrangement of numerous nucleic acid molecules into their most stable conformation. Two main features of NC's chaperone activity are its abilities to aggregate and destabilize nucleic acids. In addition, the rapid kinetics of NCp7 interaction with nucleic acids was recently proposed as another major component of NC's chaperone function based on the apparent correlation between an indirect measurement of the nucleic acid dissociation kinetics of NCp7 and its overall chaperone activity. But so far, no direct measurement of the on/off rates of NCp7 binding to oligonucleotides was performed. In the present work, we realized single molecule fluorescence resonance energy transfer (smFRET) measurements to probe the transient interactions of a TMR-labelled NCp7 with a short Cy5-labelled DNA oligonucleotide confined into nanovesicles. After confirming the efficiency of the NCp7/ODN complex encapsulation into the nanovesicles by FCS, the vesicles were tethered to the surface for immobilization. Integrity of the entrapping vesicles attached on the surface was confirmed by AFM. Finally, the association and dissociation constants retrieved from these smFRET measurements were discussed in the context of the DNA-chaperoning activity of the protein.

O-630

Optical nanoscopy: FPALM breaks the diffraction limit

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Diffraction limits resolution in optical microscopy, but many interesting biological problems occur on shorter (molecular) length scales. Recently, methods to circumvent the diffraction limit have been presented. Fluorescence photoactivation localization microscopy (FPALM) uses activation of many small subsets of photoactivatable or photoswitchable fluorescent probes (PAFPs) to generate images with effective resolution in the tens of nanometers. PAFP molecules are photoactivated, imaged, localized, and photobleached in small numbers. The process is repeated for many subsets to build up data on thousands to many hundreds of thousands of molecules. The positions of all localized molecules are used to construct an image of the sample with resolution limited not by diffraction, but by the localization precision and molecular density. Results will be shown from a variety of biological systems, including live and fixed cells expressing a variety of PAFP-tagged proteins. Bi-plane FPALM can image in three dimensions with demonstrated resolution of 30 nm x 30 nm x 70 nm. Polarization FPALM can image both molecular positions and anisotropies simultaneously with lateral resolution of ∼20-30 nm. Using these powerful capabilities, many potentially interesting biological problems can be addressed.

Abstracts
– Single molecule fluorescence –

P-631

Fast biosynthesis of GFP molecules - a single molecule fluorescence study

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Numerous studies showed that protein folding and maturation can differ substantially between *de novo* synthesized proteins and *in vitro* refolded proteins. Here we present an approach employing a two color single molecule sensitive fluorescence wide-field microscope in order to visualize surface tethered fluorescently labeled ribosomes and *de novo* synthesized GFP molecules in real time [1]. Fluorescence of co-translational folded proteins was observed from mature fluorescent GFP molecules which carry 31 additional amino acids at the C terminus remaining linked to the ribosome. Thus it was possible to co-localize fluorescence from labeled ribosomes and from GFP molecules. We demonstrate that the green fluorescence protein mutant GFP Emerald is produced with a characteristic time of five minutes. The fastest GFP molecules appeared already within one minute. Processes precedent to chromophore formation, such as polypeptide synthesis and protein folding, are fast and last not longer than one minute.

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P-632

Single-molecule FRET study of Diels-Alderase ribozyme kinetics

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RNA molecules have attracted enormous attention in recent years, and various novel roles were revealed for RNA in biological processes. Ribozymes are a class of RNA molecules capable of catalyzing chemical reactions. We have studied a Diels-Alderase (DAse) ribozyme, a small artificial 49-mer ribozyme, which is capable of catalyzing carbon-carbon bond formation between an anthracene diene and a maleimide dienophile in multiple turnovers. Single-molecule fluorescence resonance energy transfer was employed to investigate the intramolecular dynamics of this RNA molecule as a function of Mg^{2+} ion concentration. Folding into a functional state occurs via an intermediate state, and continuous fluctuations between these two states were observed on the 100-ms time-scale at the midpoint concentration of Mg^{2+} ions. An effect of substrates binding on the folding and catalytic reaction of the DAse ribozyme is in the focus of our recent research with the ultimate goal to obtain a detailed structural view of the single-molecule conformational changes that accompany the catalytic reaction.

P-633

Single molecule fluorescence microscopy of the Store-Operated Calcium channel subunit Orai1

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Store-Operated Calcium Entry (SOCE) is essential for many cellular signalling processes. The essential pore forming subunit of SOCE channels in the plasma membrane is Orai1. Here we present single molecule fluorescence microscopy of Orai1 which was performed in order to directly visualize the stoichiometry of mobile Orai1 pores. The protein was fluorescently labeled with monomeric GFP. A novel single molecule fluorescence approach, TOCCSL (Thinning Out Clusters while Conserving the Stoichiometry of Labeling), was used for the determination of the stoichiometry. This technique allows reducing the density of fluorescently labeled molecules without affecting the stoichiometry of labeling. Density reduction is achieved by completely photobleaching a defined area within the plasma membrane; non-bleached GFP-Orai1 aggregates enter the bleached region subsequently by diffusion. Our data indicate that there are different populations of Orai1 present in the cell: Most of Orai1 is located in the plasma membrane. A second population of Orai1-mGFP was found to be localized in intracellular vesicles. A significant fraction of the plasma membrane Orai1 exhibits a diffusive movement. We found by analyzing the bleaching characteristics of single Orai1-mGFP aggregates that in resting cells mobile Orai1 is predominantly dimeric.

P-634

Unraveling the membrane protein dynamics in living cells

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Membrane proteins play a fundamental role in intra- and inter-cellular functions. In particular, the proteins lateral mobility in the fluid membrane environment is crucial for the regulation of several mechanisms, as receptor-mediated signal transduction and establishment of immunological synapses. These mechanisms are controlled through protein crowding and reduced lateral diffusion, which induce macromolecular associations and limit the application of conventional single molecule fluorescence techniques. To measure proteins mobility on living cells membrane, we developed a fluorescent correlation spectroscopy (FCS) setup in which the sample illumination is obtained through near-field scanning optical microscopy (NSOM) probes. The use of NSOM probes is particularly suited for the observation of dynamics on the cell membrane and overcomes the drawbacks of other techniques. In fact, through a shear-force-based position control, the probe is kept at a fixed distance from the membrane and its sub-wavelength aperture (∼100 nm) reduces the illumination area, allowing the observation of highly crowded regions of the membrane. On the basis of preliminary results, the NSOM-FCS is expected to provide an additional insight on the proteins trafficking at the membrane level. The technique also presents several potential developments, as the further reduction of the illumination area and two-colors correlation.

Abstracts
– Single molecule fluorescence –

P-635

Fluorescence correlation spectroscopy studies of lysozyme partition to phospholipid vesicles

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Binding to membrane lipids has been increasingly recognized as an important step in the aggregation and cytotoxicity of several amyloidogenic proteins [1]. In addition, it has been recently reported that membranes containing negatively-charged phospholipids can also trigger rapid amyloid-like fiber formation by a variety of several non-amyloidogenic proteins, such as cytochrome *c* and lysozyme [2]. Our study aims to elucidate the factors that govern the formation of these lipid-protein complexes. Given the importance of electrostatic interactions between the proteins and the acidic phospholipids in the putative membrane-induced protein misfolding step, it is essential to first characterize quantitatively the protein partition behavior towards liposomes prepared with variable anionic lipid content. In this study, lysozyme was chosen as a model protein and fluorescence correlation spectroscopy (FCS) was used to monitor its binding to liposomes after its conjugation to Alexa Fluor 488. Most organic dyes labelling techniques produce a mixture of populations of molecules labelled with a different number of fluorophores. The influence of this poli-dispersity of labelled molecules on the protein partition behaviour will be explored, namely the ability of the FCS technique to detect the production of non-competent membrane-binding species.

[1] Munishkina and Fink **2007***BBA* 1768: 1862-1885; [2] Zhao *et al.* **2004** *Biochemistry* 43: 10302–10307

P-636

Fluorescently labeled ATP as a probe of the outer mitochondrial membrane barrier: role of VDAC

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Fluorescence correlation spectroscopy (FCS) was applied for studying the distribution of fluorescently labeled ATP (Bodipy-ATP) in isolated mitochondria. The setup and peak intensity analysis (PIA) was described in our recent paper (Perevoshchikova et al. 2008 Biochim.Biophys.Acta 1778:2182-90). The binding of Bodipy-ATP to mitochondria was maximal in the non-energized state, whereas the addition of succinate (respiratoty substrate) or atractyloside (adenine nucleotide translocase inhibitor) led to a decrease in the binding. NADH reduced the FCS signal from Bodipy-ATP added to non-energized mitochondria more than NAD+ did under the same conditions suggesting the control of nucleotide transport through voltage-dependent anion channel (VDAC) residing in the outer membrane. Konig's polyanion also decreased the Bodipy-ATP binding to mitochondria with the effect being reduced by alamethicin or digitonin. Control experiments showed that Bodipy-ATP did not bind to liposomes showing minor role of unspecific binding. It was suggested that Bodipy-ATP in combination with FCS can be used to monitor the functional state of mitochondrial VDAC which is considered to be a principal regulator of mitochondrial function.

P-637

Developing a fluorescent redox sensor for monitoring metal-ion mediated catalysis in biosystems

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A fluorescent redox sensor is an electron photo-switching device that can be used for the characterization of the redox state of a given environment. It combines a fluorescent fragment with a redox-active unit that senses the media by a redox reaction and controls the light emitting properties of the fluorophore. Such reversible sensor can help to examine the electrochemical state and changes in biological systems during biochemical processes in real time. Recently a new fluorescent molecular sensor with a redox-active hydroquinone-unit covalently linked to fluorophore Rhodamine B was developed. (Kierat R.M. *et al.*, Bioorg. Med. Chem. Lett., 2009 - accepted). The reduced hydroquinone-form of the sensor is fluorescent while its oxidation to benzoquinone-derivative leads to a significant decrease of the fluorescence. Although the above method shows great promise for applications in biological systems, the exact mechanism of this process is not fully understood yet.

We use fluorescence spectroscopy to investigate oxidation reactions on the ensemble and single-molecule level and study kinetic rates. The proposed strategy is to use Cu (II) complex as oxidation mediator immobilized on surface via DNA linker to examine the oxidation mechanism.

O-638

New approaches to measure interactions in the live cell plasma membrane

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In my lecture, I will show examples how to obtain insights into the organization of the cellular Nanocosm by single molecule experiments. Our primary goal is an understanding of the role of such structures for immune recognition. Brightness and single molecule colocalization analysis allows us to study stable or transient molecular associations *in vivo*. In particular, I will present results on the interaction between antigen-loaded MHC and the T cell receptor directly in the interface region of a T cell with a surrogate antigen-presenting cell. In addition, we developed a method for *in vivo* micropatterning of plasma membrane proteins to measure molecular interactions. The method allows identifying and characterizing interactions between an arbitrary fluorescence labeled protein ("prey") and a membrane protein ("bait") directly in living cells. Cells transfected with a fluorescent fusion protein of the prey are plated on micropatterned surfaces functionalized with specific antibodies to the extracellular domain of the bait; the fluorescence copatterning is used as readout for the interaction. We applied this tool for the study of the interaction between CD4 – the major coreceptor for T cell activation – and Lck, an important tyrosine kinase in early T cell signaling. In addition to the well-known zinc-clasp structure, we found strong contributions of Lck membrane anchorage for the binding of the two proteins.

Abstracts
– Single molecule fluorescence –

O-639

Dynamic multiple-target tracing probes spatiotemporal cartography of cell membranes

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In order to decipher the non random and non homogeneity of the plasma membrane organization, we had performed fluorescence correlation spectroscopy measurements on live cells. This allowed us to establish the presence of nanoscale confining structures[1] and to demonstrate their implication in signaling process[2].
Complementing these studies, we present here a new analytical method, namely multiple-target tracing (MTT)[3] which takes advantage of the high resolution provided by single-molecule sensitivity to generate dynamic maps at high densities of tracked particles. Introducing deflation by subtracting detected peaks allows detecting peaks of lower intensity. We achieved an exhaustive detection of particles with performances reaching theoretical limits, and a reconnection of trajectories integrating the statistical information from past trajectories. We demonstrate the potential of this new method of analysis by applying it to the epidermal growth factor receptor labeled with quantum dots, in the plasma membrane of live cells. This has allowed us to build up a global representation of molecular dynamics in cell membranes.

1. Lenne et al 2006 *EMBO J.* **25**:3245
2. Lasserre et al 2008 *Nat Chem Biol* **4**:538
3. Serge et al 2008 *Nat Methods* **5**:687

O-640

Dissecting the molecular dynamics of cell surface receptors in immune cells using dual-color FCCS

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Dual-color Fluorescence Cross Correlation Spectroscopy (FCCS) has been used to explore the molecular dynamics at immune cell surfaces, with a particular focus towards the regulation mechanisms of natural killer (NK) lymphocytes. NK cells are critical mediators of anti-viral immunity and protectors against cancer spread. Their activity is governed by a fine-tuned balance between inhibitory and activating receptors, where Ly49A and KIR receptors represents the inhibitory ones. Their ligands are MHC class I receptors.
FCS is a technique based on the analysis of intensity fluctuations of fluorescent molecules excited by a focused laser beam. The technique offers information about molecular dynamics at the single molecular level, in the nanosecond to millisecond range. Dual color FCCS expands FCS by correlating the intensity from two different colors. By labeling two potential interaction partners with dyes emitting at different wavelengths, the amount of interaction can be determined.
Here, we will report on recent FCCS data exploring the interaction between the inhibitory receptors and their ligands, as well as different labeling strategies used to enable these measurements.

P-641

Nucleosome structural variations characterized by single molecule FRET

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The nucleosome has a central role in the compaction of genomic DNA and the control of DNA accessibility for transcription and replication. We studied the effect of DNA sequence and selective histone acetylation on the structure, stability and disassembly of the mononucleosomes. Quantitative single molecule FRET measurements between dyes attached to different parts of the nucleosome permitted us to detect the equilibrium between several subpopulations of reconstituted nucleosomes in solution. We obtained that the heterogeneity and stability of the samples are correlated with each other and influenced both by the DNA sequence and the histone acetylation. The path of the linker DNA is more sensitive to all studied effects than the DNA on the core. Intermediates of the disassembly pathway were identified and characterized.

P-642

Investigations of photobleaching in TMR-labeled virus rods

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In fluorescence spectroscopy, photobleaching is a process which leads to irreversible loss of fluorescent properties of a dye molecule, usually due to photochemical reactions. It is especially important for FCS experiments on slow-diffusion systems since for high excitation intensities it can have a strong impact on fluorescence intensity correlation function. Usually it is observed as apparent shortening of the mean diffusion time of the dye molecules.
The behavior of TMR-labeled fd-virus rods in water solution under various excitation conditions was investigated. The experiments were conducted for low (1:1) and high (625:1) TMR:virus ratios and for increasing laser intensities. The correlation function was measured in the experiments. The results were fitted using Origin software to estimate the influence of photobleaching, and compared with computer simulations. A strong effect of photobleaching was visible for rods labeled with a single dye molecule, while rods labeled with 625 TMR molecules showed little to no bleaching. A prolongation of characteristic diffusion times for highly labeled virus rods in comparison to low-labeled ones was also observed.

Abstracts

– Single molecule fluorescence –

P-643

Addressing plasma membrane structure at the nanoscopic length-scale

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There is increasing interest in a detailed understanding of the structure and dynamics of the cellular plasma membrane, primarily based on recognizing its essential role for controlling cellular signaling processes. We employed single molecule fluorescence microscopy to study diffusion of CD59, a GPI-anchored protein, in the plasma membrane of living T24 cells at sub-wavelength resolution, both on the cell body and on tunneling nanotubules connecting cells. The lateral motion of this single fluorescence labeled molecule was imaged on a millisecond time scale. Within the experimental errors, no indications for confined diffusion for CD59 on the cell body in T24 cells have been found. Furthermore by separating longitudinal and transversal mobility, we found isotropic diffusion behavior on the surface of tunneling nanotubules. In both studies we analyzed the mean square displacement as a function of the time-lag and the distribution of displacement steps. However, a closed analytical theory for these analysis is only available for the simplest models. To address a suspected diffusion process we reasoned that a full analytical description may not be required; it may well be sufficient to compare the experimental data with Monte Carlo simulations of the process. We demonstrated the working principle for this simulation based analysis for free diffusion, hop diffusion and transient binding of the tracer molecule to slowly moving receptors.

O-644

Determination of dissociation constants in live cells and organisms by SW-FCCS

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Biomolecular interactions have been measured mostly under in vitro conditions because of higher accuracy and ease of measurement. However, it has become clear that the cellular environment has an important influence on these interactions. It was therefore necessary to develop new tools to allow the measurement of interactions in cells and organisms. Recently, we have developed a modality of fluorescence cross-correlation spectroscopy (FCCS) called single wavelength FCCS (SW-FCCS), which uses one-photon excitation to excite two fluorophores with overlapping absorption but separable emission spectra. SW-FCCS has been used to determine e.g. dimer fractions of proteins in live cells. Here we aim to extend the use of SW-FCCS to cells and organisms. In the first part we determine the dissociation constants of a small Rho-GTPase (Cdc42) with an effector domain (CRIB) and two effectors (N-WASP or IRSp53). In the second part we measure the binding between Cdc42 and a scaffolding protein (IQGAP1) in dependence of their expression levels in CHO cells and in live zebrafish embryos. By using GFP/mRFP fusion proteins we can excite both fluorophores at 514 nm and detect them separately in two different wavelength channels. We quantitatively determine the dissociation constants and compare their differences in vitro, in cells, and in embryos. These experiments demonstrate that SW-FCCS is a powerful biophysical tool for the quantitation of biomolecular interactions in cells and organisms.

Abstracts
– Folding/unfolding of proteins –

P-645

Unraveling the network structure of non-linear multi-scaled multi-component networks

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The mechanical functioning of biological tissues is important from many viewpoints such as diseases, clothing and even food. The protein collagen makes up the greater part of these tissues, and is remarkable for its many uses in the body, however there are at least two other major components. One of the most interesting properties of these tissues is their non-linear behavior under stress. This behavior is essential to prevent a catastrophic failure such as an aneurysm. At least three major theories have been proposed within the past few years to explain this behavior, but have been impossible to verify.
In order to determine a correct description of the mechanical structure of the tissue we have been using cutting edge technological solutions to address the single molecules within the extra cellular matrix. This technique combines optical methods with single molecule force spectroscopy, allowing stiffness measurements over the nanoscale as well as determining the individual protein tensions within the extra cellular matrix. The results show that this method can be used to determine the network properties even in the complicated aortic wall enabling better understanding of disease states, which in this case include Marfan's syndrome and ascending aortic aneurysms.

P-646

Complete and reversible denaturation of a helical membrane protein by urea

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In vitro protein folding studies using chemical denaturants have contributed tremendously to our understanding of the folding thermodynamics and kinetics of water-soluble proteins. This is not the case for integral membrane proteins, which constitute about one third of all eukaryotic proteins and more than half of all validated and potential drug targets. Fully reversible denaturant-induced unfolding remains limited to a few β-barrel porins, whereas the much larger and more relevant class of α-helical membrane proteins has thus far evaded this approach.
We report here the first example of an α-helical membrane protein that can be unfolded completely and reversibly by a chemical denaturant: Mistic, a 110-residue protein from Bacillus subtilis [1], dissociates from detergent micelles or lipid vesicles and assumes an unfolded monomeric state on titration with urea. Using spectroscopic and microcalorimetric techniques, we exploited this unique property to provide (i) a quantitative comparison of membrane protein stability in different membrane-mimetic systems; (ii) an experimental test of controversial predictions [2] regarding the folding core of Mistic; and (iii) a convenient setup to study the spontaneous, translocon-independent membrane insertion of this unusual membrane protein.

[1] Roosild et al., *Science* **2005**, *307*, 1317. [2] Psachoulia et al., *Biochemistry* **2006**, *45*, 9053.

O-647

Single-molecule force spectroscopy investigation of the conformational equilibria of alpha-synuclein

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Alpha-synuclein (aSyn) is an abundant Intrinsically Disordered Protein (IDP) primarily located at presynaptic terminals. Mutations in the gene encoding aSyn have been linked to early-onset Parkinson's disease (PD). By means of single molecule force spectroscopy (SMFS) experiment, we show how the conformational equilibrium of monomeric wild type (WT) aSyn shifts toward more compact structures in several unrelated conditions linked to PD pathogenicity [1]. The conformational heterogeneity of pathological alpha-Syn mutants A30P, A53T and E46K has also been characterized, revealing marked differences in the conformational behaviors of the mutants with respect to WT aSyn [2]. All the mutants show a distinctively higher propensity, with respect to WT, to acquire a monomeric compact conformation that is compatible with the acquiring of beta structure. The same SMFS experimental methodology is then used to characterize the conformational behavior of WT and mutant aSyn in a variety of conditions, in an attempt to gain insight about the multiple and contrasting parameters controlling the equilibrium.

[1] Sandal M. et al, PLOS Biology 2008 6 (1) e6
[2] Brucale M. et al, ChemBioChem 2009 10 (1) 176-183

P-648

Investigating the early stages of sheep PrP oligomerization using SAXS and MD simulations

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Prion(PrP) diseases are fatal neurodegenerative diseases affecting mammals including Human and sheep.They are characterized by the accumulation of extracellular βrich fibrillar deposits of a structurally modified form (PrP_{sc}) of the cellular PrP_c.Despite the increasing interest for PrP diseases,the mechanism of PrP_c/PrP_{sc} conversion is still unknown.Studies on PrP diseases suggest that neurotoxicity arises from small pre-fibrillar oligomers.We have used a range of biophysical techniques combined with Molecular Dynamics simulations (MD) to resolve the oligomerization pathways of sheep PrP (sPrP).We have shown that under well established conditions, sPrP oligomerizes into three oligomers, which form in parallel.In addition, we have now identified the minimal region of sPrP leading to the same oligomerization profile of the entire sPrP,namely H2H3.Low resolution shapes of sPrP, H2H3 and resulting oligomers have been determined by Small-Angle X-ray Scattering.Time-resolved studies have been used to follow the oligomerization of sPrP and H2H3 monomers into the oligomers.The conversion of $sPrP_{sc}$ at the molecular scale was studied by MD.Simulations of the H2H3 region recreating experimental conditions revealed a complete unfolding of H2 helix followed by H3 helix.These crucial steps are followed by the formation of β-sheet structures leading to a stable βrich double hairpin structure.

Abstracts
– Folding/unfolding of proteins –

O-649

Unexpected scaling laws in the mechanical unfolding of single protein molecules

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It is a question of fundamental importance to understand the response of proteins to a stretching force, particularly in the case of mechanically active proteins, such as those in muscle fibers. We aim to understand how the structure and topology of a protein affect its resilience to external forces and presumably its function. Owing to recent advances in single molecule force-clamp spectroscopy using the Atomic Force Microscope (AFM), we are now able to probe the structure and dynamics of single proteins under a constant stretching force by measuring their end-to-end length over time. The probability distribution of unfolding times at a given force allows us to estimate the strength of the protein in terms of a characteristic energy barrier, while the shape of the distribution provides a window into the microscopic mechanism by which the protein breaks apart. Here we show a novel scaling of the unfolding kinetics with the stretching force, which deviates significantly from the currently accepted Bell model. Instead, we propose a physical picture for forced unfolding that is analogous to the mechanics of fracture.

P-650

Influence of protein surface coverage on lysozyme interaction with anionic liposomes

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Recently, negatively-charged membranes were reported to catalyze amyloid fiber formation by amyloidogenic peptides/proteins and also to induce formation of "amyloid-like" fibrils by nonamyloidogenic proteins. Here, we used an approach which combines steady-state and time-resolved fluorescence measurements to obtain structural information about these supramolecular assemblies and to gain insights about the factors that control their formation. By exploring a wide range of lipid concentrations, the interaction of Alexa488-lysozyme with phosphatidylserine-containing membranes was found to be a complex multi-step process, critically dependent upon the protein-to-lipid molar ratio (P/L) used. Upon increasing the total lipid concentration in solution, there was a balance between an increased overall protein binding to the lipid vesicles and a progressive protein dilution on the membrane surface. As the surface potential of the vesicles decreased upon increasing the protein interfacial coverage of the liposomes, the protein binding mode was found to switch from a peripheral binding of lysozyme to the anionic headgroups (at low to intermediate P/L) to a partial insertion of the basic protein into the hydrophobic core of the membrane (at a high P/L). It is hypothesized that disruption of the protein tertiary structure might be a stepwise process beginning with loosening of the structure caused by its deeper insertion in the membrane bilayer.

P-651

The role of histidine 148 in the unfolding of GFPMut2

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The puzzle of the anomalously long denaturation kinetics of Green Fluorescent Protein (GFP) mutants still is largely unveiled. In this study we have followed the effect of mutation H148G on the stability of GFPMut2 (Mut2G) in the presence of guanidinium hydrochloride (GdnHCl). Different techniques of fluorescence spectroscopy have been employed in order to obtain information concerning the unfolding event: time resolved fluorescence, fluorescence correlation spectroscopy (FCS), and fluorescence anisotropy.
The substitution of the histidine with glycine affects protein stability versus pH: in particular Mut2G kinetics is not pH dependent and at basic pH values the protein is less stable. The fluorescence properties (quantum yield, lifetime) and the rotational correlation time are unchanged during the unfolding dynamics, while the number of fluorescent proteins decreases exponentially. An extrinsic probe, bound to cysteine 48, has been employed in order to gain more insights on the unfolding process, monitoring the stability of a different region of the protein. In particular, it has been found that a softer region is present around cysteine 48 in both GFP variants, showing that the unfolding process does not follow a simple two step mechanism.

P-652

Beta amyloid peptide Abetapy3-42 shows a faster aggregation kinetics than Abeta1-42

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We test directly the differences in the aggregation kinetics of three important beta amyloid peptides, the full-length Abeta1-42 and the two N-terminal truncated and pyroglutamil modified Abeta*py*3-42 and Abeta*py*11-42, found in different relative concentrations in the brains in normal aging and in Alzheimer disease.[1] We find by following the CD signal and the ThT fluorescence of the solution in phosphate buffer, a substantial faster aggregation kinetics for Abeta*py*3-42. This behavior is due to the particular sequence of this peptide which is also responsible of the specific oligomeric aggregation states, found by TEM, during the fibrillization process which are very different from those of Abeta1-42, more prone to fibril formation. In addition Abeta*py*3-42 is found here to have an inhibitory effect on Abeta1-42 fibrillogenesis, coherently with its known greater infective power. This is an indication of the important role of this peptide in the aggregation process of beta-peptides in Alzheimer disease.

1.
Schilling, S.; Zeitschel, U.; Hoffmann, T. et al. Nature Medicine 2008, 14, 1106-1111.

Abstracts
– Folding/unfolding of proteins –

O-653

Probing dynamics and folding of intrinsically disordered proteins by single molecule fluorescence

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Intrinsically disordered proteins are increasingly found to play major roles in cell biology and disease. We are utilizing single-molecule fluorescence methods to probe these complex and highly dynamic molecules, allowing more direct measurements of structural distributions and dynamics, while avoiding loss of information due to ensemble averaging. In one example, we investigated the structural dynamics of Sup35-NM, whose regulatable amyloid formation is believed to have functional significance in yeast. Using a combination of single-molecule FRET as a molecular ruler, coincidence to interrogate intermolecular interactions, and correlation analysis to probe conformational dynamics, we showed that the monomeric protein populates an ensemble of compact and rapidly interconverting conformations. A particularly interesting feature of intrinsically disordered proteins is that they are relatively unstructured in isolation, but can gain stable structure by interaction with binding partners. In this context, we used single-molecule fluorescence to characterize the complex folding pathway for the Parkinson's-related IDP alpha-synuclein induced by binding to a lipid-mimic. This combined single-molecule fluorescence methodology provides a powerful approach for detailed studies of the coupling of folding and dynamics with interactions and biology of this important class of proteins.

P-654

Protein structural changes occurring in flows

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Stresses inherent to viscous fluid flow have previously been associated with protein unfolding, although structural changes have not been well documented as a function of relevant hydrodynamic parameters. We have used Raman Spectroscopy to monitor the structure of various protein solutions in situ for multiple flow scenarios within a concentric cylinder fluidic device (1). The flows, which ranged from circular Couette to wavy Taylor-Couette flow, were characterised experimentally using Particle Image Velocimetry. Several proteins were observed to change conformation when exposed to these flows, although the nature of these changes was protein specific. Shearing hen egg white lysozyme in water altered the protein backbone structure, while similar shear rates in a 95% glycerol, 5% water solution affected the solvent exposure of the side chain residues near the exterior of the α-domain. The solvent-dependent response may be due to the flow topology, viscous stress, or the surface hydration properties. Comparison of spectra acquired at different time points, including before and after flow, confirmed that the observed changes are reversible and independent of fluid stress exposure time.

(1) Ashton, et al. *Shear induced unfolding of lysozyme monitored in situ.* **Biophysical Journal**, in press.

P-655

Mimicking metastasis by a novel microfluidic approach

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There is increasing evidence that cancer metastasis shares commonalities with thrombosis. The von-Willebrand-Factor (vWF), a mechanical active blood clotting protein appears to be a particular potent candidate to bridge the gap between clotting and cancer extravasation. Modeling the crucial physiological conditions of the blood circulatory system, for an in situ study of blood clotting-metastasis connections is not only, absolutely necessary, but also a challenging task. Here, we present acoustically driven flow as a novel microfluidics method for mimicking the blood flow. This method enjoys very beneficial advantages of possibility of handling very little volumes of fluids, together with freedom to model most of the geometries present in our microcirculatory system. One technologically challenging, yet physiologically important factor, is the hydrodynamic condition in a bifurcated vessel, where complex shear profiles arise. We present a model to mimic these conditions and discuss the impact of hydrodynamics on vWF mediated cancer cell adhesion in bifurcated vessels of our microcirculatory system.

P-656

Structural Arrangements that Support Lipid Transfer in the Cholesteryl-ester Transfer Protein (CETP)

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Studies focused on the thermodynamic and kinetic analysis have demonstrated that transfer of neutral lipids such as cholesterol esters through an aqueous phase is a highly costly biophysical event. Therefore, nature has developed a series of lipid transfer proteins such as the cholesteryl-ester transfer protein (CETP) designed to efficiently lower the energy barrier for transfer of cholesterol-esters between lipoproteins through an aqueous environment. Employing circular dichroism we evaluated the secondary structure stability of a small peptide derived from the carboxy-end of CETP (Helix Y) in a wide range of pH's. The percentage of α-helix is diminished only at extreme temperatures and acidic pH's in a reversible way. We report that while a mixture of phosphatidylcholine/cholesteryl-ester forms large aggregated particles independently of pH, inclusion of helix Y to the mixtures close to neutral pH's allows the formation of small micellar-like structures confirmed by dynamic light scattering and electron microscopy. These results suggest that helix Y when close to physiological pH values presents the ability to organize a micellar structure around itself. This type of organization allows the process to dramatically lower the energetic barrier for lipid transfer through aqueous media, phenomenon directly related with the facilitation of lipid transfer between lipoproteins.

Abstracts
– Folding/unfolding of proteins –

O-657

Super stabilization of alpha-lactalbumin: a calorimetric study

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We present an extensive calorimetric study of bovine alpha-lactalbumin for various Ca++ content. Equilibrium DSC raw data are analyzed and the melting temperature Tm, the specific heat jump DeltaCp, the heat of unfolding DeltaHm are directly extracted. The binding of calcium on the native (N) state greatly stabilizes the protein, essentially by the enthalpic difference between the unfolded (U) and N states. We show that subsequent addition of calcium in the mM range stabilizes further the N state. The equilibrium calorimetric measurements are completed with out of equilibrium stopped flow refolding kinetics by CD spectroscopy performed at different temperature and Ca++ concentrations. We discuss the possible stabilization mechanisms compatible with our measurements.

P-658

Lipid and protein organization of Hepatitis B antigen characterized by fluorescence spectroscopy

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Hepatitis B surface antigen (HBsAg) particles are 20 nm lipoprotein particles, mainly composed of the major S surface viral protein containing 13 Trp residues and yeast-derived lipids. Since the structure of these particles is still missing, we further characterized them by fluorescence techniques. FCS indicated that the particles diffuse mainly as monomers and contain about 80 proteins per particle. Fluorescence quenching and time-resolved fluorescence experiments showed that the fluorescence signal is largely dominated by the Trp residues at the protein surface. Moreover, time-resolved anisotropy measurements indicate that the protein motion is restricted and that the surface Trp residues exhibit both local and segmental motions. The lipid part of the particles has been studied by environment sensitive 3-hydroxyflavone probes and viscosity-sensitive DPH-based probes, and compared to lipid bilayers and low density lipoproteins (LDLs), taken as models. The results suggest that the lipid part of HBsAg is closer to LDLs than to model lipid bilayers.

P-659

Linker average hydrophilicity as a tool to discriminate between extended and non-extended calcium binding proteins

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The EF-hand calcium binding proteins (CaBPs) may exist either in an extended or a compact conformation, sometimes correlated with their functions. For the CaBPs with know structure and function, calcium sensors are usually extended and calcium buffers compact, hence the interest to predict the form of the protein starting from its sequence. In this study we used two different procedures, the SOSUIdumbbell algorithm and a novel procedure that is based on the linker average hydrophilicity (LAH). The two procedures were tested on 17 known-structure CaBPs and then applied to 59 unknown-structure centrins. The SOSUIdumbbell algorithm yielded the right conformations for 15 of the 17 known-structure proteins and predicted that all centrins should are compact. The LAH procedure discriminated well between the extended and non-extended forms of all the known-structure CaBPs and it reflected well the phylogenetic classification of centrins being a simple and powerful means to discriminate between extended and non-extended forms of CaBPs. Only few residues that constitute the linker are responsible for the form of the CaBP, showing that this form is mainly governed by short-range interactions. (http://u759.curie.u-psud.fr/modelisation/LAH)

P-660

Molecular dynamics study of amyloid beta-peptide unfolding

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Amyloid β-peptide (Aβ) is a 39-42 amino acid polypeptide and known to aggregate and form insoluble amyloid fibril, which is regarded as a primary cause of Alzheimer's disease (AD). The discovery of practical and effective treatments and drugs for AD has been waited eagerly. In a recent experimental study, it was suggested that stabilization of the helical conformation of the Aβ middle region, which strongly favors collapsed coil formation in the extracellular environment, would reduce the Aβ fibril formation. Based on the experimental evidence, inhibition of the unfolding of the Aβ α-helix can be a forceful strategy to repress the Aβ fibril formation resulting in prevention of AD. However, the detailed mechanism of the unfolding of the Aβ α-helix has remained unclear, because the X-ray or the NMR structure of the Aβ α-helix in aqueous solution has not been reported due to its instability. The aim of this study is to find effective ways to inhibit the unfolding of the Aβ middle region, which is a prerequisite for the Aβ fibril formation. In this study, we attempted to elucidate the molecular mechanism of the Aβ unfolding by molecular modeling and molecular dynamics (MD) simulations. The MD simulations were performed for α-helical structures of a wild-type Aβ(13-26) model and a mutant Aβ(13-26) model.

Abstracts
– Folding/unfolding of proteins –

P-661

Self-assembly of transmembrane domains in CPT1: Role of GXXXG(A) motif in possible channel formation

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Carnitine palmitoyltransferase 1A (CPT1A), a membrane protein that controls the rate of oxidation of long-chain fatty acids, is of key importance in diabetes and has recently been reported to exist as an oligomer in vivo. We have investigated full-length CPT1A and find that the protein exists as a hexamer in liver mitochondria. Using mutants of CPT1A expressed in yeast mitochondria, we have localised key protein interactions in the hexamer to the transmembrane (TM) domains of the protein. Detailed study of the TM domains in isolation, in both E.Coli membranes and detergent micelles, demonstrated that while TM1 shows little self-assembly, TM2 displayed significant self-association. Biophysical analyses of a TM2-derived synthetic peptide revealed oligomerization behaviour identical to native CPT1A in mitochondria, providing a strong link between TM2 helix-helix interactions and CPT1A hexamer formation. This is significant in light of a recent suggestion that CPT1A oligomerization may lead to formation of a channel in the mitochondrial outer membrane through which acylcarnitine gains access to the inter-membrane space. Our data supports this new theory, and we go on to demonstrate experimentally the structural determinants of hexamer (channel) formation, specifically GXXXG(A) motifs in the TM domain which pack favourably in the hexamer and stabilize the oligomer.

P-662

The role of transmembrane domain interactions in the kinetics and folding of CPT 1

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Carnitine palmitoyltransferase I (CPT 1) enzymes are polytopic integral membrane proteins in the outer membrane of mitochondria (OMM). CPT 1 controls the rate of entry of long-chain fatty acids into the mitochondrial matrix for β-oxidation. The two catalytically active isoforms, CPT 1A and CPT 1B, are different in their inhibitor binding kinetics and structure (interaction between N- and C-segments, interactions of transmembrane domains (TMD)). It has been suggested that inter- and intramolecular TMDs interactions are important for CPT 1A, but not for CPT 1B function. CPT 1A has also been implicated in formation of oligomeric complexes through its TM segments.
The study of TM helix-helix interactions in CPT 1 isoforms could lead to a better understanding of their function and inhibitor binding kinetics, and will contribute towards the design of pharmacological strategies aimed at modulating the activities of CPT 1 enzymes in conditions such as diabetes. To investigate the ability of the TMD in CPT 1 to self-associate and the order of any oligomers formed, several biochemical and biophysical techniques have been used.
We found the self-association of rCPT 1A TMDs (TM1, TM2) to be different as measured using the *in vivo* TOXCAT assay. Chemical cross-linking and analytical ultracentrifugation studies demonstrated formation of both trimers and hexamers by the rCPT 1A TM2 peptide. These results provide further evidence that TM2 plays role in formation of a channel in the OMM.

P-663

Amyloidogenic and conformational properties of ProIAPP and IAPP in the presence of lipid bilayers

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The islet amyloid polypeptide (IAPP), which is considered as the primary culprit for β-cell loss in type 2 diabetes mellitus patients, is synthesized in the β-cells of the pancreas from its precursor, the pro-islet amyloid polypeptide (proIAPP). ProIAPP is co-processed in the secretory granules and co-secreted to the extracellular matrix together with insulin as IAPP. Here, we compare the amyloidogenic and conformational properties of proIAPP and IAPP in the presence of lipid membranes, which have been discussed as loci of initiation of the fibrillation reaction. The two peptides show an enhanced amyloidogenic propensity in the presence of negatively charged membranes and similar secondary structural properties. However, proIAPP shows a much less amyloidogenic propensity, probably due to the increased net charge on proIAPP, compared to IAPP. Unlike IAPP, proIAPP forms small oligomeric structures at the lipid interface, having heights of $\sim$3.5 nm. This morphological distinction can be attributed to the presence of the pro-region, flanking the amyloidogenic IAPP. The addition of proIAPP to IAPP marginally delays IAPP fibrillation, probably by interfering with the interaction between amyloidogenic IAPP cores of distinct IAPP molecules. Thus, it appears reasonable to speculate that the pro-region of the proIAPP could serve to delay the fibrillogenesis of IAPP at negatively charged lipid bilayers.

P-664

Protein unfolding/refolding in cellular compartments: application of luciferase constructs

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Our studies show that a reporter enzyme, firefly luciferase, can be used for evaluation of the stress-induced proteotoxicity within different cellular compartments such as the nucleus, cytoplasm or mitochondria. In transfected mammalian cells, firefly luciferase is localized in microsomes. We engineered plasmid constructs encoding luciferase with inserted specific sequences that ensure its cytoplasmic or intranuclear, or intramitochondrial localization. In addition, we fused luciferase to the green fluorescent protein (GFP) that enables to visualize patterns of the compartment-targeted product. Using such GFP-labeled constructs we had a possibility to monitor protein unfolding, aggregation and refolding in the cytoplasm, nucleus and mitochondria of transfectants exposed to either stressful conditions. GFP-luciferase expressed in mammalian cells behaves as a relatively labile protein which can undergo reversible unfolding and aggregation in response to heat shock, ATP depletion or action of toxic agents. In the case of cell recovery, refolding of denatured luciferase is carried out at the chaperone machine. We explored unfolding/refolding of GFP-luciferase in the cytoplasm, nucleus and mitochondria of ischemia-stressed rat cardiac cells and in several cancer cell lines treated with hyperthermia or some chemotherapeutic drugs. The results obtained have revealed intriguing correlations between the proteotoxic impact within either compartment and the viability of treated cells.

Abstracts

– Folding/unfolding of proteins –

O-665

Thermal unfolding of small heat shock protein Hsp22

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Small heat shock protein Hsp22 was predicted to belong to the family of intrinsically disordered proteins. One of the features of these proteins is that they do not demonstrate cooperative thermal transitions on heating. We applied different methods (DSC, FTIR and intrinsic tryptophan fluorescence) to investigate the thermal unfolding of Hsp22. DSC results have shown that thermal denaturation of Hsp22 begins from 25^oC and occurs, with very low cooperativity, within a broad temperature region (up to 80^oC and above). The thermal unfolding of Hsp22 is fully reversible. The FTIR data show that heating of Hsp22 from 20 to 70^oC results in complete disappearance of α-helices (from 8–12% to 0) and the decrease in β-sheets content from 42 to 30%. Studies on the temperature dependences of tryptophan fluorescence have shown a significant red shift of the spectrum. These changes occurred within temperature region from 30 to 80^oC with midpoint at $\sim 56^o$C. Probably, this transition can be explained by destruction of β-sheets around Trp96, the only Trp residue of the α-crystallin domain of Hsp22 (other 3 Trp residues of Hsp22 are localized in the N-terminal domain). The data obtained confirm the suggestion that Hsp22 is a protein, whose significant part is intrinsically disordered. We propose that, on heating, the α-crystallin domain containing β-sheets melts at higher temperature than the N-terminal domain containing the most of α-helices.

P-666

Folding dynamics of peptides studied by time-resolved infrared spectroscopy

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Peptides are ideal model systems to study protein folding mechanisms. IR techniques provide both the necessary time resolution as well as the structural sensitivity. We initiate rapid heating by laser-excited ns temperature jumps ($\sim 10°$C) and study fast ns-to-μs relaxation dynamics [1]. The dynamics of the α-helix to random coil transition of polyglutamic acid was analyzed under reversible folding/refolding pH-conditions. The observed relaxation kinetics allowed separation of the folding and unfolding process with additional use of FTIR measurements in thermal equilibrium. Site-specific dynamics were monitored by use of isotopic labeling for a β-hairpin peptide whose conformation is stabilized by a hydrophobic core. Various single and cross-strand isotopically labeled variants were analyzed. The isotope-edited kinetics show variations in local structural stability of the hairpin backbone. Our data support a multistate dynamic behavior, and the site-specific kinetics are consistent with a hydrophobic collapse hypothesis for hairpin folding [2].

[1] C. Krejtschi, R. Huang, T.A. Keiderling, K. Hauser, Vibr. Spec., **2008**, 48, 1
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O-667

Exploring intrinsic disorder of unstructured membrane proteins by surface polymer physics

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Intrinsically unstructured proteins (IUPs) are considered as a separate class within the protein world because they lack a well-defined folded structure. Because IUP's function is indeed directly linked to structural disorder, they are assumed to be natively unfolded. Several physicochemical techniques are available to discriminate the degree of disorder. However a clear structural classification is still lacking. In this context, polymer physics emerges as a powerful tool for getting inside on the conformational abilities directly related to structural disordered of IUPs. In the present contribution, surface pressure and ellipsometry experiments in conjunction with polymer physics have been used to infer structural data in terms of molecular conformation and flexibility of a membrane protein essential for bacterial division, ZipA. This protein has been pointed to posses a high molecular flexibility and to adopt a random coil conformation.

P-668

Investigation of flexible loop role in structure and thermodynamic stability of firefly luciferase

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Protein folding, like any chemical process, consists of two fundamental components, thermodynamics and kinetics, which determine the stability of the folded state and the pathway of folding, respectively. These processes are currently too difficult to be solved *de novo* by purely computational methods. Experimental evidence is required to simplify the problem via protein engineering .In this research, the wild type firefly luciferase (*Photinus pyralis*) and some of its mutants were over expressed and purified. Then their unfolding thermodynamics were examined, using circular dichroism and conventional fluorescence measurements. The unfolding equilibrium constant were measured over a complete rang of denaturant conditions. The measurements were shown structural and physico-chemical changes between wild-type and mutant proteins.

Abstracts
– Folding/unfolding of proteins –

P-669

Cold denaturation of Yfh1 offers the clue to understand the effect of alcohols on protein stability

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Although alcohols are well known to be protein denaturants when present at high concentrations, their effect on proteins at low concentrations is much less well characterized. Here we present a study of the effects of alcohols on protein stability using Yfh1. Exploiting the unusual property of this protein of undergoing cold denaturation around 0 °C without any *ad hoc* destabilization, we determined the stability curve on the basis of both high and low temperature unfolding in the presence of three commonly used alcohols: trifluoroethanol,ethanol methanol. In all cases, we observed an extended temperature range of protein stability as determined by a modest increase of the high temperature of unfolding but an appreciable decrease in the low temperature of unfolding. We suggest that alcohols, at low concentration and physiological pH, *stabilize* proteins by greatly widening the range of temperatures over which the protein is stable. Our results also clarify the molecular mechanism of the interaction and validate the current theoretical interpretation of the mechanism of cold denaturation.

P-670

Structural characterisation and epitope binding activity of three meningococcal vaccine components

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The sensitivity to protein's secondary structure and progress in computational calculations have made Circular Dichroism (CD) an attractive technique to explore the optical properties of three promising vaccine candidates to *Neisseria meningitidis*. Clinical batches of a C-term deleted form of NadA (Genome-derived Neisseria Antigen - GNA1994) and the fusion proteins GNA2132-1030 (FP-1) and GNA2091-1870 (FP-2) were therefore studied. Increases in temperature and denaturant concentration on secondary structures and folding/unfolding profile were monitored by CD in the far and near UV regions and complemented by Trp fluorescence spectroscopy data. Furthermore, epitope conformational changes on binding activities to immune-sera were investigated. The calculated secondary structure content was in broad agreement with the available predicted or solved protein structures. Upon temperature incubation, a structural transition from a highly α-helical NadA to a more unordered conformation, with a mid point at ~40°C, was observed. FP-1 and FP-2 maintained their conformation up to 50°C or 6M GuHCl in the case of FP-1. Unfolding was not always reversible. Reductions in binding to monoclonal Ab titrated along with increasing unfolding. Folding/unfolding studies have proven useful in better understanding the solution behaviour and extent of folding of proteins.

P-671

Effects of carbonylation on the stability of proteins

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Proteins frequently become irreversibly modified by carbonylation, a process of introducing the carbonyl group (carbon monoxide) in a reaction with reactive oxygen species (ROS) such as superoxide, peroxide or ozone. The main targets for carbonylation in proteins are amino-acid side chains of lysine, arginine and proline. Products of carbonylation are aminoadipic semialdehyde from lysine (ASA) and glutamic semialdehyde (GSA) from arginine and proline. Importantly, carbonylated proteins are marked for proteolysis by the proteasome, but can escape degradation and form aggregates that can be cytotoxic. Carbonylation increases with the age of cells and it is associated with ageing and age related disorders such as Alzheimer's disease, Parkinson's disease and cancer. We have used the molecular dynamics method to study the stability of carbonylated proteins villin headpiece and ubiquitin. Simulations were run after mutations of arginine, proline and lysine into GSA and ASA had been performed. In addition, we have used thermodynamic integration on lysine, arginine, proline, ASA and GSA residues in order to estimate their solvation free energy (related to relative hydrophobicity and hydrophilicity). Our results suggest that carbonylation markedly decreases the overall stability of proteins, and that one potential reason for that may be a disruption of the balance between hydrophilic and hydrophobic regions in the protein.

P-672

Immobilization of encapsulated single protein molecules: A tool to study protein folding

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The protein folding mechanism is the missing link in the biological flow of information from the DNA to its specific function. Since most of proteins within a cell consist of more than one domain studies on this protein class are of major importance. It is a common feature of multidomain proteins to aggregate under refolding conditions, which hinders a refolding. Molecular encapsulation of single molecules prevents aggregation. By immobilizing the nanocapsule the observation period in a wide field microscope will be extended, so that slow or rare folding events can be detected. A major goal of this study is to investigate polymeric vesicles with respect to their suitability for protein folding studies [1]. Polymer vesicles maintain their structural integrity even under harsh unfolding conditions. Furthermore the nanocontainer proved to be permeable to guanidinium hydrochloride. Using encapsulated phoshoglycerate kinase, labeled with ATTO-655, a dye which experiences fluorescence quenching by photo-induced electron transfer (PET) with tryptophans, we demonstrate the remarkable properties of polymeric nanocontainers. Applying PET as a folding probe we detected multiple unfolding/refolding transitions of single proteins.

[1] Rosenkranz et al. ChembioChem, 10 (4), 702-709, 2009

Abstracts

– Folding/unfolding of proteins –

P-673

Rubredoxin mutant RdA51C unfolding dynamics

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Rubredoxins are a class of iron-containing proteins whose biological role on electron transfer processes and metal binding is still unclear. The unfolding dynamics of the rubredoxin mutant RdA51C from the mesophile Desulfovibrio vulgaris (Dv) was studied on the temperature range from 25°C to 87°C and along time at 87°C. Resonance Energy Transfer (RET) was used to determine the donor (D; Trp37) to acceptor (A; 1,5-IAEDANS) distance. From 25°C to 87°C the D-A distance increased 4 Å. However the random coil expected D-A distance was only achieved after heating the protein solution during 2.5 hours at 87°C. From UV-Vis absorption data it's clear that this protein is capable of maintaining its iron-sulfur center at 90°C. By melting the protein at the same temperature all iron-centers disintegrate and the protein unfold after 2.5 hour. The Trp37 fluorescence also shifts 13 nm to the red reflecting the partial exposition of the indole ring to the solvent. From fluorescence and anisotropy decay curves a breathing type movement of the protein structure was observed between 30°C to 60°C without lost of significant secondary structure. This structure flexibility should play an important role on the thermal stability of the DvRd

P-674

Catalytic power of partially denatured enzymes: implementation of molten-globule-like states

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Impact of nonspecific moderate denaturants, urea and DMSO, on the kinetic (functional) and thermodynamic (stability) patterns of a hydrolytic enzyme, α-chymotrypsin (α-CT) has been investigated. Furthermore, an impact of urea and GuHCl in combination with of temperature on the kinetic pattern of carboxypeptidase A has been examined. For the case of α-CT, in particular, we have observed about tenfold increase of the apparent Mickaelis constant when going from 0 to 6 M urea (25 ^{o}C), whereas the value of catalytic constant remained almost unchanged, indicating that the protein is not unfolded even under those severe conditions. The matching microcalorimetric experiments revealed that both the temperature-induced melting temperature and transition enthalpy decrease gradually with the increase of the additive concentration. With 6 M urea the peak-shaped calorimetric feature disappears totally. However, catalytic power of α-CT was preserved owing to its catalytic constant. For other studied enzyme/substrate/denaturant arrays diverse kinetic peculiarities due to MGLS have been observed.

P-675

Kinetics of intramolecular contact formation in a beta-hairpin peptide

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The experimental characterization of the elementary conformational steps constituting the protein folding pathway remains a big challenge. Quenching of the triplet state of tryptophan by close contact with cysteine has been shown to provide a new tool for measuring the rate of intramolecular contact formation - one of the most elementary events in the folding process - in peptides and proteins using only natural probes. Here we show an extensive study on a stabilized mutant of the second beta-hairpin of GB1 domain. Steady state fluorescence and kinetics of contact formation between a natural tryptophan and a cysteine added to the C-terminal are measured for different temperatures and solvent conditions. We separately address the contributions of different structural elements to the overall stability of the hairpin. We extract kinetics parameter for contact formations in the unfolded state, formation of the hydrophobic core and tails pairing in the folded state. By means of a fragment peptide terminated with a tryptophan and a cysteine, we also estimate the structural propensity of the turn region. The data coherently combine with a simple model previously developed to describe the dynamics of unstructured chain [Biophys. J. 96, 1515 (2009)], here modified with the addition of attractive interactions between specific residues.

P-676

Measuring protein-protein conformational changes using dual polarisation interferometry

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Dual Polarisation Interferometry (DPI) is a surface analytical technique capable of dynamically measuring biophysical parameters of conformational change in biomolecular interactions. The technique measures three key parameters, namely layer thickness, layer density (RI) and mass, thereby enabling the resolution of conformational changes involved during binding. A number of different applications are presented.
Protein-protein interactions: Understanding the biophysical nature of protein interactions can deliver insights into the mechanisms by which proteins interact, thereby elucidating protein function. DPI enables correlation between binding affinity and conformational change, greatly enhancing the study of structure-function relationships.
Lipid Layers: The birefringence mode of DPI can be used to study the formation of lipid bilayers and biomolecular self-assembly. It is possible to use a combination of bilayer refringence and mass to study phase transitions associated with protein or peptide binding to the lipid bilayer. The individual stages of adsorption, absorption and micellisation can be distinguished.
Carbohydrate Interactions: DPI uses a planar glass surface and a wide range of coupling chemistries. A carbohydrate-specific surface can be used to study a wide range of biomolecular interactions, such as lectins, acidic proteins, extracellular matrix signaling and interactions with complex membranes.

Abstracts
– Folding/unfolding of proteins –

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Phase diagram for lysozyme - water - ethanol solution

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Aggregation dynamics of proteins plays an important role in molecular biology and medicine as it permits explanation of several disease states. An interesting problem is to find out in which conditions the interactions of the protein molecules lead to formation of ordered structures and in which to disordered ones.
In this study, dynamic light scattering, circular dichroism and also Congo red dye experiments were performed to analyze various structural states of lysozyme induced under different concentration of ethanol solvent. At low ethanol concentration the attractive interaction between the protein macromolecules dominate. After addition of more ethanol solvent, the translational diffusion coefficient was much smaller than that for lysozyme solution at zero ethanol concentration. It can be explained by the structural transformation of the polypeptide chain leading to a partially folded conformation needed for oligomerization and fibrillation process. On the basis of the CD experiments we concluded that the ethanol solvent induces changes in secondary structure of lysozyme solution. On addition of 75% v/v ethanol solution the intramolecular hydrogen bonds were destabilized. Above this ethanol concentration, β – sheet were the dominant secondary structure of lysozyme in solution. The phase diagram illustrating the formation of: monomers, oligomers at various structural states, protofilament formation state, protofilament and amyloid fibrils was constructed.

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Dielectric method for measuring glass transition and denaturation temperatures of hydrated proteins

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The flexibility of protein structures is important in allowing the variety of motions, covering a wide range of magnitudes and frequencies, essential to biological activity. High frequency dielectric measurements can be used to study the flexibility of proteins by probing the relaxation of dipolar constituents of their structures. Hydrated proteins exhibit a broad dielectric loss extending over the frequency range from 1MHz to 10GHz which can be decomposed into a number of constituent dispersions. One of these dispersions, with a relaxation time of ∼18ns, has been attributed to the relaxation of protein backbone peptide groups in the protein interior. In the work reported here, this dielectric dispersion was investigated as a function of temperature for the enzyme Glucose Oxidase. Two critical temperatures were identified as the glass transition and denaturation temperatures, both of which were found to decrease with increasing protein water content. The results were consistent with a scheme in which the hydrated glassy protein undergoes a change in structural mobility at the glass transition temperature and experiences an irreversible change in conformation at a higher denaturation temperature. Both glass transition and denaturation temperatures are key indicators of protein stability and are important in the production and storage of protein based pharmaceuticals.

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Towards understanding the action of an antimicrobial peptide, Novicidin

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The antimicrobial peptide Novicidin (Nc) – modified from the sheep self defense peptide SMAP-29, for reduced mammalian cytotoxicity – is a cationic peptide (net charge +∼7.5) that adopts random coil structure in solution, but an α-helix in the presence of lipid vesicles. The conformation of Nc in presence of the lipids DLPC, DLPG, DMPC, DMPG, DOPC, DOPG, DOPE, and DOPS in different combinations reveal the lipid selectivity, affinity, and phase dependent changes with varying L/P ratios and temperatures, observed from CD spectroscopy. It is understood that the conformational change is dependent on chain length and head group of the lipid. Apart from the *in vivo* results on the Nc activity, studies using QCM-D, Dual Polarisation Interferometry, and Calcein Release Assay reveal the kinetics and concentration dependent activity of Nc in lipid bilayers and vesicles. Preliminary studies on orientation of Nc in various lipid environments using ssNMR, lsNMR, and Molecular Dynamics simulations apparently suggest that Nc may form toroidal pores/detergent effects depending on the chain length of the lipids. Further experiments on Nc in presence of lipids using DSC, ITC, ssNMR, Oriented CD, LD, and confocal microscopy to determine the structure, thermal stability, orientation in lipid bilayers, and thereof the action of Nc will help in proposing a comprehensive model for its mechanism of action in model membranes.