Charging and structure of zwitterionic supported bilayer lipid membranes studied by streaming current measurements, fluorescence microscopy, and attenuated total reflection Fourier transform infrared spectroscopy

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The authors report on the characterization of the charge formation at supported bilayer lipid membranes (sBLMs) prepared from the zwitterionic lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine on planar silicon dioxide substrates. The charging of the sBLMs was studied in KCl solutions of different ionic strengths between 0.1 and 10 mM by streaming current measurements. In addition, attenuated total reflection Fourier transform infrared spectroscopy and fluorescence microscopy were applied to determine the lipid concentration in the membrane and to study the influence of the harsh conditions (*p*H 9–2, shear forces) during the electrokinetic measurements on the membrane stability and the lipid diffusion coefficient. The sBLMs were found to be extremely stable. Isoelectric points of about 4 revealed that unsymmetrical adsorption of hydroxide and hydronium ions determined the charging of the outer leaflet of the membrane in the investigated *p*H range. The diffusion coefficients were found to be rather independent on the ionic strength at neutral and alkaline *p*H. However, significantly decreased lipid diffusion at $pH < 4$ indicated a charge-induced transition of the fluidic bilayer into a gel/ordered-phase bilayer. © *2009 American Vacuum Society.* [DOI: 10.1116/1.3082042]

I. INTRODUCTION

Cell membranes, the interfacial boundary between intra- and extracellular spaces, consist of numerous lipids and proteins (membrane/transmembrane proteins).[1] The interactions between lipids as well as between lipids and proteins are of fundamental interest since the cooperation between them is believed to trigger important cellular events such as signaling, apoptosis, raft formation, organization of the cytoskeleton, or protein sorting.[2,3]

The study of lipids and proteins in native cell membranes is complicated by the high complexity of the involved chemical, biological, and physical interactions. Therefore a number of biophysical approaches have been developed to study cellular membranes in simpler and well defined environments. Model systems which are commonly used include giant unilamellar vesicles (GUVs),[4] supported bilayer lipid membranes (sBLMs),[5] and free standing lipid membranes, such as black lipid membranes.[6] Every approach has its advantages in studying lipid-lipid and lipid-protein interactions. GUVs are free standing bilayer vesicles which can be used to mimic cells in less complex surroundings. The study of sBLMs allows for the use of surface sensitive techniques.[7–9] The majority of studies on sBLMs were done on solid supports, such as mica,[10] glass,[11] or titanium dioxide.[12] Methods which have been applied for the characterization of sBLMs include combinations of atomic force microscopy and fluorescence correlation microscopy,[13] impedance measurements,[14] x-ray reflectivity,[15,16] and quartz crystal microbalance.[17] Since bilayer lipid membranes are prepared and applied in aqueous environments detailed knowledge about the role of electrostatic interactions in the formation of sBLM and the ionization characteristics of membranes formed from different types of lipids is required.

The characterization of the charges resulting from lipid headgroups is essential for understanding protein-lipid interactions during insertion and binding of proteins into or onto a lipid membrane. Surface charge influences important processes such as cell adhesion, antigen-antibody binding, or cell drug delivery.[18] Knowledge about the charging of bilayer lipid membranes is of elementary importance for the evaluation of DNA transfection experiments.[13,19] Furthermore, the understanding of the membrane potential spun between bilayer lipid membranes is of major importance for many biological functions such as drug application or apoptosis.

There exist a number of techniques to determine the ionization of surface groups. Surface potential measurements,[20] various titration techniques,[20–22] conductance measurements,[22] and electrokinetic measurements[21,23,24] have been applied to study the ionization of lipid headgroups and to determine ion binding constants. The majority of electrokinetic studies have been performed at lipid vesicles in

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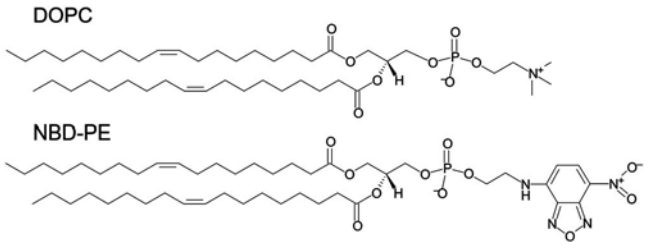

FIG. 1. Structure of DOPC and NBD-PE.

aqueous electrolyte solutions. To the best of our knowledge, results of electrokinetic measurements at sBLMs have not been published so far.

For the past decade molecular dynamics (MD) simulations were increasingly applied to study the structural and dynamical properties of bilayer lipid membranes.[25–28] By the application of this advanced method information on the lipid density and diffusion, the lipid headgroup, and alkyl chain orientation, the electrostatic potential, the dipole moment of the membrane, and ion binding to the lipid membrane were obtained.[27,28]

Streaming potential and streaming current measurements have been shown to be useful in investigating charge formation processes and ion specific effects at flat solid/liquid interfaces.[29,30] The electrokinetic or zeta potential (ζ) derived from such measurements is defined as the electrical potential at the hydrodynamic shear plane between the solid and the bulk liquid, and is often discussed in terms of electrical double layer models (i.e., with respect to the charge of the diffuse layer of ions compensating the surface charge).[31] The zeta potential, as a function of electrolyte solution concentration and *p*H, reflects the charge formation process and can be related to the intrinsic characteristics of the solid surface.

Within this study we performed streaming current measurements to get insights into the charging of sBLMs prepared from zwitterionic lipid 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC). The zeta potential of the sBLMs was determined in dependence of the solution *p*H for 0.1, 1, and 10 mM KCl solutions. The data are discussed with respect to the intrinsic ionization characteristics of the lipid headgroups and unsymmetrical adsorption of water ions. The density of the sBLMs was determined by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy to proof the stability of the bilayers under the harsh conditions (*p*H 9–2) during the electrokinetic measurements. The fluorescence recovery after photobleaching (FRAP) technique was used to study the influence of the charging processes on the lipid mobility under similar conditions.

II. MATERIALS AND METHODS

A. Liposome preparation

The phospholipid DOPC and the fluorescent probe 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) (chemical structures see Fig. 1) were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid vesicles containing DOPC were prepared by mixing the dried lipid in chloroform, evaporating the solvent with argon gas and additionally under vacuum for 4 h to remove the solvent completely, and hydrating in *p*H 4 saline (10 mM NaCl, 5 mM $CaCl_2$, adjusted to *p*H 4 with 0.1 M HCl). For FRAP measurements NBD-PE was additionally added to the lipid in the chloroform.

All chemicals were purchased from Sigma Aldrich (Munich, Germany) and used without further purification. The mixtures were extruded (miniextruder, Avanti Polar Lipids, Alabaster, AL) at least 31 times through 50 nm diameter pores in a polycarbonate membrane (Whatman Ltd., UK) following the procedure of Hope *et al.*[32] The average size of the used small unilamellar vesicles was determined to be in the range of 95 ± 25 nm by dynamic light scattering. The stock solution was stored below 4 °C and consumed within a week.

B. Preparation of substrates and sBLMs

Silicon wafers with a thermal oxide layer of 30 nm were used as sample carriers for the electrokinetic measurements. ATR-FTIR has been performed at sBLMs prepared on trapezoidal silicon plates. The substrates were freshly cleaned in a mixture of aqueous solutions of ammonia (Acros Organics, Geel, Belgium) and hydrogen peroxide (Merck, Darmstadt, Germany) (5:1:1) at 70 °C for 10 min. Prior to each preparation of a DOPC membrane the substrates were treated in a plasma chamber (Harrick Plasma, Ithaca, NY) for 2 min at high rf power. Subsequently the measuring cells were assembled and a lipid vesicle solution (0.2 mg ml^{-1} in KCl solution) was injected into the cell. The surfaces were incubated at 22 °C for 2 h. After incubation the cells were excessively rinsed at least ten times with KCl solution.

For FRAP experiments silica glass slides (Corning B.V. Life Sciences, Netherlands) have been used. Prior to the direct application of the lipid vesicle solution the silica surfaces have been treated in an oxygen plasma chamber (see above) for 2 min at high rf power in order to freshly oxidize the surface. On top of the prepared coverslips, home-built glass cylinders were glued (NuSil, Carpinteria, CA). The preparation of the samples was identical to the preparation of the samples for the electrokinetic measurements. The total applicable volume of lipid vesicle solution was 300 μl.

C. FRAP

FRAP experiments[33] were conducted on a fluorescence confocal laser scanning microscope TCS SP5 (Leica, Bensheim, Germany) by bleaching a defined spot with a short (86 ms) high power laser beam, resulting in a 10 ± 1 μm diameter bleaching spot. The recovery kinetics were recorded with a 63× objective (Leica, NA=1.4) at 128 × 128 pixels with a delay of 86 ms between each image. Diffusion coefficients were estimated following the approach of Soumpasis.[34] The mobile fraction (R_{mobile}) within the photobleached area was estimated from the ratio of the fluores-

cence intensity after full recovery (I_∞) with the fluorescence intensity before bleaching (I_{pre}) and the intensity after photobleaching (I_0) according to Eq. (1):

$$R_{mobile} = \frac{I_\infty - I_0}{I_{pre} - I_0}. \quad (1)$$

D. ATR-FTIR spectroscopy

sBLMs were prepared on trapezoidal (45°) silicon internal reflection elements ($50 \times 20 \times 2$ mm^3) as described above. ATR-FTIR spectra in parallel (p) and vertical (s) polarizations were measured on an EQUINOX 55 FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) using an *in situ* ATR-FTIR attachment operated by the single-beam-sample-reference technique,[35,36] which was purchased from OPTISPEC (Neerach, Switzerland). The surface concentration of the lipids in the bilayer was estimated as outlined in Ref. 36 using the integrated band area as well as the dichroic ratio of the symmetric CH_2 stretching vibration [$\nu_s(CH_2)$] at 2854 cm^{-1}. The following additional parameters were used for the calculation of the surface concentration: refractive index of the silicon plate: $n_1 = 3.5$, refractive index of the bilayer lipid membrane: $n_2 = 1.45$, refractive index of the electrolyte: $n_3 = 1.41$, bilayer thickness: $d = 3.5$ nm, and integrated molar absorption coefficient:[36] $\varepsilon = 5.7 \times 10^5$ cm mol^{-1}.

E. Electrokinetic measurements

The charging of the sBLMs was studied using the microslit electrokinetic setup.[30,37] In brief, the instrument permits to perform streaming potential and streaming current measurements across a rectangular streaming channel formed by two parallel sample surfaces (20×10 mm^2) at varied composition of the electrolyte. For the characterization of the sBLMs a channel height of 30 μm was used. The zeta potential was calculated from streaming current data according to the Smoluchowski equation.[31]

III. RESULTS

A. FRAP measurements

FRAP was applied to study the lipid diffusion coefficients which can be influenced by electrostatic lipid-lipid and lipid-substrate interactions. In addition FRAP was used to prove the stability of the sBLMs toward the conditions met during the electrokinetic measurements. With the help of fluorescence microscopy we were not only able to determine the diffusion coefficients of the sBLMs at different pH values but also to examine the overall fluorescence of the fluorescently labeled sBLMs. Losses of fluorescence intensity could indicate membrane instabilities arising from strong pH changes. Such significant changes in fluorescence were not observed.

In first measurements the stability of the sBLMs toward solutions of different pH was examined at constant background electrolyte (1 mM KCl) (Fig. 2). The sBLMs prepared from DOPC showed a remarkable pH dependence of the lipid mobility. At low pH a diffusion coefficient of only about 0.8 μm^2 s^{-1} was observed. The decrease in diffusion correlated with a mobile fraction of about 88%. Increasing the pH from 2 to 4 led to a sudden increase in lipid mobility to about 4.5 μm^2 s^{-1}. Diffusion coefficients between 5 and 6 μm^2 s^{-1} were found by further changing the solution to pH 6 and 9. The mobile fractions of the sBLMs did not vary significantly in this pH range (~97%).

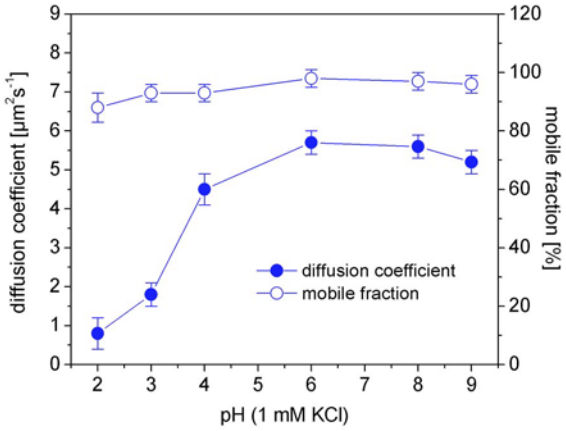

FIG. 2. (Color online) Diffusion coefficient and mobile fraction of sBLMs prepared from DOPC doped with 1 mol % NBD-PE in dependence of the solution pH (background electrolyte concentration of 1 mM KCl). The pH range was chosen according to the pH range used in the electrokinetic experiments.

The lipid mobility in the DOPC membranes remained unaffected by changing the background electrolyte concentration at a constant pH 6. Diffusion coefficients between 4.8 and 5.7 μm^2 s^{-1} were estimated for NBD-PE in the sBLMs (Fig. 3). These values are consistent with a variety of experimental results published for sBLMs containing DOPC.[38–40] The mobile fraction was found to be about 97% at pH 6 for the different KCl concentrations (Fig. 3).

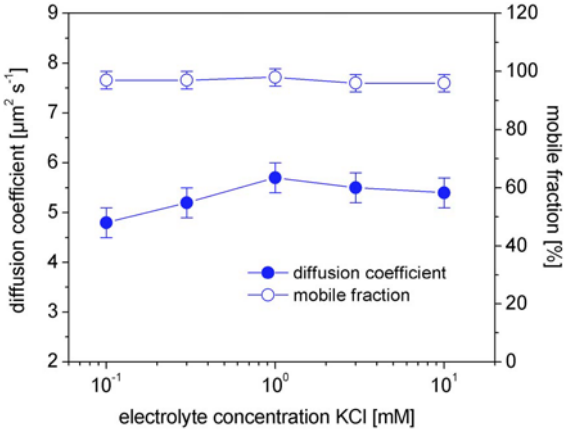

FIG. 3. (Color online) Diffusion coefficient and mobile fraction of sBLMs prepared from DOPC doped with 1 mol % NBD-PE in dependence of the background electrolyte concentration at pH 6.

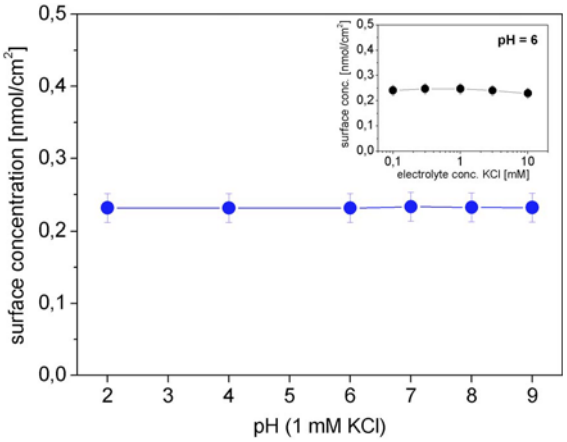

FIG. 4. (Color online) Surface concentration of lipids in DOPC membranes as a function of the solution *p*H for 1 mM KCl solution and at varied KCl solution concentration at *p*H 6 (inset).

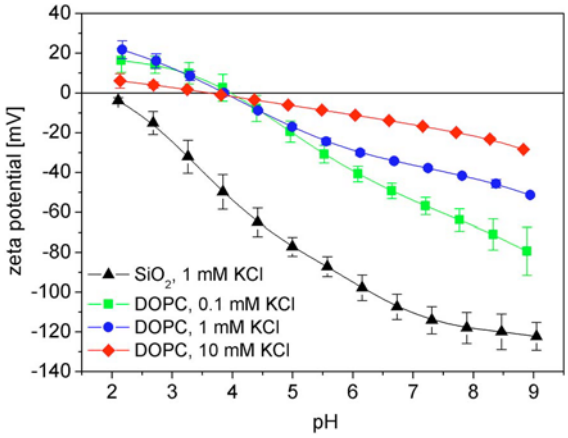

FIG. 5. (Color online) Zeta potential of sBLMs prepared from DOPC on SiO_2 substrates in dependence of the solution *p*H for different concentrations of the background electrolyte KCl. For comparison the zeta potential of the bare SiO_2 substrate is given as well.

B. ATR-FTIR spectroscopy

In order to prove the stability of the sBLMs under the conditions used for the electrokinetic measurements ATR-FTIR spectroscopy was performed at the DOPC bilayers in the range between *p*H 9 and *p*H 2 in 1 mM KCl solution and in addition at different KCl solution concentrations at *p*H 6 (Fig. 4). The average surface concentration of the lipid molecules was found to be about 0.23 ± 0.02 nmol cm^{-2}.

Based on this value the area per lipid molecule in the bilayer was calculated to be about 0.72 nm^2. This is a reasonable result as compared to the results of x-ray diffraction measurements[41] and MD simulations[28] indicating an average cross-sectional area per lipid of 0.71 nm^2 for DOPC bilayers.

The surface concentrations obtained for the different compositions of the electrolyte (Fig. 4) clearly indicate that the lipid concentration in the DOPC membranes is not significantly influenced by the *p*H and ionic strength of the liquid phase. Therefore the membranes can be considered to be stable under the applied experimental conditions.

C. Electrokinetic measurements

The charging of the DOPC bilayers was investigated in 0.1, 1, and 10 mM KCl solutions. For comparison the zeta potential versus *p*H plot of a bare silicon dioxide surface is given as well (Fig. 5). One of the prerequisites for successful bilayer formation is a hydrophilic, highly charged substrate surface.[7,10] As the low isoelectric point (IEP$<$2) of the freshly cleaned silicon dioxide surface can be attributed to a high density of OH groups this prerequisite is fulfilled in our experiments.

For the DOPC bilayers a strong and almost linear variation in the zeta potential in the intermediate *p*H range was observed (compare Fig. 5). The isoelectric point was found at about *p*H 4 independent of the salt concentration of the solution. DOPC, as a zwitterionic lipid, bears both positive and negative charges in its headgroup. sBLMs prepared from DOPC were therefore expected to show a low and almost constant zeta potential in the neutral *p*H range. The strong variation in the zeta potential indicates that the charging of the interface between the sBLMs of the zwitterionic lipid and the electrolyte solution is not only caused by ionization of the lipid headgroups but seems to be superposed by *p*H dependent adsorption of hydroxide and hydronium ions from the solution. Comparable isoelectric points were also obtained for zwitterionic lipid vesicles prepared from 1-stearoyl-2-oleoyl-phosphatidyl-choline (SOPC) by electrophoretic measurements.[24]

IV. DISCUSSION

ATR-FTIR spectroscopy and FRAP measurements confirmed the preparation of stable DOPC membranes on silicon substrates. The lipid density was shown to be not affected by harsh conditions (*p*H 9–2, shear forces) applied during the electrokinetic measurements, while the diffusion of the lipids in the bilayer decreases at *p*H values below the isoelectric point of the membrane surface. The strong variation in the zeta potential with the solution *p*H at all KCl solution concentrations indicates that hydroxide and hydronium ions play an important role for the charging of the bilayer lipid membrane.

If we just consider the ionization constants of the zwitterionic lipid headgroup the shape of the zeta potential versus *p*H plots and the IEP of 4 obtained for the DOPC membranes independent of the ionic strength are unexpected. Because of the low *p*K of the phosphatic acid group[42] and the constant positive charge of the choline group a low and invariant zeta potential was expected for the lipid membranes in the intermediate *p*H range. Instead, a strong variation in the zeta potential was found between *p*H 9 and *p*H 2 for all KCl concentrations. Therefore the data indicate that the charging of the interface between the sBLMs of the zwitterionic lipids and the electrolyte solution is not only caused by ionization of the lipid headgroup but superposed by the unsymmetrical water ion adsorption. Unsymmetrical adsorption of hydroxide and hydronium ions was found to be the origin of surface

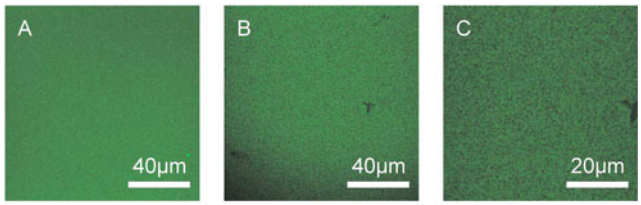

FIG. 6. (Color online) Fluorescence images of a DOPC sBLM at *p*H 6 (a) and after changing to *p*H 2 [(b) and (c)] (512×512 pixels, zoom 2 [(a) and (b)] and zoom 4 (c)).

charge for different polymer materials without ionizable surface groups,[43,44] but is also discussed for dispersed organic liquids[45] and air bubbles.[46] Typically, isoelectric points of about 4 and an almost linear dependence of the zeta potential on the solution *p*H were reported.[43–46] Because of the similarity between the zeta potential versus *p*H plot of the DOPC membranes and the pattern of charge formation earlier observed for the effect of unsymmetrical water ion adsorption we conclude that hydroxide and hydronium ions are also preferentially adsorbed at sBLMs prepared from DOPC. As the isoelectric point was found to be independent of the salt concentration a comparably strong adsorption of chloride or potassium ions can be excluded in the investigated concentration range. The experimental results are strikingly consistent with electrophoretic measurements at SOPC vesicles.[24] In this study[24] negative zeta potentials and an isoelectric point of about 4 were obtained for the SOPC vesicles. The strong affinity of the hydroxide ions for the lipid membrane was additionally verified by the *p*H dependent measurement of the membrane dipole potential.[24] It was found that the positive dipole potential in the bilayer core decreases with increasing *p*H and that the effects of the hydroxide ions are large as compared to the variation in the dipole potential by anion binding of monovalent ions from the Hofmeister series.[24,47] Altogether, the preferential adsorption of hydroxide ions dominates the charging at different zwitterionic membranes. Because of the similarity of the pattern of charge formation at SOPC and DOPC membranes one can assume that the preferential adsorption of hydroxide ions also dominates the charging at other zwitterionic membranes.

FRAP measurements showed a strong decrease in the lipid diffusion in the DOPC membranes at low *p*H. This might be correlated with the *p*H dependent variation in the bilayer charge. Träuble and Eibl studied the influence of the degree of lipid headgroup ionization on the phase transition temperature of different lipids.[48] It was shown that the phase transition temperature is sensitive to *p*H and salt dependent variations in the ionization of polar groups. For lipids with PC headgroups an increase in the transition temperature in the region below *p*H 3 was found. Therefore, we attribute the reduced lipid mobility in the DOPC membranes at low *p*H to a charge-induced transition from the fluid-phase bilayer ($p\mathrm{H}>4$) to a gel/ordered-phase bilayer ($p\mathrm{H}<4$). The occurrence of a phase transition is also supported by the fluorescence images shown in Fig. 6. While a homogeneous fluorescence intensity was observed at *p*H 6, the images taken at *p*H 2 show a substantial change in the bilayer structure. It is not possible to clearly distinguish phase separations by fluorescence microscopy because of the diffraction limit of light, but obviously the structure of the sBLM changed into a more rippled, heterogeneous topography, suggesting the presence of at least two coexisting sBLM phases (disordered/fluid →gel→ordered). At the isoelectric point and above ($p\mathrm{H}>4$) the diffusion coefficient and the mobile fraction (~97%) suggest that the force balance between the electrostatic repulsion and van der Waals interactions is at an optimum for DOPC sBLMs allowing unrestricted lipid diffusion. Obviously, the lipid motion was influenced by the solution *p*H, but remained rather unaffected by the ionic strength in the neutral *p*H range: The solution ionic strength was changed over three orders of magnitude without significant variations in the lipid diffusion coefficients. Hence, the presence of potassium ions did not influence the sBLM structure.

The transition from a disordered/fluid phase to an ordered/gel phase is usually related to a bilayer compression.[48,49] However, the surface concentrations determined by ATR-FTIR spectroscopy do not indicate significant variations in the lipid density between *p*H 2 and *p*H 4. We attribute this discrepancy to the limited amount of lipid on the ATR crystal. Since there was no lipid in the solution above the bilayer and the ATR-FTIR spectroscopy is an integral method we could not detect local variations in the lipid density during the phase transition.

V. CONCLUSION

We have shown that streaming current measurements can be used to study charging processes at sBLMs on planar silicon surfaces. The charging of sBLMs prepared from the zwitterionic lipid DOPC was exemplarily investigated to demonstrate the applicability of the technique for fundamental studies on electrosurface phenomena at sBLMs. ATR-FTIR spectroscopy and FRAP measurements showed that the sBLMs have been stable even under harsh *p*H conditions and extensive rinsing. The strong variation in the zeta potential in the neutral and alkaline *p*H range and isoelectric points of 4 found independent of the salt concentration indicate that the unsymmetrical adsorption of hydroxide and hydronium ions strongly influences the electrical potential at the interface between the membrane and the electrolyte solution. The results are consistent with earlier experimental studies on zwitterionic lipid vesicles.[24] The positive charge of the sBLMs below *p*H 4 was related to a decrease in the lipid mobility. We attribute this behavior to a charge-induced transition from the fluid-phase bilayer ($p\mathrm{H}>4$) to a gel/ordered-phase bilayer ($p\mathrm{H}<4$). Ongoing studies employ the introduced methodology to study the charging of sBLMs prepared from mixtures of anionic, cationic, and zwitterionic lipids on silicon surfaces and on polymer cushions in electrolyte solutions of varied ionic composition.

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