Chapter 7
Carbohydrates as Recognition Receptors in Biosensing Applications

Yann Chevolot, Sébastien Vidal, Emmanuelle Laurenceau, François Morvan, Jean-Jacques Vasseur, and Eliane Souteyrand

Abstract Carbohydrates are involved in crucial physiological and pathological events. One can take advantage of carbohydrate-based interaction for drug discovery, diagnosis, antibiotics, vaccine, etc. This chapter deals with biosensors and microarrays that take advantage of carbohydrates-based interactions with a special interest in devices that are designed for medical applications. A large overview of glycochemistry, followed by the biological role of carbohydrates, is given. Carbohydrate-based biosensors are then described with special emphasis on surface chemistry and signal transduction. Finally, medically relevant applications illustrate the use of carbohydrates as recognition receptors in biosensing.

Keywords Glycoarray · Carbohydrate · Glycomics · Microarray · Biosensors

Abbreviations

Ab	Antibody
Ac	Acetyl
AIDS	Acquired immune deficiency syndrome or acquired immunodeficiency syndrome
AZT	Azidothymidine or Zidovudine
BAW	Bulk Acoustic Wave
bFGF	Basic fibroblast growth factor
BSA	Bovine Serum Albumin

Y. Chevolot (✉)
Institut des Nanotechnologie de Lyon UMR CNRS 5270, Université de Lyon, Ecole Centrale de Lyon, 36 Avenue Guy de Collongue, 69134 Ecully Cedex, France
e-mail: Yann.Chevolot@ec-lyon.fr

Bz	Benzoyl
CD4	Cluster of differentiation 4
CD44	Cluster of differentiation 44
CFG	Consortium for functional glycomics
CMP-Neu5Ac	Cytidine monophosphate-N-acetylneuraminic-acid
CNS	Central nervous system
ConA	Concanavalin A
CSPG	Chondroitin-sulfate proteoglycan
Cy3	Indodicarbocyanine 3
Cy5	Indodicarbocyanine 3
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-Grabbing Non-integrin
DDI	DNA directed immobilization
DNA	Deoxyribonucleic acid
DPI	Dual polarisation interferometry
DV	Dengue virus
EC	*Erythrina crista-galli* lectin
ENFET	Enzymatic field effect transistor
EQCM	Electrochemical quartz crystal microbalance
FDA	Food and drug administration
FET	Field effect transistor
FITC	Fluorescein isothiocyanate
GAG	Glycosaminoglycans
GIPL	Glycoinositol phospholipid
GlcNAc	*N*-Acetylglucosamine
GPI	Glycosylphosphatidylinositol
GSLs	Glycosphingolipids
HA	Hemagglutinin
HIV	Human immunodeficiency virus
HIV-RT	Human immunodeficiency virus reverse transcriptase
HSA	Human serum albumin
HSPG	Heparan-sulfate proteoglycan
ICAM	Inter-cellular adhesion molecule
ISFET	Ion sensitive field effect transistor
MALDI-ToF	Matrix-assisted laser desorption/ionization time of flight
MOSFET	Metal oxide semiconductor field effect transistor
MR	Mannose receptor
MRI	Magnetic resonance imaging
MS	Mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Neuramidase
NG2	Nerve/Glial antigen 2
NHS	*N*-Hydroxysuccinimide
NIS	*N*-iodosuccinimide

NMR	Nuclear magnetic resonance
NPC	Neuronal precursor cells
NSC	Neuronal stem cells
PCR	Polymerase chain reaction
PECAM	Platelet Endothelial cell adhesion molecule
PEG	Poly(ethylene glycol)
QCM	quartz crystal microbalance
QCM-D	quartz crystal microbalance with dissipation monitoring
QD	Quantum dot
RNA	Ribonucleic acid
SAM	Self assembled monolayer
SARS	Severe acute respiratory syndrome
SDS	Sodium dodecyl sulfate
SLe	Sialyl lewis
SPR	Surface plasmon resonance
Tf	Trifluoromethanesulphonate
TMS	Trimethylsilyl
TSM	Thickness shear mode
VCAM	Vascular cell adhesion molecule
WGA	Wheat-germ agglutinin

7.1 Introduction

Glycosylation of proteins is a common post-translational modification, and eukaryotic cells are coated with complex carbohydrates associated with lipids or proteins forming the so-called glycocalix. Carbohydrates are the most abundant type of biomolecules on earth in term of biomass. Their biological role spans from the stabilisation of protein structure to their involvement in crucial biological events such as the development, growth or survival of a cell/organism (Varki 1993; Sharon and Lis 2004; Varki et al. 1999; Dwek 1996; Bertozzi and Kiessling 2001; Bishop et al. 2007). For instance, protein–carbohydrate interactions are involved in intracellular trafficking of proteins, cell adhesion, cell recognition, cell differentiation and the development of the neuronal network. They are also involved in pathological events such as inflammation, metastasis (Galonic and Gin, 2007) and host–pathogen interactions (Imberty et al. 2004; Seeberger and Werz 2007). For example, influenza virus takes advantage of the carbohydrate motifs present on the surface of the respiratory track endothelial cells for adhering to these cells (Skehel and Wiley 2000). The switch from avian influenza to human influenza sub-type is related to the subtle change of its ability to recognise the glycosylated motif of human respiratory track endothelial cell instead of the avian ones. The elucidation of these interactions is a critical issue not only in terms of fundamental research but also in terms of applied research for the development of diagnosis or new therapeutics.

Biophysical approaches such as calorimetry (Dam and Brewer 2002), nuclear magnetic resonance (Sandström et al. 2004) and crystallography (Berhe et al. 1999) have been successfully employed and have brought many valuable pieces of information. However, these techniques are not straightforward as well as are time- and material-consuming.

When dealing with carbohydrates, several limitations arise because of their chemical nature. Firstly, carbohydrate structures are extremely diverse. It has been calculated that a hexamer can lead to more than 10^{12} structures (Laine 1994). Secondly, carbohydrate synthesis is one of the most difficult and laborious synthetic work, and carbohydrates cannot be amplified enzymatically like DNA (by polymerase chain reaction) or cloned such as proteins. The consequence is that carbohydrate material is difficult to obtain in large amounts at affordable prices. Finally, carbohydrate interactions, especially with proteins, tend generally to be weak unless a multivalent interaction is involved, taking advantage of the so-called cluster effect (Lee and Lee 1995). Consequently, analysis of carbohydrate-mediated interactions requires analytical techniques with very low detection limit.

In this context, biosensors and microarrays have several advantages. They require very little amounts of biological material. In the case of microarrays, high-throughput analysis can be performed and they usually have very low detection limits (Feizi et al. 2003; Wang 2003; Jelinek and Kolusheva 2004). Almost all biosensors are based on a biological binding/recognition element (ligand) that performs a specific binding or biochemical reaction with a target. This interaction is then converted into a signal through a transducer.

The requirements for modern biosensors are sensitivity, selectivity and reproducibility. These parameters depend on a combination of many factors among which surface chemistry, ligand immobilisation, target physico-chemical properties and signal transduction are key factors.

This chapter concerns carbohydrate based biosensor in a broad sense, that is devices comprising bound bioactive carbohydrate ligands responsible for a specific interaction at the surface of a material. Here, the ligand considered will be a saccharide or an array of saccharides and more precisely mono or oligosaccharides.

At this stage, it is worth giving a few definitions. In the following, a probe is a molecule (a biomolecule) that is immobilised on the surface of a material in order to interact with a target. The target is the biological molecule to be detected in a biological fluid.

In order to avoid confusion, a biosensor is a device that can perform directly the detection of a biochemical interaction. This means that the biological interaction is measured in real time (Rasooly 2005). Surface plasmon resonance (SPR) or electrochemical detection belong to this class of biosensors. Another class of biosensors relies on indirect detection such as fluorescence detection (Rasooly 2005).

A biochip or microarray is a special class of biosensor as it allows for the simultaneous detection of multiple interactions. Common to both cases, the ligands are immobilised on the surface of a material (plane or particles) that can be active as a transducer (electrochemically active or optically active for SPR) or inert (glass in the case of fluorescence).

In this chapter, we are concerned with carbohydrate-based biosensing. Therefore, we will first give an overview on the physico-chemical properties of carbohydrates, as these are critical for obtaining and designing the probes. We will then focus on their biological role. We will then give an overview of carbohydrate-based biosensing from their immobilisation, as this is critical for obtaining reliable devices and to the signal transducer. Finally, we will give some applications described in the literature.

7.2 General Aspects of Glycochemistry

7.2.1 Introduction

Carbohydrates were named after their composition as *hydrates of carbon,* but they can be also called saccharides from greek σάκχαρον meaning sugar. They are polyfunctional biomolecules with the general formula $C_n(H_2O)_n$ (e.g. $n = 6$ for glucose). The biological aspects of carbohydrates have long been underestimated because of their complexity in comparison to the three other classes of biomolecules. Polypeptides (proteins) and polynucleotides (e.g. DNA or RNA) are linear polymers, whereas carbohydrates are either linear or branched polymers, thereby increasing drastically the number of possible combinations (Laine 1994; Werz et al. 2007). Recent advances in glycobiology have demonstrated the numerous roles of carbohydrates in a series of biological processes such as storage and transport of energy, signal transduction, fertilisation, immune responses, pathogenesis, blood clotting, virus infection and cell-to-cell communication (Bertozzi and Kiessling 2001; Sharon and Lis 2004; Dwek 1996; Varki 1993; Varki et al. 1999). These fundamental discoveries found several applications in the design of carbohydrate-based drugs and vaccines against various diseases (Werz and Seeberger 2005b) such as malaria (Hölemann and Seeberger, 2006; Seeberger and Werz 2007), cancer (Galonic and Gin, 2007; Livingston and Ragupathi 2006) and AIDS (McReynolds and Gervay-Hague, 2007; Scanlan et al. 2007). The ever-increasing number of chemists, biochemists and biologists who are involved in research with carbohydrates is testimony to the fact that there is still a lot more work to be achieved in glycochemistry and glycobiology.

7.2.2 Structural Aspects and Chemistry of Carbohydrates

Monosaccharides are classified according to the number of carbon atoms, which ranges from 4 (tetroses), 5 (pentoses), 6 (hexoses) and up to 10 atoms. Aldoses (Fig. 7.1) and ketoses (Fig. 7.2) are polyhydroxylated aldehydes or ketones, respectively, which can cyclise intramolecularly into hemiacetals. The nucleophilic attack of a hydroxyl group on the carbonyl will provide two diastereoisomeric hemiacetals
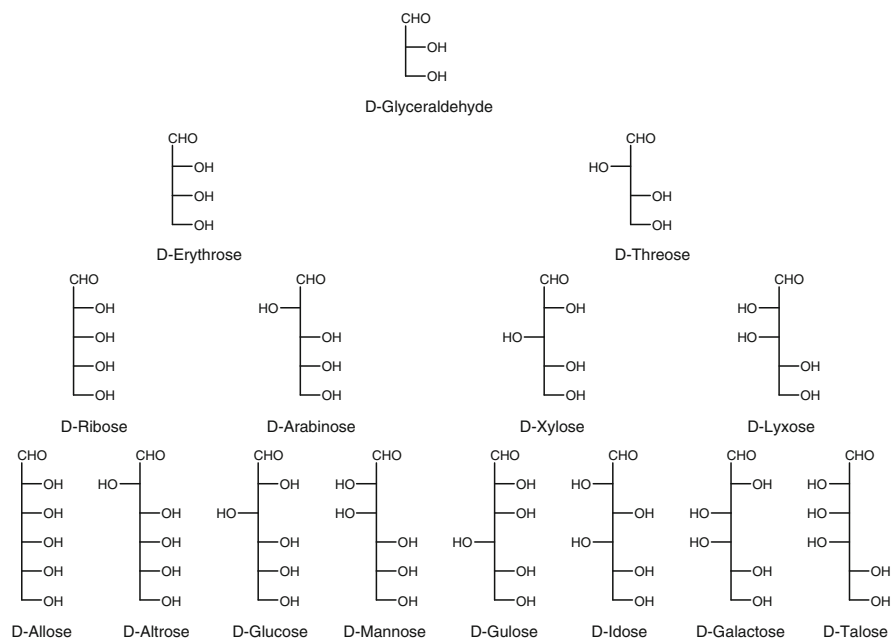
CHO
—OH
—OH

D-Glyceraldehyde

CHO
—OH
—OH
—OH

D-Erythrose

CHO
HO—
—OH
—OH

D-Threose

CHO
—OH
—OH
—OH
—OH

D-Ribose

CHO
HO—
—OH
—OH
—OH

D-Arabinose

CHO
—OH
HO—
—OH
—OH

D-Xylose

CHO
HO—
HO—
—OH
—OH

D-Lyxose

CHO
—OH
—OH
—OH
—OH

D-Allose

CHO
HO—
—OH
—OH
—OH

D-Altrose

CHO
—OH
HO—
—OH
—OH

D-Glucose

CHO
HO—
HO—
—OH
—OH

D-Mannose

CHO
—OH
—OH
HO—
—OH

D-Gulose

CHO
HO—
—OH
HO—
—OH

D-Idose

CHO
—OH
HO—
HO—
—OH

D-Galactose

CHO
HO—
HO—
HO—
—OH

D-Talose

Fig. 7.1 Structures of acyclic forms of D-aldoses

—OH
=O
—OH
—OH

D-Tetrulose

—OH
=O
—OH
—OH
—OH

D-Ribulose

—OH
=O
HO—
—OH
—OH

D-Xylulose

—OH
=O
—OH
—OH
—OH
—OH

D-Psicose

—OH
=O
HO—
—OH
—OH
—OH

D-Fructose

—OH
=O
—OH
HO—
—OH
—OH

D-Sorbose

—OH
=O
HO—
HO—
—OH
—OH

D-Tagatose
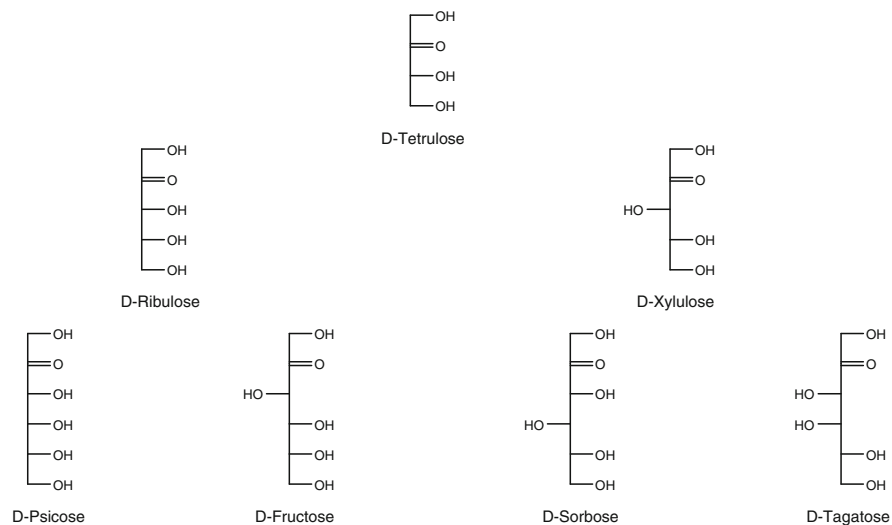
Fig. 7.2 Structures of acyclic forms of D-ketoses

called α- and β-anomers. Cyclisation can occur between O-4 and O-5 to form a
five-membered ring designated as furanose or a six-membered pyranose ring,
respectively (Fig. 7.3). D-Aldoses are derived from D-glyceraldehyde and their
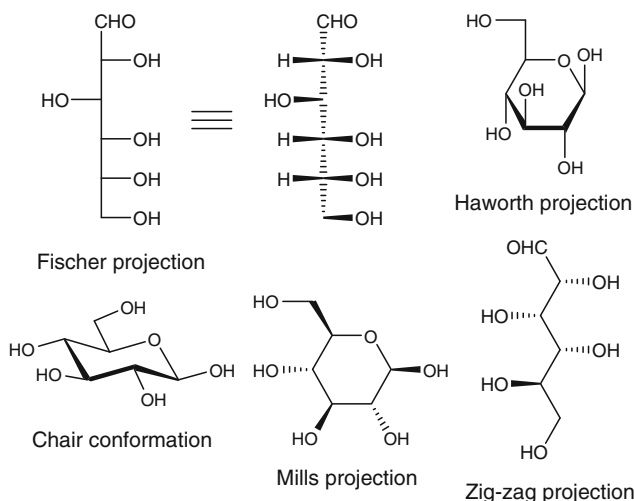
Fig. 7.3 Cyclic and acyclic forms of D-glucose

enantiomers (L-aldoses) from L-glyceraldehyde. Atom numbering starts at the most oxidised end of the carbon chain (e.g. carbonyl group for aldoses) and increases along the carbon chain.

The cyclic structures of monosaccharides can be represented according to several projections (Fig. 7.4). The Fischer projection was the first one to be applied to acyclic forms of sugars, where the carbon chain is drawn vertically with the most oxidised carbon atom (carbonyl) at the top. Later, Haworth designed a projection for a more realistic description of cyclic forms of carbohydrates. The chair conformation provides the most accurate description of the geometry of the carbohydrate ring and should be preferred to other projections. Mills and zig-zag projections are useful when considering the stereochemistry of each carbon atom along the chain.

7.2.2.1 Mutarotation

Carbohydrates are single stereoisomers in their crystalline form. Nevertheless, optical rotation of a single anomer in aqueous solution slowly changes with time to reach a constant value. Mutarotation (Pigman and Isbell 1968; Isbell and Pigman 1969) refers to this equilibration process where an α-anomer would equilibrate to a mixture of α- and β-anomers through the ring-opened aldehyde form and re-cyclisation into its opposite anomer (Fig. 7.5). This equilibrium is catalysed by acids or bases. Carbohydrates are therefore present as a mixture of acyclic and

Fig. 7.4 Projections used for the description of carbohydrates

Fig. 7.5 Mechanism of mutarotation

five- or six-membered cyclic forms with two different anomers in equilibrium in aqueous solutions with ratios that can be determined by NMR spectroscopy (Table 7.1).

7.2.2.2 The Anomeric Effect and the *Exo*-anomeric Effect

The anomeric effect (Edward and Puskas 1962) was studied by Lemieux (Praly and Lemieux 1987) as the tendency for an electronegative substituent at the anomeric centre to prefer the axial orientation instead of the less hindered equatorial position (Edward and Puskas 1962; Praly and Lemieux 1987). Two explanations can be invoked based on dipolar moments or hyperconjugation (Fig. 7.6). Equatorial

Table 7.1 Composition of D-aldoses in aqueous solution at equilibrium (Angyal 1984)

Carbohydrate	Temperature (°C)	α-Pyranose (%)	β-Pyranose (%)	α-Furanose (%)	β-Furanose (%)	Acyclic form (%)
D-Ribose	31	21.5	58.5	6.5	13.5	0.05
D-Arabinose	31	60	35.5	2.5	2	0.03
D-Xylose	31	36.5	63.0	< 1	< 1	0.02
D-Lyxose	31	70	28	1.5	0.5	0.03
D-Allose	31	14	77.5	3.5	5	0.01
D-Altrose	22	27	43	17	13	0.04
D-Glucose	31	38	62	–/–	0.14	0.02
D-Mannose	44	64.9	34.2	0.6	0.3	0.005
D-Gulose	22	16	81	–/–	3	–/–
D-Idose	31	38.5	36	11.5	14	0.2
D-Galactose	31	30	64	2.5	3.5	0.02
D-Talose	22	42	29	16	13	0.03
D-Fructose	31	2.5	65.0	6.5	25.0	0.8

Disfavorable   Favorable   Disfavorable   Favorable
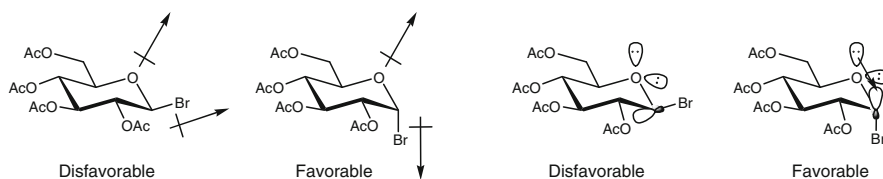
Fig. 7.6 The anomeric effect through two explanations
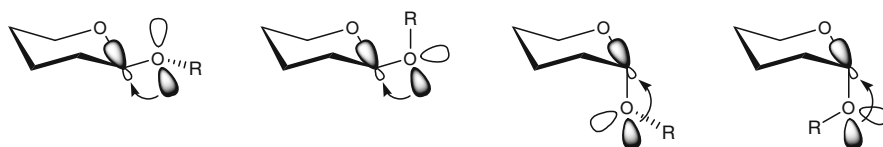
Fig. 7.7 Conformations stabilized by the *exo*-anomeric effect

configuration of an electronegative anomeric substituent results in the formation of two similar dipoles repelling each other and destabilizing the molecule. On the contrary, axially orientated substituents create roughly opposed dipoles accounting for a lower energy state. The second explanation to the anomeric effect is the presence of a stabilizing interaction through hyperconjugation between an unshared electron pair of the endocyclic oxygen atom and the σ* orbital of the anomeric bond (e.g. C–Br).

The orientation of the substituent at the anomeric centre (aglycon) can be described by the *exo*-anomeric effect (Fig. 7.7) (Praly and Lemieux 1987; Perez and Marchessault 1978; Thoegersen and Lemieux 1982) In this case, the exocyclic anomeric oxygen atom's lone pair is interacting with the anti-bonding orbital of the endocyclic C–O bond. The *exo*-anomeric effect can be observed for both α- and β-anomers.

7.2.3 Glycosylation Methods

The formation of a glycosidic bond occurs through the nucleophilic displacement of
a leaving group at the anomeric centre of a glycosyl donor by an alcohol of a
glycosyl acceptor (e.g. ROH) to provide the corresponding *O*-glycoside (Fig. 7.8)
(Fügedi 2006; Schmidt 1986; Veeneman 1998; Whitfield and Douglas 1996). This
reaction is usually promoted by a third partner (promoter) increasing the reactivity
of the glycosyl donor.

The substitution at the 2-position plays an important role in the stereochemical
outcome of the glycosylation process (Fig. 7.9). When a non-participating group is
present at the 2-position, the major glycoside obtained is the α-anomer because of
the stabilization through the anomeric effect. However, a participating group at the
2-position creates a cationic *bis*-acetal intermediate hindering the α-face of the
pyranose ring and therefore favours the nucleophilic attack of the glycosyl acceptor
from the β-face to give rise to the 1,2-*trans* glycoside. During this process,
the alcohol could also form an orthoester which can be rearranged into the desired
1,2-*trans* glycoside under Lewis acid catalysis (Kochetkov et al., 1967, 1971).

Each glycosylation method can be distinguished through the glycosyl donor/
promoter system involved (Table 7.2). Acetalation of a reducing sugar with an
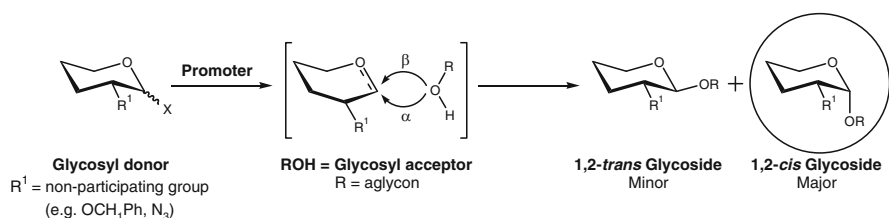
Fig. 7.8 General mechanisms for glycosylation
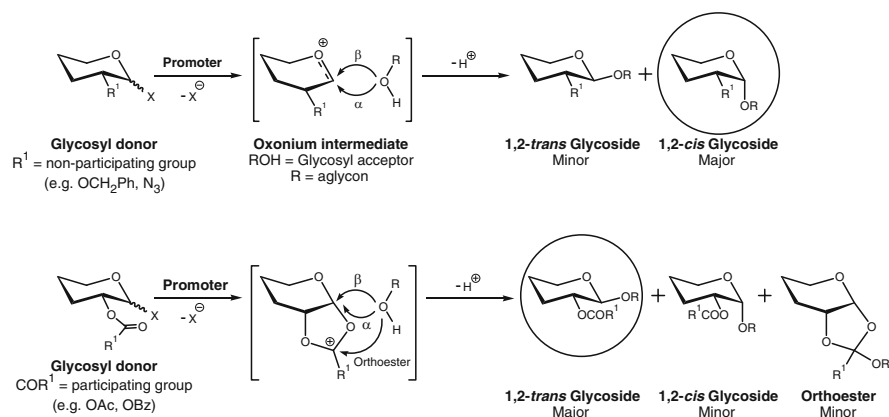
Fig. 7.9 General strategies used for the formation of 1,2-*cis* or 1,2-*trans* glycosides

Table 7.2 General glycosylation methods for the synthesis of *O*-glycosides

Glycosyl donor (name)	Leaving group(X=)	Promoters	Comments
Hemiacetals (Fischer)	OH	H^+, $BF_3 \cdot Et_2O$, $FeCl_3$	Provides the α-anomer from the native carbohydrate
Glycosyl halides (Koenigs–Knorr)	Cl, Br	Ag_2O, AgOTf, HgO, $HgBr_2$, $Cu(OTf)_2$	Requires expensive silver or toxic mercury salts
Glycosyl fluorides	F	$SnCl_2$/AgOTf, $LiClO_4$, $Cu(OTf)_2$	Glycosyl fluorides are glycosidase substrates
1-*O*-acyl glycoside (Helferich)	OAc	$BF_3 \cdot Et_2O$, $SnCl_4$, TMSOTf	Mixture of anomers can be used as donor
Trichloroacetimidate (Schmidt)	$OC(NH)CCl_3$	$BF_3 \cdot Et_2O$, I_2, TMSOTf, AgOTf, TfOH	Usually requires the addition of molecular sieves
Arylthioglycosides	SAr	MeOTf, NIS/TfOH, I_2	High stability to chemical reactions and time
Glycosyl sulfoxides and sulfones (Kahne)	$S(O)_nAr$ ($n = 1$ or 2)	TMSOTf, Tf_2O, I_2	Requires the corresponding arylthioglycoside
n-Pentenyl glycosides (Fraser-Reid)	$O(CH_2)_3$ $CH = CH_2$	NBS, NIS/TfOH, iodonium dicollidine perchlorate (IDCP)	Fairly stable to functional group manipulations
Glycosyl phosphites	$OP(OR)_2$	TMSOTf, $BF_3 \cdot Et_2O$, I_2, NIS/TfOH	Diethyl and dibenzyl phosphites commonly used
Glycosyl phosphates	$OP(O)(OR)_2$	TMSOTf, $BF_3 \cdot Et_2O$, TfOH	Applied to both solution and solid phase synthesis
Selenoglycosides	SeAr	$AgOTf/K_2CO_3$, IDCP, NIS, I_2	Can be selectively activated in the presence of arylthioglycosides

alcohol in the presence of a protic acid promoter was used as one of the first glycosylation method by Fischer (Fischer 1893; Fischer 1895). It is probably the most appropriate method for the synthesis of simple glycosides (e.g. methyl, benzyl, allyl). Glycosylation was then performed from glycosyl halides in the presence of heavy metal salts by Koenigs and Knorr (Igarashi 1977; Koenigs and Knorr 1901). Glycosyl fluorides can also be used as glycosyl donors and are much more stable than their chlorinated or brominated analogues (Mukaiyama et al. 1981). They can be activated under specific conditions compatible with most protecting groups. Peracetylated carbohydrates are particularly convenient for large-scale synthesis and can be also involved in a glycosylation as glycosyl donors but their activation sometimes requires drastic conditions (Helferich and Schmitz-Hillebrecht 1933). Glycosyl trichloroacetimidates were then introduced by Schmidt and are activated under acidic conditions with either Brønsted or

Lewis acids usually in the presence of molecular sieves and at low temperature (Schmidt and Michel 1980; Pougny and Sinay 1976). Arylthioglycosides are highly stable even when stored for long periods on shelves and can also accommodate a large number of chemical reactions on the pyranose ring. They can be activated by thiophilic reagents under mild conditions, and formation of the oxonium intermediate proceeds through the addition of the electrophilic promoter to the sulphur atom (Garegg 1997; Ferrier et al. 1973; Fügedi et al. 1987). Activation can also take place under single electron transfer, electrochemical or even high pressure conditions. *n*-Pentenyl glycosides were introduced by Fraser-Reid as glycosyl donors and are activated through halogenation of the terminal double bond followed by intramolecular cyclisation to provide the oxonium intermediate and a halomethyl-2-furane (Fraser-Reid et al. 1988; Fraser-Reid et al. 1992). Glycosyl phosphites (Kondo et al. 1992; Martin and Schmidt 1992; Zhang and Wong 2000) react with alcohols at low temperature in the presence of Lewis acids affording the corresponding glycosides, and glycosyl phosphates (Hashimoto et al. 1989; Vankayalapati et al. 2002) can be activated by protic acids. Activation of selenoglycosides (Mehta and Pinto 1991; Mehta and Pinto 1993) can be achieved under similar conditions as for thioglycosides, by photo-induced or single electron transfer or iodine. They can be selectively activated in the presence of thioglycosides.

All these glycosylation methods have found numerous applications for the synthesis of natural products or for the selective formation of the desired anomer. One should also consider the influence of protecting groups on the pyranose ring, which sometimes affect the glycosylation outcome.

7.2.4   *Chemo-enzymatic Glycosylation Methods*

Among the powerful methods of glycosylation listed above, only Fischer glycosylation can accommodate a non-protected carbohydrate where the hydroxyl functions are not masked by protecting groups. Glycosyltransferases and glycosidases are natural enzymes capable of creating or hydrolyzing glycosidic bonds in an aqueous medium, using native sugars and with high stereo- and regioselectivities. Their use as "glycosylating agents" or promoters has attracted a lot of interest in the scientific community and a series of applications are now available for the synthesis of complex oligosaccharidic structures (Qian et al. 2001). This technology requires both biochemical and chemical skills since enzymes sometimes need to be mutated for obtaining the expected result. Sialylation is one of the most difficult glycosylation because of the poor stability of activated sialic acid derivatives. The biochemical engineering of sialyltransferases can readily provide a large series of sialylated derivatives ranging from the glycosylation of simple alcohols to mono- or disaccharides (Drouillard et al. 2006; Blixt et al. 2005; Huang et al. 2006b; Dumon et al. 2006; Muthana et al. 2007; Yu et al. 2006).

7.2.5 Glycoconjugates

Carbohydrates play an important role in a large series of biological processes and are usually present in nature either in their native form or as glycoconjugates where a sugar residue is associated to a lipid (glycolipids), a protein (glycoprotein) or a peptide (glycopeptides), along with several other natural products containing sugars. The chemical conjugation of carbohydrate moieties to proteins or polyvalent species will create neoglycoproteins or neoglycoconjugates, respectively, which can find numerous applications in biology and biochemistry. After introducing a functionalised linker at the anomeric position of a carbohydrate through standard glycosylation methods, the other end of this arm will be used for its conjugation to the desired multivalent scaffold (Fig. 7.10).

The conjugation of carbohydrate derivatives to multivalent species has been achieved through a wide range of chemical ligation techniques. When considering
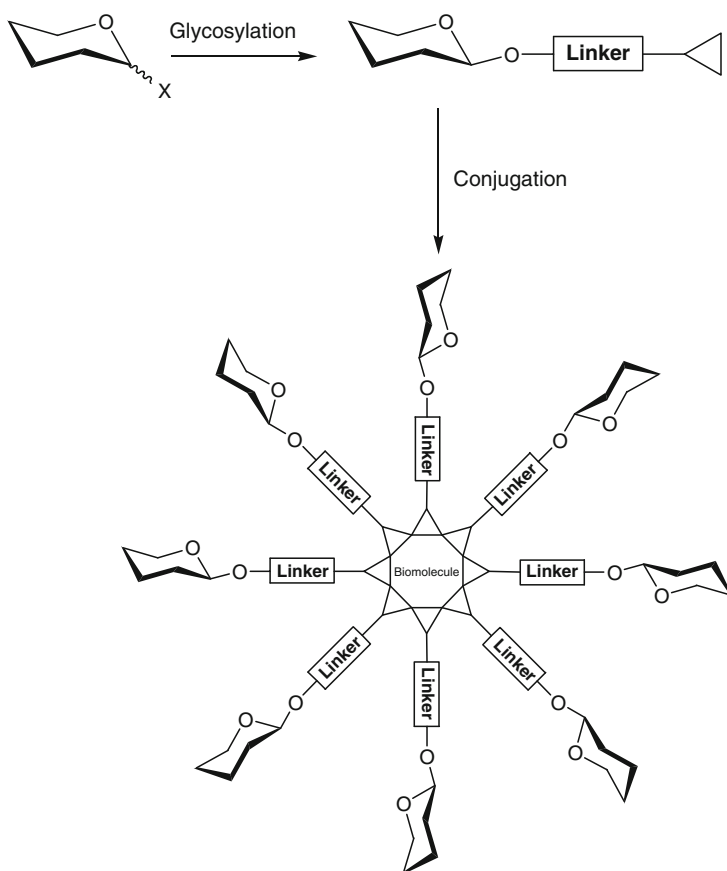
Fig. 7.10 Schematic representation of the synthesis of neoglycoproteins
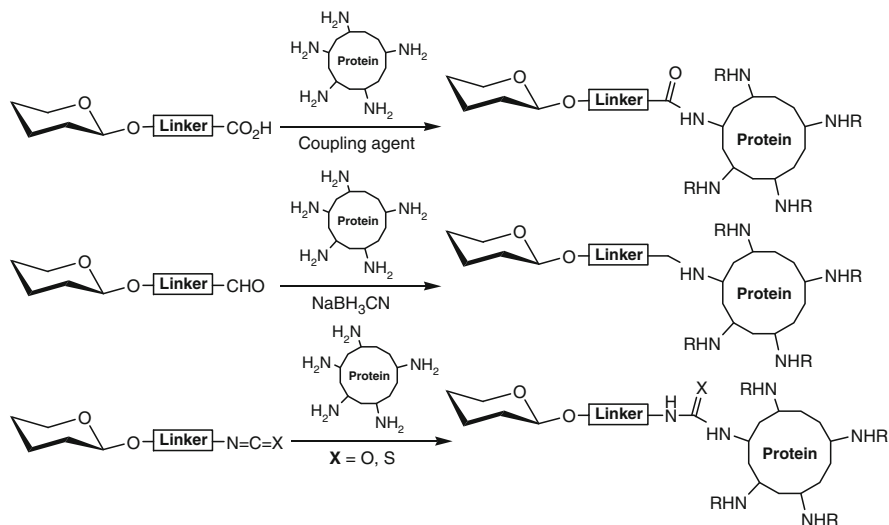
Fig. 7.11 Examples of conjugation of carbohydrates to proteins through amidation, reductive amination and urea/thiourea ligation
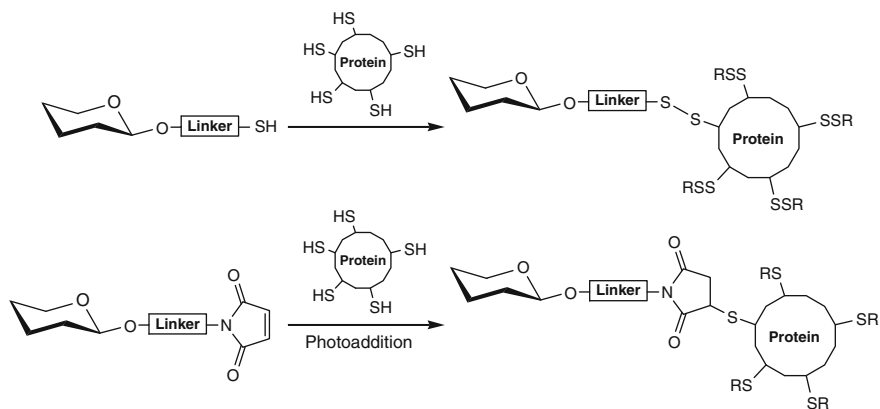
Fig. 7.12 Examples of conjugation of carbohydrates to proteins through thiol functions

the formation of neoglycoproteins, the chemist will take advantage of the reactive amino group from the lysine residues present at the surface of proteins and therefore use a series of reactions involving an amine function (Fig. 7.11). Similarly, neoglycoproteins can be prepared by reacting the thiol groups of cystein residues of proteins with thiol or maleimide functions (Fig. 7.12).

The above-mentioned techniques can be also applied to multivalent non-natural scaffolds for the preparation of neoglycoconjugates. In these cases, the nature of functionalities accessible is much broader. 1,3-Dipolar cycloaddition between an
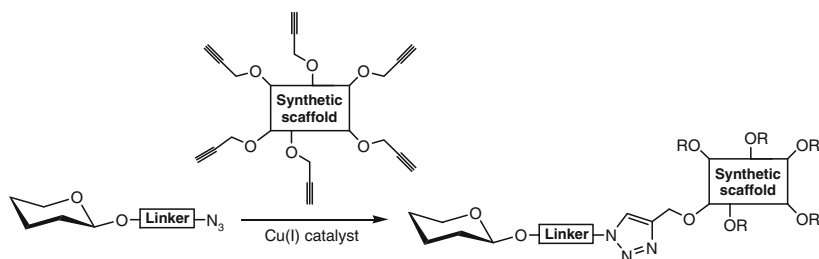
Fig. 7.13 Conjugation of carbohydrates to proteins through 1,3-dipolar cycloaddition

alkyne and an azido derivative to form 1,2,3-triazoles, developed by Huisgen (Huisgen et al., 1965, 1969) in the late 1960s, was recently introduced into the realm of techniques for conjugation and rapidly found a large number of applications in glycobiology (Fig. 7.13). This methodology is now accessible due to the improved Cu(I)-catalysed processes developed more recently (Kolb et al. 2001; Rostovtsev et al. 2002; Bock et al. 2006; Tornoe et al. 2002; Gil et al. 2007; Bouillon et al. 2006; Morvan et al. 2007). This chemical ligation technique is highly attractive since the reactive species involved are pretty much unreactive to the chemical functions present in biological media, the reaction does not create any by-product and they can be performed in water, at low temperature and on reasonable time scales.

7.2.6 Examples of Naturally Occurring Carbohydrates

7.2.6.1 Structures of Some Mono- and Disaccharides

A large number of monosaccharides incorporating a single carbohydrate residue are present along with several disaccharides composed of two sugar moieties (Fig. 7.14). Lactose is a disaccharide present in milk and fructose in fruits, while saccharose is the most common food sweetener. Ribose is a major component of RNA-forming nucleotide building blocks. Xylose is also called the *wood sugar* as it is one of the main components of trees and plants along with the disaccharide cellulose. Fucose is a 6-deoxy sugar found in mammalian, insect and plant cell surface.

7.2.6.2 Nucleosides

RNA and DNA can be differentiated through the nature of the carbohydrate involved in their structural subunits (nucleosides). Ribose is the sugar involved in RNA, while DNA is based on 2-deoxy-D-ribose lacking one hydroxyl group at the 2-position. The four constituents of RNA are based on a ribose residue linked to a
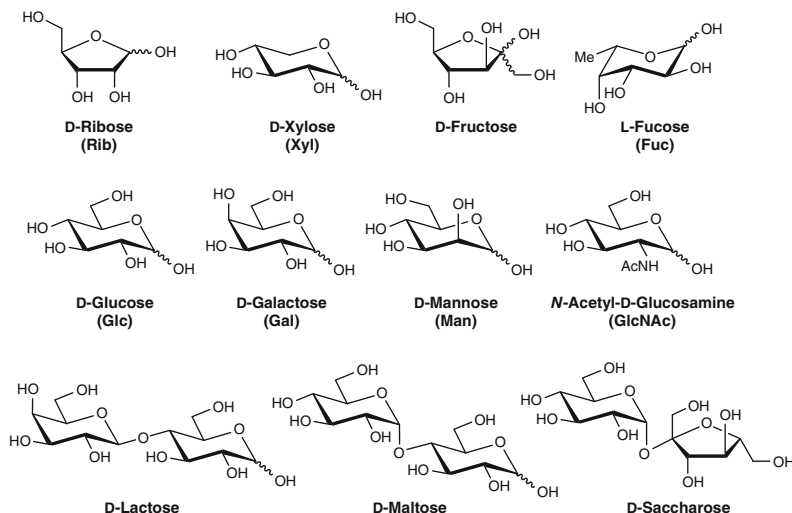
Fig. 7.14 Selection of naturally occurring mono- and disaccharides. Conventional abbreviations are mentioned in parentheses when appropriate
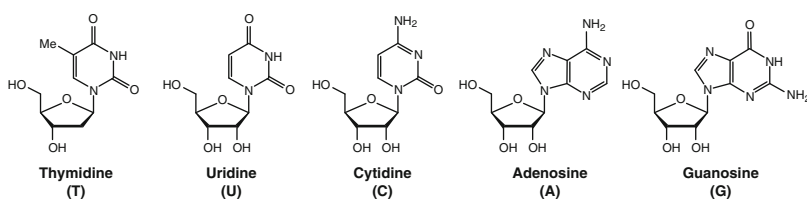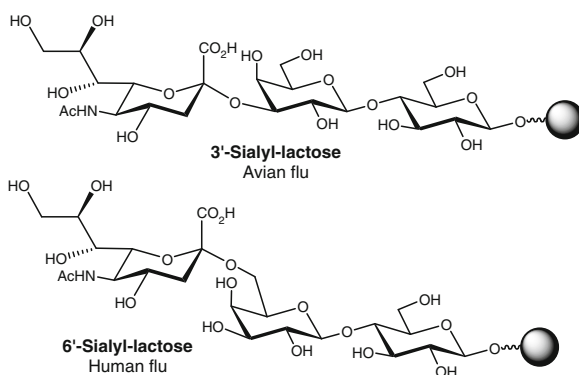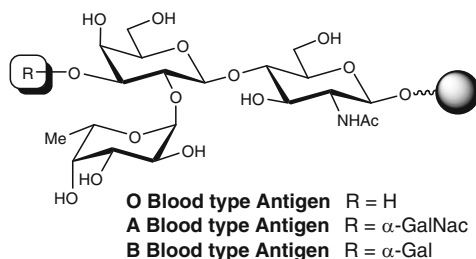

Fig. 7.15 Thymidine and the four natural nucleosides as RNA polynucleotide repeating units

pyrimidine: uracil (U) or cytosine (C), or to a purine: adenine (A) or guanine (G). In the DNA series, thymine (T) replaces uracil while the three other nucleobases are similar (Fig. 7.15).

7.2.6.3 Oligosaccharides

Carbohydrates can be associated through glycosidic linkages to form oligosaccharides possessing a wide range of biological aspects. The Lewis blood group antigens are oligosaccharides present on the surface of red blood cells and distinguish the human blood groups. These oligomers differ by the substitution (R) at the 3-position of the non-reducing galactosyl residue (Fig. 7.16).

Sialylated oligosaccharides are involved in a series of viral infection events. The substitution of lactose by sialic acid either at the 3′ or 6′ position differentiates between avian and human flu, respectively (Fig. 7.17) (Shinya et al. 2006).

Fig. 7.16 Structure of the ABO blood antigens

O Blood type Antigen R = H
A Blood type Antigen R = α-GalNac
B Blood type Antigen R = α-Gal

3'-Sialyl-lactose
Avian flu

6'-Sialyl-lactose
Human flu

Fig. 7.17 Structure of the 3′- and 6′-sialyl-lactose oligosaccharides involved in *influenza virus* infection

Oligosaccharides can also adopt cyclic forms conferring a very different set of chemical and physical properties. Cyclodextrins are natural cyclic oligomers composed of six or more glucose units linked through an α-1,4-glycosidic bond (Fig. 7.18) (Nepogodiev and Stoddart 1998). Their inside cavity is highly hydrophobic and can form host–guest complexes with a series of molecules of various interests in the pharmaceutical industry (drug release) or for environmental applications (interactions with toxic compounds).

7.2.6.4 Polysaccharides

Polymeric carbohydrates are also widely present in nature. Cellulose is a β(1→4)-glucose polymer with a linear shape present in plants, while amylose is the corresponding α(1→4)-polyglucoside and is organized in a helical structure (Fig. 7.19). Polysaccharides are also employed for energy storage, like glycogen which is a branched polysaccharide based on the same α(1→4)-glucose polymeric chain as in amylose. Chitin is a polymeric β(1→4)-*N*-acetyl glucosamine and is the main component of the exoskeletons of crustaceans and insects.
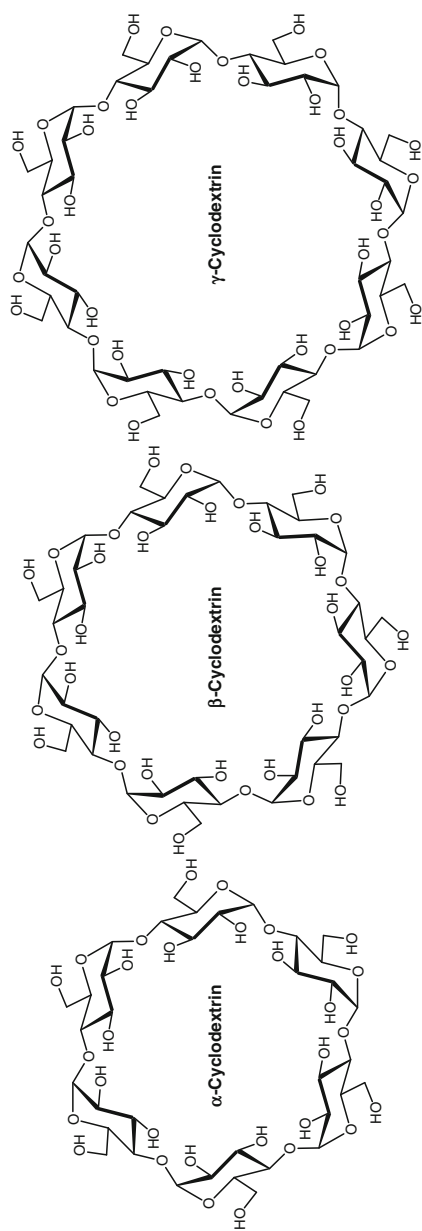
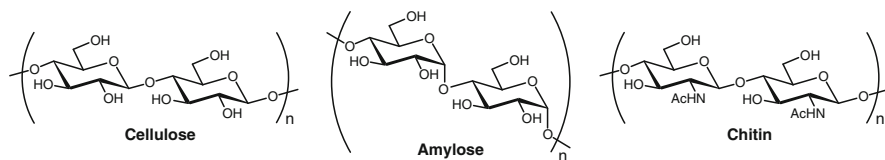**Fig. 7.18** Structure of the α-, β- and γ-cyclodextrins

Fig. 7.19 Structure of a selection of polysaccharides
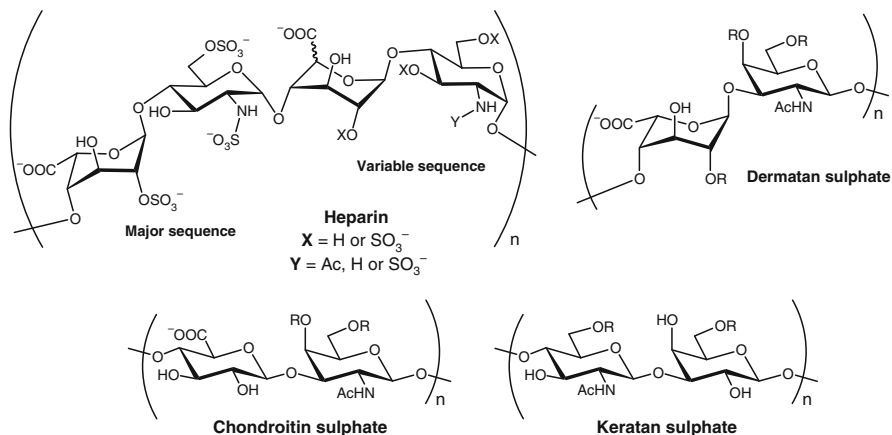

Fig. 7.20 Structure of heparin and other glycosaminoglycans (R = potentially sulphated sites)

7.2.6.5 Glycosaminoglycans

Heparin and heparan sulphate are the most complex members of the glycosamino-glycans (GAGs) family, which also includes chondroitin sulphate, keratin sulphate and dermatan sulphate (Fig. 7.20) (Bishop et al. 2007; Capila and Linhardt 2002). These highly negatively charged polysaccharides are displayed on the extracellular matrix or on the membrane of the cell. Heparin is an anticoagulant and is used for the treatment of heart diseases. It is composed of a tetrasaccharidic repeating unit with a major sequence highly conserved throughout the polymer chain and a variable sequence in which the sulphation/acetylation sites are not systematically functionalised. Heparan sulphate is a less sulphated version of heparin where the major sequence of heparin is not sulphated and the amino group acetylated.

7.2.6.6 Carbohydrate-Based Drugs from Natural and/or Synthetic Sources

A series of natural products containing carbohydrates have been isolated and their structure elucidated. Some of them are now used as drugs for the treatment of various diseases. Nojirimycin is produced by *Streptomyces* strains (Inouye et al. 1966). It is used as an antibiotic and inhibits α-glucosidases, thereby preventing the
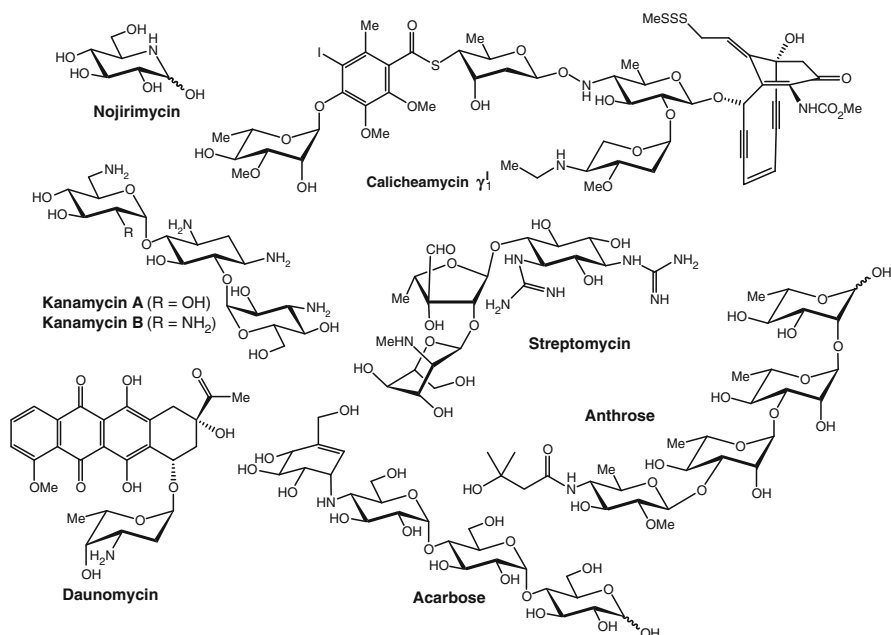
Fig. 7.21 Structures of natural products incorporating carbohydrate, one or more residue(s)

normal glycosylation of proteins (Fig. 7.21). Calicheamicin γ_1^I is isolated from *Micromonospora echinospora* and is used as an antibiotic (Nicolaou and Smith 1992). It binds to the minor groove of DNA showing a high degree of base pair sequence specificity which can be attributed to the oligosaccharide residues. Kanamycins are antibiotics affecting the 30S ribosomal subunit and preventing the correct translation of RNA (Maeda et al. 1957). Indeed, the proteins are biosynthesized but fold incorrectly, thus destroying the bacterium. Daunomycin is an anti-cancer chemotherapy agent of the anthracycline family and isolated from *Streptomyces peucetius* (Arcamone et al. 1964). Acarbose is an inhibitor of α-glucosidase used for the treatment of type 2 diabetes mellitus (Junge et al. 1984). Streptomycin is an antibiotic drug obtained from the actinobacterium *Streptomyces griseus* and inhibits bacterial growth by damaging cell membranes and blocking protein synthesis (Majumdar and Kutzner 1962). Anthrose is found on the surface of *Bacillus anthracis*. This antigen is a very promising target for the development of an anti-anthrax vaccine (Tamborrini et al. 2006).

A series of nucleoside analogues have been approved by the Food and Drug Administration (FDA) for the treatment of HIV infection (AIDS) (Fig. 7.22). Zidovudine (AZT) was one of the first nucleoside approved by FDA and acts as a chain terminator for HIV reverse transcriptase (HIV-RT). Although resistance to AZT frequently develops, the association of Lamivudine (3TC) and AZT can cause AZT-resistant HIV strains to revert to AZT-sensitive strains. Abacavir is a carbocyclic nucleoside analogue significantly less toxic than AZT and appears to have a
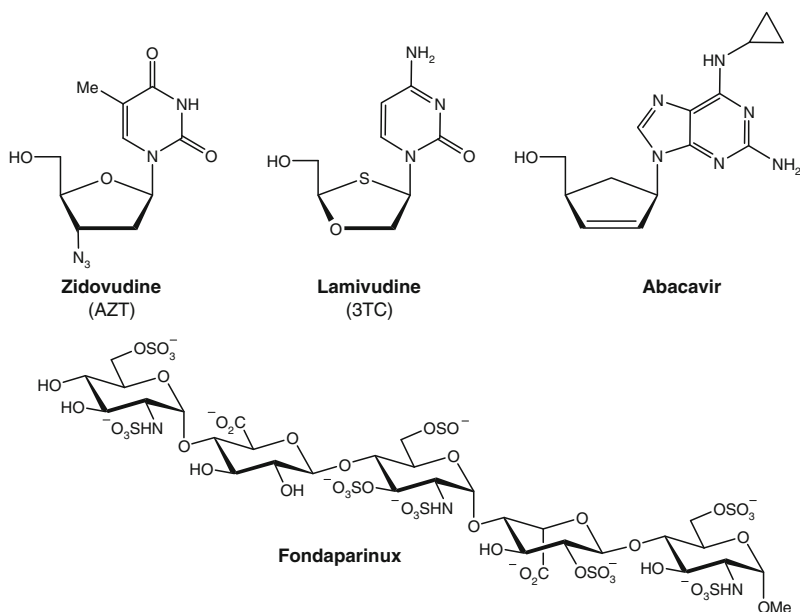
Fig. 7.22 Structures of synthetic carbohydrate-based drugs

stronger anti-HIV effect than any of the approved nucleoside analogues. The association of AZT, 3TC and Abacavir in a tri-therapy strategy improves considerably the life expectancy of AIDS (or HIV-infected) patients.

Fondaparinux has been recently marketed by Sanofi-Aventis as an heparan sulphate analog for the treatment of heart diseases (Fig. 7.22) (Petitou and van Boeckel 2004).

7.3 Biological Role

The carbohydrates are probably best known for their role in energy metabolism. But like proteins, some carbohydrates perform structural functions, providing scaffolding for bacterial and plant cell walls (cellulose, pectin), connective tissues such as cartilage in animals, and exoskeleton shells (chitin) in arthropods. Carbohydrates are also covalently bound with proteins (glycoproteins) or lipids (glycolipids) on cell surfaces. The glycoproteins and glycolipids play important roles in cell–cell recognition processes and in signal transduction

7.3.1 The Glycocalix and Extracellular Matrix Polysaccharides

The surface of most types of cells is covered by a carbohydrate-rich coat, the glycocalyx, which plays an important functional role in cell–cell and cell–matrix

interactions. These specific contacts are realized by using attractive forces between cell-surface oligosaccharides and proteins (lectins) (Hakomori 1991; Hakomori 2002; Rojo et al. 2002). It is now accepted that these interactions strongly depend on divalent cations and show high specificity and low affinity. However, its mechanism is not yet clarified because of the difficulty in analysing weak affinity interactions. The main challenge to understand and control interactions between carbohydrates and lectins is the low affinity of the interactions. Nature overcomes this problem by a polyvalent presentation of ligands and receptors at the cell surface (Mammen et al. 1998).

Studies of the terminal carbohydrate residues of the glycocalyx of immune cells have shown that cell–cell and cell–matrix interactions, which play an important functional role in various processes of the immune response, are mediated at least in part by these carbohydrate residues (Hounsell 2000). The use of lectin histochemistry in lymphatic organs of chickens demonstrated that carbohydrate residues are differentially expressed in the cells of the immune system of chickens (Jorns et al. 2003). For example, in the thymus, mannose as well as N-acetyl glucosamine (GlcNAc)-specific lectins labelled macrophages, epithelial reticulum cells and lymphocytes within the cortex. In the bursa of Fabricius, the brush border of the lining epithelium, the macrophages and the endothelium were labelled by mannose-specific lectins.

Because glycoconjugates, including proteoglycans, glycoproteins and glycolipids, are mainly localized in the plasma membrane and in the extracellular matrix of cells, they are considered as useful cell-surface biomarkers and play crucial roles in signal transduction and cell adhesion. To illustrate this, the vascular endothelium is presented in the following.

The endothelial glycocalyx is considered to be connected to the endothelium through several "backbone" molecules, mainly proteoglycans and also glycoproteins. Proteoglycans consist of a core protein to which one or more glycosaminoglycan chains are linked. There are five types of glycosaminoglycan chains: heparan sulfate, chondroitin sulfate, dermatan sulfate, keratan sulfate and hyaluronan (or hyaluronic acid). They are linear polymers of disaccharides with variable lengths that are modified by sulfation and/or (de)acetylation to variable extents. The disaccharides are each composed of a uronic acid and a hexosamine. In vasculature, heparan sulfate proteoglycans represent 50–90% of the total amount of proteoglycans present in the glycocalyx (Pries et al. 2000). Hyaluronan, another important glycosaminoglycan, differs from others because it is not linked to a core protein. Its exact link to the cell membrane is not known, but it can be bound to the receptor CD44 (Nandi et al. 2000).

Besides the proteoglycans, well-defined glycoproteins bearing small (2–15 sugar residues) and branched carbohydrate side chains connect the glycocalyx to the endothelial cell membrane. These endothelial cell adhesion molecules play a major role in cell recruitment from the bloodstream and in cell signalling. The three families of cell adhesion molecules present in the endothelial glycocalyx are the selectin family, the integrin family and the immunoglobulin superfamily. Selectins are carbohydrate-binding glycoproteins. These glycoproteins were

designated as E-, P- and L-selectin, according to the cell type on which the selectin was found (i.e. endothelium, platelets, and lymphocytes, respectively). E-selectin and P-selectin are both involved in leukocyte–endothelial cell interactions (Sperandio 2006). The carbohydrate ligands that are recognised by these selectins have been identified. E-selectin recognizes sialyl Lewis X (sLe^X) on the surface of neutrophils. P-selectin also binds sLe^X on neutrophils or leukocytes with a lower affinity. L-selectin weakly recognises sLe^X on endothelial cells, but the affinity is higher with a sulphate group on the 6-position of Gal or, perhaps more likely, on the 6-position of the GlcNAc residue. Integrins are heterodimeric molecules composed of non-covalently bound α (18 different α-subunits) and β (8 β-subunits) subunits. Integrins are found on many cell types, including endothelial cells, leukocytes, and platelets. These integrins, such as α2β1, α5β1 and α6β1, bind to multiple extracellular matrix ligands and are responsible for interactions with laminin, fibronectin, and collagen. Many studies have focused and still focus on the interactions between these integrins and the sub-endothelial matrix during angiogenesis (Ruegg and Mariotti 2003). The best known immunoglobulin superfamily of glycoproteins includes intercellular adhesion molecule 1 and 2 (ICAM-1 and -2), vascular cell adhesion molecule 1 (VCAM-1) and platelet/endothelial cell adhesion molecule 1 (PECAM-1). They act as ligand for integrins on leukocytes and platelets and are crucial mediators of leukocyte homing to the endothelium and subsequent diapedesis. In addition, glycoproteins of the endothelial glycocalyx display functionality in coagulation, fibrinolysis and homeostasis.

Leukocyte adhesion to endothelial cells is a complex process involving capture of free-flowing leukocytes from the bloodstream, rolling on the endothelial surface, deceleration and, eventually, leukocyte immobilization (firm adhesion). Studies have shown that disruption of heparan sulphate proteoglycans of the endothelial glycocalyx stimulates firm adhesion of leukocytes (Constantinescu et al. 2003). Indeed, heparan sulfate proteoglycans are abundant on the endothelial cell surface, are highly negatively charged due to the presence of numerous sulfate groups, and bind numerous plasma proteins, contributing to the glycocalyx charge and thickness (Esko 1999; Yanagishita and Hascall 1992). The integrity of vascular endothelium depends on the number of negatively charged molecules at the endothelial cell surface. In myocardial tissue oedema, the decrease of microvascular fluid is associated with a reduction of negatively charged molecules (Gotloib et al. 1992).

Glycoconjugate antigens are also utilized as unique markers in the developing brain and central nervous system (CNS) (Yu and Yanagisawa 2006; Yanagisawa and Yu 2007; Zhang 2001). Indeed, basic fibroblast growth factor (bFGF) is important for sustaining multipotency and enhancing proliferation of neural stem cells (NSCs)/neural precursor cells (NPCs). The signal of bFGF is mediated by receptors localized in the cell surface, but additional co-factors are also required. One of these co-factors is heparan sulfate proteoglycan (HSPG; Fig. 7.1). In addition to HSPGs, chondroitin sulfate proteoglycans (CSPGs) are also expressed in NSCs/NPCs. Nerve/glial antigen 2 (NG2) is a CSPG expressed in heterogeneous cell populations, including glial precursor cells and/or NSCs (Belachew et al. 2003). Furthermore, NG2 displays high affinity for bFGF and platelet-derived

growth factor (PDGF)-AA, which are mitogens of NSCs/NPCs and oligodendrocyte progenitor cells (Goretzki et al. 1999).

Another class of carbohydrate-bearing molecules are glycosphingolipids (GSLs) which are composed of a core lipid moiety (ceramide) linked to a carbohydrate chain (Yu et al. 2004). GSLs having one or more sialic acid residues are named gangliosides. GSLs are most abundant in nervous tissues and are localized on the plasma membrane of cells. Studies have shown that certain GSLs are expressed in NSCs/NPCs like GD2 in human and mouse NSCs (Klassen et al. 2001) and GD3, GT1b and GQ1b in mouse NPCs(Yanagisawa et al. 2005).

Because of the biological significance of NSCs in neurogenesis, glycoconjugates are expected to play a significant role in developing clinical uses of NSCs for the treatment of a variety of neurodegenerative disorders. First, glycoconjugates can be used as biomarkers to define and isolate NSCs and their progenies. They also may be used for enriching NSCs. In addition, glycoconjugates may be useful for inducing differentiation of NSCs into specific lineages.

7.3.2   Carbohydrates in Host–Pathogen Interactions and Metastasis

Carbohydrates can serve as key elements in various molecular recognition processes, including bacterial and viral infections, cell adhesion in inflammation and metastasis.

The glycosylated phosphatidyl inositols (GPIs) are ubiquitous eukaryotic glycolipids covalently linked to the C-terminus of membrane-associated proteins. They provide an alternative mechanism for the attachment of proteins to the plasma membrane. However, studies have shown that glycoinositol phospholipids (GIPLs) not linked to either protein or polysaccharide are major cell-surface glycolipids in trypanosomatids, and are closely related to the structure of the GPI–protein anchor (Ferguson 1999). The GIPL of the parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, contains a unique glycan chain expressing slight variations among distinct *T. cruzi* isolates, and linked through a non-*N*-acetylated glucosamine residue to an inositol phosphorylceramide, mainly composed of an *N*-lignoceroyl-dihydrosphingosine (Carreira et al. 1996). The β-D-Gal*f*-(1→3)-α-D-Man*p* substructure is involved in its antigenicity (Mendonça-Previato et al. 1983). Biological effects of *T. cruzi* GIPL and its isolated glycan and lipid moieties were investigated on cells of the immune system. Dos Reis et al. (2002) found that GIPLs are bioactive against every cell type that has been tested.

Leishmania spp. are protozoan parasites that cause a spectrum of diseases in humans, ranging from self-limiting cutaneous infections to visceral leishmaniasis. Control of this disease, which affects more than 20 million people worldwide, has been hampered by the absence of a vaccine, limitation with frontline drugs and the increased transmission as a result of co-infections with HIV (Croft et al. 2006). The promastigote stages of *Leishmania* are coated by a thick glycocalyx composed

of glycosyl phosphatidyl inositol (GPI)-anchored proteins, the GPI-anchored phosphoglycan, lipophosphoglycan (LPG) and GIPLs (Naderer et al. 2004). Analysis of *Leishmania* mutants lacking individual or multiple surface components indicates that only LPG plays a critical role in macrophage infection, while loss of other surface components has relatively little affect on promastigote virulence in the mammalian host (McConville et al., 2007). The plasma membrane of *Leishmania* amastigotes is unusual in several other respects. It contains relatively high levels of externally exposed phosphatidyl serine, which may provide a mechanism for entering host cells via apoptotic cell receptors without activating microbicidal processes or proinflammatory responses (Wanderley et al. 2006).

The study of N-linked glycosylation has become an area of intense interest because of its importance in viral virulence and immune evasion for several prominent human pathogens such as Hendra (Carter et al. 2005), severe acute respiratory syndrome coronavirus (SARS-CoV) (Oostra et al. 2006), influenza, hepatitis C (Goffard et al. 2005) and West Nile (Hanna et al. 2005). HIV and influenza, two clear threats to human health, have been shown to rely on expression of specific oligosaccharides to evade detection by the host immune system.

Influenza viruses express two envelope proteins that are involved in virulence, neuraminidase (NA) and hemagglutinin (HA). Influenza hemagglutinin binds to sialic acids attached to membrane glycosphingolipids, proteoglycans and glycoproteins. It was shown that the linkage between sialic acid and the second sugar (usually galactose) could determine whether an influenza virus binds to avian or human cells (Carroll et al. 1981; Rogers and Paulson 1983), and that a single mutation in the hemagglutinin (HA) was sufficient to change binding from α-2–3′ to α-2–6′ sialyl-lactose and host cells from avian to human, or vice versa (Rogers et al. 1983; Rogers et al. 1985). Now it is generally accepted that human influenza viruses bind to α-2–6′ sialic acids and avian viruses bind to α-2–3′ sialic acids (Baum and Paulson 1990; Matrosovich et al. 1997), while swine influenza viruses are reported to bind mainly to α-2–6′ sialic acids and also to α-2–3′ sialic acid receptors (Ito et al. 1998; Rogers and D'Souza 1989).

Infection by dengue virus (DV), a mosquito-borne flavivirus leading to haemorrhagic fever in humans, has undergone a global resurgence in the past 40 years such that almost half the world's population are currently living at risk in dengue-endemic areas. The four serotypes of DV (DV1–DV4) bind to a number of opsonic and non-opsonic receptors on cells of the mononuclear phagocyte lineage including DC-SIGN (Navarro-Sanchez et al. 2003; Tassaneetrithep et al. 2003) and glycosaminoglycans (Chen et al. 1997). Recently, Miller et al. (Miller et al. 2008) have shown that the mannose receptor (MR) is a functional receptor for DV infection of human macrophage. MR and DC-SIGN both contain lectin domains, but differ distinctly in terms of ligand specificity. MR binds to terminal mannose, fucose and *N*-acetyl glucosamine, whereas DC-SIGN binds to mannose within high-mannose oligosaccharides and fucosylated glycans (Taylor et al. 2005; Feinberg et al. 2001).

Carbohydrate antigen sialyl Lewis a (CA19-9) is the most frequently applied serum tumour marker for diagnosis of cancers in the digestive organs. Moreover, malignant transformation is associated with changes in the glycosylation

of cell-surface proteins and lipids. In tumour cells, alterations in cellular glycosylation may play a key role in their metastatic behaviour. Indeed, GD2 ganglioside is a major constituent of neuroectodermal tumors (Livingston 1995). Studies suggest that peptides mimicking GD2 ganglioside inhibit tumour growth through antibody and/or CD4+ T-cell-mediated mechanisms.

Oligosaccharide expression is highly relevant to many cancers. Many studies show that alterations in N-linked oligosaccharides of tumour cells are associated with carcinogenesis, invasion and metastasis (Varki 1993; Dwek 1996). Numerous studies have demonstrated a correlation between hyaluronan expression and the malignant properties of various kinds of cancer. Furthermore, hyaluronan oligosaccharides have been shown to inhibit several tumour cell types via disruption of receptor–hyaluronan interaction. So hyaluronan oligosaccharides could have potent antitumour effects due in part to the abrogation of hyaluronan-rich cell-associated matrices (Hosono et al. 2007).

Recent work has highlighted the importance of altered cell–matrix interactions in the acquisition of malignant characteristics of glioblastoma (Yamada and Araki 2001). Several studies have provided evidence that hyaluronan–cell interactions may play a role in glioma invasiveness. For example, addition of exogenous hyaluronan enhances glioma cell migration and invasion in vitro, and this effect is blocked by antibodies or anti-sense oligonucleotides to CD44, a cell-surface hyaluronan receptor (Merzak et al. 1994; Koochekpour et al. 1995; Okada et al. 1996; Tsatas et al. 2002).

7.4   Carbohydrate-Based Biosensors

Saccharide probes have been immobilised on a variety of substrates ranking from plane substrates such as polymer (Fukui et al. 2002), glass (Blixt et al. 2004), diamond (Chevolot et al. 1999) or gold (Seo et al. 2007; Miura et al. 2002; Smith et al. 2003) to nanoparticles or quantum dots (Larsen et al. 2006; Robinson et al. 2005; Huang et al. 2005). Both plane and spherical substrates are important to consider, as both can be used for biosensing.

7.4.1   Obtaining Saccharide Probes

Custom-synthesised peptides or nucleic acids are commercially available, and powerful synthetic tools such as automatic synthesisers are on the market. Furthermore, they can be amplified by PCR or cloning. On the other hand, oligosaccharides are tedious to synthesise and cannot be amplified. They are of synthetic or natural origin. Consequently, they are often available in limited quantities and expensive in the market.

Also, as mentioned in the introduction, since they have a very high structural diversity because of the fact that they can be linear or branched and the anomeric

carbon can be α or β, they can be in the chair or boat conformation and the carbon involved in the glycoside linkage can vary.

Furthermore, all the functional groups are hydroxyls with special reactivity for the one carried by the anomeric carbon atom and for the primary alcohol. This means that the synthesis of oligosaccharides requires selective protections and deprotections of the desired hydroxyl function for coupling of monosaccharides through a glycosyl bond. Some authors have developed automated solid-phase synthesis, while some others have used a "one-pot" approach (Feizi et al. 2003; Werz and Seeberger 2005a; Seeberger 2008; Plante et al. 2001). Still others take advantage of enzymes (glycosidases or glycosyltranferases) to perform the synthesis of oligosaccharides. Glycosidases are enzymes that under usual conditions hydrolyse the glycosidic bond. However, as this reaction is reversible, under defined conditions, the enzyme can catalyse the formation of the linkage. Glycosyltransferases are enzymes that catalyse the glycosidic linkage formation between two saccharides. They are more efficient than glycosidases, giving better yields. However, the number of different enzymes is limited and expensive. The natural substrate of the enzyme is usually expensive as well. These reasons have motivated some authors to engineer whole bacteria with genes coding for the different enzymes required for oligosaccharides synthesis (Fort et al. 2005).

Nevertheless, synthesis of a considerable number of complex carbohydrates has been achieved but often in limited amounts and at high cost.

In the following section, the different methods employed for surface immobilisation of carbohydrates are described, followed by a paragraph on signal transduction and finally some applications.

7.4.2 Surface Physicochemistry: Non-specific Adsorption and Immobilisation

One can picture the saccharide–protein interaction as a key–lock interaction. In consequence, like protein arrays (Gordus and Mac Beath 2006), carbohydrate arrays require the preservation of the 3-D conformations of the saccharide ligand in order to perform specific biomolecular recognition with their receptor. In addition, their accessibility and orientation are major issues especially in the case of immobilised probes where the degrees of freedom are fewer than in solution. Therefore, the surface chemistry employed for immobilisation (the chemical reaction for immobilisation, but also the structure of the spacer arm) of the saccharide and the physico-chemical properties of the surface (and ligand) are major issues in maintaining the biological activity, as they will play key roles not only in the preservation of the 3-D conformation but also in the probe availability. For example, weak interactions between the surface and the probe can influence the orientation of the probe. Surface organisation of the probe (dense packing which depends among other things on the spacer arm structure) can also influence the probe

availability. Organisation of the water molecules at the surface of the material may also play a role in the thermodynamics of biological recognition at the surface of the material.

The immobilisation should

- proceed with good and reproducible yield for a given probe;
- proceed with the same yield independently of the probe (especially in the case of glycoarray);
- be oriented in order to preserve the "key–lock" interaction between the probe and the target and to obtain a reproducible device;
- allow the accessibility of the probe by the target although the degree of freedom is reduced; and
- allow quality control of immobilised probes.

The performances (lower detection limit, reliability, robustness, etc.) of the micro-devices will greatly depend upon this step, as the target capture by the probe will depend on the probe availability (not taking into account diffusion and microfluidic-related phenomena (Raynal et al. 2004)).

The detection technique will determine the choice of the substrate to be used and, therefore, the subsequent coupling reactions. For example, gold is the substrate of choice for SPR detection, whereas glass is often preferred with fluorescent detection. In one case, mercapto-based coupling agents have been used (Schreiber 2000), whereas silane-based coupling agents are preferred with glass.

Furthermore, the background signal depends on non-specific adsorption of the target. It should be reduced to avoid high background.

In the following, we will discuss the various strategies employed for reducing non-specific adsorption, as well as the various strategies for immobilising carbohydrates, on surfaces of materials, more specifically mono and oligosaccharides.

Finally, the collective behaviour of the molecules such as surface organisation of probe molecules can influence the affinity of a target for a probe as discussed in section "Surface Cluster Effect or Not."

7.4.2.1 Non-specific Adsorption

A protein in solution adsorbs on a surface if the total Gibbs free energy is negative. In a simple medium where only one protein is present, the entropic factor (Norde 1985; Norde et al. 1986) seems to be the driving force for protein adsorption. Entropy is affected by the dehydration of both the surface and the protein, rearrangement of the protein structure and rearrangement of charged groups. The dehydration effect on hydrophobic surfaces can be viewed as the disruption of an ordered ice-like layer of water at the surface of the materials by the incoming protein. This disruption is due to surface–protein hydrophobic interactions that are stronger than the surface–water and protein–water interactions. This leads to an increase in entropy. On hydrophilic surfaces, the driving force is the change in protein structure (Norde et al. 1986).

In case of complex media, such as biological fluids, where different proteins coexist, the adsorption phenomenon can be regarded as a competitive adsorption (Vroman effect). The composition of the adsorbed layer may be very different from that of the bulk solution (Vroman 1962; Bamford et al., 1992).

From the above considerations, the following conclusions can be made:

- Protein adsorption on a surface depends on a combination of factors such as the solution physico-chemical characteristics (pH, ionic strength, temperature, composition, etc.), on the physico-chemical properties of the protein(s) and on the physico-chemical properties of the material surface (surface energy, roughness, surface chemistry, etc.).
- Protein adsorption can be reduced by two major means:
- By pre-adding a protein that will readily adsorb on the surface and displace the protein that one wants to analyse: BSA (bovine serum albumin), HSA (human serum albumin), casein, etc. The choice of one blocking protein over another will depend on the protein to be analysed.
- By developing a surface on which protein adsorption is thermodynamically not favoured. Neutral hydrophilic polymers such as polyethylene glycol (PEG) (Desai and Hubbell 1991; Jeon et al. 1991; Lee et al. 1997), polyhydroxyethyl methacrylate, polyvinyl alcohol, poly-*N*-vinyl 2-pyrrolidone, etc. seem to be ideal in this case. Some authors have described the use of polymers mimicking the lipid double layer (Ishihara et al. 1998; Ruiz et al. 1999).

In the case of carbohydrate-based biosensors, Table 7.3 summarises the blocking strategies commonly described in the literature. It has to be ensured that

Table 7.3 Reducing non-specific adsorption requiring passivation of the surface

Blocking	Substrate	Target	Refs
Denhardt	Glass	ConA, tetraglonolobus; EC	Zhou and Zhou(2006)
PVP K30	Polymer	Glycosyltransferase R	Seibel et al.(2006)
BSA 0.5%	Polymer	Lectin	Seibel et al.(2006)
BSA 1%	Code link-activated glass slides	Fibroblast growth factor	de Paz et al.(2006)
BSA 1%	Maleimide-modified glass slides	ConA tetraglonolobus; EC	Brun et al.(2006)
BSA 3%	NHS ester glass slides	Haemaglutinin	Stevens et al.(2006)
BSA 1%	PhotoChips	Plant lectin	Angeloni et al.(2005)
BSA 3 %	Epoxide-modified glass slides	Lectin	Manimala et al.(2006)
PVA	Hydrogel-modified glass slides	Ab	Dyukova et al.(2005)
BSA 3%	Glass	Lectin	Pei et al.(2007)
BSA0.005%	Alcyne-modified Gold	Lectin	Zhang et al.(2006)
BSA 0.1%	Polystyrene		Chevolot et al.(2001)
No blocking	NHS ester glass slides	Globo H/Ab	Huang et al.(2006a)
	Glass	Lectin and Ab	Bryan et al.(2004)
Casein/BSA	Nitrocellulose	Micronemal protein	Blumenschein et al. (2007)

The table summarises some strategies described in the literature

the blocking protein does not have glycosylation motifs that can compete with the saccharide ligands and may interfere with the target molecule. Some authors have demonstrated that BSA can hide the probe immobilised at the surface from the target (Lesaicherre et al. 2002; Mac Beath and Schreiber, 2000).

7.4.2.2 Immobilisation of Saccharide Ligands

The immobilisation of the saccharides at the surfaces of the biosensor has been achieved through three main strategies:

- Physisorption
- Covalent immobilisation
- Biochemical-based interaction

7.4.2.2.1 Physisorption

Physisorption (Fukui et al. 2002; Wang et al. 2002; Satoh et al. 1999; Stoll et al. 2000; Palma et al. 2006) of carbohydrate ligands relies on the weak interactions between the molecules to be immobilised and the surface. This is a convenient technique, as it does not require surface functionalisation. Nitrocellulose or polystyrene are mainly used as the substrates.

In the case of low molecular weight species such as oligosaccharides, their interactions with the surface are too weak and require the modification of the saccharide with an anchoring tail for increasing the interaction, e.g. a lipid, a fluorescent tag (Jaipuri et al. 2008), a protein or a polymer such as polyacrylamide. Such a tail can be introduced by the condensation of the azido functional group with sp2- and sp3-bearing molecules (Fazio et al. 2002), by aminoreduction (Fukui et al. 2002) or by the condensation of amine with isocyanate (Fazio et al. 2004). The group of Wang at Stanford University and the group of Feizi at Imperial College, London, have developed neoglycoconjugates bearing such tails for their immobilisation on nitrocellulose membranes or nitrocellulose-coated glass slides. Oligosaccharides (2–20-mers) are linked to amino phospholipid 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine or its anthracene derivative by reductive amination. The anthracene derivative allows quality control of the relative surface density of the probes (Palma et al. 2006). The group of Wong has derivatised oligosaccharides with a C14 alkyl chain through Cu[(I)]-catalysed Huisgen 1,3-dipolar cycloaddition. A similar strategy was used by Huang et al. (2005). These two groups have demonstrated in various publications that this strategy is very efficient, allowing the immobilisation of femtomoles of compounds and with a very good signal-to-noise ratio.

7.4.2.2.2 Covalent Immobilisation

The covalent immobilisation of the saccharide has been achieved by reacting with functionalised surfaces with unmodified saccharides or functionalised glycosides (saccharide bearing a functionalised aglycon).

7.4.2.2.2.a Immobilisation of Underivatised Saccharides (Fig. 7.23)

Activation of the hydroxyl functions can be achieved with a compound leading to good leaving groups under nucleophilic attack. Activating agents (CNBr, CNCl, divinylsulphone, isocyanate, etc.) have been reported for immobilisation of oligo- and polysaccharides. However, divinylsulphone may lead to intra-reactions between two adjacent hydroxyl groups.

Jouan et al. (1996) have described the immobilisation of saccharides by divinylsulfone cross-linking of amino groups and oligosaccharide hydroxyl groups. The main drawback is that the hydroxyl groups react randomly with the amino groups.

Reductive amination takes advantage of the reaction of the reducing end of the carbohydrate (or aldehydes obtained after oxidation under mild conditions agent such as nitrous acid) with an amine or an hydrazine followed by the reduction of the Schiff base by hydrides. Many examples have been reported. However, it requires long reaction times and the yields are low. To circumvent these drawbacks, other authors have immobilised saccharides through hydrazide (Lee and Shin 2005; Satoh and Matsumoto 1999) or oxime (Zhou and Zhou 2006) linkages. Thiazolidine and glycosyl amide bonds have also been described (Larsen et al. 2006). The hydrazide linkage is poorly stable at low pH, while oximes are stable over a wider range of pH (Larsen et al. 2006). Nevertheless, the immobilisation of unmodified saccharides is advantageous with complex carbohydrates from natural sources. However, the main drawbacks are that the immobilisation reactions are limited to reactions involving the reducing end of the saccharides (and consequently cannot be used with non-reducing sugars) and they often lead to the opening of the reducing end ring while it may be crucial to maintain the structure of the molecule (Larsen et al. 2006).

7.4.2.2.2.b Immobilisation of Saccharide Derivatised with a Functional Aglycon

It can be advantageous to derivatise the saccharide with a functionalised aglycon allowing for the subsequent immobilisation. Two main strategies can be envisioned. One is the use of an aglycon bearing a function that can react directly with the surface, such as thiolated or disulfide aglycons, which permits the direct immobilisation of the glycoside onto the metal surfaces (gold in particular, Fig. 7.24). The second is chemically modifying the surface with a hetero cross-linker bearing a functional group for reaction with the surface and a second functional group for reaction with the aglycon (Fig. 7.24).
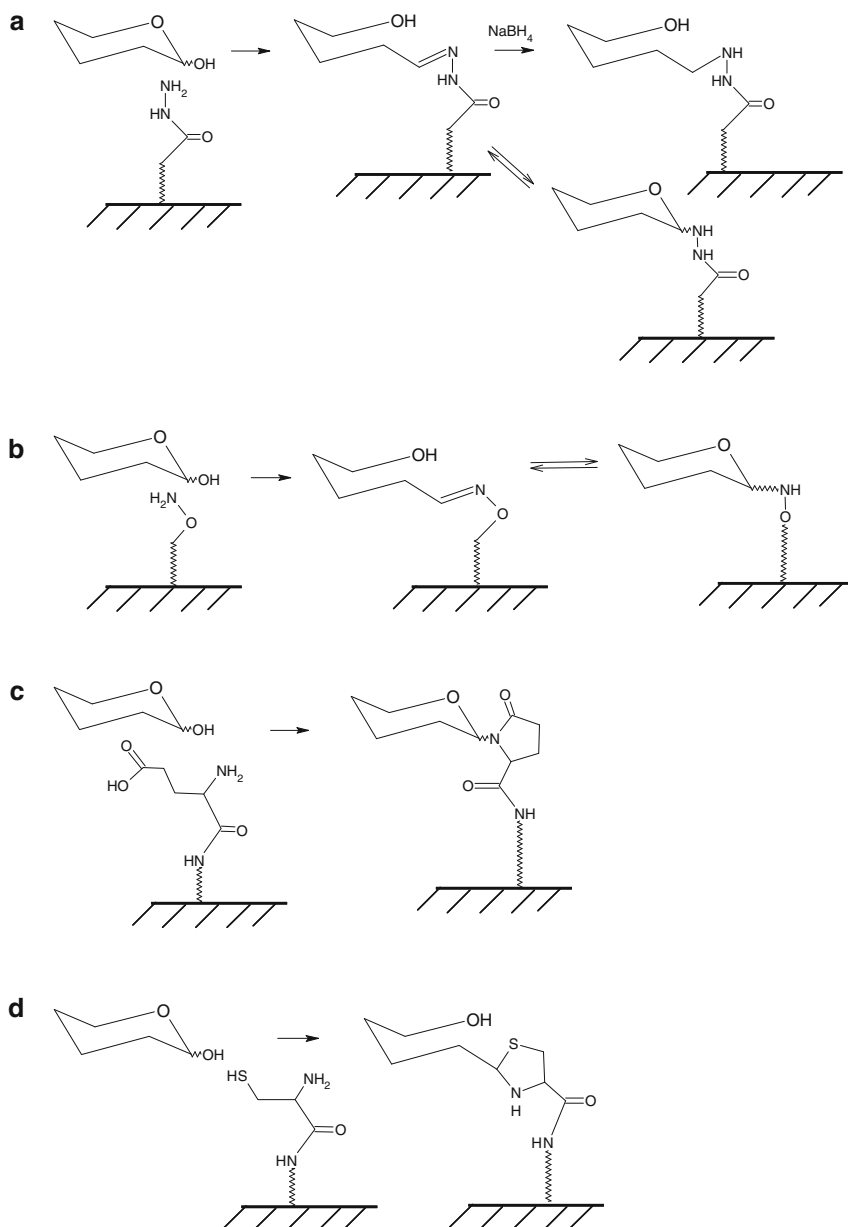
Fig. 7.23 Immobilisation of carbohydrate through their reductive end: **a** base Schiff or reductive amination, **b** oxime, **c** glycosyl amide bound formation and **d** thiazolidine. Immobilisation of underivatised saccharides usually take advantage of the reducing end of the saccharides as anchoring point, though immobilisation by the O-3, O-6 and N-2 have been described (Larsen et al. 2006; Seo et al. 2007)
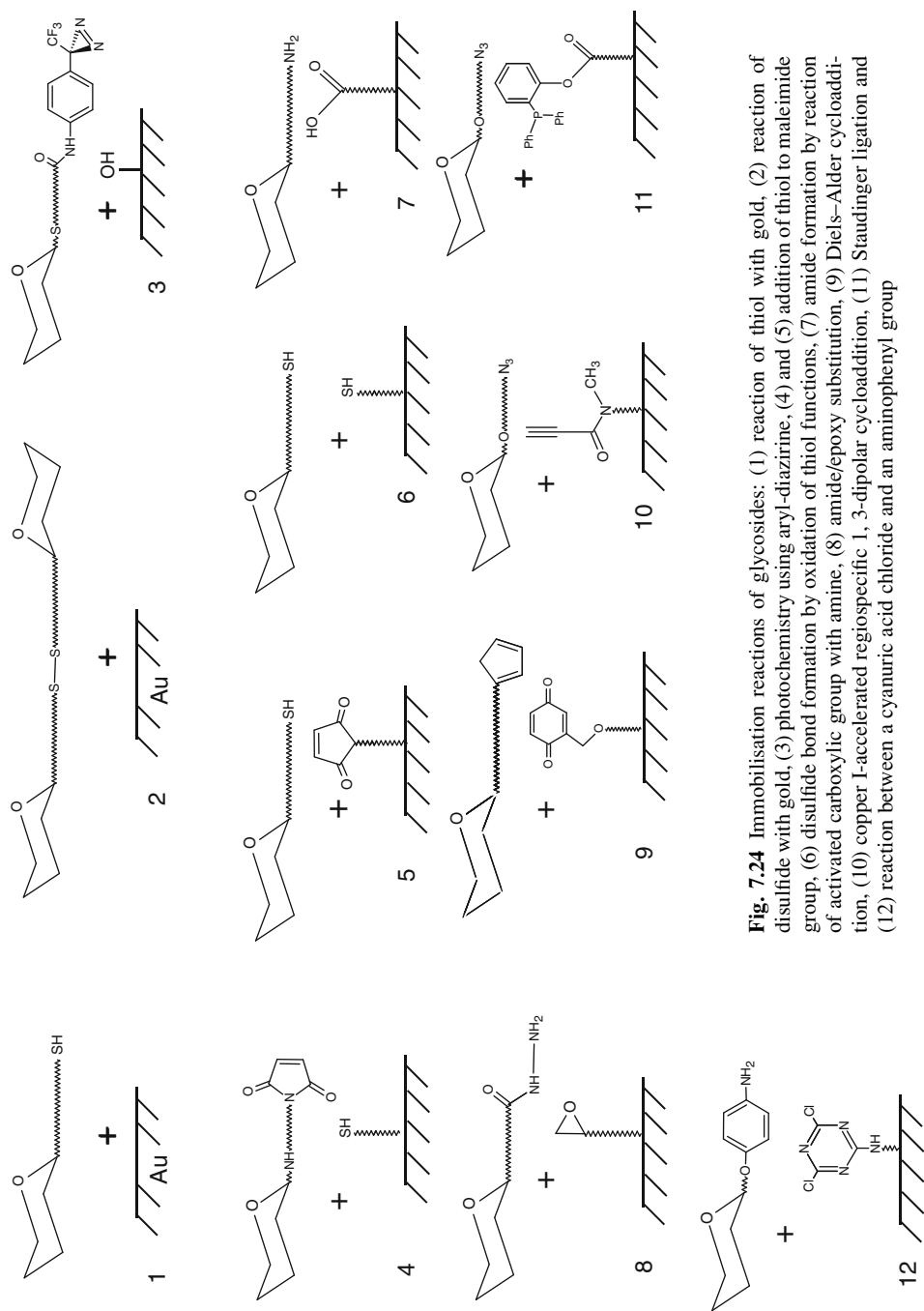
Fig. 7.24 Immobilisation reactions of glycosides: (1) reaction of thiol with gold, (2) reaction of disulfide with gold, (3) photochemistry using aryl-diazirine, (4) and (5) addition of thiol to maleimide group, (6) disulfide bond formation by oxidation of thiol functions, (7) amide formation by reaction of activated carboxylic group with amine, (8) amide/epoxy substitution, (9) Diels–Alder cycloaddition, (10) copper I-accelerated regiospecific 1, 3-dipolar cycloaddition, (11) Staudinger ligation and (12) reaction between a cyanuric acid chloride and an aminophenyl group

Direct reaction. Thiol functions have strong affinity for noble metals such as gold, silver, indium, etc. (see the corresponding chapter 2). Voltametric studies (Wink et al. 1997) have shown that upon adsorption thiols are deprotonated, leading to a covalent bond between the thiolate and the gold metal. Disulfide can also strongly bind to gold; however, the resulting films are less compact and less ordered. Many authors have taken advantage of these interactions for the immobilisation of thiol- (or disulfide-) containing biomolecules. In the case of carbohydrates, a mercapto aglycon should be introduced into the molecule.

Miura et al. (2002) have reported the immobilisation of Gb3 mimics (a saccharide ligand of the shigatoxins 1 and 2) bearing a disulfide aglycon on a gold substrate. The surface density of the immobilised Gb3 mimics was evaluated with quartz micro-balance measurements. The area per molecules was estimated to be 54 $\mathring{A}^2$, leading to an estimated surface density of approximately 2×10^{14} molecules per cm^2. Ratner et al. have studied the affinity of the protein cyanovirin-N with mannosyl oligosaccharides. Immobilisation on gold substrates for SPR-binding affinity measurements was achieved with thiol derivatives of mannosyl oligosaccharides (Ratner et al. 2004).

Gold nanoparticles are often functionalised with saccharide ligands by mean of thiol reaction with gold (Chen et al. 2005). Robinson et al. (2005) have reported the synthesis of N-acetyl glucosamine encapsulated quantum dots (QDs). CdSe/ZnS core shell QDs were synthesized and subsequently encapsulated with pyridine. They were then allowed to react with disulfide-functionalised GlcNAc in the presence of $NaBH_4$, leading to GlcNAc-modified QDs. They have demonstrated by NMR an IR spectroscopy the reaction between the sulphur atom and the QD and the presence of the GlcNAc.

The synthesis of mannose-containing CdSe/ZnS QD has also been reported using mannose-containing phosphine oxide (Larsen et al. 2006). CdS QD modified with maltose or Lewis X was achieved using thiol or disulfide glycosides in the presence of sodium sulfide and cadmium nitrate (Larsen et al. 2006).

Other authors (Chevolot et al. 2001; Chevolot et al. 1999) have derivatised mono- and di-saccharides with photoactivatable aryl-diazirine. Immobilisation was performed by irradiation at 350 nm, leading to a reactive carbene, which reacted with the substrate. The advantage of this chemistry is the versatility of the reaction with respect to the substrate and the possibility of using photolithography for obtaining specific patterns of carbohydrate domains on the surface. In such a way, the immobilisation of galactose was performed on silicon, silicon nitride, diamond and polystyrene. Time-of-flight secondary ion mass spectroscopy (ToF-SIMS) analysis (Leonard et al. 1998; Léonard et al. 1998; Léonard et al. 2001) demonstrated that the hydroxyl groups present on diamond surfaces were involved in the immobilisation reaction. The resulting surface density was estimated to be in the range of 10^{12}–10^{13} molecules per cm^2. Square features of 25 μm were obtained using photolithography.

Surface immobilisation of saccharides can be achieved by the surface co-polymerisation of saccharide-containing monomers with monomers (without

saccharides). Dubois et al. (2005) have developed an electrochemical approach based on a polypyrrole-coated electrode displaying pendant carbohydrates.

Reaction with functionalised surfaces. Several covalent immobilisations of oligosaccharides through reaction between an aglycon and a functionalised surface are described in the literature. The introduction of the aglycon can be performed by various means (refer to the corresponding paragraph on glycoside formation 7.2.3).

Surface functionalisation with the desired function such as carboxylic acid, amine, thiol and maleimide is achieved with a hetero-bifunctional cross-linker or coupling reagent. These molecules possess two functions: one that reacts with the surface and the other with the function of the aglycon. If necessary, the latter function can be protected during the reaction with the substrate. The coupling reaction may require the activation of one function, such as NHS activation of a carboxylic function, for reaction with an amine (see the corresponding chapter 2).

The usual yields are between a few percent and 10–20%, leading to a surface density between 10^{12} and 10^{13} molecules per cm^2. Therefore, high biomolecule concentrations are used for the immobilisation (mM range). To increase the yield, the saccharide solution is allowed to dry. This can lead to spot non-homogeneity, such as donut-like spots. This can be circumvented by adding moistening agents and surfactants (Dugas et al. 2005) and/or with good control of the evaporating conditions (relative humidity, temperature).

After performing the coupling reaction, the unreacted surface function should be disabled to perform further reaction. This is done by reacting the surface with a molecule bearing the same function as the aglycon but at a higher concentration. Usually this molecule is commercially available and cheap. This operation is sometime called "capping" of the surface. For example, NHS-activated surfaces after reaction with an amine-bearing carbohydrate are allowed to react with ethanolamine to deactivate the unreacted NHS esters (Fig. 7.25).

Immobilisation of carbohydrate have been achieved by taking advantage of

- thiol reactivity towards double bonds such as maleimido groups (Park et al. 2004; Brun et al. 2006; Ratner et al. 2004; Houseman et al. 2003) or towards thiol groups under oxidative condition, leading to the formation of a disulphide bond (Shin 2007);
- amine reactivity (or hydrazine) towards carboxylic (Blixt et al. 2004) (Consortium for functional glycomic (CFG)), towards aldehydes (Biskup et al. 2005) or towards epoxy groups (Lee and Shin 2005);
- cycloaddition reaction such as Diels–Alder (Houseman and Mrksich 2002) by copper I-accelerated regiospecific 1,3-dipolar cycloaddition (Huang et al. 2006a; Bryan et al. 2004; Seibel et al. 2006);
- Staudinger ligation (Kohn et al. 2003);
- the reaction between a cyanuric acid chloride and an aminophenyl group (Schwarz et al. 2003); or
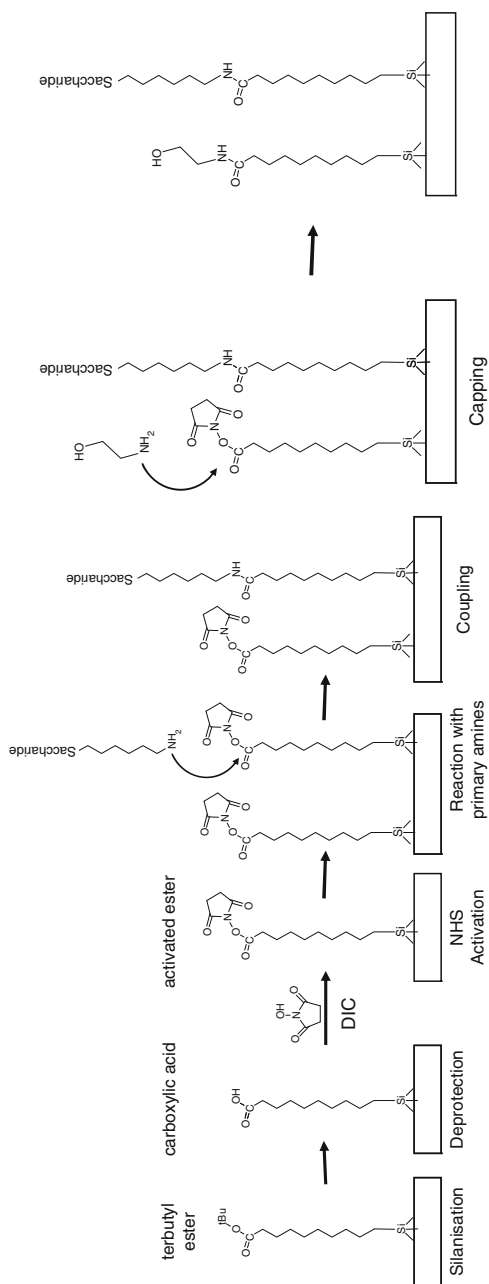- functional macromolecules such as glycoproteins, neoglycoproteins or polysaccharides.

Fig. 7.25 Coupling of amine-derivatised saccharide with ester-activated surface. After the coupling reaction, the unreacted activated esters are capped with ethanolamine

Biskup et al. (2005) have reported the synthesis of amino, aldehyde and carboxylic acid glycoside for their immobilisation on amino- or formyl-functionalised glass slides.

A tremendous effort from the CFG led to a library of 200 (now 400) amino glycosides for subsequent immobilisation on NHS ester-activated glass slides. These slides have been used for a variety of applications such as antibody to viral protein affinity profiling.

The group of Seeberger in Zurich has reported the immobilisation of amine-derivatised heparin oligosaccharides onto NHS ester surfaces (de Paz et al. 2006), thiol oligomannosyl onto maleimide-functionalised surface (Brun et al. 2006) or aminoglycosides with disuccinimydyl carbonate or disuccinimidyl tetrapolyethyleneglycol cross-linker (Disney and Seeberger 2004a).

For 1,3-cycloaddition as well as Staudinger ligation, one takes advantage of azido-functionalised carbohydrates. In the latter case, Schwartz et al. have generated amine-functionalised surfaces using fourth-generation PMAM, a fourth-generation dendrimer, for increasing the number of reaction sites (amines). The surfaces were further treated to form the phosphane group, allowing subsequent Staudinger ligation (Kohn et al. 2003).

Macromolecules have been used as spacers for the immobilisation of carbohydrates taking advantage of functional groups such as amines, carboxylic acids, thiols or diazirines.

For example, BSA glycoconjugates and glycoproteins were immobilised on epoxy-derivatised glass slides (Manimala et al. 2006; Ratner et al. 2004; Adams et al. 2003). The saccharides were covalently link to BSA by reaction of thiol-modified carbohydrates with maleimide-derivatised BSA (Adams et al. 2003; Ratner et al. 2004) or by reaction of NHS ester-modified carbohydrate with BSA (Manimala et al. 2006; Manimala et al. 2005). The neoglycoprotein was then immobilised on NHS ester-activated surfaces (Ratner et al. 2004; Adams et al. 2003) or epoxy-derivatised glass slides (Manimala et al. 2005). Seeberger's group has used these strategies for immobilising carbohydrates on plane substrates as well as on microspheres (Adams et al. 2003).

Angeloni et al. (2005) described the immobilisation of oligosaccharides after surface functionalisation with Optodex. The latter is a dextran molecule bearing aryl-diazirine, allowing surface immobilisation by converting the diazirine into a reactive carbene under light activation.

As one can see, many different reactions have been employed for achieving the immobilisation of saccharides. However, it is difficult to draw a general conclusion, as the surface densities of the probes are rarely given and the washing protocols are often not the same. Dugas et al. (2004) have demonstrated that the final surface density of aminolinker-derivatised oligonucleotides immobilised on the NHS-activated ester surface is greatly dependent on the washing step. A quick rinsing with water led to 10^{14} molecules per cm^2, whereas rinsing with hot water led to 10^{13} molecules per cm^2. A further rinsing with SDS led to 3×10^{11} molecules per cm^2. This means that a significant amount of the probes can be adsorbed rather than

covalently linked depending on the washing steps. In consequence, surface-oriented immobilisation can also be effected.

Linker effect. Affinities of targets for surface-immobilised probes depend on the saccharide structures and also on the aglycon structure. Miura et al. (2002) have demonstrated that shigatoxins 1 and 2 affinities for immobilised Gb3 mimics (a saccharide ligand of the shigatoxins 1 and 2) were affected by the aglycon alkyl chain length. Gb3 mimics were derivatised with a disulfide bearing 2 or 10 carbon alkyl chains as a spacer. The resulting molecules were self-assembled onto gold surfaces and the affinity of Shigatoxins 1 and 2 were studied. They found that Shigatoxin 1 binds to 2-carbon-derivatised Gb3 five times more strongly than 10-carbon-derivatised Gb3, while Shigatoxin 2 binds more strongly to 10-carbon-derivatised Gb3. Some authors (Sato et al. 1998) have demonstrated that the availability of the carbohydrate probe can be spoiled by steric hindrance due to compact packing. It may that the observed difference is related to the surface organisation of the probe. Indeed, it was demonstrated that the C10 long spacer forms compact layers compared to the C3 long spacer (Grubor et al. 2004).

Huang et al. (2006a) compared the affinity of antibodies for Globo H analogues. Globo H is a glycosphingolipid which is highly expressed on a wide range of tumoral cell lines. They synthesised four analogues of increasing complexity of the saccharide moiety of Globo H with two different aglycons bearing either an amine function or an azido function. The amino derivatives were immobilised on NHS ester-functionalised surfaces, while the azido derivatives were immobilised on alkyne-bearing surfaces through 1,3 dipolar cycloaddition. They found that the affinity of the mouse antibodies (MBr1 or VK-9) for the derivatives linked through an amide function was higher than those linked through a 1,2,3-triazol ring. They interpreted the observation as a difference of solubility between the two types of derivatives, a difference in the coupling yield or the better suitability of the shorter linker for binding.

The overall affinity of the target for the immobilised probe is a complex process, which should be viewed as the combination of the contribution of probe structure, probe surface density, probe availability (which is related to the probe orientation and to the physico-chemical properties of the spacer), the surface physico-chemical properties of the substrate and the target physico-chemical properties (Yeung and Leckband 1997).

7.4.2.2.3 Biochemical Interaction Based Immobilisation

Bochner et al. (2005) reported the use of streptavidin–biotin interaction for the immobilisation of sialic acids. One hundred and eighty biotinylated glycosides were immobilised on streptavidin-coated polystyrene titer plates and on streptavidin-coated gold surfaces for SPR measurements (CFG). Linhardt's group immobilised biotinylated heparin for studying the affinity and the kinetic of factor P/heparin interaction (Muñoz et al. 2005). Heparin was conjugated with biotin by

the reaction of sulfo-NHS-LC-biotin with the free amino groups of unsubstituted glucosamine residues in heparin.

Chevolot et al. reported the use of DNA hybridisation for surface immobilisation of glycomimetics (Bouillon et al. 2006; Chevolot et al. 2007). Glycomimetics were synthesised as follows: azido glycosides were attached to pendant propargyl residues on a phosphorylated scaffold by means of solid-supported 1,3-dipolar cycloaddition. The architecture (distance between two saccharides residues, hydrophilic–lipophilic balance) was designed by adjusting the linker between two pendant propargyl residues. Each glycomimetic was tagged with a DNA sequence bearing a cyanine 3 (Cy3) dye. The resulting molecules were immobilised on a DNA array (Dugas et al. 2004) bearing complementary sequence by means of DNA/DNA hybridisation. The fluorescent dye borne by the oligonucleotide permits a quality control of the immobilised molecules. Hybridisation was performed with a 1 μM solution of Cy3 –DNA glycomimetic, leading to a probe surface density of $1–4 \times 10^{10}$ molecules per cm^2 as estimated by Dugas et al. with radiolabelled oligonucleotides immobilised under the same conditions. The resulting analytical device had a lower detection limit in the nanomolar range (albeit the use of a 1 μM solution of glycomimetic), which is similar to what is described in the literature where typical immobilisation solution concentrations are in the millimolar range.

In the field of protein array, Niemeyer et al. (Wacker et al. 2004) have used a similar strategy called DNA directed immobilisation (DDI). They also found that immobilisation by means of hybridisation give lower detection limit of the device, which is improved, as opposed to direct covalent immobilisation. They hypothesised that the improvement was due to the rigid structure of the DNA duplex and to the reversibility of the hybridisation allowing the denser packing of the probes.

Another advantage of the DDI strategy is that biological recognition between the glycomimetics and the lectin can be first performed in solution before immobilisation of the complex on the solid support by hybridisation, circumventing the limitations due to the surface proximity.

7.4.2.2.4   Surface Cluster Effect or Not?

Individual protein–carbohydrate interactions have binding constants in the millimolar to low micromolar range, with apparent low specificity. However, under multivalent interactions in a Velcro-type mode, these interactions can be enhanced. This is the so-called cluster effect as defined by Lee in 1994(Lee and Lee, 1994, 1995): "binding affinity enhancement exhibited by a multivalent carbohydrate ligand over and beyond that expected from the concentration increase resulting from its multivalency."

Two different mechanisms have been proposed to explain the cluster effect (Pohl and Kiessling 1999):

- Due to multivalency, the local concentration of the ligand is high at the receptor-binding site allowing an increase of 5- to 10-fold in binding affinities.
- Due to intra molecular binding, which is the chelate effect, a multivalent ligand binds with greater affinity for a multivalent receptor than the monovalent ligand, similar to the mechanism described in inorganic chemistry. It leads to an exponential increase of the affinity.

Several reviews have been published on this topic (Lundquist and Toone 2002; Mammen et al. 1998; Turnbull and Stoddart 2002).

In the case of biosensors, packing of carbohydrate at the surface of a material should permit taking advantage of the cluster as opposed to solution assays. For example, in the case of neoglycolipid-based glycol array (Jelinek and Kolusheva 2004; Stoll et al. 2000), lipid clustering and surface oligosaccharide organisation allowed higher affinity of proteins for the saccharides. On the contrary, Sato et al. observed a decreased WGA affinity by the high probe surface. They studied the affinity of WGA (Wheat Germ Agglutinin) for different glycolipids and found that the maximum affinity of WGA for GM 3 and GM4 was for a monolayer containing 20% GM (Sato et al. 1998). Similar results were found by Ebara (Ebara and Okahata 1994) with concanavalin A and glycolipids. This phenomenon was attributed to steric hindrance due to the compact packing of the carbohydrate heads in gangliosides monolayers (not mixed) limiting the access to WGA.

These apparent contradictory results suggest that the surface-based cluster effect is dependent on surface density of the probes and their surface organisation. This latter parameter is dependent on interactions between the probes through van der Waals interactions, polar, ionic forces and hydrogen bonds. Usually, when immobilising carbohydrates via a functional aglycon with a functionalised surface, the surface densities of the probes are below that of a densely packed monolayer. On the other hand, direct immobilisation of glycosides leads to denser layers.

7.4.3 Transduction

The specific biological interaction between the probe and the target can be studied by optical, gravimetric or electrochemical detection and more recently by mass spectroscopy (MS) or by the use of nanoparticles-based assays.

The detection of biomolecular interactions may require the pre-labelling of the target prior to detection or may be label free and exploit the change of physico-chemical properties induced by the biological recognition.

In the first case, strategy consists in labelling the molecule to be detected (directly or via sandwiches) or grafting different markers on probes (Cy3) and targets (Cy5) for discriminating the specific molecular interaction in a better way and requires appropriated techniques for detecting the signal associated with the label. The markers can be fluorescent, radioactive or electroactive species. Detection by fluorescence is a semi-quantitative and sensitive technique with high spatial

resolution but the quenching and blenching phenomena must be monitored. Nowadays, read-out and analyses are made with standard commercial fluorescence scanners. Detection using radioactive labels such as ^{125}I, ^{32}P or ^{33}P is a more quantitative method but the weak spatial resolution and both the inherent safety and waste disposal problems have directed studies towards alternative solutions. Spe-cificity of the recognition signal must be ensured by careful washing protocols to remove non-specifically labelled probes adsorbed out of the surface without breaking the specific binding. A drawback is that the detection is performed a posteriori, out of the liquid environment and in dry conditions, which hides the analysis of the kinetics of the interactions. Advantage of these methods is that they are particularly well adapted for the parallel and massive detection analysis of numerous spots, as for instance with the DNA biochips performed by Affymetrix containing more 400,000 spots per cm^2.

In the second case, the detection strategy is based on the measure of the change of properties such as temperature, mass, electrical, optical and chemical (pH) parameter. The necessary development of new and reliable tools designed for detecting low parameter change has considerably increased on the two last decades. The release of commercial systems such as microcalorimeter, quartz crystal microbalances (QCM), field effect transistors (FETs), surface plasmon resonance (SPR) and, more recently, platforms based on piezoelectric acoustic sensors (principally bulk acoustic wave (BAW)) and thickness shear mode (TSM) sensors are leading a large number of studies determining binding specificities, affinities, kinetics and conformational changes associated with a molecular recognition event. The investigations concerned a large variety of interactions involving small molecular weight ligands, carbohydrates, proteins, nucleic acids, viruses, bacteria, cells as well as lipidic and polymeric interfaces. Beyond the direct and in situ interaction detection, the main advantage of this strategy is its ability to obtain kinetics informations on molecular interactions.

7.4.3.1 Optical Detection

7.4.3.1.1 Fluorescence

Fluorescence is one of the most commonly used detection techniques in the field of microarrays. It relies on fluorescent dyes (see Table 7.4). These molecules are capable, under illumination with a monochromatic light corresponding to their absorption maximum, of reaching an excited state. They return to the ground state by emitting a photon. The energy of the outcoming photon is less than that of the absorbed photon due to loss of energy by a non-radiative process. The difference between the maxima (in wavelength) in the excitation spectrum and the emission spectrum is called the Stokes shift. The ratio between absorbed photon and emitted photon is the fluorescence quantum yield. Table 7.4 gives some of the commonly used fluorescent dyes. Fluorescein and related molecules (Alexa, Oregon green, rhodamine, etc.) are four-ring structures with conjugated double

Table 7.4 Some of the physical properties of commonly used fluorophores (Schena 2003; http://www.invitrogen.com/)

Fluorescent probe	Exitation (nm)	Emission (nm)	Fluorescence quantum yield	ε
Fluorescein	490	519	0.71	67,000
Rhodamine 6G	525	555	0.9	85,000
Tetramethylrhodamine	555	580		176,000
Indodicarbocyanine Cy3	548	562	0.14	150,000
Indodicarbocyanine Cy5	646	664	0.15	250,000
Alexa 488	495	519	0.92	
Alexa 568	578	603	0.69	
Alexa 647	647	668	0.33	
Texas red	596	620	0.51	85,000
Bodipy650/665	646	660		

This is not an exhaustive list. Data were collected according to Invitrogen catalogue (for Alexa and microarray analysis)

bounds. FITC (fluorescein isothiocyanate) is affordable but has several drawbacks (Schena 2003). It is sensitive to self-quenching and photobleaching, and the fluorescent signal is influenced by pH (Schena 2003). The parent molecules of FITC (Alexa, Texas red, Oregon green, etc.) have additional polar, aliphatic and/or hetero cyclic groups that influence their excitation and emission spectra. These molecules are in general photostable. Furthermore, Alexa dyes are insensitive to pH change over a broad range, have high fluorescence quantum yield and a long half-life of the excited state (Schena 2003).

A second family of fluorescent dyes is of the indocarbocyanine series. The most used ones are Cy3 and Cy5. The cyanine dyes are bright, are stable to photobleaching, and are commercially available with chemical function allowing their conjugation with biomolecules. Their fluorescent signal is affected by ozone exposure as low as 5–10 ppb for Cy5 and above 100 ppb for Cy3 (Fare et al. 2003).

A third family of organic dyes used in microarrays applications is the BODIPY (boron-dipyrromethene) series. Next to these organic dyes, QDs are emerging as an alternative. Huang et al. (2005) have compared the lower detection limits for a FITC-labelled lectin plant and a QD-labelled lectin plant (Con A) interacting with immobilised D-glucopyrannoside. They found that the QD-label-based method was at least a 100 times more sensitive than the FITC approach in detecting ConA interaction.

A biosensing experiment relies on (a) the labelling of the target or (b) the detection of the target through a second interaction using a sandwich technique with antibodies, for example (Fig. 7.26).

Dyes have been modified for allowing their direct conjugation with molecules bearing amine, thiol, etc. In a fluorescent experiment, after incubation of the device with the protein of interest, the substrate is washed and dried and the remaining fluorescent signal is detected with a microarray scanner.

One of the main advantages of fluorescence is that the technique is very sensitive, allowing the detection of plant lectin with concentration as low as a few tens of picomolars (Manimala et al. 2006). However, generally no real-time experiment can be performed. Furthermore, the washing step is compulsory and
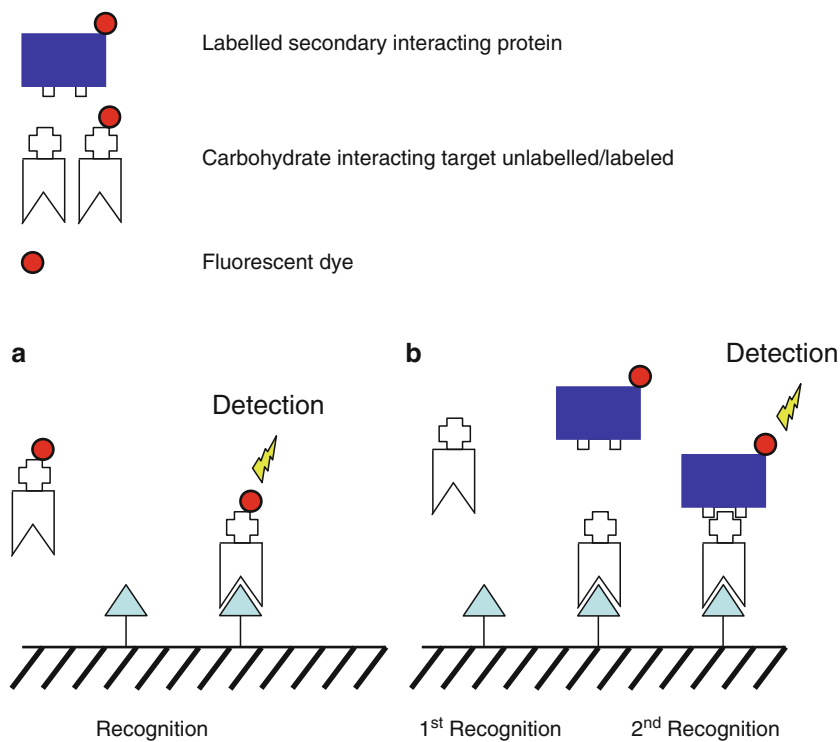
Labelled secondary interacting protein

Carbohydrate interacting target unlabelled/labeled

Fluorescent dye

a

Detection

Recognition

b

Detection

1st Recognition   2nd Recognition

Fig. 7.26 Principle of fluorescent detection: (**a**) When target is previously labelled, then the recognition interaction is directly detected. (**b**) Sandwich technique consists in a first recognition step between probe and unlabelled target following by a secondary recognition step between the formed complex and a labelled interacting protein

critical in terms of background levels. It is tricky to find an optimum between removing non-specifically adsorbed protein and maintaining a protein that interacts weakly with the carbohydrates.

Instruments on the market (Zeptosens (http://www.zeptosens.com/en/; Disney and Seeberger 2004b)) take advantage of evanescent field for fluorescent dye excitation. By this means, real-time experiments can be performed without washing the surface. Indeed, evanescent field decreases exponentially with the distance to the surface. Consequently, only the fluorescent dyes next to the surface are excited.

Fluorescent-labelled plant lectins have been widely used as model protein for the validation of carbohydrate microarrays. The best lower detection limits, depending on the considered model, are in the nanomolar range.

Stevens et al. (2006) have used a double sandwich for probing the interaction of the influenza virus hemagglutinins of different sub-types for more than 200 saccharides displayed on a microarray. Due to the low affinity of the hemagglutinin–carbohydrate interaction, they developed an approach based on two antibodies. The hemagglutinins (HA) bear a His6 tag. First, hemagglutinins are allowed to
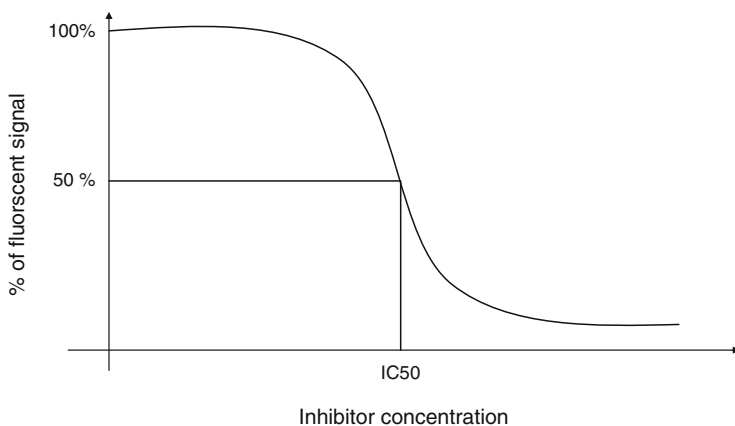
Fig. 7.27 Basic principle of an IC_{50} experiment

interact with the microarray. Next, the slides are incubated with a labelled anti-His6 mouse antibody followed by the incubation with a second labelled antibody directed against mouse antibody. With this approach, the signal is amplified, allowing the probing of weak interactions.

Usually, fluorescence give semi-quantitative results. The group of Shin (Shin 2007) has developed a procedure for quantitative analysis by determining the IC_{50} based on inhibition experiments (Fig. 7.27). In brief, the microarray was incubated with increasing amounts of a soluble inhibitor. The concentration that produces 50% of inhibition is called the IC_{50}.

An original glycoarray was developed using internally encoded microspheres (Adams et al. 2003). Each population of microspheres has a unique carbohydrate molecule covalently attached and a unique spectral signature due to an internal fluorescent dye. The microspheres are randomly spread over an imaging optical fibre. Due to the spectral signature, each microsphere is identified at the surface of the fibre with regard to the immobilised carbohydrate and position. Interactions with labelled Concanavalin A, a plant lectin, and Cyanovirin A, an HIV-inactivating protein issued from a cyanobacterium, are performed and analysed by fluorescent imaging.

7.4.3.1.2 Surface Plasmon Resonance and Surface Plasmon Resonance imaging

Surface plasmons are surface electromagnetic waves propagating at the metal/dielectric interface. Surface plasmons can be excited by an incident polarised light especially by means of an evanescent field. The total internal reflectance of the incident light will be affected as a function of the incident or reflection angle leading to a sharp minimum.

The position of this minimum is dependent on the refractive index and thickness of the surface layer. Change in the surface refractive index on the surface of the metal will thus be probed by either measuring the change in total internal reflectance or by the change in the minimum position.

Two configurations can be used: namely, the Otto set-up and the Kretschmann set-up. The Kretschmann set-up is the most widely used. In this set-up, a thin layer of gold, typically 50 nm, is evaporated on a glass substrate (with an intermediate adhesive layer such as chromium). An incident polarised light travels across the glass, and an evanescent field penetrates through the metal film and excites the surface plasmons of the outer side of the metal film.

Its use for biosensing began in the late 1980s (Lundquist and Toone 2002) and was further implemented in the early 2000s with the first commercially available surface plasmon resonance imaging (SPRi) apparatus.

SPR for detecting carbohydrate-based interactions has been reported with antibodies (Zou et al. 1999; Young et al. 1999), membrane proteins, viruses proteins (Ratner and Seeberger 2007), chemokine (de Paz et al. 2007), fibroblast growth factor (de Paz et al. 2007), plant lectin (Smith et al. 2003; Satoh and Matsumoto 1999), hSiglec7 (Karamanska et al. 2008) and enzyme (Park and Shin 2007).

SPR offers many advantages: firstly, it is a label-free detection technique; secondly, it provides rates (K_{on} and K_{off}) allowing the determination of affinity constants; and thirdly, it is a real-time measurement. The main drawback relates to its lower detection limit, which is not as good as that of fluorescence. Also, the on and off rates seem to be affected by the flow vector (mass transport) and by the orientation of the molecules at the surface of the sensor (Lundquist and Toone 2002). Recent developments have focused on coupling SPR plates with matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Lundquist and Toone 2002).

7.4.3.1.3 Dual Polarization Interferometry

Yates (Yates et al. 2006) has pointed out another optical technique based on dual polarization interferometry (DPI), which measures real-time changes in the mass and the geometry of molecules bound to a surface. DPI measures in phase both TE (transverse electric) and TM (transverse magnetic) polarizations of light. Using Maxwell's equations, one unique value for thickness and for refractive index of the layer of molecules is true for both phases of light. DPI gives absolute measurements and allows full characterisation in terms of mass, thickness and refractive index of the layer to be formed. It allows measurements in real time of probe coverage and probe orientation and then to follow in real time the biological interaction. The measurement by DPI immobilization of streptavidin on a biotinylated surface followed by the capture of the reducing end biotinylated heparin oligosaccharides is given as an example.

7.4.3.2 Gravimetric Detection: Quartz Crystal Microbalance

Among the analytical tools available to investigate the biological interactions, QCM holds a special place because this direct measurement does not require probe-labelling and allows monitoring the binding affinity. In their recent review, Cooper and Singleton (2007) register a total of 1,404 publications referencing QCM in the Web of Sciences database, of which 569 specifically involved molecular recognition studies in the period 2001–2005.

In 1959, QCM was developed by G. Sauerbrey (Sauerbrey 1959) for correlating changes in the oscillation frequency of a piezoelectric crystal with the mass deposited on it. He simultaneously developed a method for measuring the characteristic frequency (resonant frequency) and its changes by using the crystal as the frequency-determining component of an oscillator circuit. His method continues to be used as the primary tool in QCM experiments for the conversion of frequency to mass and is valid in nearly all applications under vacuum or gas.

The Sauerbrey's equation is given below:

$$\Delta f = \frac{-2\Delta m f_0^2}{A\sqrt{\rho_q \mu_q}} = -\frac{2f_0^2}{A\,\rho_q v_q}\Delta m$$

where f_0 is resonant frequency (Hz), Δf is the frequency change (Hz), Δm is the mass change (g), A is the piezoelectrically active crystal area (m^2), ρ_q is the density of quartz ($\rho_q = 2.648$ g/cm^3), ρ_q the shear modulus of quartz for AT-cut crystal ($\mu_q = 2.947 \times 10^{11}$ g/cm s^2) and v_q is the transverse wave velocity in quartz (3,340 m/s).

QCM measures the mass per unit area by measuring the change in frequency of a quartz crystal resonator. The resonance is disturbed by the addition or removal of a small mass on the surface of the acoustic resonator. When a thin film is attached to the sensor crystal, the frequency decreases. If the film is thin and rigid, the decrease in frequency is proportional to the mass of the film. In this way, the QCM operates as a very sensitive balance. The mass of the adhering layer is calculated by using the Sauerbrey's relation. For instance, with a crystal of resonant frequency 6 MHz, and considering an active crystal surface of 0.2 cm^2 and a frequency resolution of 1 Hz, we can detect a change of 2.46 ng. For a long time, the main application of QCM consisted in monitoring thin-film deposition in vacuum or in gas phase (King, 1964). When QCM was used in liquid environments (Bruckenstein and Shay 1985), the number of applications increased dramatically, in particular in the biology field. For instance, an adsorbed protein monolayer on a surface electrode of 1 cm^2 adds a mass of a few hundred nanograms (Muratsugu et al. 1993). But, the Sauerbrey equation developed for oscillation in air must be applied only to rigid masses attached to the crystal. Besides the mass, other physico-chemical parameters (such as viscosity, roughness, temperature) can induce a change in the resonant frequency. Thus, in liquid environment, Kanazawa and Gordon (1985) showed that quartz crystal microbalance measurements can be performed, in which case a viscosity-related decrease in the resonant frequency will be observed:

$$\Delta f = -f_0^{3/2}(\eta_l \rho_l / \pi \rho_q \mu_q)^{1/2}$$

where ρ_l is the density of the liquid and η_l is the viscosity of the liquid.

The advantage of QCM in biological measurements is connected with its ability to monitor the changes of small masses in real time, and such measurements can be performed by use of the native or synthetic molecules without any supplementary labelling.

Specifically, a QCM is constituted of a thin quartz crystal resonator disc attached with metal electrodes plated onto the upper and lower faces of the disc. Gold is usually used as the electrode material. When an alternating electric field is applied across the quartz crystal between the upper and lower electrodes, a mechanical oscillation of the characteristic frequency (f) is generated in the crystal. This resonant frequency shifts as a function of the additional bound material occurring at the upper surface. For adapting this system to molecular recognition, the metal surface is specifically biofunctionalised with a selective, passivating layer on which the analyte-specific receptor is immobilized. Appropriate flow channels with monitored microfluidics allow controlled introduction of the analyte. Nowadays, commercial QCM integrate user-friendly software for characterising the biomolecular interactions at the surface. A large variety of surface coatings have been proposed, allowing several optimized chemical treatments and biomolecule as the receptor grafting depending on the aimed interactions. Apart from the standard gold material, which is the most commonly used sensor surface because Au is chemically inert and easy to modify in different ways, we can find in product catalogues (Q Sense) electrode materials such as silicon dioxide whose main advantage is the ability to form lipid bilayers which act as a platform for further immobilisation of molecules; stainless steel surfaces used for studies of biofilm formation; titanium-coated surfaces mainly used for implant research; metals and oxides (aluminium trioxide, platinum, tantalum, tantalum nitride, tungsten, copper, chromium, iridium, iron, silicon carbide, iron carbide, silver and cobalt); and more recently commercially available polymers (polysterene, PMMA) deposited by spin coating.

QCM-D. In biochemical applications, as macromolecules (proteins, polymers, etc.) deposited on the surface do not constitute a thin rigid layer, their viscoelastic behaviour leads to a vibrational energy loss by dissipation. That is why, in addition to measuring the frequency, the dissipation is often measured to help with the analysis.

The dissipation (D) is defined as:

$$D = \frac{E_{lost}}{2\pi E_{stored}}$$

where E_{lost} is the energy lost (dissipated) during one oscillation cycle and E_{stored} is the total energy stored in the oscillator.

The QMC-D consists in measuring the dissipation in the crystal by recording the response of a freely oscillating crystal at its resonance frequency. This also gives the opportunity to jump between the fundamental frequency and overtones (e.g. 15,

25 and 35 MHz). By working at multiple frequencies and applying a viscoelastic model (the so-called Voight model), the adhering film can be characterized in detail; viscosity, elasticity and correct thickness may be extracted even for soft films when certain assumptions are made.

The QCM can also be combined with other surface-analytical instruments. The electrochemical QCM (EQCM) is particularly advanced (Schumacher 1990; Buttry and Ward 1992). Using the EQCM, one can determine the ratio of mass deposited at the electrode surface during an electrochemical reaction to the total charge passed through the electrode. This ratio is called the "current efficiency." This methods allows one to compare the deposited mass value estimated from current flowing between the working electrode (which is also the resonant electrode of QCM) and the counter electrode with the value deduced from the frequency shift of the QCM. The shrewdness of the method concerns the double use of the electrode (as the resonant QCM electrode and as the electrochemical working electrode) and the possible electrical cutting of the two functions (electrochemical and piezoelectric transducer) thanks the two different ranges of frequency.

The binding between two molecules can be quantitatively described by two parameters: the association equilibrium constant and the association rate. QCM measurements give access to these parameters. But we must keep in mind that theses values are strong depending on the experimental conditions (temperature, solution ionic strength and pH value). They also reflect structural properties of the binding sites. For instance, conformation of proteins on the surface affect drastically their recognition activities, the spacer between the surface and receptor appears as crucial factor and, in the case of lectin–carbohydrate interaction, the number of sugar monomers in the oligosaccharide chain strongly influence the interaction. Thus, uncontrolled immobilisation of receptor on biosensor surface could lead to meaningless results. The large number of parameters influencing the experimental results make a direct comparison of values (sensitivity, assay time, detection limit, association rate)reported in the literature very difficult . In their review, Cooper and Singleton (2007) have collected several tables comparing QCM assays and results for DNA sequences, for virus and phage, for selected pathogenic bacteria and for small-molecule detection. This drawback is also noticed by Lebed et al. (2006). Therefore, they discuss carefully the lectin–carbohydrate affinity values obtained by QCM measurement and compare them with others' previously published results.

Nevertheless, this technique used in the similar and controlled conditions allows comparison of the binding behaviour of a set of monosialogangliosides (GM1, GM2, GM3, GM4) towards wheat germ agglutinin as described by Sato et al. (1998) as far back as 1998. More recently, Pei et al. (2007) demonstrated the great interest in the use of QCM sensor for rapid identification of glycosyldisulfide lectin inhibitors from a dynamic combinatorial library. Gold-plated quartz crystals were drop-coated with polystyrene before being covered with mannan in phosphate-buffered saline (PBS), and complete blocking was achieved with BSA to obtain a stable baseline. The plant lectin concanavalin A was injected as target species alone or in presence of potential inhibitor library components. Between

each screening of libraries, the Con A binding to the surface must be carefully released.

Yates et al. (2006) affirm that QCM-D provides a unique means to explore the change in mass and the molecular flexibility of oligosaccharides, GAG (glycoaminoglycans) chains and entire proteoglycans and their complexes with proteins thanks to the ability to form membranes mimetics on the sensor surface in an environment that approximates that of the cell surface.

Nagase et al. (2003) used a lectin coated on the silver face of a piezoelectric crystal biosensor for oligosaccharide analysis. They used it for the detection of dissolved sugars and identification of erythrocytes, and obtained a detection limit approaching 100 cells.

Miura et al. (2004) used the QCM for investigating the biological functions of their synthetic glycoconjugate polymers with respect to lectins and Shiga toxin-1 (Stx-1) produced by the pathogen *Escherichia coli*. QCM detector was covered by a Gb3C10 SAM. First they demonstrated that the addition of Stx-1 to aqueous solutions induced a frequency reduction depending on the concentration. The binding affinity of Stx-1 to Gb3C10 SAM was estimated to be 1.2×10^8 (M^{-1}). Secondly, upon addition of their glycoconjugates, they exhibited a notable inhibitory activity for the adhesion of Stx-1 to the Gb3C10 SAM (degree of inhibition going from 15% up to 70% versus glycoconjugate).

7.4.3.3 Electrochemical Detection

The use of electrochemical methods involves the molecular recognition-induced redox reaction. This is particularly true for enzymatic systems. The most basic amperometric enzyme electrode concerns glucose, which was first oxidized within an immobilized layer of glucose oxidase and then determined at a conducting (platinum or carbon) electrode by measuring the current resulting either from the oxidation of hydrogen peroxide or reduction of diatomic oxygen consumed by the enzymatic reaction (Updike and Hicks 1967).

An electrochemical biosensor for determination of the tumour marker-bound sialic acid (b-SIA) was reported by Aubeck et al. (1993). This sensor consisted of a copolymer-immobilized bilayer containing the enzyme sialidase, placed in contact with a H^+ selective indicator membrane. The release of sialic acid following enzymatic cleavage resulted in a local pH change monitored by the proton-sensitive indicator electrode.

Potentiometric detection can be used if the binding of biomolecules induces large non-equilibrium potentiometric responses. One type of device named ISFET (ion sensitive field effect transistor) sensors uses this event and is derived from MOSFET (metal oxide semiconductor field effect transistor), the most important component in integrated circuits. In 1970, Bergveld et al. (Van Kerhof et al. 1993) showed the sensitivity towards ions H^+ of a MOSFET without metallic grid when the blocking silicon layer is directly in contact with the solution.

ISFET is made of a p-type silicon substrate with two areas of n-doping (called source and drain) on which metallic electrodes are applied. The channel between the source and the drain is covered of a thin blocking SiO_2 layer. Metallisation of this layer forms the gate, which is the electrode monitoring the conductivity in the channel. In the case of ISFET, an ion-sensitive membrane is put in place of the metallic layer. This structure is intrinsically blocked, so no current flows between source and drain because whatever the potential applied at this two areas, one of the p-n junction is in reverse polarisation. When a positive voltage V_G is applied between the gate and the source, the majority of holes tend to be repelled while the minority electrons of the p-substrate move to the channel. For $V_G > V_T$ (V_T is the threshold voltage), the electron density becomes larger than the hole density and the formation of n-type channel ensures electrical continuity between the source and the drain; the current flows from source to the drain. I_D is a function of V_G and V_D voltages. If $V_D < V_G - V_T$, the FET is in the linear range; for $V_D > V_G - V_T$, FET is in the saturation range.

$V_T = V_{T0} + y_0$, where V_{T0} is relative to the ISFET characteristics and y_0 is the potential between the sensitive membrane and the electrolytic solution.

Taking into account the equilibrium between the ion analyte in solution and its chemical conformation inside the sensitive membrane, y_0 is dependent of the activity of the ions to be detected. The following figure gives the shape of I_D variation for ISFET pH with an alumina sensitive membrane.

The high concentration of negative charges on heparin makes the detection of the variations in charge density in porous membranes mounted on ISFET easy. Thus, heparin biosensors based on the ISFET concept exhibit very high sensitivity thresholds of between 0.1 and 1 units per millilitres (Van Kerhof et al. 1993).

A similar transducer can be used for detecting enzymatic reactions. In fact, catalysed enzymatic reactions of hydrolase or oxidase type, respectively, consume or produce protons. The presence of the resulting products from the enzymatic reactions can be detected through the pH variation induced at the ISFET surface on which specific enzymes have been grafted. In this case, ISFET is called ENFET (enzymatic field effect transistor). Numerous molecular species can be detected by ENFET: for example, urease, glucose, organophosphorous pesticides. This type of transducer was also adapted for the detection of DNA hybridization. In this case, the SiO_2 thin film was coated with an APTS (3-aminopropyltriethoxysilane) layer on which single-strand DNA was immobilised. This FET put in contact with a solution containing complementary DNA strands shows a strong drain current resulting from the field effect induced by the arrival of the negative charge borne by DNA strands under hybridisation (Souteyrand et al. 1997). This device was called GENFET. To exploit this field effect on a microarray, Souteyrand et al. (2000) described a technique based on the optoelectrochemical impedance measurement in which electrical excitation is replaced by modulated light for scanning the surface of a electrolyte/DNA modified dielectric layer/semiconductor and for detecting directly the hybridisation on spots.

7.4.3.4 Mass Spectrometry

Mass Spectroscopy used as a detector has the advantages of being label free and of allowing the determination of the target structure after interaction in a complex medium. Bryan et al. (2004) reported the use of cleavable linker (disulfide) for subsequent electron spray ionisation MS. The use of porous silicon as a matrix for MALDI-TOF analysis was also proposed. Chen et al. (2005) used carbohydrate encapsulated gold nanoparticles for capturing proteins. After purification of reacted material from the unreacted one by centrifugation, the proteins were subjected to protease digestion. The remaining peptides were analysed by MALDI-TOF for the determination of the amino acid involved in the binding. Enzymatic reaction by enzymes can also be monitored by MALDI-TOF MS (Park and Shin 2007; Su and Mrksich 2002; Laurent et al. 2008).

7.4.3.5 Nanoparticles

The use of saccharide-modified nanoparticles (polymeric, metallic or semiconductor) for biosensing purpose is attractive, as it allows purification from a complex mixture of reacted products (by centrifugation, or magnetic field) and the multivalent display of carbohydrate. They can be used advantageously for in vivo bioimaging in transmission electron microscopy or confocal microscopy, or for glycoprofiling by mass spectroscopy (after purification and release of reacted molecules) (Larsen et al. 2006).

Carbohydrate-modified core magnetic nanoparticles could be advantageously employed for in vivo imaging by magnetic resonance imaging (MRI). Indeed, in the literature (Harris et al. 2006; Perez et al. 2002; Perez et al. 2003; Perez et al. 2004), the detection of biomolecules using super paramagnetic iron nanoparticles (and MRI) is reported. The assay is based on modified nanoparticles that self-assemble in the presence of the targeted biomolecule. The spin–spin relaxation time of surrounding water molecules is then enhanced and can be imaged by MRI, allowing the detection of the target even in complex media.

The multivalent nature of carbohydrate interactions should be suitable for this type of biosensors. However, to our knowledge, this has not been reported for probing carbohydrate–protein interaction. This would be useful for in vivo imaging in diagnosis where abnormal carbohydrate–protein interactions take place.

7.5 Biosensors: Applications

7.5.1 Antibody/Antigen

Carbohydrate biosensing can be applied to the design of sugar-based vaccine or for antibody profiling for diagnosis (IgMs profiling for tumor detection, natural auto-antibodies profiling, etc.).
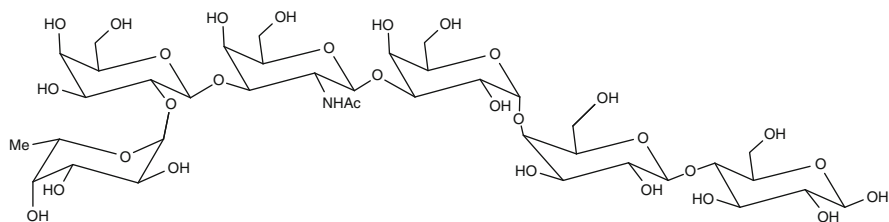
Fig. 7.28 Structure of Globo H

The cell sphingolipid Globo H possesses a hexasaccharide moiety. It is an antigenic carbohydrate such as T antigen, which is highly expressed on a variety of tumoral cell lines. Huang et al. (2006a) reported the design of a glycoarray based on Globo H and truncated Globo H ligands for understanding Globo H interaction with antibodies MBr1 and VK-9. MBr1 and VK-9 are mouse monoclonal antibodies targeting the Globo H epitope. The epitopes (including Globo H) were synthesised using "one-pot" programmable synthesis and modified with an aglycon bearing an amino or an azido function. The glycosides were covalently immobilised on a glass support using ester-activated or alkyne-modified glass slides. Affinities for MBr1 and VK-9 were probed with FITC-labelled secondary antibodies. It was shown that the monoclonal antibodies bind the terminal tetrasaccharides as well as the natural hexasaccharides and that the fucose residue is compulsory for binding. However, serum from patients with breast cancer binds both the fucosylated and defucosylated pentasaccharide, probably due to the polyclonal nature of the serum. According to the authors, their carbohydrate platform was more effective and more sensitive than ELISA for probing the affinity of antibodies for Globo H (Fig. 7.28) with the use of very minute amounts of the material.

7.5.2 Enzyme/Carbohydrates

Another application of carbohydrate microarray is to understand the structural features that govern substrate specificity of enzymes processing carbohydrates. The group of Mathieu (Chevolot et al. 2001) demonstrated that α-2,6-sialyltransferase was active on lactose-modified polystyrene. Detection was performed using radiolabelled CMP-Neu5Ac. The group of Shin (Shin 2007) showed, using *N*-GlcNAc and *O*-Fuc-α-modified glass slide, that LacNac could be successfully synthesised on chip using β-1,4-galactosyltransferase. They further illustrated that complex oligosaccharides could be synthesised using sequential β-1,4-galactosyltransferase followed by α-2,3′-sialyltransferase. Each step of the synthesis was characterised using FITC-labelled specific lectin.

Aminoglycosides are a broad-spectrum class of antibiotics due to their interaction with bacterial 30S ribosome. Their efficiency has decreased as a result of the

emergence of resistant strains. The resistance mechanism is usually due to enzymatic modification of the molecules such as aminoglycoside acetyl transferases. Disney et al. have used aminoglycoside microarrays for probing the affinity of three acetyl transferases (from *Salmonella enterica* and from Mycobacterium tuberculosis) towards aminoglycoside antibiotics (Disney and Seeberger 2004a; Disney et al. 2004). They also studied new aminoglycosides as inhibitors of these enzymes. Based on their microarray experiment, they identified new aminoglycosides with low affinity towards the acetyl transferases, meaning they are less susceptible to enzymatic transformation by the bacteria, as well as being a potent inhibitor.

7.5.3 Carbohydrates/Lectins

Plant lectins are widely used usually as a model to assess biosensor/microarray in terms of specificity. Here, we will focus on three examples where carbohydrate biosensing was used advantageously with the perspectives of medical applications for

- analysing ligand affinity of Dectin-1, a C-type leukocytes lectin involved in phagocytosis and inflammation (Palma et al. 2006);
- analysing the specificity of hemagglutinins from modern and pandemic influenza virus (Stevens et al. 2006); and
- determining the affinity profile of GP120-binding protein. GP120 is a hyper-mannosylated protein of the HIV envelope. These mannose residues are involved in the binding of the virus to the host cells (Adams et al. 2004).

Plama et al. studied the binding of Dectin-1, a C-type leukocytes lectin involved in phagocytosis and inflammation that binds both β-1,3 and β-1,3/β-1,6 rich β-glucans (Palma et al. 2006). They developed a bioassay based on neoglycolipids oligosaccharides immobilised through physisorption onto nitrocellulose membranes. Oligosaccharides comprise mammalian oligosaccharides and oligosaccharides issued from the depolymerisation of natural polysaccharides. Fluorescence was used for detection. They showed that Dectin-1 binds exclusively β-1,3-linked glucose oligosaccharides. Ten to eleven glucose residues per oligosaccharides were compulsory for good binding.

GP120 is a hyper-mannosylated protein of the HIV envelope. The mannose residues are involved in the entry of the virus into the host cells by interacting with CD4, a chemokine family receptor present at the surface of T-lymphocytes, macrophages, dendritic cells, etc. Furthermore, the mannose residues are involved in protecting the virus from antibodies directed against GP120 by hiding the targeted epitope. This mannose oligosaccharide has been considered a potential target for the design of vaccine (it interacts with the 2G12 antibody (Calarese et al. 2005)) or as potential target for prophylactic measures. In the latter case, proteins interacting with the hyper-mannosylated moiety of GP120 would prevent the entry of the virus

into the host cell. Adams et al. designed a glyco array bearing different mannose and studied the affinities of 2G12, CD4, Scytovirin (a potential anti-HIV protein), Cyanovirin N (CVN, a cyano bacteria protein with potential anti-HIV properties) and the dendritic cell lectin DC SIGN involved the internalisation of the virus. Detection was performed with FITC labelling (Adams et al. 2004). It was shown that each protein has specific requirements concerning the structure of the mannosyl oligosaccharide. For example, an α-1–2 linkage alone is necessary for the interaction of 2G12. The binding profile of the Scytovirin showed that the terminal α-1–2 mannose linkage at the terminal non-reducing end is required.

Stevens et al. took advantage of the CFG platform for studying the affinities of hemagglutinin of different strands of the influenza virus (Stevens et al. 2006). Influenza virus is an enveloped virus with a segmented negative RNA genome. Its envelope bears two proteins: hemagglutinin (HA, around 5,000 copies) and neuraminidase (3–500 copies). HAs interact with the sialic acids present at the surface of the host cell, permitting adhesion of the virus. It is the specificity of the HA towards the sialic acid linkage that determines the host specificity of the influenza virus. Human influenza viruses preferentially bind the α-2–6 linkage present at the surface of the epithelial cells of the upper respiratory track and lungs, while the avian ones recognise the α-2–3 linkage present in epithelial cells of the intestine of birds. Therefore, the subtle switch from avian to human subtype is related to the HA affinity to α-2–6.

Stevens et al. used a glycoarray bearing 200 oligosaccharides for studying the specificity of HAs from modern and pandemic influenza virus for α-2,6′/α-2′,3 linkage, and also the influence of sulfation, fucosylation and sialylation at position 2. They showed that the species barrier of the New York 1918 variant (human pandemic variant) could be reverted to an avian virus by a single mutation of the Asp190Glu. The species barrier from avian to human pandemic, as defined by the specificity of 1918 viruses (New York and South Carolina), can be jumped by the likely avian progenitor by only changing two amino acids of their HA.

7.5.4   Whole Cells

Using whole cells, the biosensor takes advantage of the natural multivalency of carbohydrate binding on the cell surface. Indeed, in nature, multivalency is often achieved by the self-association of lectine at the cellular surface upon interaction with carbohydrates (Nimrichter et al. 2004). Several authors have reported the use of whole-cell detection by means of carbohydrate/whole cell interactions.

Disney and Seeberger (2004b) have reported the use of microarrays as a means for probing bacteria (*E. coli*) affinities for immobilised oligomannose. *E. coli* strains were stained with a fluorescent cell-permeable nucleic acid dye and incubated on the microarray. They demonstrated that the platform allows discriminating strains according to their affinities for mannose residues. This issue is critical since the ability of screening cells' affinity for different carbohydrate affinities is related

to their pathogenicity. The ability of the microarray to sense multiple carbohydrate-based interactions in one single experiment allows profiling pathogen–carbohydrate affinities. This should permit establishing a "finger print" like identification of the pathogen present in a complex medium. They also showed that they could identify *E. coli* from complex media such as a serum containing erythrocytes. Anti-adhesive molecule activities were evaluated using such arrays. They compared the IC_{90}s of mannose, *p*-nitro mannose and mannose-containing polymers.

Chevolot et al. (2001) tested the effect of galactose and a lactose-derivatised surface on primary rat hepatocytes. They found that their viability and xenobiotic metabolism were not affected, while neutral red uptake and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) activity increased with galactose surface density. Neutral red uptake is related to the lysosomal activity. They hypothesised that this may related to the natural lysosomal activity of hepatocytes in correlation with the asialoglycoprotein receptor.

Adhesion of primary chicken hepatocytes and CD4+ human T cell onto glycoarrays (bearing 200 and 45 structures, respectively) were studied by Nimrichter et al. (2004) using fluorescent staining of the cells. Primary chicken hepatocytes express a C-type lectin that binds to *N*-acetyl glucosamine (GlcNac) present at the non-reducing terminal position of oligosaccharides. They found that the cells did bind as expected onto spots bearing GlcNAc at the terminal non-reducing end independently of the linkage and orientation. The hepatocytes did not adhere onto spots bearing galactose residues at the terminal non-reducing end. CD4+ human T cell from healthy individual express affinity towards Lewis X structure mediated by an L-selectin. On a 45-structure-bearing chip, they showed that the presence of the fucosyl residues was compulsory for obtaining good adhesion. They also showed that the adhesion was influenced by the staining protocol. According to the authors, the main limitations are the control of detachment forces, the low compatibility of cells with the robotics and high-throughput liquid and long-term storage. Adhesion of pathogens such as *E. coli* (Nimrichter et al. 2004) or viruses (Amonsen et al. 2007) was also reported.

7.6 Concluding Remarks

Carbohydrate biosensors have demonstrated to be powerful tools for studying glycobiolgy. They should find applications in fundamental research, diagnosis or drug discovery. However, availability of carbohydrate ligands in terms of synthesis, biosynthesis or extraction from natural sources remains a key issue. Furthermore, fundamental understanding should be addressed. For example, the influence of surface chemistry on the subsequent protein–carbohydrate interaction has not been widely studied, even though it may influence the overall results.

Most reported biosensors have so far focused on carbohydrate–protein interactions. However, other interactions may be of crucial interest such as carbohydrate–carbohydrate interactions, DNA–carbohydrate interactions, etc. Finally, to our

knowledge, very few integrated (i.e., with integrated microfludic) systems have been reported. Such systems could in theory improve applications by reducing the amount of needed material, decreasing the experimental time, etc.

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