Unravelling Carbohydrate Interactions with Biosensors Using Surface Plasmon Resonance (SPR) Detection

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Surface plasmon resonance (SPR) is an optical phenomenon occurring at a metal coated interface between two media of different refractive index, e.g., water and glass. Exploitation of this phenomenon for investigating biomolecular interactions has occurred since biomolecules can be attached to a chemically modified gold surface and that an interaction between the surface and other biomolecules in solution will affect the SPR of the system. A great deal of the literature on this subject has involved an investigation of protein-ligand interactions, but this chapter will review the use of this technique for investigating carbohydrate-ligand interactions.

Keywords. Surface plasmon resonance, Carbohydrate interactions, Kinetics

List of Abbreviations

BSA   bovine serum albumin
ConA   *Concanavalin A* agglutinin
DMPC   dimyristoylphosphatidylcholine
DSA   *Datura stramonium* agglutinin
GAG   glycosaminoglycan
Gal   galactose
GalNAc   *N*-acetylgalactosamine
GlcNAc   *N*-acetylglucosamine
LPL   lipoprotein lipase
LPS   lipopolysaccharide
MAA   *Maackia amurensis* agglutinin
MM   molecular mass
MP   *Microciona prolifera*
Neu5Ac   sialic acid/*N*-acetyl neuraminic acid
PSA   polysialic acid
RCA   *Ricinus communis* agglutinin
RI   refractive index
RU   response unit
SAM   self-assembled monolayer
SNA   *Sambucus nigra* agglutinin
SPR   surface plasmon resonance

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Introduction

Carbohydrate recognition is a subject of increasing importance with the fine specificity of these molecules now being increasingly recognized and exploited. Carbohydrates are molecules carrying an enormous amount of information and have been implicated in a number of biochemical events including cellular adhesion and mediation of protein folding [1–8]. Techniques for studying these interactions vary depending on the interactions of interest.

Biosensors using surface plasmon resonance (SPR) as detection were introduced to the scientific community in the early 1990s [9–13]. These biosensors are used to derive kinetic and affinity data for ligand-analyte interactions in real-time [14, 15]. SPR is an optical phenomenon occurring at a metal coated interface between two media of different refractive index (RI). Under conditions of total internal reflection of a plane polarized light source, an evanescent wave will penetrate into the medium of lower RI, causing free electrons in the metal layer to oscillate – the generation of surface plasmon waves. This phenomenon is induced by one specific angle of the incident light, and can be monitored in the reflected light since, at that angle, a reduction in intensity occurs.

The SPR of the system is dependent, amongst other things, on the RI of the two media. If the RI of one medium changes then the SPR of the system will also change, indicated by reduction in intensity of a different angle of the reflected
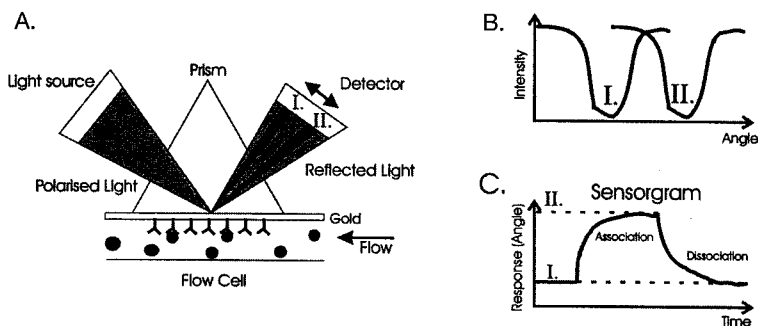
Fig. 1. A Diagram of an SPR system containing a prism as one medium and a solution of analyte (●), in the flow cell, as the other. At the interface is a gold surface containing substrate (Υ). A light source is internally reflected at the interface and monitored at the detector. **B** The angle of the incoming light at which SPR occurs is indicated by reduction in intensity of the same angle in the reflected light (I; see A and B). As more analyte binds to substrate the refractive index of the solution close to the surface will change, with the result that the angle of light at which SPR is induced is changed (II; see A and B). **C.** The speed and extent of this gradual change is monitored in real-time in a plot known as a sensorgram

light source (Fig. 1 A, B). The difference between the angles is indicative of the amount of substrate bound to the surface, whereas the rate at which the change occurs depends on the kinetics of the interaction. Biosensors based on this technique contain a prism as one medium and usually an aqueous solution as the other, with a thin layer of gold at the interface (Fig. 1 A). One biomolecule (substrate) of interest is attached to the surface of the gold in contact with the solution, whilst a second biomolecule (analyte) is dissolved in the aqueous solution. If an interaction between these two biomolecules occurs the local refractive index at the surface will change, thus affecting the SPR of the system. A large amount of data involving protein-ligand interactions has been derived from this type of system [16], whereas that of carbohydrate-related interactions has produced less, and until now has not been evaluated and reviewed. In this review we will consider some of the common problems encountered when analyzing carbohydrate interactions, such as multivalency, weak affinity interactions, and handling low molecular mass (MM) compounds. Studies illustrating the scope and limits of SPR detection systems for investigating carbohydrate interactions will be especially highlighted.

1.1
Experimental Set-Up

Biomolecules can be attached to the gold surface of the sensor in one of several ways. One of the most common means of attachment (or immobilization) is to couple the molecules to a carboxymethylated dextran-coated gold surface by covalent attachment to the carboxyl groups of the dextran [17]. This can be achieved in several ways (Fig. 2).

Other immobilization procedures are direct coupling of thiol-containing molecules to a gold surface and incorporation of molecules into immobilized
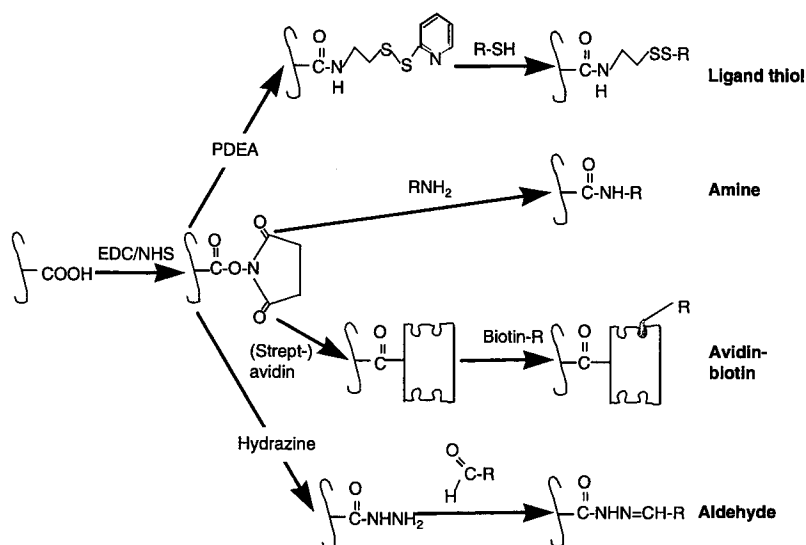
Fig. 2. Chemical approaches for attaching biomolecules to a carboxymethylated dextran coated surface. NHS, N-hydroxysuccinimide; EDC, N-ethyl-N'-(dimethylaminopropyl)carbodiimide; PDEA, 2-(2-pyridinyldithio)ethaneamine hydrochloride

lipid membranes [18]. Careful choice of experimental conditions and immobilization reactions is a prerequisite. It is essential that reference surfaces are used so that non-specific binding effects are taken into account, avidity binding is avoided or at least taken into account, mass transport and aggregation are minimized, regeneration of the surface is complete, and data processing is exact [19, 20]. The majority of experiments in this review have been performed on systems in which the analyte is continuously flowed across the gold surface, as opposed to the cuvette system, which is static. The sensorgram (Fig. 1 C) is a graphical representation of the change in SPR angle, or response, with time as the interaction between substrate and analyte proceeding in a continuously flowing system. The response, measured in number of response units (RU), is a representation of the amount of surface bound substance: 1000 RU = 1 nmol l^{-1} mm^{-2} of protein. The kinetics of the interaction can be derived from the change in response with time recorded in the sensorgram.

    The kinetics [20–22] are governed by the real-time on- and off-rates which are described by the association rate constant (k_A; M^{-1} s^{-1}) and the dissociation rate constant (k_D; s^{-1}). The overall affinity of the system is expressed as the equilibrium association or dissociation constant, K_A (M^{-1}) or K_D (M), respectively, in which the one is the reciprocal of the other. Equilibrium kinetic constants can be calculated either from the level of binding recorded at equilibrium, and so can be derived from results obtained with static SPR biosensors as well as continuously flowing SPR systems, or can be obtained by dividing the real-time rate constants k_A and k_D by each other. Good agreement between K_A (or K_D) derived in both ways is an indication that the experimental set-up is of high quality.

Within this review equilibrium constants will be quoted in the form that they appear in the relevant publication. This will mean that both K_A and K_D will be interchanged throughout the text. K_D may be referred to as a power of ten (e.g., 10^{-3}, 10^{-6}, 10^{-9} mol l^{-1}), or as the abbreviated unit (e.g., mmol l^{-1}, μmol l^{-1}, nmol l^{-1}).

2
Carbohydrate-Protein Interactions

The most commonly studied carbohydrate interaction is that with protein, a recognized and abundant recognition process throughout nature. The specificity and intensity of these interactions have been studied by numerous methods including microcalorimetry [23], fluorescence anisotropy [24, 25], NMR spectroscopy [26, 27], atomic force microscopy [28], mass spectrometry [29, 30], and molecular modeling techniques [31], of which all have their relative merits. The attraction of being able to monitor binding events in real-time, without the use of labels, has facilitated the introduction of SPR biosensors into the field of carbohydrate-protein interactions.

The affinity (K_D) of this type of interaction ranges from mmol l^{-1} to μmol l^{-1} for lectin-carbohydrate interactions, mmol l^{-1} to nmol l^{-1} for antibody-carbohydrate interactions, and even lower values for the interaction of glycosaminoglycans with protein, largely because these interactions tend to involve multiple, and co-operative, binding events. Within this section data will be discussed that has been acquired using this technique, including comparisons with other techniques when data are available.

2.1
Carbohydrate-Lectin

Lectins are carbohydrate binding proteins of non-immuno origin, occurring in both animals and plants, involved in cellular recognition [8, 32, 33]. By using SPR detection the specificity, affinity, and stoichiometry of several lectin-based interactions have been investigated, as well as exploitation of this technique for characterizing glycans of unknown structure with lectins of known specificity. Exploring the possibilities of developing a system for determining oligosaccharide structures began in 1994. Hutchinson [34] investigated the interaction of a number of lectins with the glycoprotein bovine fetuin, and subsequent glycosidase treated bovine fetuin. The lectins, of known specificity, were shown to bind to the correct epitopes, for example the sialic acid (Neu5Ac) specific lectin from *Maackia amurensis* (MAA) bound to intact fetuin but not to sialidase treated fetuin. The level of binding was calculated as a percentage of the maximum change in SPR response, the kinetics of the interactions were not calculated or taken into account. In a similar study, Shinohara et al. investigated the interaction of surface-bound sialidase treated fetuin with a group of lectins [35]. Both the galactose (Gal) binding lectin from *Ricinus communis* (RCA) and the lectin from *Datura stramonium* (DSA), which recognizes *N*-acetylglucosamine (GlcNAc) but also polylactosamine, bound but as expected not the Neu5Ac binding

lectins. RCA had a k_A of 3.4×10^5 M^{-1} s^{-1} and a k_D of 2.1×10^{-3} s^{-1}, which gave a K_A of 1.6×10^8 M^{-1}. DSA had a k_A of 5.7×10^5 M^{-1} s^{-1}, a k_D of 1.3×10^{-3} s^{-1}, and a K_A of 4.3×10^8 M^{-1}. These K_A values are higher than expected for common lectin-saccharide interactions, and values 100-fold less would be more probable. One possible explanation for these high values, particularly in the case of RCA with two Gal binding sites, is that the interaction may have been stabilized by a second interaction. Co-operative binding due to one extra binding site can increase the affinity by a factor of 100 (see throughout the review). However, it could be as high as 10,000-fold, as was shown for the interaction of wheat germ lectin with chitooligosaccharides, the difference in affinity being derived from experiments in which both lectin and oligosaccharide were immobilized to a gold surface, enabling the comparison of the affinities and calculation of the effect of co-operativity [36]. Co-operative binding will be seen throughout this review to play both positive and negative roles in the investigation of carbohydrate interactions.

Since it was clear that lectin-carbohydrate interactions could be measured using SPR, it became important to evaluate and assess the different possibilities for recording accurate and reproducible data. Kalinin et al. revealed *Concanavalin A* (ConA) lectin to bind (K_A 2.5×10^5 M^{-1}) the carboxymethylated dextran matrix commonly used on sensor surfaces [37]. They also stressed that this interaction did not follow first order kinetics, and that care should be taken when using multivalent solutes: many lectins are dimers or tetramers, and often contain more than one carbohydrate binding site (e.g., RCA and ConA). This point was further emphasized in work utilizing carbohydrate-derivatized self-assembled monolayers (SAMs) [38]. Not only did multivalent binding take place, but as the surface density of the carbohydrate ligands increased, the binding selectivity of *Bauhinia purpureas* lectin was shown to switch from one carbohydrate ligand to another. At low surface densities [mole fraction of sugar (X_{sugar}) ~ 0.1] the lectin bound more strongly to mixed SAMs containing 1 than to those containing 2 or 3 (Fig. 3). As the surface density increased (X_{sugar} ~ 0.6), the avidity of the lectin for monolayers containing 1 decreased and that containing 2 increased. It would appear that secondary interactions play a significant role on the binding of the lectin at high surface densities, and that this mechanism may have a real influence on biological recognition processes.

In order to avoid 'non-specific' binding to the carboxymethylated dextran surface, Mann et al. generated synthetic glycolipid surfaces (see Sect. 5), which avoided the use of the dextran, in their development of a system for rapidly evaluating the ability of various inhibitors to block ConA binding [39]. The affinity of ConA tetramer, containing four possible carbohydrate binding sites, to a lipid membrane layer containing 10% glycolipid (containing terminal α-mannose) had a K_A of 2.7×10^4 M^{-1}. This value appears to be lower than would be expected for a carbohydrate-lectin interaction involving co-operative binding, although multivalent binding was assumed to occur. A reduced k_A value could possibly be due to either performing experiments at a lipid layer or hindered presentation of the glycolipid at the surface (see Sect. 2.5). The interaction could be inhibited by methyl α-mannoside or methyl α-glucoside, for which the calculated K_D values were 92 and 290 µmol l^{-1}, respectively. These values were
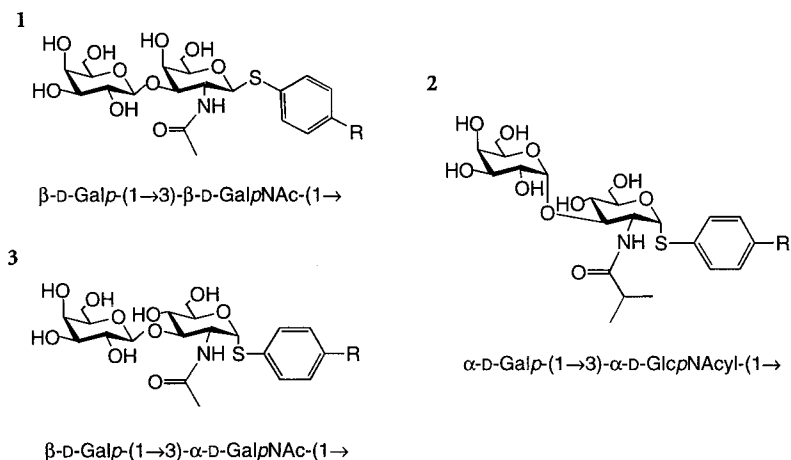
Fig. 3. The structure of ligands **1, 2,** and **3** used in the study of the binding selectivity of *Bauhinia purpureas* lectin at carbohydrate-derivatized self-assembled monolayers. R denotes a hydrophobic spacer

only marginally different to those derived from microcalorimetry (132 and 416 µmol l^{-1}, respectively) [40], and the ratio of the two was identical in both cases. These conclusions would appear to support a lack of co-operative binding, at least in the sense of multivalent binding as opposed to that in terms of communication between binding sites [41, 42], since microcalorimetry should only facilitate elucidation of the monovalent interaction. The results were also very similar to those derived from fluorescence anisotropy (181 and 588 µM, respectively) [40]. The 'multivalent properties' of this system were illustrated by the increased inhibitory potency (up to 40-fold higher than that for methyl α-mannoside) of mannose polymers containing more than 15 monosaccharide residues: the proposed minimum length required to span two sub-units of the lectin.

In order to develop a system for accurately calculating the monovalent interaction kinetics of lectin-carbohydrate binding, Haseley et al. have devised and evaluated a method in which the lectin and the respective denatured lectin, established as being the most suitable blank surface, are immobilized to the gold surface [43]. They also investigated buffer composition and both immobilization and regeneration conditions. The definitive system proved very successful, with determination of kinetic data for the binding of ten pure oligosaccharides to the Neu5Ac binding lectins from *Sambucus nigra* (SNA) and MAA, in which certain recurring changes in oligosaccharide structure brought about similar subtle changes in the kinetic parameters. This effect is nicely illustrated by the values in Table 1 recorded for four of the oligosaccharides (Fig. 4). The lectin SNA, which recognizes Neu5Ac linked to the 6-position of Gal, had a similar affinity for all four oligosaccharides. This was as expected since all oligosaccharides contained this Neu5Ac epitope in very similar chemical environments. The lectin MAA, however, which recognizes Neu5Ac linked to the 3-position of

Table 1. Equilibrium association rate constants (K_A) for the binding of oligosaccharides **1 – 4** to *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA). Average values from five experiments, error between 10 – 20 % for each result. The closeness of fit for each estimated parameter is indicated by the statistical value of χ^2 in brackets

Oligosaccharide	SNA K_A (M^{-1})	MAA K_A (M^{-1})
1	1.36×10^6 [0.054]	2.92×10^5 [0.16]
2	1.58×10^6 [0.41]	3.83×10^5 [2.94]
3	7.88×10^5 [0.74]	1.42×10^6 [0.71]
4	1.86×10^6 [0.34]	2.40×10^6 [2.46]

Gal, was able to differentiate between oligosaccharides containing this Neu5Ac epitope on both the upper (as indicating in Fig. 4) and lower branches of the glycan and those only containing the epitope on the lower branch. The 5- to 10-fold increase in K_A brought about by the addition of Neu5Ac to the upper branch may be the result of increased availability, or reduced steric hindrance, of the epitope at this position, which in turn could cause an increase in the overall affinity of these oligosaccharides for MAA.
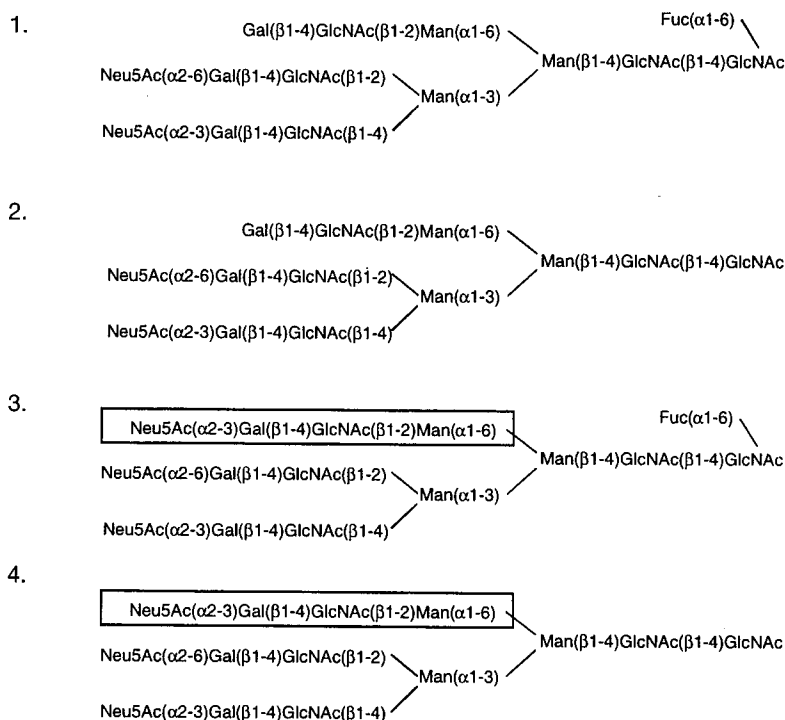
Fig. 4. The structure of oligosaccharides **1 – 4** used to study the specificity of lectins from *Sambucus nigra* and *Maackia amurensis*. The *circled epitopes* denote structural motifs of particular interest (see text)
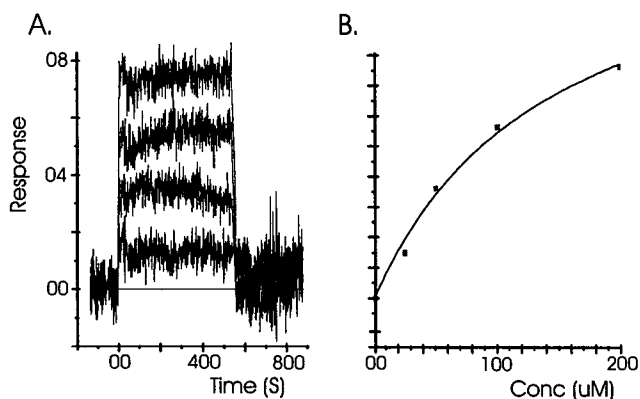
Fig. 5. **A** Overlaid sensorgrams produced by the interaction of different concentrations (0, 25, 50, 100 and 200 μmol l^{-1}) of the Sd^a-determinant tetrasaccharide with immobilized *Dolichos biflorus* lectin. **B** A plot of equilibrium response against concentration (steady-state analysis). The interaction K$_D$ was derived from this plot using known mathematical equations [71]

This system has now been employed for the analysis of several lectins, including that from *Dolichos biflorus* [44] for which the affinity of the Sd^a-determinant, a tetrasaccharide, has been determined via steady-state analysis (Fig. 5 B), i.e., from a plot of the level of binding at equilibrium (Fig. 5 A) against concentration. The interaction had a K$_D$ of 1.44×10^{-4} mol l^{-1}. Biosensors based on SPR usually require the analyte to have a molecular mass larger than 1000 Da (SPR response is related to the size of the molecule). The sensitivity of this system has enabled determination of the affinity of a trisaccharide mimic of the Sd^a-determinant (MM 600 Da) for *Dolichos biflorus* [44]. Regeneration of lectin surfaces can, in the majority of cases, be successfully achieved by using the methyl glycoside based on the monosaccharide epitope recognized.

Other studies involving lectin-carbohydrate interactions are as follows.

A mouse C-type macrophage lectin was revealed [45] to bind glycopeptides and oligosaccharides containing terminal *N*-acetylgalactosamine (GalNAc) residues with relatively high affinity (K$_A$ 6.2×10^7 M^{-1}).

An investigation of P-selectin binding to immobilized P-selectin glycoprotein ligand-1 (PSGL-1) has revealed [46] that this Ca^{2+}-dependent interaction has both a rapid on- (k$_A$ 4.4×10^6 M^{-1}s^{-1}) and off-rate (k$_D$ 1.4 s^{-1}). These features are in agreement with the fact that leukocytes use PSGL-1 to tether and roll on P-selectin on activated endothelial cells. The affinity of this interaction (K$_D$ 320 nmol l^{-1}) was relatively high for a lectin-carbohydrate interaction.

Hamster galectin-3 binds to laminin with a k$_A$ of 1×10^4 M^{-1} s^{-1}, and a k$_D$ of 0.2 s^{-1} resulting in a K$_A$ of 1×10^5 M^{-1} [47], whereas calreticulin, a molecular chaperone thought to possess lectin-like properties, bound to laminin with a K$_A$ of 2.1×10^6 M^{-1} [48]: it did not show any binding to deglycosylated laminin.

2.2
Carbohydrate-Antibody

Carbohydrate-antibody interactions are in many ways similar to interactions with lectins. The analyses follow similar rules, and have similar properties to take into account when designing an experiment, especially the problems of multivalency, which lead to co-operativity [5]. The interaction of an O-specific polysaccharide from *Salmonella* serogroup B bacteria with a monoclonal antibody specific for the tetrasaccharide repeating unit has been rigorously studied by MacKenzie et al. [49].

In this study a BSA-O-polysaccharide conjugate, containing up to 25 repeating units, was immobilized to the surface (via carboxymethylated dextran). Whereas the wild type antibody (SE155–4) gave distinct biphasic association and dissociation data, monomeric fractions of the antibody showed little evidence of biphasic behavior with both fast on- and off-rates (Table 2), with overall K_D values of between 4.1×10^{-6} and 7.5×10^{-6} mol l^{-1}. Dimeric forms of the antibody showed off-rates approximately 20-fold slower and association rates 5-fold faster than the monomers, and therefore at least a 100-fold increase in the affinity (Table 2). These values were slightly higher than that of the wild type antibody (K_D 2.3×10^{-7} mol l^{-1}).

The dissociation stage of all experiments was performed in the presence of the free trisaccharide epitope to minimize rebinding of the antibody (dissociation is commonly performed only in the presence of the system buffer). The effect of this was to increase the rate of release, or alternatively minimize rebinding, of the antibody, although whether dissociation in the presence of free trisaccharide resembles more the natural dissociation situation is debatable. It is important for this phenomenon to be further investigated in order to develop a standard protocol to secure comparable SPR biosensor results. Equilibrium binding analyses provided K_D values (Table 2) in good agreement with those derived from active association and dissociation rate constants, thus confirming the quality of the data and supporting the models proposed. In addition, the

Table 2. Comparison of the kinetic values calculated for the active rate constants k_A and k_D, K_D derived from k_A and k_D, and K_D calculated from Scatchard analysis of the responses at equilibrium (final column) for wild-type and monomeric and dimeric fractions of antibody SE155–4 specific for the *Salmonella* serogroup B O-polysaccharide

Antibody fractions		k_A ($M^{-1}s^{-1}$)	k_D (s^{-1})	k_D/k_A (M)	K_D (M)
Monomeric	SK4	3.0×10^4	2.0×10^{-1}	4.1×10^{-6}	6.8×10^{-6}
	3B1	3.8×10^4	2.4×10^{-1}	7.5×10^{-6}	7.6×10^{-6}
	B5–6	4.8×10^4	2.9×10^{-1}	6.5×10^{-6}	6.1×10^{-6}
	B3–20	4.3×10^4	2.5×10^{-1}	5.3×10^{-6}	5.8×10^{-6}
Dimeric	SLA-1	1.5×10^5	1.1×10^{-2}	4.5×10^{-8}	7.2×10^{-8}
	B5–1	3.2×10^5	1.3×10^{-2}	8.1×10^{-8}	4.1×10^{-8}
	B4–3	1.6×10^5	7.8×10^{-3}	5.6×10^{-8}	5.0×10^{-8}
Wild-type IgG	SE155–4	8.7×10^4	1.2×10^{-2}	2.3×10^{-7}	1.4×10^{-7}
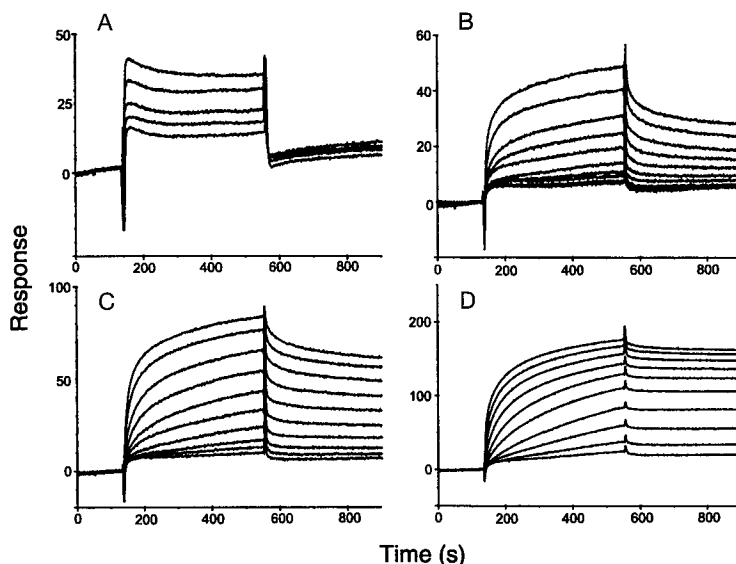
Fig. 6A–D. Sensorgrams produced by the interaction of different concentrations of PSA with antibody: A Neu5Ac$_{16}$; 50, 25, 12.5, 6.25, and 3.125 µmol l^{-1}; B Neu5Ac$_{30}$; 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 nmol l^{-1}; C Neu5Ac$_{100}$; D Neu5Ac$_{200}$; 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.195 nmol l^{-1}

binding affinities for the monovalent antibodies were in good agreement with data acquired from titration microcalorimetry [50]. This SPR investigation was evaluated using the BIAevaluation software supplied by BIAcore and is a good example of the data that can be produced.

In an analysis of the interaction between (α2–8)-linked polysialic acid (PSA) with a monoclonal antibody [51] in which the antibody was bound to the surface, both fast on- and off-rates (average K$_D$ of 8 µmol l^{-1}) were obtained for molecules containing up to sixteen residues of Neu5Ac (Fig. 6A). Molecules containing a higher number of Neu5Ac residues however, appeared to possess some biphasic binding characteristics (Fig. 6B, C), whereas molecules with ~ 200 residues appeared to follow a triphasic interaction mechanism with tight binding (average K$_D$ of 900 pmol l^{-1}) and negligible dissociation of the complex (Fig. 6D).

This phenomenon is due to the presence of co-operativity and illustrates the care that should be taken when performing experiments, and how quickly co-operative binding can enhance the affinity, or avidity, of the system. Since 1 ng protein per mm^2 on the sensor surface results in an SPR response of ~1000 RU, it was possible to calculate the amount of surface bound antibody corresponding to the increase in SPR response (6000 RU) obtained following immobilization of the antibody and therefore the average distance between single molecules on the surface. The calculated average of one antibody molecule every 100 Å denoted that at least 16 residues of Neu5Ac were needed to span two antibodies, and thus needed to initiate a biphasic binding mechanism, a value in good agreement with the experimental data.

In a similar study, but in which glycoconjugates were bound to the surface, multivalency was again seen to make an appearance. In this case the emergence and level of epitope multivalency could be observed and determined by monitoring R_{max}, the SPR response required for saturation of the surface. A fixed density (200 RU) of different glycoconjugates, each containing oligosaccharides with differing numbers of the type III group B *streptococcus* capsular polysaccharide repeating unit, were immobilized to the surface, and the binding of a Fab fragment of an antibody was observed [52]. The conformational epitope of the capsular polysaccharide recognized by the Fab fragment was identified as between two and three pentasaccharide repeating units (K_D 4.6 × 10^{-6} mol l^{-1}), and saturation of the surface was identified as occurring at an R_{max} of 148 RU. Above seven repeating units of surface bound oligosaccharide the R_{max} of the system began to increase, indicating binding of more than one Fab fragment to each oligosaccharide, and at 20 repeating units the R_{max} was 486 RU, binding of approximately 3 Fab fragments per oligosaccharide. Epitope multivalency therefore occurred at between six and seven repeating units. Moreover, the affinity of the monovalent interaction was shown to increase threefold from 7 to 20 repeating units (K_D 6.5 × 10^{-7} mol l^{-1}), indicating optimization and further stability of the conformational epitope.

Evaluation of antibody recognition of synthetic oligosaccharide fragments of the gut-associated circulating anodic antigen excreted by the *Schistosoma* worm has led to the development of a rapid and efficient system, based on SPR detection, for evaluating titers of antibodies from humans [53]. This system has been proposed as a means of diagnosing *Schistosoma mansoni* infection.

2.3
Carbohydrate-Enzyme

Only one study exists to date for the analysis of the interaction between carbohydrate and enzyme. The main reason for this is that enzyme interactions are not simple monovalent interactions, since once the reaction has been catalyzed the product vacates the enzyme. Laroy et al. [54] have avoided this problem by cloning mutants of a sialyltransferase in which the catalytic domain, but not the binding site, has been deactivated. However, they do report that binding of wild type sialyltransferase to sialidase treated fetuin does occur at low salt concentrations (<25 mmol l^{-1} NaCl) and that binding can be inhibited with CMP-Neu5Ac, the natural donor substrate. It should be noted, however, that SPR experiments are generally performed in buffer containing 150 mmol l^{-1} NaCl to avoid non-specific ionic interactions with the surface. One of the mutants bound at similar salt concentrations to those required for the wild type enzyme but, since CMP-Neu5Ac did not inhibit the binding, the sialyltransferase activity had most likely been lost. A further two mutants bound at physiological salt concentrations. As SPR biosensors become more refined it is likely that the analysis of enzyme catalyzed reactions, such as the rather elegant analysis of a DNA-polymerase [55], which may also involve carbohydrate interactions, will become more common.

2.4
Glycosaminoglycan-Protein

Glycosaminoglycans (GAGs) are highly heterogeneous, highly sulfated glycans coating animal cells and tissues. Included within this group are heparin and heparan sulfate, molecules highly sulfated by N-deacetylation and N-sulfation of N-acetylglucosamine (GlcNAc), and various O-sulfation patterns. This coat can be modified during differentiation, development, and during disease, and so it is important to investigate the functional role of these molecules. There has been a considerable amount of research using SPR into the interactions of heparin and other glycosaminoglycans [56–69]. One particularly interesting study, which highlights many of the advantages and disadvantages of this technique, is that covered in two publications by Lookene et al. [56, 57]. They initially investigated the interaction of lipoprotein lipase (LPL) with heparin and heparan sulfate [56]. Attached to the vascular endothelium, LPL binds circulating lipoproteins and hydrolyses their triglycerides. Attachment to the endothelium most likely occurs via interaction with heparin or heparan sulfate. To prepare a heparin or heparan sulfate coated surface the GAG was biotinylated via free amino groups [70], and bound to previously immobilized streptavidin. The interaction between LPL and heparan sulfate was found to be a fast exchange process with a k_A of 1.7×10^8 M^{-1} s^{-1}, and a k_D of 5×10^{-2} s^{-1}, resulting in a K_D of 3×10^{-10} mol l^{-1}. These results were obtained at 150 mmol l^{-1} NaCl. In order to calculate the electrostatic contribution of the interaction, the kinetics were determined at NaCl concentrations between 150 mmol l^{-1} and 900 mmol l^{-1} (the concentration at which binding to the GAG surface was shown to be identical to that to the blank surface). A plot of log K_D against log [NaCl] indicated a linear relationship. Using known equations the electrostatic contribution of the interaction of heparan sulfate with LPL was calculated to be 44%, while that to heparan sulfate was 49%. Other interesting and subtle points included the observation that the dissociation rate followed a biexponential decay in buffer containing less than 300 mmol l^{-1} NaCl, but that at higher concentrations it followed a single exponential decay process. Of all the possible explanations for this phenomenon, the most likely were either heterogeneity of the heparan sulfate chains, resulting in heterogeneous binding along the polymer, or rebinding of LPL during dissociation. To determine whether rebinding occurred, free heparin fragments were injected into the system during dissociation (see [49]). This increased the dissociation rate by a factor of 100; therefore the biexponential decay process was due to rebinding of LPL. To determine the affinity of size-fractionated heparin fragments (tetra-, hexa-, octa- and decasaccharides) and low molecular mass heparin for LPL, a solution-affinity experiment [71] was performed, whereby the concentration of LPL was kept constant, and the concentration of the heparin fragments in solution, varied. In this way the affinity of these fragments for LPL could be determined by inhibiting the interaction of LPL to heparan sulfate. The change in K_D from tetra- to decasaccharide was large, while that between decasaccharide and low molecular mass heparin (mean length of 24 monomers) was significantly less: the decasaccharide had a K_D only 4.5-fold lower than that of the low molecular mass heparin, which had a

K_D of 0.2 nmol l^{-1}. LPL is a dimer in rapid and reversible equilibrium with its inactive monomer, and it is known that heparin protects LPL from inactivation, i.e., from returning to the monomeric form. Since both octa- and decasaccharides blocked LPL binding to heparin at a molar ratio of 1:1 with LPL, the authors concluded that an octasaccharide was the minimal length heparin fragment sufficient for maximum stabilization of the dimer. This conclusion was also reflected in the calculated K_D values. This led to the conclusion that an octasaccharide possessed the minimum length required to span both monomers at the same time, therefore stabilizing the dimer. Monomeric LPL had an association rate constant for heparan sulfate 1000 times lower than dimeric LPL. To summarize, the interaction of LPL dimer with a heparan sulfate surface is a rapid process with a K_D of 0.3 nmol l^{-1}. It is partially electrostatically driven, and leads to accumulation of the enzyme close to the surface, i.e., it rebinds to the surface rather than dissociating. Heparin fragments longer than octasaccharide span the binding sites of both subunits of the dimer. Previously reported values for the interaction of low molecular mass heparin to LPL were 13 nmol l^{-1} [72] and 43 nmol l^{-1} [73]. The differences between these two results and the values obtained by SPR detection (K_D 0.2 nmol l^{-1}), are factors 40- and 100-fold lower, respectively. These may be due to heparin binding to immobilized LPL in the first experiment [72], or perhaps partial inactivation of LPL during both of the previous studies, resulting in an average binding K_D between inactive monomer (K_D 1.2 µmol l^{-1}) and active dimer.

In their second publication the interaction between lipoprotein and heparan sulfate, in both the presence and absence of LPL, was investigated [57]. In short, they identified that lipoprotein binds to heparan sulfate, but that the binding is amplified in the presence of LPL, and that heparin has a higher binding capacity for lipoproteins than does heparan sulfate. They also discovered that two to four lipase dimers per lipoprotein were necessary for efficient binding to the heparan sulfate surface, indicating a role for co-operativity in the interaction. The association rate constants were about tenfold higher for the interaction between lipoprotein and LPL bound to heparan sulfate than for lipoprotein to immobilized LPL, demonstrating a contribution of heparan sulfate to the interaction. The role of LPL in the interaction of lipoprotein with heparan sulfate, was not to increase the affinity of the interaction, but actually to increase the number of binding sites at the surface. These conclusions were all derived from SPR biosensor experiments and further illustrate the power of this technique.

Among the many SPR studies investigating glycosaminoglycan interactions the following are of particular interest.

The binding of human acidic fibroblast growth factor (aFGF) to heparin was found to have an affinity K_D of 50–140 nmol l^{-1} [58], which was in agreement with results obtained from affinity electrophoresis experiments [74].

The interaction of platelet-derived growth factor with heparin had an affinity K_A of 1.7×10^8 M^{-1}, and was shown to involve two heparin binding sites of which the carboxy-terminus played an important role [59].

Shuvaev et al. showed that hyperglycation of apolipoprotein E, as in diabetic patients, impairs lipoprotein-cell interactions, which are mediated via heparan sulfate proteoglycans [60, 61].

The interaction between heparin and heparin binding growth-associated molecule had a K_D (4 nmol l^{-1}) 100-fold lower than that determined by isothermal titration calorimetry [62]. This tighter binding was suggested to have been the result of immobilizing the growth-associated molecule to the surface, as opposed to the interaction in solution.

2.5
Glycolipid-Protein

Investigating glycolipid interactions with SPR biosensors is an almost completely different and separate subject to other carbohydrate-related interactions as these studies usually employ membranes or vesicles attached to the gold surface. The presence of a lipid monolayer in the analysis can not only produce quite different results to those using molecules attached to carboxymethylated dextran, but may also produce data more closely resembling the in vivo situation. Using a liposome-capture method, MacKenzie et al. have analyzed in great depth the interaction of cholera toxin with a selection of gangliosides [75]. These glycolipid receptors were incorporated into artificial liposomes that also contained a small amount of lipopolysaccharide (LPS) to allow capture of these liposomes by an immobilized anti-LPS monoclonal antibody (immobilized to a carboxymethylated dextran surface). Cholera toxin demonstrated an absolute requirement for terminal galactose and internal sialic acid residues (as in G_{M1}; Fig. 7) with tolerance for substitution with a second internal sialic acid (as in G_{D1b}, Fig. 7).

One of their major findings was that the percentage of glycolipid incorporated into the liposomes affected the affinity of the toxin interaction. Both the association and dissociation rates decreased as the percentage of glycolipid changed from 2% to 4%, the K_D values for G_{M1} being 1.7 nmol l^{-1} and 6.8 nmol l^{-1}, respectively. The reason for this was that two distinct on-rates existed at the surface containing 4% G_{M1}, but only the faster on-rate was observed at G_{M1} concentrations of 2%, resulting in a slight increase in the calculated affinity. Compared to conventional assays, the liposome-capture method showed highly restricted specificities for bacterial toxin binding to glycolipids, and the data were in good

G_{M1}

β-D-Gal$p$-(1→3)-β-D-Gal$p$NAc-(1→4)-β-D-Gal$p$-(1→4)-β-D-Glc$p$-(1→

                                  3

                                  ↑

                                  2

                       α-Neu$p$5Ac

G_{D1b}

β-D-Gal$p$-(1→3)-β-D-Gal$p$NAc-(1→4)-β-D-Gal$p$-(1→4)-β-D-Glc$p$-(1→

                                  3

                                  ↑

                                  2

α-Neu$p$5Ac-(2→8)-α-Neu$p$5Ac

Fig. 7. Structures of the glycolipids G_{M1} and G_{D1b}

agreement with the majority of previous studies [76–81]. The results were inconsistent with an earlier SPR study [82] which observed the order of binding strength $G_{M1} > G_{M2} > G_{D1a} > G_{M3} > G_{T1b} > G_{D1b} >$ asialo-G_{M1}, with the interaction of G_{M1} possessing a K_D (4.61×10^{-12} mol l^{-1}) 1000-fold lower than that reported by MacKenzie et al. (1.7×10^{-9} mol l^{-1}). The binding of G_{D1a}, G_{M3}, and asialo-G_{M1} contradicted binding specificities by other means, particularly the high affinity reported for G_{D1a} (K_D 31.8 pmol l^{-1}). This ligand was previously used as a negative control in titration experiments of cholera toxin with gangliosides [78]. MacKenzie et al. suggested that the specificity differences may have been the result of different presentation of the two lipid bilayer environments. The glycolipid surfaces used in the latter experiment [82] were prepared by immobilizing glycolipid: palmitoyloleoylglycero-3-phosphocholine (5:95 mol%) to an alkylthiol monolayer on the gold surface to form hybrid bilayer membranes. There is considerable evidence that glycolipid carbohydrate can be modulated by the bilayer microenvironment, although non-specific binding, which is rife in analyses using exposed alkane-thiol groups, may also have contributed [82, 83].

In a study characterizing the binding of an IgM antibody to glycolipid asialo-G_{M1} Harrison et al. compared the affinity recorded using liposomes fused to an alkane-thiol layer (formation of a hybrid bilayer) and liposomes captured to an antibody previously immobilized to a carboxymethylated dextran surface [84]. The sensorgrams indicated that the hybrid bilayers had approximately ten times the capacity for binding, lower association rates (factor of ~ 5) and lower dissociation rates (factor of ~ 10), resulting in derivation of similar K_Ds by both methods. By injecting asialo-G_{M1} tetrasaccharide during dissociation the K_D value decreased 60-fold from 3.0×10^{-8} mol l^{-1} to 5.2×10^{-10} mol l^{-1}. It is believed that this unusual effect (the dissociation rate is usually increased [49, 56]) is due to selective inhibition of lower valency binding, which promotes higher affinity antibody-carbohydrate interactions.

The most interesting result from the interaction of murine R24 IgG with G_{D3}-bearing liposome surfaces was that saturation of the surface was never attained [85]. This was the result of additional homophilic binding of the antibody, as had been previously reported for the interaction of this antibody to human melanoma cell lines expressing G_{D3} [86]. The binding kinetics were complex at all concentrations of antibody. Data collected at low IgG concentrations fitted relatively well to a one-to-one interaction model with a k_A of 1.3×10^5 M^{-1} s^{-1}, a k_D of 2.3×10^{-3} s^{-1}, and a K_D of 18 n. Calculation of the interaction kinetics for the homophilic binding was not performed.

The interaction of the lectin from *Ulex europaeus* (UEA) with H-fucolipid embedded in liposomes attached to immobilized ricin [87] had a k_A of 2.1×10^4 M^{-1} s^{-1} and a k_D of 0.09 s^{-1}, and therefore a K_A (2.3×10^{-5} M^{-1}) in accordance with the expected affinity for carbohydrate-lectin interactions. The K_A was shown to decrease from 2.42×10^{-5} M^{-1} to 1.72×10^{-5} M^{-1} by increasing the temperature of the system from 15 °C to 30 °C. The activation energy for the association phase was calculated, with the aid of Arrhenius plots, to be 29.1 kJ/mol, indicating that a large amount of energy needed to be expended in order for binding to occur. Thus, thermodynamic data may also be derived from SPR experiments, although this is the only example for a carbohydrate-related interaction.
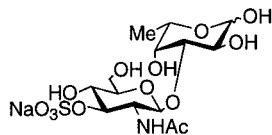
The interaction of an IgA antibody with a lipid monolayer containing LPS from *Vibrio cholerae* (Inaba strain) was determined [88] to have a K_A of 1.0×10^8 M^{-1}. The interaction of detergent solubilized LPS had a K_A of 6.6×10^5 M^{-1}, thus indicating the care that should be taken when designing experiments to investigate the interactions of glycolipids.

3
Carbohydrate-Carbohydrate Interactions

The study of carbohydrate-carbohydrate interactions is a subject of immense interest in cellular recognition as it is a relatively unexplored field even though some groups have postulated that this type of interaction may play an important role in a number of cellular binding events [7, 89–94]. Since carbohydrate-carbohydrate interactions are of low affinity, careful choice of the techniques and experimental methods used is essential. The majority of investigations performed have focused on an analysis of these weak interactions in the context of multivalent models [7, 89–91] since co-operative binding will in general result in an increased avidity of interaction [5]. Monovalent interactions have also been investigated, for example for the self-interaction of Lewis X epitopes [95, 96], although the affinity was difficult to evaluate and has been reported to have a K_A as low as 2 M^{-1} [95]. Another example of carbohydrate self-interaction, which has been implied but never proven, is that of defined carbohydrate epitopes at the surface of marine sponge cells [7, 92–94]. For the red beard sponge, *Microciona prolifera*, two carbohydrate epitopes, a sulfated disaccharide (Fig. 8) [97] and a pyruvylated trisaccharide [98], have been implicated in Ca^{2+}-dependent cellular adhesion. In order to investigate this phenomenon a model system, using SPR detection, is being developed [99].

In this system a BSA-conjugate (MP-conjugate), containing on average 7.8 moieties of the sulfated disaccharide [100] has been immobilized to a carboxymethylated dextran coated gold surface, along with BSA and underivatized carboxymethylated dextran as control surfaces. The interaction of these three substrates with the analytes BSA and MP-conjugate will then be monitored both in the presence and absence of 10 mmol l^{-1} Ca^{2+} ions. The system buffer consists of 20 mmol l^{-1} tris.HCl (pH 7.4) and 500 mmol l^{-1} NaCl, the concentration of salt commonly found in sea-water. The presentation of multiple epitopes of disaccharide per conjugate means that the specific interaction of carbohydrate to car-

β-D-GlcpNAc3S-(1→3)-L-Fucp

Fig. 8. Structure of the sulfated disaccharide implicated in the Ca^{2+} dependent cellular adhesion of the marine sponge *Microciona prolifera*

B. Multilayer formation

+10 mM Ca²⁺

A.

MP-conjugate (analyte)

MP-conjugate (substrate)
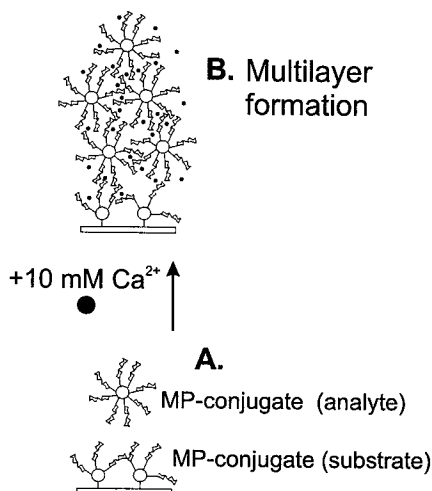
Fig. 9A, B. Illustration of the polyvalent multilayer formation of MP-conjugate: A before addition of Ca^{2+} ions; B upon addition of Ca^{2+} ions

bohydrate will result in polyvalent multilayer formation of MP-conjugate at the surface (Fig. 9): the interaction of two conjugates will result in the presentation of further carbohydrate epitopes at the surface. In addition the binding profile of the sensorgram should have characteristics very different to the standard profile as saturation of the surface cannot be attained. If successful, this system may provide a powerful means of investigating other types of carbohydrate interactions, such as the self-interaction of Lewis X.

4
Other Carbohydrate-Related Interactions

In addition to the subjects mentioned above, SPR and carbohydrate-related interactions have also been used for the development of detection assays, such as the detection of molecules in human serum [101], and detection of oligosaccharides by continuous sampling [102], which exploits weak-affinity interactions (immediate on- and off-rates) between oligosaccharides and antibodies [103, 104]. Within our group the recombination of two sub-units of a glycoprotein has been performed at the surface of a SPR biosensor, in which the role of the glycans and the question of whether a correctly folded protein can be formed, have been investigated [105].

5
Preparation of Carbohydrate Surfaces

The surface of the gold can be coated by carbohydrate in several ways. The most common method is to attach neo-glycoconjugates to a carboxymethylated dextran surface, often via amine functions on the surface of the protein (Fig. 2).

Alternatively, biotinylated carbohydrate can be attached to a streptavidin coated surface, previously attached via the amine coupling procedure. Heparin has been biotinylated in several ways, these include labeling of amine functions using NHS-LC-biotin [61], or sulfo-NHS-biotin [56, 58], or carbonyl functions, after periodate oxidation of *cis*-diol groups, using biotin hydrazide [58] or amino biotin [17]. A comparison of the k_As for the interaction of acidic fibroblast growth factor with heparin biotinylated either at amine or carboxyl functions, derived from periodate oxidation, has shown a marked (tenfold) decrease due to carboxyl labeling [58]. The k_A for the *cis*-diol biotinylated product had a value of 1.9×10^5 M^{-1} s^{-1}, as opposed to 1.1×10^6 M^{-1} s^{-1} for the amino biotinylated heparin. Chondroitin sulfate has been attached to a carboxymethylated surface by activating the surface with a 1:1 mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC), followed by attachment of adipic acid dihydrazide (ADHZ) to the activated carboxyl functions, and finally reaction of formyl groups at the reducing end of the carbohydrate to form Schiff bases (as indicated in Fig. 2: aldehyde). The Schiff bases were then reduced, by reductive amination using $NaBH_3CN$ to give stable alkylamine bonds [106]. Alternatively, carbohydrate may be directly coupled to a dextran surface via a carboxyl or amine containing spacer molecule.

There are several ways of preparing glycolipid surfaces. One way is to capture liposomes, via an independent molecule, to the carboxymethylated dextran surface. An example of this is the attachment of glycolipid/DMPC liposomes containing a low percentage of *Salmonella* group B LPS to an antibody specific for the LPS [75, 84, 85]. In an alternative approach liposomes are fused to an alkane thiol monolayer present on a gold sensor chip [39, 82, 84]. This results in a hybrid bilayer in which the ligand of interest is presented to the analyte. As already mentioned, different immobilization protocols for similar problems can result in contrasting results [83, 84].

6
Conclusions and Outlook

Biosensors using surface plasmon resonance detection have been employed to investigate and solve some interesting carbohydrate-related problems. It is clear that the reliability of the results depends on the type of experiment performed, and the care taken in designing the experiment, e. g., the use of blank surfaces in the analysis. This technique would at present appear to be a suitable alternative to other techniques, but perhaps as the instruments available are improved it may become even more powerful. Possible improvements could involve the design of gold-bound lipid membrane bilayers more closely resembling cell surfaces, and also fine-tuning of the sensitivity of the system, in order to analyze low molecular mass compounds. One major downside of this type of system is that one biomolecule or the other is likely to be changed or modified in some way during the experiment, e. g., by attachment to the surface. However, the use of lipid membranes may also, in the future, avoid this problem. In conclusion, these systems obviously offer something more to the scientific community than

was already available, which is clear from the thousands of articles that have appeared over the last decade [16]. It can be anticipated that they will be utilized to their full extent for characterizing interactions involving or mediated by carbohydrates.

7
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