Carbohydrate Biosensors

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Carbohydrate Biosensors

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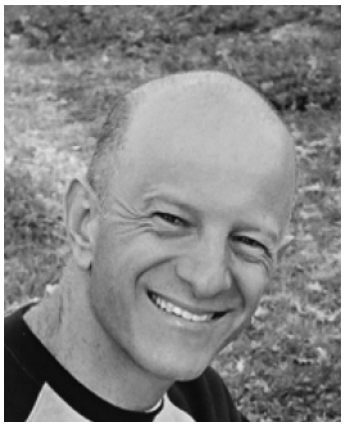

Raz Jelinek was born in 1964 in Beersheva, Israel. He obtained his B.Sc. in chemistry (summa cum laude) at the Hebrew University, Jerusalem, Israel, in 1988 and went on to get his Ph.D. in chemistry at the University of California, Berkeley, in 1993, where he did research under the guidance of Alex Pines on solid-state NMR of zeolites and molecular sieves. During his postdoctorate at the University of Pennsylvania, he switched to structural biology, doing NMR on peptides in membrane environments. In 1996, Raz returned to Beersheva to take a position at Ben Gurion University, where he is conducting research on biosensor development and biomimetic cellular systems.

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1. Introduction

Carbohydrates (denoted also oligosaccharides or polysaccharides) constitute a large and diverse class of compounds present in varied materials and have major roles in applications in chemistry, biology, materials science, and related fields. In the context of biological systems, in particular, carbohydrate research has emerged as the "new frontier" for elucidating fundamental biochemical processes and for identifying new pharmaceutical substances. Beside nucleic acids and proteins, carbohydrates appear to play critical roles in determining biological functions and affecting wide-ranging physiological processes, thus, their study and characterization have become increasingly important.

This review aims to provide a comprehensive overview of recent scientific activity pertaining to systems, methods, and devices designed to detect carbohydrates. In addition, we discuss biosensor assemblies in which carbohydrates comprise essential

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parts of the biological recognition systems. We have particularly tried to discuss in depth several topics that we believe define the current status of the carbohydrate biosensors field and point to possible future avenues. We have not attempted to cover all aspects of carbohydrate chemistry and biology, carbohydrate-detection methods, or issues concerning molecular processes involving carbohydrates; these aspects are broad and prolific fields of study, and the reader is referred to relevant literature.[1] We have also not discussed here the highly technologically and commercially important field of glucose sensing, an active area of research because of the profound health effects of aberrant glucose levels in diabetes, as glucose is technically a *mono*saccharide rather than a carbohydrate. We do, however, include a description of monosaccharide biosensors, where such devices represent important concepts in biosensor designs, for example, biosensors employing carbohydrate−lectin recognition. Similarly, this review does not address the large body of commercially oriented literature (i.e., patents) related to polysaccharide biosensors. Overall, we tried to limit the scope of this review to more recent published reports, rather than providing a historical perspective of the field. Related reviews on the subject have appeared in the literature in the past.[2]

Biosensors are generally defined as multifunctional assemblies composed of matrix-bound bioactive substances responsible for the specific recognition of the species of interest, which are directly coupled to a physicochemical transducer supplying the output signal. In this review, however, the term "biosensor" has been used in a somewhat broader sense, including systems that can be characterized as biochemical "assays". Because of space and scope considerations, we have not provided here a complete description of all biological assays in which carbohydrates have been directly or indirectly involved; we have focused instead on assemblies in which carbohydrates constitute the critical or central sensing components and discussed in more depth systems which define or represent special and novel functions of their carbohydrate constituents. Similarly, only representative publications were cited in the text when we discuss assay systems that are widely applied.

The review is divided into two sections. In the first part, we discuss schemes for detection of carbohydrates, where the sensors are designed to detect the sugar molecules by themselves or as parts of larger biological or chemical entities (for example, glycolipids and glycoproteins). Subsections focus on the significance of lectin−carbohydrate interactions in biosensor design (section 2.2) and the analysis of carbohydrate derivatives such as lipopolysacharides (LPS) and other glycolipids and glycoproteins for toxin, pathogen, and cancer detection (section 2.4). Certain overlap exists among the subtopics; for example, some pathogen-detection schemes utilize carbohydrate−lectin recognition. A subsection was devoted to the emerging field of "polysaccharide nanobiosensors" (section 2.5), recognizing the contributions and unique scientific and technological potential of "nanotechnology" in carbohydrate biosensor research.

The second part of the review summarizes biosensors and bioassays intended not to *detect* carbohydrates but in which the carbohydrates constitute essential components in the biosensor design, either as recognition elements or as the building blocks within the sensor template. We discuss biosensor schemes that employ specific biomolecular interactions such as lectin−carbohydrate affinities, carbohydrates as substrates in enzymatic processing reactions, and solid carbohydrate matrixes incorporated within sensing devices. A subsection is devoted to the large body of experimental work utilizing surface plasmon resonance (SPR) biosensors that have been widely used in recent years for studying interactions and molecular recognition processes involving carbohydrates.

2. Detection and Analysis of Carbohydrates in Biological Systems

2.1 Identification of Carbohydrates and Carbohydrate Derivatives

2.1.1 General Procedure

The primary requirements facing new biosensor technologies include maximization of the sensitivity, selectivity, and reproducibility within the experimental setup. These issues have been predominant in biosensor design and construction. In that regard, the complexity of carbohydrate structures and the diversity of their chemical properties and molecular context pose particular and significant bioanalytical challenges. These have led to the development of a large number of biosensors and bioassays for carbohydrate identification and analysis using spectroscopic, biochemical, or electrochemical methods.

The use of enzymatic digestion has been among the first and most common assay approaches for carbohydrate analysis.[3−7] In such techniques, carbohydrate detection generally relies on enzymatic catalysis of saccharide substrates by immobilized glycoenzymes.[3−5] Because the detected signal in enzyme-based biosensors originates from the reaction products of the enzyme action, a critical requirement in such sensors is the maintenance and optimization of the biological activity of the enzyme. This could be particularly demanding because in most sensing applications and devices the enzymes have to be immobilized on solid supports.[3] Immobilization of glycosylated enzymes through binding to lectins has been reported.[3−5] This approach has certain advantages over surface binding of the enzymes using means of covalent linkage, particularly because the latter technique might interfere with the stability and biological viability of the enzyme. Furthermore, the high lectin−carbohydrate affinity constituting the basis for the immobilization procedure contributes to the stability of the biosensor assembly and its resistance to varied external degrading factors, such as heat or chemical/biological denaturation.

Frequently, the output signals produced by enzyme-based-detection methods are relatively low, and

amplification of the sensor response is necessary. Magnification of enzymatic signals has been achieved through multiple glycoenzyme layering.[5] Such lectin-based immobilization methods have opened the way to assemblies with higher glycoenzyme affinities and better load factors. Varied synthetic developments have focused on identification of solid matrixes that facilitated repetitive layering of glycoenzymes and lectins.[3−5] Such "multiple bioaffinity layering" exhibited superior analytical capabilities compared to other glycoenzyme-immobilization approaches.[3−5] Some technical challenges, however, are inherent in detection schemes utilizing multiple glycoenzyme layering using immobilized lectins, primarily the need for several preparative steps while retaining the catalytic activities of the enzymes in the solid-supported environments.

The multiple glycoenzyme-layering technique further necessitates careful selection of the solid supports. The appropriate matrixes should be sufficiently reactive to allow derivatization with the lectin, have to exhibit relatively large and accessible surface area, and should not interfere with the catalytic domains of the immobilized glycoenzyme layers.[5] Biospecific sorbent matrixes were reported.[3−5] Importantly, from a biosensing point of view, lectin-based multilayering methods do not dictate which *detection* schemes are to be used for measuring the enzymatic activity. Thus, different sensing methods based on enzymatic digestion in lectin-based "multilayer" environments were described in the literature, including flow-microcalorimetry, in which changes in heat capacity induced by the catalytic action of a glycoenzyme were recorded.[5] Other methods employed coupling between several enzymatic processes that produce spectroscopically detected species.[7]

Signal amplification inherent in the multilayering approach was employed toward achieving sensor prototypes that could identify carbohydrates within complex mixtures. An important consequence of this property is the feasibility of miniaturization within devices based on multienzyme assemblies. Technical advances in this field have led to fabrication of microfabricated biosensors containing lectin-bound glycoenzyme layers coupled to silicon chips.[6] Such achievements could open the way to diverse biosensing applications, such as the fabrication of flow channels within the biosensor chip.[6]

Immobilization of sugar-digesting enzymes within miniaturized biosensor devices has been achieved by other methods. An enzyme-based disaccharide microdetector sensor prototype included enzyme-derivatized agarose beads placed within wells etched on a silicon chip. Miniaturization in this kind of device allows the simultaneous analysis of carbohydrate mixtures.[7] Practical advantages of enzyme-based biosensor chips include the very low sample volumes required (often in the range of nanoliters), the feasibility for presentation of different enzymes on a single chip, which facilitates parallel analysis of complex oligosaccharides or multicomponent systems, translated into significant cost reduction, and the availability of mass production.[6−10]

Varied electrochemical methods have been applied for carbohydrate detection in biological and pharmaceutical samples. A major impetus for development of electrochemical approaches as compared to other bioanalytical techniques has been the observation that carbohydrates do not generally contain intrinsic chromophores (neither fluorescent nor emitting in the UV−visible range). Recent advances in the design and application of electrochemistry in saccharide assays were extensively reviewed.[11] Two electrochemical carbohydrate-detection strategies, in particular, have been thoroughly explored: enzyme-based electrodes and direct oxidation at electrode surfaces, mostly employed in postseparation analysis in liquid chromatography or capillary electrophoresis schemes.

Historically, enzyme-based electrochemical detection strategies for carbohydrates were developed because *direct* analyses of saccharide compounds were traditionally hampered by the unfavorable redox properties of many sugars.[11] In the most basic amperometric enzyme electrode, glucose was oxidized within an immobilized layer of glucose oxidase and then determined at a conducting platinum or carbon electrodes by measuring the current resulting either from oxidation of hydrogen peroxide or reduction of diatomic oxygen consumed by the enzymatic reaction.[12] Overall, the underlying concept of enzymatic electrodes for carbohydrate analysis involves the highly specific conversion of mostly monosaccharide analytes into more conveniently oxidized species (such as H_2O_2).

Immobilization of carbohydrate-digesting enzymes onto electrode surfaces without impairing their functionalities and mediation of the electron transfer to the electrode surface have been among the practical impediments for implementation of enzyme-based electrochemical techniques. Accordingly, a number of electrochemical biosensing approaches have utilized direct oxidation of carbohydrates at electrode surfaces. Many of these techniques require for the oxidation to occur electrochemical potential conditions for which many electrode materials are inadequate.[11] Accordingly, a critical issue in such applications has been the proper selection of electrode composition.[13] Most direct-oxidation detection schemes have combined electrochemical processing of the carbohydrates with liquid ion-exchange chromatography for compound separation.[14,15]

Complex carbohydrates or multicomponent mixtures pose particular challenges for application of electrochemical detection methods. In such systems, the issue of selectivity and/or separation often has to be addressed in parallel with the actual detection process. Varied methods have been developed for achieving these goals, roughly divided into two main approaches: the first relies on the actual selectivity of the chemical/biological component recognizing or reacting with the carbohydrate to be analyzed (such as the enzyme for which the carbohydrate is the substrate); the second group of detection schemes combines the electrochemical analysis with separation techniques such as capillary electrophoresis (CE) or liquid chromatography (LC).[11] Development of

separation methods for carbohydrates is particularly important, because it has been found that, unlike proteins or other macromolecules, a large size or high molecular weight does no significantly impair the capability of electrochemical methods to accurately detect the molecule.[11]

Coupling of electrochemical detection to CE has attracted interest in recent years because of the power of the technique to resolve and identify carbohydrates in complex mixtures.[16,17] Actual analysis of the carbohydrates is similar to other electrochemical methods in which the redox reactions take place at metal electrode surfaces, while the function of CE is separation of the compounds within the mixtures. However, a specific technical problem that has to be surmounted in such devices concerns the electronic separation between the electrophoretic and electrochemical processes. This is due to the fact that current leakage between the two electrical circuits has to be avoided and minimized, because the detection potential is usually much smaller than the capillary electrohoresis voltages.[11]

Several techniques were described in the literature that similarly rely on compound separation but use detection schemes other than electrochemistry, for example, UV absorbance.[18] A carbohydrate-detection technique employed in conjunction with a separation method was denoted polarized photometric detection (PPD).[19] The sensor apparatus of the PPD unit consisted of placing two light polarizers at opposite sides of a conventional UV−vis spectrophotometer flow cell. This arrangement allowed the measurement of optical rotation of chiral compounds through the change in absorbance. Despite its crude mechanism, application of PPD was claimed to achieve extremely high detection sensitivity for oligosaccharides through the different rotations exerted by the molecules.[19]

Even though carbohydrate detection schemes that are combined with compound separation are generally satisfactory in achieving high-sensitivity compound identification, their main drawback is the ultimate dependence upon the separation technique for efficient application. Thus, many of the generic prototypes and published experimental data required, to some degree, prior knowledge of the type of oligosaccharide mixture to be analyzed. Accordingly, the majority of reported differential-elution/detection methodologies have been applied toward analysis of simple sugars.

Fluorescence spectroscopy has had an important contribution to development of carbohydrate biosensors, mostly through the use of fluorescent labels.[20−22] Such "carbohydrate fingerprinting" techniques usually consist of several stages. The analysis includes attachment of nonspecific fluorescent tags that bind to monosaccharide building blocks within the sugar molecule, breaking the larger saccharide into smaller fluorescent-tagged units mostly by enzymatic digestion and application of separation procedures (for example, liquid chromatography) for complete assignment.[20,22] The coupling of the carbohydrate analyte with additional molecular entities (the fluorescent tags) might interfere with both the fragmentation of the larger molecule (a prerequisite for separation and analysis), as well as affect the elution of the components. These constraints naturally pose challenges to the successful use of this methodology. Other fluorescence-based assays were developed not only for identification of individual oligosaccharides but also to characterize biochemical processes in which carbohydrates participate. A fluorescence-labeling technique has been introduced to study the gelation properties and cell-wall localization of alginate, the major cell-wall carbohydrate of brown algae.[23] Specifically, the fluorescent dye fluorescein was conjugated to short polygluronate chains and used to target the gelling subunits of the carbohydrate. The method allowed rapid labeling and probing of distinct cellular regions from varied algae sources.

Several carbohydrate-detection schemes based on boronic acid were reported in the literature, often utilizing fluorescent tags attached to the boronic acid moieties.[24−27] The three primary building blocks comprising such photoinduced electron transfer (PET) biosensors are the fluorophore, the carbohydrate receptor, and a molecular spacer separating them.[27] In particular, saccharide detection achieved with the use of these molecular assemblies rely on the reactivity of boronic acid with vicinal *cis*-diols of carbohydrates.[24,27] Boronic acid PET biosensors exhibit notable advantages and disadvantages. On one hand, the criteria for molecular design allow significant flexibility in determining saccharide ligand binding thorough shape selectivity, chiral recognition, allosteric discrimination, and other factors.[27] On the other hand, functionality of the biosensor generally requires high pH environments to produce ionization of the boronic acid units (yielding boronate anions), a feature that limits the usefulness of such assays.

Varied boronic-acid-based biosensor designs included polymer hydrogels coupled to pendant boronic acid units.[24] Such biosensors conform to the "classic" biosensor design in that the recognition event between the sensor (the hydrogel−boronic acid conjugate) and the carbohydrate analyte gives rise to a detectable physical change in the system, a shift of the visible wavelength of light diffracted by the hydrogel.[24] Swelling of the hydrogel (responsible for the change in the diffraction wavelength) is induced in the sensor assembly by the increased osmotic pressure occurring from the decrease of pK_a of the boronic acid following binding to the carbohydrate. This carbohydrate-detection scheme is simple, robust, and quite sensitive (lower than 50 μM carbohydrate analyte detected).[24] The system was demonstrated primarily for detection of simple sugars, such as glucose, although conceptually, it could be generally applied for more complex carbohydrates. Similar biosensor constructs utilizing boronic acid derivatives consisted of a fluorophore and boronic acid attached to an amine moiety.[25,26] When a saccharide analyte binds to the boronic acid, the boron atom becomes more acidic, leading to an enhanced Lewis acid−base interaction with the amine nitrogen. This reduces the interaction of the nitrogen lone pair with the fluorophore, thus suppressing the PET process and increasing the fluorescence.

Detection methods employed in boronic-acid-based biosensors were not limited to fluorescence techniques. Indeed, the use of the chemical reaction between the saccharide and boronic acid as the defining feature of a biosensor facilitates the application of a plethora of sensing approaches that were recently reviewed.[25] Specific bioanalytical techniques included chiral saccharide recognition using circular dichroism (CD) and liquid crystalline suspensions,[25,28] the use of colorimetric carbohydrate receptors,[29,30] electrochemical detection via coupling of the boronic acid recognition assembly to a redox unit such as ferrocene,[31,32] and others.

Several carbohydrate-sensing schemes have been based on recently developed chemical and biophysical techniques. Molecularly imprinted polymers (MIPs), for example, have attracted an increasing interest as templates for carbohydrate-detection assays. Molecular imprinting creates recognition sites in polymers by using template molecules; the templates are prepared by initiation of the polymerization processes, while molecules of a particular analyte are incorporated within the solidifying material.[33,34] Following the removal of the embedded analyte molecules, the polymer essentially becomes a porous framework that selectively adsorbs only the analyte molecules within the pre shaped binding sites.[35] This kind of "templating biosensing" approach could be particularly well-suited for carbohydrate detection and analysis because the imprinting procedure might be able to distinguish between different functional units and/or saccharide moieties within complex carbohydrates.

"Proofs of concept" for the application of MIPs for detection of simple sugars were described in several publications. Quartz crystal microbalance (QCM, see more in-depth description of the technique below) coated with MIPs was recently synthesized for detection of sialic acid, the cell-surface receptor of influenza virus.[36] That design built upon template imprinting of sialic acid moieties via boronic acid-derivatized polymer for construction of a QCM sensor. The significance of this type of study lies in the demonstration of a MIP as viable technology for use in fundamental biosensor design. In fact, the representative QCM−MIP sensor points to a primary criterion in the design of MIP-based biosensors, which is the choice of the detection scheme. Specifically, one of the important issues underlying MIP sensors is how would the sensors respond to the specific binding of analytes in general, carbohydrate analytes in particular, and how would the signal produced within the biosensor be recorded. Detection of binding interactions to the polymer template through embedded fluorescence tags could be the technology of choice, albeit this approach could pose significant technical and synthetic challenges. Coupling between imprinted polymer technology and fluorescence-based detection of carbohydrates was reported in a representative study.[37] In that work, the researchers synthesized a fluorescent monomer that facilitated detection of *cis*-diols, which was then successfully assembled into an imprinted polymer, preserving its fluorescence-sensing capabilities for carbohydrate derivatives adsorbed onto the polymer framework. The key methodological requirements successfully demonstrated in that report were, first, the synthesis of a functional fluorescent monomer displaying strong binding interactions with particular structural elements in carbohydrates (*cis*-diols) and, second, retaining the discrimination capabilities of the fluorophore and its fluorescence sensitivity inside the polymer framework. Gao et al. attained these goals for detecting fructose with the use of a monomer-conjugated boronic acid monomer.[37]

A practical weakness encountered in MIP applications, particularly saccharide-templated materials, has been the low reloading capacity of the analytes. To overcome this limitation, some studies proposed to enhance the binding capability of the polymer matrix through chemical manipulations, for example, by increasing the polarity of the polymer backbone, thus enabling multiple hydrogen bonding between the polymer framework and the incorporated carbohydrate guest molecules.[38] Specifically, the researchers explored the effects on saccharide rebinding of inclusion of multiple metal cations, such as Cu^{II}_2, within the polymer template and the use of polar cross linkers such as pentaerythritol within the polymer matrix. Improved performance of the MIP was indeed demonstrated for several polysaccharides, indicating that varied synthetic routes could be employed to optimize the bioanalytical performance of MIP-based sensors.

Carbohydrate detection using whole-cell biosensors has been also an active field of research in recent years. Even though the technique was so far employed almost only for detection of mono- and disaccharides,[39] it holds promise as a highly generic approach for carbohydrate analysis in natural samples. In contrast to simple, modular carbohydrate biosensors, the interest in development of cell-based carbohydrate is precisely due to the intrinsic sophisticated, cooperative properties of whole cells. Indeed, living cells are routinely engaged in converting complex substrates into smaller molecular units through distinct metabolic pathways. Cells are also capable to continuously repair their enzymatic cascades, including those involved in carbohydrate digestion.[39−41] Whole-cell biosensors could have advantages over simplified carbohydrate-detection methods such as enzyme-based sensors because cell assays generally monitor *sum parameters* such as toxicity or oxygen uptake, rather than individual molecular analytes in solution.

Held et al. constructed a microbial biosensor array consisting of immobilized *Escherichia coli* bacterial mutants lacking specific metabolic systems for individual carbohydrates.[40] The sensor components included an electrode for monitoring electrochemical potential arising from the reduction of molecular oxygen. The oxygen for its part was produced by *E. coli* mutants immobilized within a solid matrix and was indicative of the metabolic activity of the bacteria. In particular, the bacterial mutants used were deficient in translational pathways for specific carbohydrates; thus, addition of those carbohydrates resulted in increased metabolic activities and higher

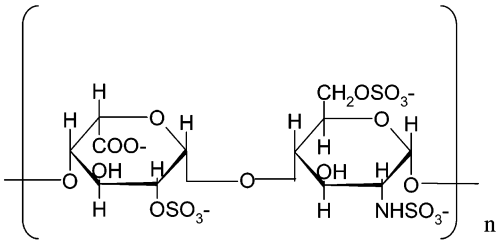

Figure 1. Most common disaccharide unit in the heparin structure.

production of O_2. This microbial biosensor featured high selectivity among different saccharides and was also noteworthy for its overall stability, a general difficulty encountered when working with living systems. A different method was proposed by Svitel et al. consisting of a cell-based biosensor, which utilized an oxygen electrode coated with membrane-containing microorganisms (such as *Gluconobacter oxydans* and *Saccharomyces cerevisiae*) in which the carbohydrate analytes were enzymatically oxidized.[42]

2.1.2 Heparin Detection

An example of an intensive biosensor research focusing on a particular family of carbohydrates is the effort to achieve sensitive and real-time monitoring of the anticoagulant compound heparin. Heparin, a linear sulfated carbohydrate (Figure 1) and its heparinoid derivatives are abundant constituents of the extracellular matrixes of most cell types.[43] Heparin and its derivatives function as modulators and effectors for the activities of important signaling molecules such as chemokines, extracellular matrix proteins, growth factors, and cellular receptors mostly through binding to these biological macromolecules.[43] In a clinical setup, it is critical to maintain heparin levels that on one hand are sufficient to prevent thrombosis but on the other hand avoid risks of bleeding. Real-time monitoring of heparin concentrations was reported during cardiopulmonary bypass surgery and other invasive procedures.[44] However, a limitation for practical commercial and mass use of heparin biosensors has been the requirement for additional reagents and/or specialized laboratory equipment. Considering the fact that more than half of a billion doses of heparin are used annually, there have been intensive efforts to develop simple sensor systems that could detect heparin directly in blood or serum samples.[45]

Several methods for heparin detection were described in the literature. A particular emphasis in the efforts to develop new detection methods has been the introduction of rapid, preferably one-step methods that would facilitate detection of heparin through simple means, for example, a visible color change. An indicator displacement assay for heparin was developed in the laboratory of Anslyn.[46] A colorimetric displacement assay of the type described in that work, shown schematically in Figure 2, is based on generation of colorimetric changes induced by replacement of a receptor-embedded indicator molecule by the analyte.[47] The critical requirement of such a biosensor is the design of a synthetic receptor that would display satisfactory selectivity between the

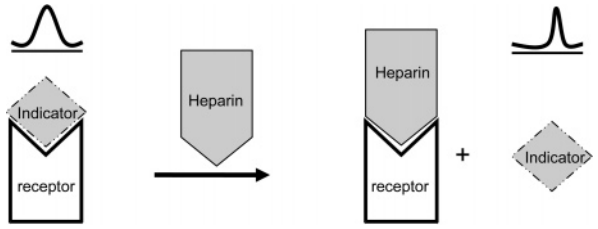

Figure 2. Schematic representation of a colorimetric displacement assay for heparin detection. Different visible spectra are recorded when the indicator molecule is bound to the synthetic receptor (left) and when the indicator is ejected by heparin from the binding site (see the text).

desired analyte and compounds similar to it (as well as the initially incorporated indicator molecule). In the reported heparin biosensor,[46] the synthesis of a receptor containing a boronic acid derivative made possible both an easy replacement of the colorimetric dye by heparin as well as a sufficient selectivity among saccharides with a similar structure. The order-of-magnitude differences between the binding constant of heparin and other glycosaminoglycan derivatives examined were ascribed to the anionic charge densities on the compounds, pointing to negative charge as an important determinant affecting heparin binding and affinity.

Electrostatic interactions indeed played a significant role in other heparin biosensor designs. Heparin detection was carried out through interactions between the negatively charged carbohydrate (average charge of −70) and positive electrode surfaces in ion-channel sensor assemblies.[48] Binding of fibroblast growth factor (FGF) to specific heparin sequences was analyzed by using a radioactive-labeling technique.[49] FGF selectivity among particular glycosaminoglycans was determined through displacement of H^3-labeled heparin by unlabeled carbohydrates, complemented by competitive binding assays using radioactive-labeled and unlabeled saccharide moieties.[49] SPR (see subsection 3.4 below) has been often used as a sensor technique for heparin detection and analysis.[50]

The QCM technique has been applied for heparin detection. QCM biosensors drew interest because of technological progress in producing precisely cut piezoelectric quartz crystal resonators.[51] The QCM sensor is constructed by immobilization of a recognition element (antibody, receptor, carbohydrate-binding protein, and others) onto the surface of a transducer. Selective binding of the desired molecule to the QCM transducer results in mass and consequent changes of its oscillation frequency, which could be detected electrically.[52] Detection of minute changes in the mass of the films allows application of the QCM biosensor for studying varied biomolecular recognition events.[53] QCM has been applied for detection and kinetic analysis of heparin binding to protamine-adsorbed surfaces.[54] This report examined the effect of receptor (protamine) coverage on the gold electrode on the sensitivity of the biosensor toward heparin and the adsorption profiles of heparin at different concentrations. Even though the QCM frequency reached a steady-state condition after a relatively long time (several minutes), admittedly a

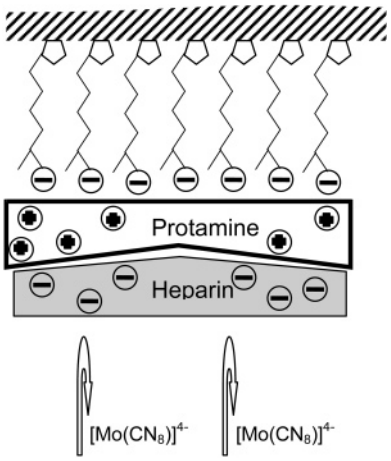

Figure 3. Schematic representation of an ion-channel biosensor for heparin. Heparin−protamine binding at the electrode surface disrupts the attachment of the metal complexes, thus modifiying the redox potential of the electrode.

deficiency of the system, the sensitivity achieved using that sensor was within the clinically required range.[54]

Achieving sufficient detection sensitivity has been a major factor in shaping development of heparin biosensors. Ion-channel sensor methods were shown to detect heparin in very low concentrations in various solutions.[55] The principle of ion-channel sensing, depicted schematically in Figure 3, resembles the displacement assay described above in that detection relies on replacement of a signal-producing molecular species by the analyte. Specifically, negatively charged heparin modulates the electrical voltage developing at an electrode surface through prevention of redox reactions between ionic metal complexes, such as $[Mo(CN)_8]^{4-}$, and an electrode.[55] In the case of heparin, the physical or electrostatic exclusion of the electroactive ions or molecules (referred to as markers) is usually carried out through covalent or electrostatic attachment of specific receptors of the saccharide, such as protamine, to the electrode.[55,56] Heparin is particularly amenable to such applications because of its high negative charge facilitating a low-concentration detection threshold. Gadzekpo et al., for example, demonstrated a dynamic range of between 0.6 and 3.0 μg/mL for heparin, which is well below the values encountered in biomedical applications.[55] A recurring problem of using ion-channel electrodes for heparin biosensing has been the decrease in reproducibility and precision of the determined concentrations after repeated use of the electrode.[55] A partial solution to this deficiency was the removal and reincorporation of the protamine receptor in addition to the heparin analytes. Naturally, this electrode regeneration method could not be applied in the case of covalently attached protamine, leading to development of specially designed surface-attached heparin receptors having superior detection limits and reproducibility properties.[55]

Potentiometric detection methods using polymeric membrane-based electrodes were used for heparin detection. These devices originate from the observation that binding of biological polyions, such as heparin, to the electrode surfaces induce large non-equilibrium potentiometric responses.[57] Such electrodes have been utilized extensively in clinical studies because of their adaptability for rapid and selective detection of ions in blood and plasma liquids.[58] Modification of polymer ion-selective membrane electrodes for heparin detection was reported.[59] Methods for construction of ion-selective heparin biosensors based on irreversible exchange of the carbohydrate polyanion with monovalent ions at the electrode surface were also described in the literature.[60,61] Binding of heparin to the electrode was facilitated in such sensors through doping the electrode surface with lipophilic substances such as quaternary ammonium salts.[57,59] Indeed, the choice of the ion-exchanger dopant incorporated within the polymer determined the biosensor sensitivity and performance to a large extent. One of the important advantages of polymeric-based potentiometric biosensors has been the possibility to use such techniques for determination of heparin levels in whole blood samples, in actual operation environments. An obvious disadvantage is the irreversibility of detection, which mandates that the technology be used mainly in disposable devices.[57]

The irreversibility of heparin binding requires carrying out electrode renewal using varied chemical or physical means, such as heparin displacement by high-concentration Cl^- solutions, that are often elaborate and cumbersome. To overcome this limitation, a reversible heparin sensor employing an additional H^+ ionophore within the electrode membrane was reported.[62] Specifically, doping the biosensor membrane with the ionophore allowed displacement of heparin from the electrode surface by simply increasing the pH of the solution. The addition of the H^+ carrier might make the sensor somewhat more complex; however, the reversibility of the heparin-detection scheme through this elegant design is a noteworthy advantage.

The high concentration of negative charges on heparin has been a basis for other sensing techniques, such as the detection of variations in charge densities in porous membranes mounted on an ion-selective field effect transistor (ISFET).[45,63] The ISFET essentially measures the stepwise change in the potential between the membrane and the bulk solution, following the binding of the analyte (in this case heparin) to the affinity receptor, generally protamine.[45] Indeed, the strong electrostatic attraction between protamine and heparin amplifies the change in surface-charge densities within the ISFET-placed membrane. Heparin biosensors utilizing the ISFET concept exhibited very high sensitivity thresholds of between 0.1 and 1 units/mL;[63] however, drifts of the recorded potential occurred at long incubation times, and optimal pH conditions of the analyte solutions (such as blood plasma) had to be determined before application of the device.[45,63]

A recent study pointed to the feasibility of heparin biosensing applications based on binding of the carbohydrate to glycoproteins.[64] In that study, Borza and Morgan examined the properties and the re-

markable pH sensitivity of the interaction between heparin and plasma histidine−proline-rich glycoprotein (HPRG). The extraordinary abundance of histidine residues in the protein sequence makes it highly sensitive to the solution pH through protonation of the histidines. Because the heparin-binding site spans some of the histidines, the sensitivity of the protein to heparin association could be fine-tuned through controlling the pH. The researchers further demonstrated that heparin binding to HPRG was highly dependent on metal ions; little binding of HPRG to heparin was detected at physiological pH in the absence of metals, but the interaction was promoted by nanomolar concentrations of zinc and copper.[64] Indeed, the frequently encountered high affinities between particular protein classes (such as lectins, see below) and their carbohydrate ligands (heparin or others) has been thoroughly exploited for carbohydrate analysis.

2.1.3 Carbohydrate Structures

Deciphering the organization and order of the monosaccharide units within oligosaccharides poses as one of the most formidable analytical challenges in glycobiology. Varied approaches and generic techniques were applied to facilitate accurate analysis of the individual monomers in complex carbohydrates.[65] Gel electrophoresis methodologies were modified for extraction, separation, and analysis of bacterial cell-surface (capsular) polysaccharides.[66] Enzymatic processing of carbohydrates and glycoconjugates has been frequently used for determination of carbohydrate structures and sequences because of the overall accuracy of the technique and the requirement of small sample quantities.[67] Recent studies have concentrated on the integration of advanced separation and detection methods for achieving fast and accurate oligosaccharide sequencing. Simultaneous detection by UV absorbance and electrospray ionization−mass spectrometry (ESI−MS), for example, provide important structural information on the oligosaccharide components of mixtures.[68]

Detailed structural analysis of bacterial capsular carbohydrates has been achieved by "enzymatic fingerprinting" procedures combining high-performance anion-exchange/pulsed-amperometric detection liquid chromatography, fluorophore-assisted carbohydrate electrophoresis, and matrix-assisted laser-desorption ionization time-of-flight (MALDI−TOF) mass spectrometry (MS).[69] This carbohydrate profiling technique made possible rapid identification of plant-cell-wall mutants and was proposed as a viable alternative for more cumbersome genetic or biochemical phenotyping methods.[69] Specifically, Lerouxel et al. explored the advantages and disadvantages of application of the bioanalytical techniques for the capsular oligosaccharide analysis, particularly in terms of speed, reliability, and accuracy. The researchers asserted that MALDI−TOF MS offers an efficient and rapid method for carbohydrate analysis.[69] This claim could be somewhat problematic because of the fact that prior knowledge of specific carbohydrate components is necessary for the correct interpretation of MALDI−TOF MS. On the other hand, the technique could indeed serve as an excellent tool for initial fast analysis of cell-wall carbohydrates. Combining MALDI−TOF MS with other separation and detection methods and the construction and use of relevant databases could make enzymatic fingerprinting a powerful tool for analysis and sequencing of complex carbohydrates.

Enzyme digestion was also used in a high-throughput assay by which *Arabidopsis thaliana* stems were hydrolyzed with driselase or trifluoroacetic acid (TFA).[70] Specifically, driselase, a mixture of fungal enzymes, hydrolyzes cellulose (to glucose) and all of the major matrix carbohydrates, while TFA hydrolyzes the matrix carbohydrates but not cellulose to monosaccharides. The application of the two substances together yielded a carbohydrate profile of the cell wall, facilitating, for example, identification of mutants with differing compositions of cellulose, xyloglucan, or xylan.[70]

Enzymatic digestion and electrochemical detection of the enzymatic cleavage products have been widely utilized for determination of oligosaccharide structures.[71−73] The chemical profiles of carbohydrate moieties expressed on several glycopeptides were determined by enzymatic desialylation and deglycosylation combined with analytical separation.[73] Another representative report described identification and analysis of carbohydrates by using an enzyme array/amperometric-detection scheme.[72] The technique could decipher structures of complex carbohydrates by direct quantification of monosaccharides released by enzymatic reactions (carried out within the "enzyme array") through pulsed amperometric detection at a gold electrode, rather than determination of the uncleaved carbohydrate moieties. The enzyme array electrochemical detection method does not require any separation or prior labeling of oligosaccharides.[72] However, this method faces several limitations. First, the ultimate resolution power of the sensor is determined by the size and diversity of the enzyme array, and one could anticipate a situation when similar oligosaccharides would produce nondistinguishable cleavage products. Moreover, correct interpretation of the sensor output depends on the assumption that the tested carbohydrates are pure, rather than complex mixtures. The use of an array setup, however, is promising in that it opens the way to high-throughput screening applications and the inclusion of database analysis as an integral part of the biosensor usage. Array-inspired bioanalytical methods in which enzymatic digestion was coupled to fluorescence detection of specific attached markers were applied for carbohydrate structural analysis.[20−22]

Other bioanalytical techniques were developed to elucidate carbohydrate sequences. Nuclear magnetic resonance (NMR) spectroscopy has been highly useful for determination of carbohydrate and glycoconjugate sequences, conformations, and dynamics.[74,75] CD spectroscopy is another important bioanalytical technique that was applied for analysis of oligosaccharide secondary structures and conformational dynamics.[76] Similarly, MS was also applied for obtaining structural information on oligosaccharides.[77] The use of permethylation combined with gas chromatography−

mass spectrometry (GC−MS) for linkage and sequence analysis of oligosaccharides was reviewed.[78] SPR was also successfully applied to glycoconjugate analysis (see detailed discussion in section 3.4 below).

A generic and elegant methodology for carbohydrate biosensor design has been the construction of *neoglycolipids*. These new molecular composites, based on the coupling of oligosaccharides to lipid residues, constitute a chemical-synthesis route for deciphering carbohydrate sequences and structures. The attachment of hydrophobic lipid moieties to carbohydrates opens the way for applications of versatile immobilization methods.[79,80] There are several important advantages of the neoglycolipid approach for biosensor purposes. First, neoglycolipids contain preselected *single* lipid residues rather than the heterogeneity of acyl chains encountered in natural glycolipids, which often adds to the complexity of analysis of saccharide derivatives from natural sources. Another inherent strength of neoglycolipid-based assays is the selective reactivity of different carbohydrates in heterogeneous mixtures following their chemical derivatization, facilitating their separation through varied analytical means. In addition, surface display of carbohydrates immobilized through their lipid chains is well-suited to probing directly the biological roles of oligosaccharide sequences as antigens, ligands, or other recognition elements, thus providing valuable information on the "glycome", the entire spectrum of glycans produced by the cell. Furthermore, neoglycolipids are particularly adaptable for modern microarray applications for high-throughput evaluation of the specificities of oligosaccharide-recognizing proteins (see below).

In an extension of the original neoglycolipid concept, chemical derivatization techniques utilizing *fluorescent* glycoconjugates were developed to decipher carbohydrate components in complex mixtures, particularly focusing on ligand discovery within varied mixtures of neutral and acidic oligosaccharides.[81] The important advantage of this approach is that it adds to the *detection* capabilities for employing neoglycolipids, which by themselves do not contain chromophores other than the saccharides. Further strength of the technique is the analysis of carbohydrates through fluorescence emitted *directly* from the saccharide-coupled fluorophore (rather than indirect detection of fluorescent substances that bind to the neoglycolipid). A recent report described conjugation of an aminolipid 1,2-dihexadecyl-sn-glycero-3-phosphoethanolamine (DHPE) and the fluorescent label anthracene.[81] This reagent is highly fluorescent and can form neoglycolipids by reaction with diverse oligosaccharides through reductive amination. Such conjugates can be resolved by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) and quantified either spectroscopically or through scanning densitometry.

Overall, neoglycolipid technology offers a comprehensive carbohydrate characterization approach, whereby an oligosaccharide ligand population is detected and isolated through selective chemical derivatization. The construction of new and discrete chemical entities containing hydrophobic lipid moieties and saccharides can thus be complemented by analytical methods such as MS and enzymatic digestion for complete structural analysis. In reality, the limitations of neoglycolipids for generic biosensor applications can be traced to their origin in synthetic organic chemistry. For example, one has to verify the sufficient yields of the lipid-coupling reactions, as well as the efficient immobilization of the neoglycolipid products onto the solid matrixes, prior to putative application as carbohydrate-detection devices. In addition, the technique generally requires several preparative and analysis steps that limit its applicability in faster biosensing uses.

While neoglycolipids are created synthetically, studying carbohydrate structures and properties within naturally occurring glycoconjugate entities, such as glycoproteins or glycolipids, is often critical for understanding the biological functions of such assemblies. Evaluation of carbohydrate organization and structures within aggregates of collagen, an abundant fibrous protein localized in various tissues, has been carried out by photometric measurements of textural birefringence.[82] That research has shown that the extent of optical retardations because of birefringence was indicative of the ordering conferred to collagen fibers by the attached carbohydrate moieties. The birefringence measurements exposed the important role played by collagen-bound carbohydrate molecules in the ordered aggregation of collagen fibers and subsequent attachment of other structured macromolecules to the fibers.

2.2 Lectin-Based Biosensors

Lectins constitute a broad family of proteins involved in diverse biological processes, occasionally having potent toxic properties.[83−85] Lectins generally exhibit strong binding to specific carbohydrate moieties (glycans), and this property has been extensively exploited as a basis for biosensor design. Furthermore, particular structural profiles of glycans and their recognition by lectins have been attributed to disease progression, making analysis of saccharide−lectin binding processes important as a diagnostic tool.[86] Glucose biosensor designs, for example, have frequently utilized the specificity and high affinity of different lectins to this monosaccharide. Varied detection methods based on lectin−glucose recognition have been reported in the literature, including electrochemical detection of the monosaccharides via immobilization of lectins on electrode surfaces,[87] and glucose-sensing based on the competitive reversible binding of a mobile fluorophore-labeled lectin concanavalin-A (con A) to immobile pendant glucose moieties within Sephadex beads.[88] Lectins also exhibit high potential in peripheral biotechnology industries, such as food safety; their unique recognition properties are finding promising applications in detecting microorganisms and carbohydrate additives in foods. Reported data suggest that the use of certain lectins may provide a simple and rapid alternative to traditional methods of bacterial analysis and screening.[89]

The high affinity of lectins to saccharide units has been attributed to multivalency and spatial organiza-

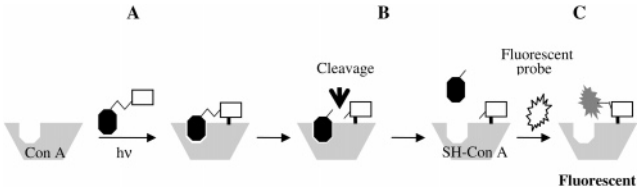

Figure 4. Schematic description of a biosensor design using the post-photoaffinity-labeling modification technique. A synthetic guest is incorporated within the binding site of con A and covalently linked by UV irradiation (A). After cleavage (B), the guest is released and the fluorescent moiety is attached through covalent bonding with a free thiol (SH) residue in proximity to the binding site (C). Binding of the actual carbohydrate guest to con A would change the fluorescent emission from the dye.

tion of the oligosaccharide ligands.[90] The selective binding of lectins to terminal carbohydrate moieties on cell surfaces and protein aggregates has been widely exploited in physiological and pathological research.[91,92] A number of histo- and cytochemical assays have used a series of lectin−enzyme (generally horseradish peroxidase) conjugates, which yielded color reactions upon enzyme processing, following the occurrence of lectin−carbohydrate binding.[93] These techniques enable not only visualization of carbohydrate distribution patterns within tissues and cell systems but also probe the different saccharide compositions expressed by the cells examined. Various staining techniques have been similarly based on lectin−carbohydrate binding. Lectin-based histochemical assays, for example, provide a platform for tissue visualization through binding between stained lectins and cells expressing lectin-reactive glycoproteins.[94]

Lectin-based immunosensor techniques have been routinely used for identifying pathogen and viral species expressing particular carbohydrates on their surface. For example, ELISA methods utilizing immobilized lectins were developed for detection of the human immunodeficiency virus (HIV).[95] Such techniques rely upon the high selectivity of particular lectins, for example, con A or the snowdrop lectin GNA, for capturing the carbohydrate antigens of the envelope glycoproteins displayed on the surface of the virus.[95,96]

con A, a disaccharide-binding lectin, is one of the most widely used lectins in sacchride-detection schemes. Several schemes have used synthetic organic chemistry pathways for coupling of con A to fluorescent moieties, thus creating fluorescence biosensors. The goal of these efforts was to combine the ligand selectivity of con A (as well as other lectins) with the intrinsic sensitivity of fluorescence phenomena, thus forming powerful platforms for carbohydrate biosensors. The laboratory of Hamachi reported the construction of saccharide biosensors in which a fluorescent label was attached in proximity to the binding site of con A, yielding a fluorescent con A in which the degree of fluorescence was modified by saccharide binding[97−99] (Figure 4). The technique, denoted "post photoaffinity labeling modification", relied on UV-induced coupling of a carbohydrate fluorescent dye (having a photoreactive site) within the binding site of con A.[97,98] The fluorescent con A

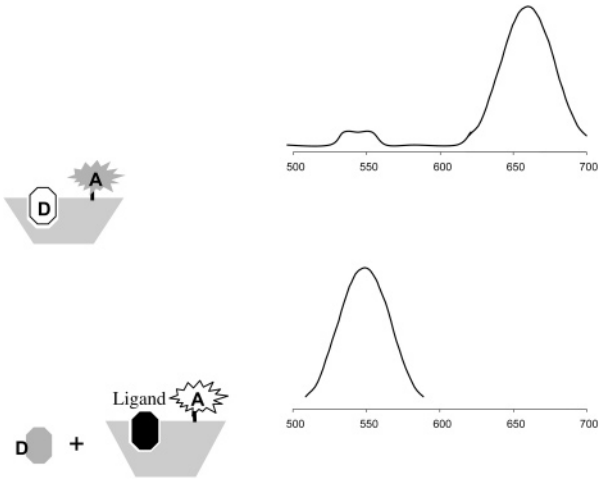

Figure 5. FRET experiment. Initially, a fluorescent donor molecule (D) is located within the binding site of the lectin, leading to a fluorescence energy transfer to an acceptor molecule (A) in close proximity and the observation of an emission spectrum from the acceptor (top). After binding of the ligand analyte (bottom), the donor molecule is released and fluorescence energy is no longer transferred to the acceptor. Thus, the emission spectrum observed (at a lower wavelength) is from the donor.

thus contained the chromophore within the binding site; however, the modified lectin retained its high affinity to various saccharides.[97] Accordingly, the interaction of the fluorescent lectin with its native saccharide ligands led to displacement of the fluorophore, which resulted in a significant decrease of the fluorescence signal.

Lectin-based carbohydrate biosensors have taken advantage of advanced fluorescence techniques, such as fluorescence resonance energy transfer (FRET, Figure 5).[100,101] Application of FRET in the carbohydrate biosensor context hinges on labeling a lectin molecule (for example, con A) with a fluorescent donor close to the binding site, while a lectin-bound carbohydrate ligand (dextran) is labeled with a fluorescent receptor. In the absence of a saccharide analyte, the binding between the lectin and the labeled carbohydrate allows a high FRET efficiency. However, the fluorescence energy transfer is decreased upon displacement of the bound ligand by the carbohydrate analyte, thus facilitating sensing of the soluble saccharide.[100,102]

The selectivity of saccharide binding to con A was the basis of an electrical-oscillation biosensor.[103] In the experimental setup reported in that research, the electrical oscillations across two electrode plates immersed in a con A solution were recorded. These oscillations were shown to depend on the presence of different carbohydrates in solution, presumably because of changes in solution capacitance following carbohydrate-lectin binding. These empirical data are intriguing because they indicate that the different mobility of bound versus free carbohydrate in aqueous solution are significant enough to form a basis for a biosensor. An interesting question arises as to whether different lectin−carbohydrate pairs would produce different signals in the electrical oscillator sensor.

A flow-microcalorimetry bioassay based on mutiple layers of con A and the glycoenzyme invertase was shown to give rise to superior catalytic response of this affixed enzyme.[5] The research demonstrated an almost 10-fold increase of the catalytic activity of the immobilized invertase through the alternating bioaffinity layering of lectin and glycoenzyme and amplification of the microcalorimetry signal. Inhibition tests of hemagglutinating activity were used as assays for monitoring oligosaccharide−lectin binding.[104] The hemagglutinating inhibition technique examines the retardation of erythrocyte agglutination by influenza virus hemagglutinin (HA), a sialic-acid-binding protein.[105] The same assay has been used to assess lectin binding to carbohydrate derivatives on a synthetic glycopeptide.[106]

Lectin−carbohydrate biomolecular recognition constitutes the core of other oligosaccharide biosensor designs. A lectin-coated piezoelectric crystal biosensor was developed for oligosaccharide analysis.[97] Piezoelectric-crystal-detection methods rely on the response of a resonating crystal following analyte binding because of either the increased mass of the crystal or changes in its viscoelastic properties.[107] The piezoelectric crystal sensor developed by Nagase et al., in which the lectin was immobilized on a quartz surface connected to a silver electrode, was used for detection of dissolved sugars and for erythrocyte identification with a detection limit approaching 100 cells.[97] Indeed, the use of lectin−carbohydrate binding in biosensor design offers significant detection sensitivity. A lectin-based biosensor capable of detecting subnanomolar concentrations of glycogen was reported.[108] The sensor concept underlying that study was the occurrence of rapid transients of the surface potential at bi- and monolayer lipid membranes. The selective binding between the lectin and its carbohydrate ligand was shown to induce sizable, rapid potassium ion current fluctuations across the bilayer membranes in a manner that was periodic and reproducible.

Lectin-based biosensors are routinely used as essential tools in biochemical research. Resonant mirror biosensor technology, for example, was applied for sophisticated kinetic rather than thermodynamic analysis of molecular interactions involving carbohydrates. The technique facilitated evaluation of the binding profiles of a carbohydrate antigen with five different lectins.[109] The experiment determined kinetic parameters such as the on rate (k_{on}) and off rate (k_{off}) of the oligosaccharide, as well as the extent of binding at equilibrium. Other applications of the resonant mirror biosensor technology concentrated on probing the difference among the binding strengths of several lectins and proteins with particular carbohydrate ligands.[110−112]

Some studies have expanded upon the concept of lectin−carbohydrate recognition as the basis for original sensory and diagnostic methods. Saccharide-presenting neoglycoprotein probes were introduced to measure the cellular capacity for binding glycan epitopes and human lectins.[108] These newly developed chemical constructs could serve as sensors for endogenous binding sites and as diagnostic tools.[113] Another application employed incorporation of a heparin-specific lectin into a temperature-sensitive gel, in which the presence of the carbohydrate could be detected through modification of the temperature-dependent shrinkage properties of the gel following binding.[114]

The binding selectivity of lectins among related carbohydrate structures forms the basis for varied biosensor designs. Hasegawa et al. evaluated the saccharide composition of several glycopeptides by using SPR (see section 3.4 below).[115] The glycopeptides were immobilized on the sensor surface, and their binding properties to various lectins were monitored following progressive trimming of their carbohydrate moieties by glycosidase digestions.[115] The use of lectins as recognition elements in diverse biosensor applications is also discussed in other sections in this review (for example, sections 2.3 and 3.4).

2.3 Glycoprotein and Glycosylation Biosensors

Glycoproteins and protein glycosylation have attained prominence in recent years as key constituents in varied cellular processes.[116,117] The exact roles of the carbohydrate moieties in such molecules, however, have not been determined yet. Protein-bound saccharides were suggested to contribute to nonprimary functions of proteins, such as nonspecific interactions with other carbohydrates or macromolecules, stabilization of protein conformations, or protection from proteolysis. Nonspecificity of the expressed saccharides is consistent with both the similarity of carbohydrate structures appearing within diverse glycoproteins and the frequent structural microheterogeneity of carbohydrate chains at given sites.[116] This concept is further supported in its overall outline by the viability of cells whose glycosylation processes have been globally disrupted by pharmacological inhibitors.[116,118] Other studies, on the other hand, have revealed the existence of specific receptors for discrete oligosaccharide structures on glycoproteins. Such receptors seem to be either important for compartmentalization of the glycoprotein or for positioning of the cells on which the glycoproteins are located.[116,119] N-linked glycans are believed to play pivotal roles in targeting, transport, and compartmentalization of glycoproteins in cells.[120] Oligosaccharides were also proposed as antigenic determinants of glycoproteins.[121]

Varied schemes for glycoprotein detection have been reported.[122] Such applications are particularly important from a therapeutic standpoint because changes in expression and abundance of glycoproteins in cellular environments are often associated with tumor proliferation (see section 2.4.3 below). Several immunosensing techniques were used for glycoprotein analysis. Ma et al. reported the application of an amperometric impedance biosensor for detection of a human mammary tumor-associated glycoprotein through binding to a monoclonal antibody.[123] The experiment demonstrated that the alternating current from an antibody-functionalized gold electrode was modified after binding of the specific carbohydrate antigen to a monoclonal anti-

body immobilized on the sensor surface. Other approaches utilized more conventional constituents for saccharide recognition and binding such as lectins (section 2.2) for assaying glycoprotein composition and glycosylation. Several reviews summarize lectin overlay assays in which the sugar moieties were initially detached from the protein residues by enzyme digestion procedures.[124,125] The effectiveness and clinical potential of lectin-based assays for studying subtle changes in serum protein glycosylation, particularly associated with disease onset, have been reviewed.[126]

An intriguing technique for creating potential glycoprotein sensors based on Langmuir−Blodgett films of fullerene−glycodendron conjugates was described by Cadullo et al.[127] The authors constructed monolayers at the air−water interface that were comprised of fullerene−dendrimers covalently attached to glycodendron headgroups. The noteworthy achievement of the researchers was the prevention of fullerene aggregation within the monolayers, accomplished by optimization of the hydrophilic/hydrophobic structure of the fullerene−dendrimer conjugates. The absence of aggregation and consequent display of the carbohydrate units at the film surface could be potentially applied to glycoprotein detection.

Interactions between viral envelope glycoproteins and host cells play fundamental roles in viral penetration into cells and viral pathogenesis.[128,129] Accordingly, studying the molecular recognition and interactions between cellular receptors and viral envelope glycoproteins showing receptor-binding activity are of great importance both for understanding the molecular basis of virus entry, as well as for developing antiviral drugs and diagnostic tools. Bertucci et al. have used an optical biosensor to study the binding of recombinant glycoproteins of herpes simplex virus (HSV) to an immobilized recombinant form of the human cellular receptor for HSV.[130] The mode of action of the biosensor was based on detection of changes in the refractive index close to the sensor surface, which was dependent upon the mass of the adsorbed species. The resonant mirror technology utilized in the research represents a class of biosensor technologies that essentially detect binding events and biomolecular interactions in real time. The strengths of the resonant mirror biosensor are mainly traced to the increased sensitivity (nano- to microgram range for glycoproteins), the short time required to perform the experiment (less than an hour), and the fact that there is no need for additional labeling of the analytes.[131] The biosensor could be regenerated after measurements through washing of the bound species, although some decrease of the reproducibility of the results was observed after repeated use.[130]

Envelope glycoproteins of the HIV, in particular gp41 and gp120, have been implicated in viral entry to various cell types.[132,133] Glycosylation of these two proteins is believed to play an important role in their antigenicity and cell-surface interactions, and specific assays were developed to decipher the structure and molecular interactions of the carbohydrates attached to these glycoproteins.[134] The association of gp120 with glycopeptides and glycolipids and contribution of the carbohydrate moieties to gp120 interactions were evaluated with bioanalytical techniques such as enzyme-linked immunosorbent assay (ELISA).[135] Assays measuring the effect of glycosylation on the immunoreactivity of glycoprotein hormones were also evaluated.[136] Different techniques have been developed to determine hemoglobin glycosylation, believed to provide an accurate index of long-term blood glucose control in diabetes mellitus, including ion-exchange chromatography, electrophoresis, isoelectric focusing, thiobarbituric acid colorimetry, and affinity chromatography.[137,138]

Protein glycosylation by chemically modified oligosaccharides ("oligosaccharide tags") could become a useful tool for investigating protein and peptide targeting in cellular processes. Analysis of glycosylation patterns of glycopeptide enzyme substrates was carried out by glucosylation of a set of the glycan substrates *in vitro*, followed by determination of glucose composition by MS.[139] Synthesis of maleimide-activated carbohydrates as site-specific tags for peptides and proteins was also reported.[140] This work built upon the high reactivity of maleimide with thiol groups, making possible attachment of maleimide-activated mono- and polysaccharides to cysteine-containing peptides. Even though technically this method essentially creates "artificial glycopeptides", tagging peptides with different saccharide moieties could be useful for detection of carbohydrate-recognition sites and carbohydrate receptors on cell surfaces.

Diverse glycosylation processes occur on cell surfaces, and elucidating cellular carbohydrate expression and glycosylation pathways is essential for understanding varied cellular events.[92,141] Elegant biochemical techniques were developed for probing oligosaccharide compositions and carbohydrate processes at cell surfaces. Bertozzi and others have expanded upon the concept of "chemical glycobiology" as a generic approach for deciphering biochemical processes in which carbohydrates constitute central components and for studying structure−function relationships involving surface-expressed oligosaccharides.[92,142] The approach, which was also denoted "metabolic oligosaccharide engineering" involves chemical modification of specific saccharide units. These unnatural carbohydrates could then be incorporated into various cell compartments and locations via the biosynthetic machinery of the cell.[142] In particular, it was shown that interference with biochemical and metabolic pathways contributing to oligosaccharide biosynthesis could shed light on the progression and significance of such processes.[92,143]

Chemical intervention in biochemical processes occurring at cellular levels has other important features. The method allows, for example, insertion of varied reactive functional groups and labels onto the cell; some studies demonstrated incorporation of glycoconjugates containing sensor probes into the cell wall, facilitating analysis of distinct reactions and transformations involving the carbohydrate molecules.[143] Charter et al. showed that unnatural sialic acid analogue containing levulinoyl moieties can be incorporated into neuronal cell surfaces. The ketone

group within levulinoyl could then be used for cell imaging using biotin, facilitating insight into metabolic pathways involving adhesion molecules (containing sialic acid) on the cell surface.[143]

Biosynthetic construction of unnatural saccharide assemblies in surfaces of living cells could aid exploration of complex processes involving carbohydrates and contribute to the search for inhibitors, agonists, and antagonists to various carbohydrate and glycoconjugate receptors. Predetermined and controlled modification of cell-surface glycans might lead to promising diagnostic applications, particularly because varied diseases are associated with altered cell glycosylation patterns (see section 2.4.3 below). A possible metabolic carbohydrate engineering approach can be conceived for discrimination of tumor cells through their altered surface glycan expression.[142,144] Additionally important in term of carbohydrate biosensor development, the ability to chemically modify glycoproteins on cell surfaces could open the way for molecular or whole-cell imaging and high-throughput screening in proteomics, "glycomics", and "cellomics" applications.

The compositions and structural features of carbohydrates expressed on cell surfaces have been employed as a tool for cell visualization and physiological research. Cytochemical methods have been applied to probe the localization and distribution of glycoproteins expressed on cell surfaces by utilizing the targeting of specific carbohydrate moieties by lectins or antibodies.[91,145] Researchers utilized both lectins that bind specifically to terminal disaccharides as well as monoclonal antibodies against carbohydrate epitopes.[91] Comparative staining based on these molecular systems differentiated and partially characterized several glycoconjugates in various sites and allowed evaluation of the relationship between chemical heterogeneity and neural speciation.

Advanced high-sensitivity MS approaches have been increasingly used for deciphering glycoprotein structures. MS has been capable to elucidate the primary structures of highly complex glycoprotein mixtures, and the technique could provide an insight into post-translational protein modification processes in particular and structural glycobiology in general.[146] Recent technical advances in MS, specifically fast atom bombardment (FAB), ESI, and MALDI considerably increased the analytical capabilities of the technology to analyze complex carbohydrates and glycoconjugates. For an in-depth discussion of the subject, the reader is referred to a recent comprehensive review.[146]

2.4 Pathogen and Cancer-Detection Assays

2.4.1 Pathogen Identification

Development of biosensors and rapid detection kits for microorganisms such as *E. coli*, *Salmonella typhimurium*, and others are highly desirable because of the adverse and often devastating health effects of pathogen infection.[147] In recent years, diverse techniques have been introduced aiming to detect pathogens in shorter times and with maximal potential sensitivity.[148] Varied techniques for pathogen detection are based on the use of antibodies specific to enzymes or other proteins expressed by the microorganism to be examined.[149] Such methods, however, often require prior knowledge of the identity of the pathogenic species to be analyzed. The search for rapid, low-cost diagnostic pathogen techniques has also focused on the use of oligosaccharides, which constitute primary molecular components and markers on pathogen surfaces. The diversity and broad knowledge base regarding surface-displayed carbohydrates could aid the design of diagnostic tests for specific bacteria. Rapid agglutination assays have been routinely used for detection of microorganisms through binding of their surface carbohydrates to varied external substances, such as antibodies and receptors. The latex agglutination test (LAT), for example, utilizes latex beads coated with polyclonal antibodies against the capsular carbohydrate of particular bacteria. Aggregation of the beads can be observed via the solution turbidity, indicating the presence of bacteria. The technique facilitated, for example, identification of *mycoplasma* in an early development stage within farm animals.[150]

Optimization and enhancement of conventional agglutination tests were reported. Application of ultrasonic standing waves in conjunction with immunoagglutination has significantly enhanced the speed and sensitivity of the assay.[151,152] In that diagnostic technique, the researchers suspended antibody-coated microparticles in an acoustic field, physically promoting interactions between the antibodies and sugar antigens and accelerating formation of aggregates. Using the ultrasound-enhanced agglutination procedure, more than a 50-fold increase in sensitivity was observed for bacterial carbohydrates, approaching the detection levels obtained by the polymerase chain reaction (PCR).

Other immuno-based techniques, such as the widely used ELISA, were applied for pathogen detection by employing cell-displayed (capsular) carbohydrates. While some assays were designed to detect the capsular carbohydrates themselves, most ELISA applications utilize the carbohydrates within the sensor framework as recognition elements designed to bind to carbohydrate-specific antibodies.[153] Published ELISA methods employing saccharide−antibody binding have mostly used carbohydrate immobilization onto the solid support, while variations exist regarding the immobilization procedures. Among the methods summarized were biotinylation of the carbohydrates,[153] conjugating to poly-L-lysine polypeptide for coating the microtiter plates,[154] and others.

Varied techniques have been developed to facilitate rapid detection of pathogen-displayed carbohydrates that could also be applied in field conditions at high sensitivity. A fluorescence polarization assay (FPA) was successfully applied for serological diagnosis of brucellosis in cattle and other farm animals through antibody binding of the capsular carbohydrate epitopes of several *Brucella* strains.[155] The FPA technology is based on the rotational differences between a solubilized fluorescent-labeled free antigen and the antigen molecule bound to its antibody. In principle, a small molecule will rotate randomly at a rapid rate,

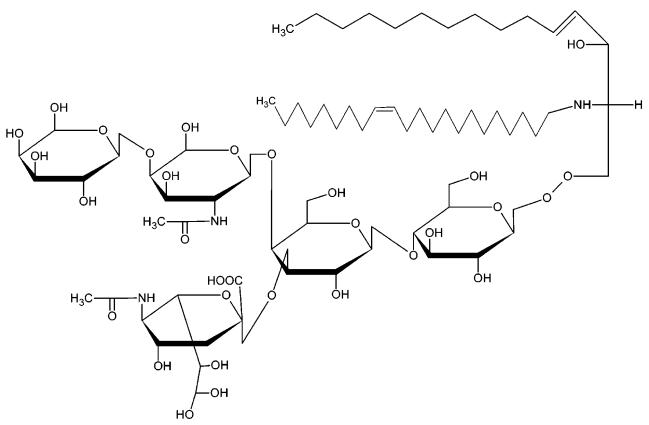

Figure 6. Ganglioside GM1 structure.

resulting in fast depolarization of light, while a larger complex would depolarize light at a reduced rate because of the slower reorientation in water.

An optical biosensor based on a resonant mirror technology was applied for studying physiological interactions of *Helicobacter pylori*, a human pathogen colonizing the gastrointestinal tract.[156] The biosensor technique could be used for actual detection of the bacterial presence. The sensing assembly described in the study utilized immobilized human gastric mucin as the recognition element, and modification of the surface refraction index resulting from bacterial binding could be easily detected. This microbiological study suggests that the suitable choice of molecular recognition determinants has a significant effect on the performance of the resonant mirror biosensor (and other technologies for that matter). The data further demonstrated that addition of sialylated and sulfated oligosaccharides, generally displayed on gastric mucins, interfered with the bacterial binding, confirming the important role of carbohydrates in bacterial surface interactions.[108]

Pathological conditions and bacterial infection could be also detected through analysis of protein glycosylation patterns. Specifically, it was shown that inflammations and infections often lead to alterations in glycosylation patterns of glycoproteins and that such modifications are generally dependent upon the particular disease encountered.[157] Carbohydrates and increased levels of particular glycoproteins have been also used as indicators of disease progression. Laminin and the high-molecular weight carbohydrate hyaluronate, for example, were suggested as biochemical markers of liver fibrosis in clinical practice.[158] Production of specific immunogenic carbohydrates was also observed by molds, a major cause for food deterioration and consequent adverse health effects.[159] Varied ELISA-based assays employing secreted immunogenic carbohydrates have been developed for detecting molds in foods.[160]

Cholera toxin (CT) is the universal marker and binding ligand of the cholera-inducing pathogen.[161,162] The cell-surface ligand of CT is the ganglioside GM1 (Figure 6), and many methods for detection of CT were based on the multivalent binding between the toxin and GM1. Indeed, the strong binding and recognition specificity of this ligand/receptor pair have made the use of this system particularly attractive both as a basis for actual biosensor design and also for demonstrating the proof of concept for putative biological- and pathogen-detection schemes. Several representative reports are described herein. Cooper et al. developed a SPR sensor chip to which ganglioside-displaying vesicles were attached, facilitating the binding of CT to the chip surface.[163] GM1 was reconstituted within model lipid bilayers in other vesicle-based assays.[164] Several studies presented sensor arrangements in which GM1 molecules were incorporated within phospholipid-covered microspheres, onto which specific binding of CT occurred.[165]

Detection of CT using FRET (Figure 5) as the generator of optical signal was reported in several biosensor schemes.[166,167] One example was a flow cytometry assay based on glass beads coated with phospholipids, which served as the scaffold for the fluorescence-labeled GM1 units.[166] Binding of CT to the GM1-coupled donor and acceptor dyes modified the distance between the fluorophores and consequently affected the fluorescence energy transfer. This biosensor arrangement achieved a high detection sensitivity of the toxin, less than 10 pM. Other studies employed the CT−GM1 pair as a model system for construction of biosensors based on FRET pathways.[166−169] Song et al. have presented several sophisticated detection schemes exemplified with the CT−GM1 system. An elegant experiment showed CT detection by FRET, where a protein−carbohydrate binding event induced distance-dependent fluorescence self-quenching and/or resonant-energy transfer processes.[169] Another study focused on the design of a "two-tier FRET" biosensor, in which the excitation spectra of the donor and acceptor were sufficiently separated to minimize the background fluorescence signal because of indirect excitation of the acceptor fluorescence.[167] Energy transfer in that arrangement was achieved through an intermediate fluorophore, also covalently bound to GM1. These reports point to the feasibility of very high detection sensitivities, specificities, and reliability when advanced fluorescence techniques are employed within an integrated detection system consisting of an appropriate biological recognition system.

A gravimetric sensor in which GM1 was incorporated as the target molecule on a QCM surface chip was reported.[170] In that application, a biomimetic film containing glycolipids was shown to attract bacterial toxins and whole cells, facilitating binding and kinetic analysis. A similar device in which self-assembled monolayers (SAMs) of GM1 were immobilized on gold surfaces was used in a QCM setup for detection of CT and the closely related heat-labile enterotoxin of *E. coli* in a continuous-flow cell.[171] Because of its high intrinsic sensitivity, QCM could be a useful reporter technology in pathogen-detection schemes based on carbohydrate recognition. However, like other techniques that rely on specific binding, applicability of the method requires a sufficiently high concentration of the capture agents for the analytes, in this case GM1.

The latter study by Spangler and Tyler[171] points to the utilization of SAMs as a promising design feature of carbohydrate biosensors. Indeed, deposition

of glycoconjugate films on solid surfaces has been a generalized technique for pathogen biosensor designs, and the construction of biofunctional and biocompatible interfaces on solids to generate models of cell and tissue surfaces may have numerous scientific and practical applications.[172,173] Mixed monolayers of thiol-terminated poly(ethylene glycol) (PEG) and thioacetyl GM1 deposited on gold crystals might be used as potential biosensor arrays.[172] Such assemblies could satisfy several key prerequisites in biosensor design: the display, flexibility, and accessibility of the recognition elements (the ganglioside moieties in the case of CT detection), the relatively facile transduction of the analyte-binding signal (through the PEG residues) to the reporting unit, which is associated with the solid surface, and the possibility for surface regeneration. The SAM architectures reported by Nyquist et al. are robust and readily controlled to provide a network of the receptor GM1 in the PEG-terminated matrix.[172] However, the extent of nonspecific protein binding to such film assemblies is still a primary concern for future biosensor applications.

An original approach for detection of pathogenic toxins via thin films of lipid and glycolipid mixtures deposited on the surface of a resonant mirror biosensor was reported.[174] The small quantities of glycolipid ligands incorporated within the films were responsible for generation of the optical signals following binding of the protein receptors. The experimental analysis indicated that the response of the films was sensitive to external parameters, such as pH. On the other hand, lipid/glycolipid deposition generally afforded surface regeneration through simple chemical procedures, enhancing the potential applicability of the biosensor. Another sensing device exploited optically tagged glycolipid ligands embedded within a fluidic phospholipid bilayer formed on the surface of a planar optical waveguide.[175] Multivalent binding of the CT to the film triggered FRET, resulting in a two-color optical change that was monitored through recording the emitted luminescence above the waveguide surface.

A significant hurdle for biosensor applications based on molecular recognition is the amplification of the signal because of the binding event over nonspecific background interactions. Several surface-biosensor designs introduced a transduction concept that relied on induction of structural modifications within biomimetic films in which the recognition events have occurred.[157] Specifically, Bardeau et al. have developed sensor devices that detect signals generated by shifts in the phase-transition temperatures of phospholipid/ganglioside films.[176] Such transitions, probed by IR vibrational spectroscopy, were induced by the highly specific GM1−CT interactions within the hybrid assembly of the glycolipid receptors and phospholipids. This method could have potential applications for signal amplification in biosensor design.

Original pathogen colorimetric sensors that respond to molecular recognition phenomena through the occurrence of rapid color transitions have been recently reported.[177,178] Several laboratories have

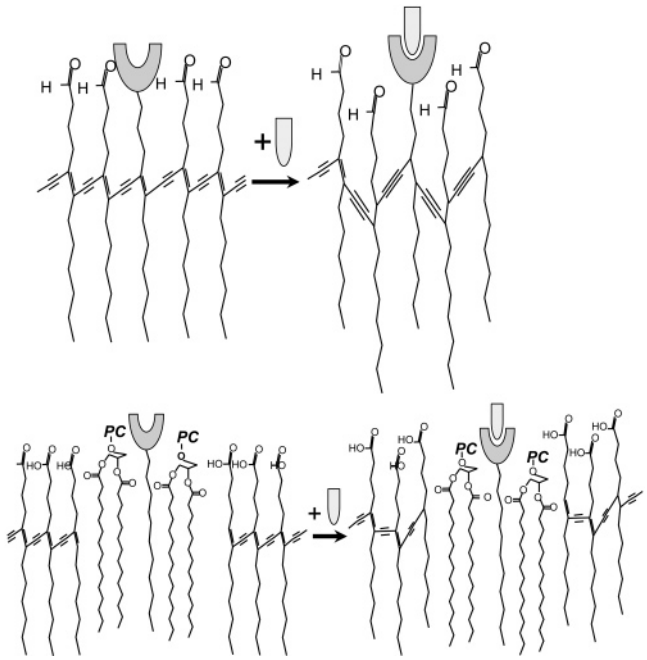

Figure 7. Schematic figures of colorimetric biosensors based on polydiacetylene (PDA). (A) Recognition element is covalently attached at the surface of the PDA framework (which appears blue to the eye). Interaction with the ligand induces a structural transition within the conjugated ene−yne polymer backbone, changing the conjugation length within the polymer network, with a consequent blue−red transition (see the text). (B) Recognition molecule is physically incorporated within phospholipids (phosphocholine, PC) domains in the PDA matrix. Ligand−receptor interaction indirectly induces the structural transformation of the polymer (see the text).

demonstrated that artificial cell membranes made from conjugated lipid polymers (polydiacetylene or PDA) can, on a simple level, mimic membrane surfaces allowing both the occurrence and consequent detection of molecular recognition processes (Figure 7).[177,179−185] Specifically, the ene−yne conjugated backbones of several polydiacetylene species absorb light at the visible region of the electromagnetic spectrum, thus exhibiting visible colors (in most cases, appearing intense blue). Furthermore, it was shown that external perturbations to the polymer induce structural transformations within the conjugated backbone of PDAs, giving rise to dramatic colorimetric transitions (blue−red). In a biological context, it was demonstrated that the blue−red transitions of PDA can be induced by ligand−receptor interactions occurring between soluble molecules and ligands embedded within the PDA matrix. The display of the ligands could be either achieved through covalent binding at the PDA headgroup region (Figure 7A)[177−179] or through physical incorporation of the recognition element within lipid domains assembled in the PDA framework (Figure 7B).[182−185] In PDA-based biosensors, the conjugated polymer backbone essentially acts as a built-in reporter of binding events, measurable by a chromatic change in the visible absorption spectrum. Such assemblies may provide a general approach for direct assays and biosensing devices for varied biological substances and biomolecular recognition events.

Some PDA-based biosensor applications reported on the covalent attachment of the ganglioside GM1 within polydiacetylene liposomes. In this arrangement, specific interactions between GM1 and CT at the interface of the liposomes resulted in a change of the vesicle color (from blue to red) because of conformational changes in the conjugated (ene−yne) polymer backbone induced by the molecular binding.[178,186] Such "chromatic liposomes" might be used as simple colorimetric sensors for screening of recognition processes involving carbohydrates and other biomolecules. A similar PDA-based colorimetric sensor was constructed in a Langmuir−Blodget film format, rather than the vesicle assemblies discussed above.[187] The film assay exhibited the blue−red transformations induced by biomolecular recognition and by other lipid-perturbing processes occurring at membrane surfaces. Song et al. have similarly reported the incorporation of gangliosides or sialic acid moieities in thin films, which permitted the colorimetric detection of CT or influenza virus, respectively.[187]

Sialic acid, the primary ligand for the hemagglutinin coat protein of influenza virus, has been also employed as a key component in colorimetric biosensor designs. Langmuir−Blodgett films as well as liposomes of polydiacetylenes derivatized with sialic acid were shown to undergo blue−red transitions that were specifically induced by binding to influenza virus particles.[179] Construction of sol−gel biosensors containing sialic acid as the recognition element for influenza virus was also reported.[188] In that technique, the researchers have incorporated blue PDA liposomes (see above) functionalized with sialic acid on their surface within transparent sol−gel matrixes. The entrapped liposomes still exhibited the blue−red transition following interaction of the sol−gel biosensor with influenza virus.[188] Indeed, the sol−gel matrix provided higher stability to the colorimetric biosensor compared to the more conventional soluble vesicle assemblies.

2.4.2 LPS Biosensors

Carbohydrate-based pathogen biosensors increasingly rely on detection of LPS moieties (also denoted endotoxins) on pathogen surfaces. LPS molecules, which consist of carbohydrates covalently attached to a lipid A moiety (Figure 8),[189] are located on the outer cell surface of various pathogens.[190] LPS plays a major role in conferring resistance of Gram-negative bacteria toward toxic agents, most likely by participating in the formation of an effective permeability barrier at the outer membrane.[191,192] Varied biosensor assemblies have utilized biomolecular recognition between surface-expressed LPS and lectins or other proteins. Ertl et al. described electrochemical biosensor arrays that facilitated *E. coli* subspecies detection through con A−LPS interactions[193] or through LPS binding to other lectins.[194] In particular, the researchers examined whether different lectins could *selectively* bind LPS moieties on surfaces of different bacteria. The construction of devices based on lectin recognition took advantage of the selective and reversible binding between the surface-immobilized lectins and the oligosaccharide groups. Electrochemical detection was facilitated through changes in the redox potential within the bacterial respiratory chain, following pathogen surface immobilization through the lectin−carbohydrate binding.[193,194] It should be emphasized that, even though carbohydrate recognition served in these biosensors only as an *indirect* means for bacterial detection, the high affinity between LPS and the lectin could generally ensure high sensitivity and fidelity of the sensor. Furthermore, the availability and diversity of known lectins and their carbohydrate ligands could facilitate the construction of sensor arrays for identification of several pathogens through their "signature response" in such arrangements.

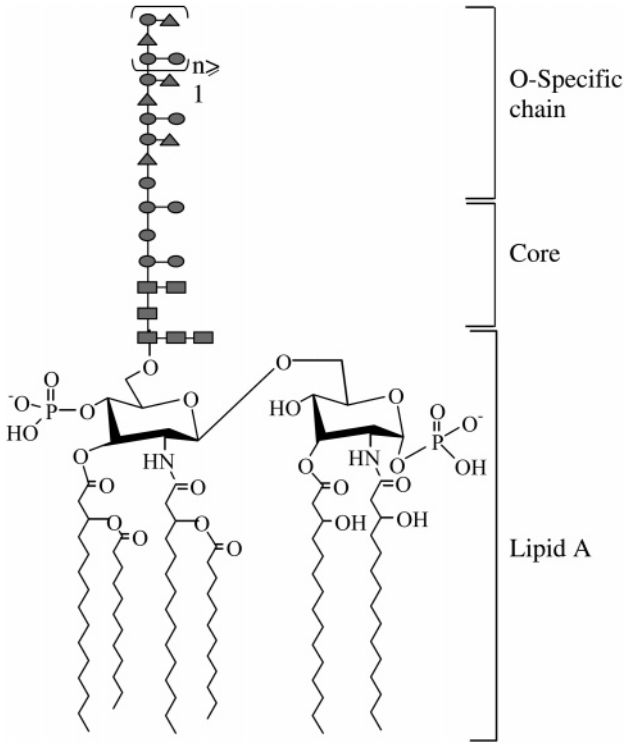

Figure 8. LPS structure.

Innovative LPS biosensors based on protein engineering have been developed.[195] A recent study illustrated a sensor concept in which the sequence of green fluorescent protein (GFP), a common fluorescent marker for protein targeting in intact cells, was modified to accommodate binding sites for either the lipid A moiety or the saccharide headgroup of LPS.[195] The engineered binding sites were localized in the vicinity of the chromophore of GFP, thus inducing fluorescence quenching following LPS binding. The research demonstrated the occurrence of a decrease of the fluorescence yield through association of the mutant proteins with lipid A or with LPS, exhibiting dissociation constants at the micromolar range.[195] The technique suggests that the use of genetic and protein engineering methods could assist in designing novel fluorescence carbohydrate biosensors. This approach could be attractive because it would take advantage of the vast knowledge base on protein structures and *de novo* structure design, increasing ligand affinities in protein-binding sites through residue modification and other factors. In

principle, integrating protein chemistry into carbohydrate-detection schemes could truly revolutionize the development of carbohydrate and pathogen biosensors.

A modified disposable QCM sensor for detection of LPS was reported.[196] The strategy undertook by the research was conceptually different than most other carbohydrate-detection schemes, focusing on detecting changes in the *solution viscosity* close to the sensor surface, rather than recording actual binding to *surface-immobilized* species. Specifically, it was shown that binding between the endotoxins and soluble *Limulus amebocyte* lysate (LAL) led to alterations in the acoustic load impedance at the sensor surface. One of the technical questions in this detection method concerns its intrinsic sensitivity, the extent of modification (damping) of the quartz oscillations induced by the changes in solution viscosity and density. The researchers claimed detection of LPS concentrations approaching 10 fg/mL, which is a rather low threshold. Increasing the hydrophilicity of the sensor might even improve the sensitivity further. Other bioanalytical assays in which LPS was an essential component were described. An ELISA approach using a phage LPS antigen was developed for identification of immunoglobulin antibodies to *Salmonella*.[197]

2.4.3 Cancer Diagnostics

Modification of carbohydrate expression and glycosylation patterns on cellular surfaces is a common feature of cancer cells.[198,199] A majority of human carcinomas are associated with altered expression of oligosaccharides on membrane glycoproteins, for example, in breast cancer,[200] adenocarcinomas of the pancreas,[201] cervical cancers,[202] and others. There have been intensive efforts toward development of diagnostic techniques for tumor identification utilizing carbohydrate markers on cancer cells.[199,203] Dwek et al., for example, have reported an immunohistochemical approach for early tumor detection.[199] Monoclonal antibodies (mAbs) identifying altered glycosylation of specific glycoproteins associated with tumor appearance were used as a diagnostic tool.[200,204]

Changes in the localization and relative abundance of carbohydrate species on cell surfaces can be monitored with the aid of specific carbohydrate-binding proteins, such as lectins. Lectin histochemistry has been utilized to identify modulation of the expression of sialic acid on human cervical carcinomas.[202] Plzak et al. employed biotinylated galactoside-binding (metal-ion-independent) animal lectins (galectins) to detect domains of increased differentiation in human carcinoma tumors.[205]

Sialylated Lewis antigens (SLeAs) and their enhanced cell-surface expression are recognized markers for various malignancies and metastatic processes.[206] SLeAs (a representative antigen, Silaylated Lewis^x, is shown schematically in Figure 9) have been frequently used as molecular targets in immunohistochemical and serological cancer assays.[207,208] MAbs have been raised and tested against SLeAs with the goal of developing immunoassays for the detection and management of malignancies.[209] An electrochemical biosensor approach for determination of the tumor-marker-bound sialic acid (b-SIA) was reported.[210] The sensor consisted of a copolymer-immobilized bilayer containing the enzyme sialidase, placed in contact with a H^+-selective poly(vinyl chloride)−poly(vinyl acetate) indicator membrane. The release of sialic acid, an α-ketocarboxylic acid with a pK_a of 2.6, following enzymatic cleavage resulted in a local pH change monitored by the proton-sensitive indicator electrode. This electrochemical sensor was shown to be capable of differentiating between pathological and nonpathological levels of b-SIA within a relatively short detection time (3−5 min) and in reasonable accuracy. The cancer marker Sialylated Lewis^x antigen and its mimetic structures have been comprehensively characterized through analysis of their binding to selectin, a natural lectin.[211]

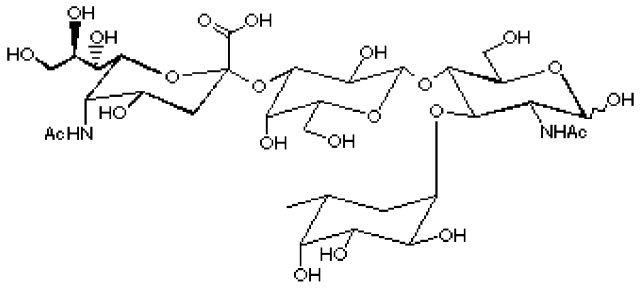

Figure 9. Silaylated Lewis^x structure.

Mucins, a class of highly glycosylated circulating proteins, were also investigated and utilized as biological markers of cancer.[212] Mucins have attracted particular attention as highly specific serum tumor markers because they could differentiate between epithelial ovarian carcinoma and benign growths. Mucin-based assays have significantly increased the specificity and sensitivity of cancer detection, having a significant potential for cancer patient management and tumor detection.[212]

2.5 Carbohydrate Nanobiosensors

The recent emergence of "nanotechnology" as a promising scientific and technological avenue has led to an expanding activity toward development of "nanobiosensors". Some studies have focused on the integration between carbohydrates and nanometer-size systems and devices, while other efforts have attempted to integrate advanced nanotechnology-oriented instrumentation within carbohydrate biosensors. You et al. described an amperometric biosensor facilitating high-sensitivity detection of sugar moieties through embedding nickel nanoparticles within a graphite-film electrode.[213] The authors reported that the dispersion of Ni nanoparticles within the carbon film yielded an order-of-magnitude improvement in the sugar-detection limit compared to conventional electrode arrangements. The use of nanoparticles was not directly related to the actual detection of the carbohydrate molecules but rather as a way for improving the technical performance of the electrode. Another study has employed nanosize amphiphilic C60 dendrimers for achieving better interactions between the sensor surface and the

carbohydrate analytes.[127] Binding was achieved through deposition of ordered Langmuir monolayers of the bucky-ball conjugated with glycodendron headgroups at the air−water interface. The films could be further transferred to solid quartz surfaces, pointing to their potential applications in biosensor design.

Atomic force microscopy (AFM) has been a major driving force in nanotechnology research and development. AFM is conceptually similar to the way old "long-play" records were read by the stylus of a phonograph, where the AFM tip acts like a "stylus" capable to image molecules and atoms on solid surfaces.[214] Among the most widespread applications of AFM in carbohydrate research has been imaging of single carbohydrate molecules and surface characterization of oligosaccharide assemblies.[213,214] An example of the practical application of AFM was its use for evaluation of the structure and texture of food carbohydrates.[215]

AFM has been explored as a tool for varied biosensor-related applications, such as determination of carbohydrate heterogeneity on bacterial surfaces[216] or the observation of a nonhomogeneous distribution of specific oligosaccharide units on the surface of yeast cells through derivatization of the AFM tip with lectins.[217] Such studies illustrate both the capabilities as well as the significant hurdles for application of single-molecule imaging and force measurements in biosensors. On one hand, the atomic-level resolution of AFM could provide unique "carbohydrate imaging fingerprinting" for bacterial and other cellular surfaces. One can conceive, in principle, the construction of an AFM image database for bacterial surfaces that might be used for rapid pathogen identification. Further contributions could be envisaged from integration of computer-aided image analysis into AFM-biosensor applications. However, the particular strength of AFM as a single-molecule-imaging technique rather than characterizing large-population ensembles could raise formidable difficulties in using this method for sufficiently fast and reliable detection. For example, the carbohydrate heterogeneity exposed by Camesano and Abu-Lail[216] could make any interpretation of AFM images of unknown pathogens inconclusive and highly complex. Furthermore, the very high sensitivity of AFM to environmental factors, such as temperature, salt types, and concentrations, etc., might lead to impracticality as a biosensing method.

The capability of AFM to resolve chemical and physical events involving single molecules has led to exploration of other potential biosensor applications. AFM was used for characterizing structural properties of a single xanthan molecule on a solid surface.[218] AFM was also employed for detection of bacterially secreted carbohydrates in river sediments.[219] A novel saccharide "force fingerprinting" technique, based on the single-molecule-imaging capabilities of AFM was reported.[124] The method has built upon the variability of force-induced conformational transitions of the pyranose ring, which are also dependent upon the glycosidic linkages in the molecules. These transitions yield characteristic force-spectrum fingerprints for specific carbohydrates.[124]

The AFM methodology can further identify individual carbohydrate molecules in solution, contributing to its bioanalytical applicability. For example, the capability of AFM to distinguish among chair-twist−boat conformational transitions of the pyranose ring within different α-(1,4)-linked carbohydrates could serve as a "nanomechanical" fingerprinting of different oligosaccharides.[220] AFM was also used to evaluate minute differences in the forces between carbohydrate moieties on bacterial cell walls and biopolymer surfaces, pointing to its use as a tool for bacterial detection.[221]

2.6 Miscellaneous Carbohydrate Bioassays

A large number of bioanalytical techniques are used for routine carbohydrate analysis. Detection methods of carbohydrates in food products, particularly fruit, have been reviewed.[222] The majority of saccharide analysis schemes in food processing combine compound separation, mostly chromatography, and detection. GC has been popular for carbohydrate analysis because it has the advantage of speed, although this technique generally requires carbohydrate prederivatization.[223] TLC is relatively inexpensive; however, it lacks in resolution and quantification information.[224,225] Other techniques have been used, primarily HPLC using polar and nonpolar columns,[226] anion-exchange columns,[222] or cation-exchange columns.[227,228]

Theoretical analyses have been employed in conjunction with carbohydrate biosensor studies. Fractal analysis was used to characterize the binding kinetics between cell-surface receptors (such as bacterial-displayed oligosaccharides) and external soluble analytes.[229] Such theoretical treatments could be of use in interpreting oligosaccharide-binding data and for optimization of sensor performance.

3. Carbohydrate Components in Biosensors

3.1 Carbohydrate Recognition Elements

Carbohydrates often constitute fundamental parts within biosensor devices, either comprising the recognition elements or as scaffold components of the sensor matrixes. Such applications take advantage of two important (and unrelated) properties of carbohydrates. The first is the participation of numerous oligosaccharides in molecular recognition phenomena, which could make them ideal for targeting specific analytes. Another oft-encountered characteristic of molecular framework arrays constructed from saccharide assemblies is their stability and rigidity, making them attractive components in biosensor design.

Films composed of synthetic saccharide derivatives for potential biosensor applications have been constructed.[230] That study presented a detailed physicochemical characterization of the organization and cooperative properties of lipo/glycohomopolymer and random lipo/glycocopolymer monolayers assembled at the air−water interface. The researchers have further proposed utilization of the molecular recognition properties of such films in carbohydrate-based biosensor designs.[230] Similar surface-deposited films of

glycopolymer conjugates were prepared.[231] This research achieved adsorption of SAMs of glycopolymers, specifically polystyrenes carrying maltooligosaccharides with different chain lengths and lactose-carrying polymers with polystyrene and polyphenylacrylamide main-chain structures, which were investigated by QCM.

Other innovative approaches for fabrication of glycoconjugate-containing monolayers and films were described. A recent study focused on the insertion of P glycoprotein (P-gp) into planar lipid bilayers formed either by liposomes disassembled on amorphous carbon film surfaces or as Langmuir−Blodgett monolayers.[232] Specifically, P-gp, a membrane drug pump, was incorporated in model membranes obtained by fusing P-gp-containing vesicles onto two hydrophobic supports: amorphous carbon films or Langmuir−Blodgett lipid monolayers. The researchers demonstrated that the glycoprotein retained its functionality and recognition properties in these model systems, most likely because of the supported lipid bilayer scaffolding. Another important factor contributing to the recognition capabilities of the films and potential utilization is the type and quality of the solid surface, which was shown to intimately affect the vesicle fusion and protein display.[232]

"Surface glycoengineering" methods that could produce carbohydrate-recognition films were reported. Chevolot et al. demonstrated a strategy for chemical immobilization of saccharides through diazirine derivatization.[233] The technique employed synthesis of aryl diazirine coupled to mono- and disaccharide moieties; illumination of the diazirine resulted in covalent binding of the carbohydrates to the polystyrene surface. Even though covalent binding of carbohydrates to surfaces has been achieved using varied methods,[234] *photo*immobilization has the potential to produce well-defined patterns, feasible through the advanced microprinting technologies prevalent in the electronic and semiconductor industries. Further pointing to the integration of electrooptics and carbohydrate biosensors, chemical conjugation of carbohydrates to a sensor surface was carried out for construction of the chemiluminescence-based optical fiber immunosensor.[235] This assembly was designed to identify anti-pneumococcal antibodies, in which pneumococcal cell-wall carbohydrates were covalently attached to optical fiber tips. The optical immunosensor system was shown to be an accurate and sensitive method for detection of antipneumococcal antibodies in specimens such as saliva and urine.

A generic design for biosensor application using immobilized carbohydrates for molecular binding is shown in Figure 10. The key for construction of such biosensor is the efficient immobilization and display of the carbohydrate ligands on the surface, facilitating both interactions with soluble analytes, as well as transduction of the analyte binding to the sensor surface for generation of a measurable signal. Optimization of the deposition and adhesion properties of carbohydrates on solid surfaces are critical to construction and applicability of biosensors. Several investigations focused on characterization of the

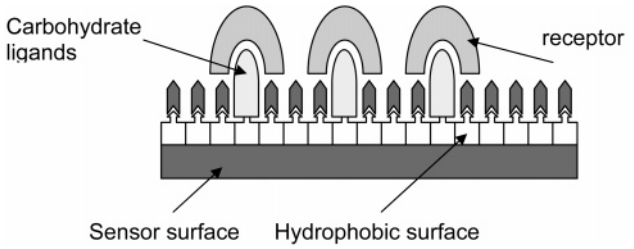

Figure 10. Schematic representation of a generic binding assay containing surface-immobilized carbohydrates. The carbohydrate ligand is displayed within a biocompatible layer, which is placed on the transducer surface of the biosensor.

factors affecting carbohydrate attachment onto solid surfaces and the binding specificity and adhesion properties of biomolecules onto carbohydrate-derivatized surfaces.[236,237] A comparative study has identified key properties that make specific carbohydrate coatings resistant to the adsorption of proteins.[237] Dutra et al. have devised a method for immobilization of a pneumococcal carbohydrate onto silicon oxide wafers for use in surface acoustic wave biosensors in which the sugar molecules were attached to the surface through their specific binding to protein A, which was chemically adsorbed to the solid surface.[238]

Some applications have utilized the strong affinity of ionic carbohydrates to particular metal ions in the design of voltammetric-sensing devices.[239] The electrochemical biosensor was composed of an ion sensor for copper and lead by means of incorporating pectic and alginic acids and heparin onto copper electrodes. The accumulated metal ions in such assemblies modified the recorded voltage, thus allowing high sensitivity and reproducible cation detection. Even though (or perhaps because) this method reports upon the presence of the carbohydrate indirectly (through density of the metal ions), the sensitivity of the biosensor was quite satisfactory.

Novel colorimetric detection methods for toxins and pathogens based on the affinities of carbohydrate ligands embedded within sensor assemblies to their soluble molecular counterparts were reported.[181,240] The sensing schemes consisted of carbohydrate derivatives (lipopolysaccharides and gangliosides) incorporated within a polydiacetylene matrix undergoing dramatic blue−red transitions induced by binding between the embedded carbohydrate moieties and soluble macromolecules or intact pathogens (Figure 7, above). Examples of the applications of this technology include the detection of CT,[178] endotoxin binding,[186] and screening of LPS-binding antimicrobial peptides.[181] Other sensors consisting of chromatic scaffold materials were developed, including a family of glycopolythiophenes containing sialic acid or mannose ligands that exhibited binding to lectins, influenza virus, and bacteria.[241] Similar to the PDA-based sensors, the ligand−receptor binding in these polymers resulted in an unusual red shift of the visible absorption spectra.

Other design principles employed in biosensor development take advantage of the unique assembly properties of cell-surface glycoconjugates. Several publications reported on the construction of ampero-

metric enzyme sensors for sucrose based on bacterial cell-surface layers (S layers) as immobilization matrix for the biological recognition elements.[242,243] S layers, consisting mainly of identical glycoprotein (or other protein) subunits displaying organized and oriented functional groups,[244] represent ideal matrixes for display of enzymes and other functional macromolecules as required for biosensor development and applications. A recent study demonstrated immobilization of S-layer glycoproteins through activation of the hydroxyl groups of the carbohydrate chains with cyanogen bromide or their conversion into carboxyl groups by succinylation.[245] These S-layer-mimicking templates were further coupled to saccharide-degrading enzymes such as glucose oxidase, β-fructosidase, and mutarotase and incorporated within amperometric and fiber-optic biosensor prototypes. Similar experiments utilized two-dimensional glycoprotein crystals as patterning elements and immobilization matrixes for the development of biosensors.[246]

Construction of oligosaccharide arrays has opened the way for coupling glycochemistry with high-throughput screening applications. A novel carbohydrate array has been recently used for profiling and identifying anti-glycan antibodies.[247] In this study, an immobilized glycan array was created by covalently linking the oligosaccharides to a solid surface via a long linker at their reducing ends. The carbohydrates were thus presented to the medium with a well-defined orientation and were accessible for specific binding by glycan-binding proteins, antibodies, and lectins. In preliminary experiments, the researchers used the technique to identify a novel anticellulose antibody that binds specifically to β4-linked saccharides with a preference for glucopyranose over galactopyranose residue. Also discovered in that study were antibodies against mono- and oligosaccharides displayed on surfaces of common bacteria. The introduction of this array biosensor emphasizes the crucial role of clever chemistry for achieving optimal recognition and sensing conditions. The carbohydrate array approach could be employed for high-throughput screening of glycan-binding proteins, pathogen detection, and putative bacterial adhesion substances.

Molecular recognition of oligosaccharides by specifically raised antibodies constitutes the basis for new chemiluminescence-based optical fiber immunosensors.[235] In that technique, chemiluminscence was induced within derivatized antibodies following their binding to immobilized carbohydrates. The concept was demonstrated by chemically conjugating pneumococcal cell-wall carbohydrates to an optical fiber tip, detecting accurately anti-pneumococcal antibodies. This optical immunosensor system might be applied to monitor antibodies in specimens such as saliva and urine.

3.2 Carbohydrate Scaffolds

A number of carbohydrate molecules have been used as rigid components in biosensors. The glycopolymer agarose and cellulose are likely the most widely used constituents of rigid matrixes and gels

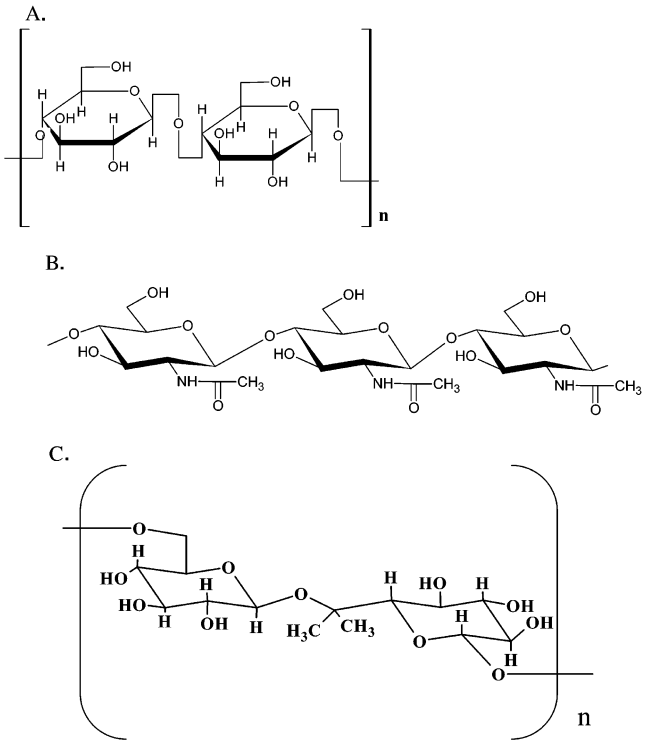

Figure 11. (A) Cellulose structure. (B) Chitin structure (fragment). (C) Dextran structure.

for numerous bioanalytical applications.[248] Cellulose is an abundant natural glycopolymer (Figure 11A), and its distinct physicochemical properties, in particular, its rigid structure, have made it an abundant component in biosensors. Hartmann et al. reported the fabrication of cellulose−antibody films for highly specific evanescent wave immunosensors.[249] Cellulose was recruited for this particular biosensor design as a substrate for deposition of highly stable antibody films on the sensor surface.

Chitin (Figure 11B), a major component of the outer shells of crustaceans, has been also used as a framework constituent for immobilization of recognition elements in various sensor devices.[250,251] The excellent biocompatible properties and relative biological and chemical inertness of chitin and chitosan, its deacetylated derivative, have made these compounds attractive as matrixes for enzyme sensors [250] and potentially implantable devices.[252] Chitin-constructed membranes further exhibit attractive protein entrapment properties and favorable oxygen and glucose permeability profiles, making them highly compatible for biosensor design.[253] The use of chitin in sensor frameworks assists in maintaining the stability and durability of the biosensor system.

Similar to chitin, dextran has been employed as a template and backbone component in numerous biosensor designs. Dextran (Figure 11C) has a repeating uncharged glucose chain structure making this carbohydrate particularly attractive as an inert structural element in biosensors. Dextran assemblies are mostly incorporated as layers or films supporting biological interactions essential to the sensing capabilities.[254] Derivatized dextran polymers have been used as matrixes for affinity biosensors.[255] In another application, highly wettable, covalently grafted, dex-

tran coatings were applied to flat silicon wafer surfaces to be used in potential sensor devices.[256]

Other representative biosensor applications of dextran include its use as a substrate for β-cyclodextrin immobilization in immunosensors,[254] as a material used for functionalization of novel carbon nanotubes in electronic sensors,[257] and for enzyme immobilization.[258] Several schemes utilized fluorescently labeled dextran. Dextran labeled with fluoroscein isothiocyanate (FITC) was used as a framework for a glucose biosensor using the FRET technique.[259] Dextran was co-entrapped with a hydrolytic enzyme in sol−gel films developed for pH sensing.[260] Fluorescently labeled dextran was deployed in conjunction with the lectin con A in a hydrogel arrangement for glucose sensing,[102] employed as a surface-functionalizing agent facilitating antibody immobilization in chemoluminescent immunosensors,[261,262] and as the constituent of a coating layer in surface acoustic waveguide (SAW) biosensors.[263]

Cyclodextrins, macrocyclic carbohydrates with nonpolar internal cavities that participate in numerous chemical systems and applications, have been also widely used in biosensor design.[264] These inclusion compounds have generally appeared in sensor schemes as framework elements facilitating immobilization of other molecular species that are essential for the functionality of the sensor. A representative cyclodextrin-based biosensor was described by David et al., constructing an immunosensor by grafting amino-β-cyclodextrin onto functionalized gold surfaces.[254] The incorporation of additional dextran-derivatized adamantyl groups (adamantane derivatives being the common ligand of cyclodextrins) enabled the coupling of antibodies as the biological recognition elements within the biosensor. Other cyclodextrin-templated biosensors were reported, including cross-linked cyclodextrin films within dopamine biosensors[265] and β-cyclodextrin derivates impregnated in graphite paste for enzyme immobilization in amperometric enantioselective drug biosensors.[266]

Other carbohydrates have been used as substrates in gel constructs for detection of reactant species in the mobile phase. Carboxymethyl (CM)-curdlan, a carbohydrate linked with a chromatic dye, was assembled within polyacrylamide gels for facilitating rapid colorimetric detection of glucanases.[267] Beside applications in which carbohydrates have been directly involved in biochemical reactions, saccharides have been incorporated in sensor assemblies as chemically inert species, albeit essential to the functionality of the systems. Brinkman et al., for example, reported on the construction of hydrogels comprised of poly(vinyl alcohol) and heparin.[268] On one hand, the cross-linked assembly was shown to resist nonspecific protein permeation, an important requirement for biosensor design but, on the other hand, facilitated slow release of the incorporated heparin, thus pointing to potential biosensor applications. Saccharide derivatives were also examined for their ability to form solid gels for cell-based biosensors.[269] O'Connor et al. examined the entrapment of neuronal cells in a three-dimensional matrix constructed from a novel sugar poly(acrylate) hydrogel.[269] A significant hurdle to such biosensor applications has been an insufficient adsorption of the cells to the saccharide template.[269]

Novel uses for carbohydrates as templating agents were reported in the framework of molecular-imprinting technology.[270,271] Shi et al. described a template-imprinted matrix for protein recognition in which the protein-binding sites were molded by a disaccharide framework.[270] The carbohydrate molecules were particularly important in that setup, providing added synthetic flexibility and analyte specificity. This experimental achievement is noteworthy because it points to a generic synthetic pathway for constructing molecularly imprinted protein biosensors, a highly challenging goal in recent years.

Several studies have addressed theoretical aspects pertaining to carbohydrate-containing biosensors. Griesser et al. investigated the interfacial forces between carbohydrate surfaces and adsorbed proteins.[237] When theoretical predictions and experimental approaches such as X-ray photoelectron spectroscopy (XPS), MS, and AFM are combined, the researchers have established key parameters responsible for the resistance of particular polymer coatings to the adsorption of proteins, an important feature of varied biosensor arrangements.

3.3 Biosensors Utilizing Protein–Carbohydrate Interactions

Molecular recognition and interactions between carbohydrates and proteins play key roles in many biochemical processes. The participation of specific oligosaccharide sequences in protein targeting and folding and in propagating infection and inflammation processes through interactions with receptors and antibodies have become increasingly apparent.[1] Studying such interactions is also desirable for development of therapeutic substances that would mimic or interfere with the recognition process. Various approaches have been introduced to probe carbohydrate−protein binding and to utilize such recognition events in the action mechanism of biosensors. However, elucidation and understanding of the bioactive domains within oligosaccharides and their protein-binding properties pose distinct bioanalytical and chemical challenges.

From the standpoint of biosensor design, protein−carbohydrate binding has been employed as a platform for extraction and analysis of varied proteins. In most of these applications, the biosensor operation relies on immobilization of carbohydrate species, which generally function as the recognition elements, followed by generation of measurable signals induced by association with their complementary macromolecules. Construction of carbohydrate-modified recognition surfaces is synthetically demanding because of the structural complexity of oligosaccharides. Distinct problems have been encountered because of the multiplicity of hydroxyl groups that might make specific binding difficult, as well as the requirement of appropriate linker systems to facilitate display and access to the immobilized oligosaccharides.[250] The two most common carbohydrate immobilization tech-

niques employed in such sensors exploit the high affinity of the biotin−avidin pair[272] or the deposition of alkane thiolate monolayers on gold surfaces.[273]

Biosensor technologies based on surface immobilization of oligosaccharides have to address critical technical and fundamental issues. A primary requirement concerns the feasibility of attaching the generally hydrophilic carbohydrate molecules to solid transducer surfaces. In that regard, most surface-layering strategies use hydrophobic chemical interactions. Consequently, many sugar immobilization methods require chemical modification of the saccharide molecular units. Such chemical treatments, however, should not interfere or adversely affect the biological properties of the examined carbohydrates, in particular, molecular recognition by soluble macromolecules. Furthermore, any proposed biosensor design has to exhibit high sensitivity and sufficient versatility for allowing detection of a wide range of proteins and other biomolecular analytes.

A recent development with potentially significant implications for glycobiology research in general and studying carbohydrate−protein interactions in particular has been the fabrication of carbohydrate arrays as a tool for rapid analysis of sugar-binding events and carbohydrate interactions. Examples for such applications include array carbohydrates that are first immobilized on pretreated surfaces, followed by addition of fluorescently labeled carbohydrate-binding proteins; binding occurrence can then be monitored by fluorescence spectroscopy.[274] The challenges for wide applications of such methodologies, however, are mostly synthetic, i.e., the construction of diverse enough, analyte-accessible immobilized carbohydrate arrays.

An elegant and important demonstration of noncovalent immobilization of a carbohydrate antigen array on glass surfaces was recently reported.[26] The researchers assembled dextran polymers produced by *Lactobacillaceae* bacteria on nitrocellulose-coated glass slides and examined binding of anti-dextran antibodies to the slides using fluorescence scanning. Immobilization and specific antibody−antigen binding were detected in this configuration. Glass-immobilized carbohydrate microarrays could have significant diagnostic and clinical applications, including rapid detection of specific antibodies in physiological solutions and "antibody profiling" of such solutions, identification of cross-reactive antibodies and antigens, and quantitative determination of carbohydrate diversity within microorganisms. The platform developed by Wang et al. is particularly robust and involves relatively straightforward preparative steps, facilitating rapid analysis of complex solutions through simple and sensitive detection schemes. Furthermore, this method intrinsically enables the display of a large repertoire of cellular carbohydrates and carbohydrate antigens on a single slide, approaching the capacity to include oligosaccharides encountered in most common pathogens.

Varied chemical strategies were introduced for fabrication of carbohydrate arrays for high-throughput screening applications. As a parallel to the more widely used "DNA chips", "carbohydrate chips" could facilitate rapid evaluation of protein−saccharide interactions. Carbohydrate chips for evaluation of lectin binding and glycoenzyme substrate specificities were prepared by saccharide immobilization onto SAMs of cyclopentadiene conjugates via the Diels−Alder reaction[275] or through coupling of the carbohydrates to thiol moieties.[276] The functionalized monolayers in those studies contained chemical entities such as benzoquinone[275] or maleimide[276] for covalently bonding the carbohydrate derivatives but also displayed ethylene glycol for minimization of nonspecific protein attachment to the surface. Such surface engineering strategies might find uses in sensor applications for analysis of complex carbohydrate structures. However, the ultimate utility of such "biochip" designs would most likely depend on the detection method to be used, its sensitivity, reproducibility, and technical limitations. For example, fluorescence microscopy could provide high sensitivity and spatial resolution; however, this technique might incur problems of bleaching, background signals, and surface regeneration.

Progress in carbohydrate array research has been also achieved through the creation of microarrays of *neoglycolipids* and their display on solid surfaces.[277] Neoglycolipids, comprised of oligosaccharides chemically conjugated to lipids, can be readily immobilized on solid matrixes through their hydrophobic lipid residues, thus facilitating the surface display of the carbohydrate molecules for rapid screening of binding interactions.[81] Immobilized neoglycolipid assemblies could achieve higher avidity of protein analytes because of lipid clustering and surface oligomeric organizations of the oligosaccharides.[81] The microarrays constructed by Fukui et al.[277] contained neoglycolipids prepared from diverse physiological and synthetic sources (including extracts from whole organs). That exploratory and potentially groundbreaking study demonstrated that carbohydrate-recognizing proteins bound their ligands not only within arrays of homogeneous oligosaccharides but also within mixture of heterogeneous carbohydrate species. The technology could have much more general diagnostic appeal, as a tool for profiling carbohydrate-binding proteins from different sources, for discovery of new carbohydrate-binding proteins within cellular targets, and for large-scale analysis of protein-binding characteristics of the glycome.

A recently reported screening assay for protein−carbohydrate recognition utilized surface immobilization of sulfated carbohydrates.[278] The technique, denoted sulfated carbohydrates coating ELISA (SPC-ELISA) employed initial coating of sulfated carbohydrates followed by binding with different target proteins, consequently detected by a conventional ELISA method. Complementing carbohydrate immobilization and immunosorbent detection in SPC-ELISA has some advantages over other frequently applied immunosensing techniques, including its compatibility with automation in general and high-throughput screening methodologies and equipment in particular and the versatility of the technique with regard to molecular-target variability and detection methods.

A novel technique for the screening of carbohydrate−peptide interactions through phage-display selection of peptide binding to mirror-image sugars has been developed.[279] The researchers used phage display to identify peptides that bind to surface-immobilized synthetic L-type saccharide enantiomers; the corresponding *mirror image* peptides that bind the D-type saccharides could then be identified through application of SPR. The technique was demonstrated for detection of saccharide binding to high-affinity antibodies.

Interactions between proteins and glycolipids are of particular importance in carbohydrate-based biosensor design. The lipid moieties of glycolipids are generally buried within the hydrophobic membrane bilayer, leaving on one hand, the oligosaccharide components exposed to the solution but, on the other hand, close enough to the bilayer surface facilitating ligand presentation. Furthermore, the structural features of immobilized glycolipids might play pivotal roles in shaping carbohydrate−protein binding. This is mostly due to the observations that *multivalent* interactions rather than the relatively weak monovalent affinities are prevalent between proteins and carbohydrates.[280] Studies of protein−saccharide recognition and the effects of the membrane environment on these phenomena are in their infancy.[281] The presence of the acyl chains could be further advantageous for immobilization of the carbohydrate recognition elements within varied hydrophobic surfaces in potential membrane-mimic biosensor designs. The creation of surface patterns of glycolipid targets and biosensor arrays[282] would be a natural extension of the immobilization capabilities.

Pathogen detection is an important field in which glycolipid−carbohydrate interactions could be of particular importance. The interactions between gangliosides and CTs have been widely studied and included in biosensor designs, in many instances using surface immobilization of GM1 (see section 2.4.1, above). A multiarray evanescent wave biosensor for detection of CT was described in which gangliosides immobilized at discrete locations on the surface of an optical waveguide.[282] Rapid and easy detection of the fluorescent-labeled CT or tracer antibodies was achieved using the same technique.[157] Other examples for the use of the CT−GM1 recognition pair in biosensor design are described above (see section 3.3, pathogen detection). The binding between globotriaosylceramide (Gb3) and *E. coli* verotoxins could similarly constitute the core of diverse bacterial detection schemes.[281]

Heparin−protein binding constitutes the basis of varied peptide and protein bioassays. A range of techniques has exploited surface expression and the selective protein-binding properties of heparin and its derivatives in biosensor devices and as vehicles for diverse detection schemes. "Heparin biochips" were constructed for applications in techniques such as SPR to measure the extent of heparin−protein interactions.[283,284] In such applications, it was observed that the biosensor response was often affected by the method of heparin immobilization on the solid surface.[284] Covalent attachment of glycosaminoglycans such as heparin and heparin derivatives has been problematic because of the presence of only a single reducing-end amine group.[283] Original methods for surface immobilization of heparin were proposed, including covalent attachment of heparin on an evanescent wave biosensor cuvette,[285] binding as an albumin conjugate on a functionalized polystyrene surface,[286] and on a SPR biochip.[283] Evanescent wave biosensors have been used for studying heparin−protein interactions.[285] Optical sensing of heparin/albumin thin films was used to measure modifications of film thickness by the pH of the solution.[287] SPR analysis was also carried out to systematically evaluate interactions between collagens and different heparin derivatives.[288]

QCM has been applied for detection of various biological saccharide-binding reagents. There has been, however, some skepticism as to the accuracy and applicability of the technique for analysis of molecular recognition, partly related to problems arising from immobilization and positioning of large biomolecules on the sensor surface.[52] A procedure for incorporation of α-galactose antigen on a microbalance surface resulting in a rigid and sensitive recognition biofilm was recently described.[289] In that work, SAMs of α galactose were prepared by thiol-tail derivatization, allowing construction of a highly reproducible and selective lectin sensor.

Carbohydrate−protein binding has an additional advantage when utilized in biosensor design. This is due to the fact that one of the most important criterion for efficient, reversible surface immobilization of biomolecules in sensor devices is whether such molecules retain their biological functions. This issue is particularly important in biosensors based on enzymatic reactions.[290] The optimal design should permit high affinity of the enzyme to the surface to avoid loss; however, the attachment should not be too strong as to not allow enzyme elution and regeneration.[291] Chemical or physical adsorption techniques are often inadequate for such requirements, and biospecific methods are also problematic. For example, binding based on the avidin−biotin system is too strong (binding constant K_{ass} in the order of 10^{15}), and antibody−hapten association, while in the correct binding-strength range, is highly dependent upon the immuno system and solution conditions. Lectin−carbohydrate binding ($K_{ass} = 10^6-10^7$), on the other hand, offers a practical route for reversible immobilization of enzymes and recognition elements in biosensors.[292] Koneke et al. demonstrated the use of con A for reversible immobilization of glucoenzymes within a fluoride ion-sensitive field-effect transistor (FET).[291] Enzyme-reloading in the biosensor assembly was achieved through removal of the lectin-bound glucoenzymes by elution with soluble mannosides.[291]

Carbohydrate−lectin binding is central to other biosensor designs. Galanina et al. have synthesized and compared radioactively and fluorescently labeled carbohydrate conjugates for detection of cell-expressed lectins.[293] The technique was based on the coupling of the saccharide moiety to a soluble polyacrylamide spacer, onto which were attached the

reporter molecules. This synthesis approach was reported to achieve optimal binding of mammalian cells to the carbohydrate ligands, without interference from the spacer arms and the molecular labels. The different molecular components further aided in prevention of nonspecific binding of the cells. The technique was easily amenable to conducting experiments in a standard 96-well plate setup and also for cell- and tissue-staining applications. Organic chemistry synthesis approaches have been employed for creation of carbohydrate conjugates used in electrochemical biosensors.[294,295] Kitov et al. have synthesized a hexadecanyl−polysacchride conjugate to facilitate incorporation of the carbohydrate recognition element within a hydrophobic film.[294] The carbohydrate−protein recognition pair in other designs perturbed the electron-transfer properties of a self-assembled alkaneoid film that constituted the biosensor.[295]

3.4 Carbohydrates in SPR

SPR has become a powerful and widely used analytical technique for evaluating and quantifying biomolecular interactions. SPR measures binding interactions between molecules immobilized on the surface of a biosensor chip and their soluble counterparts through optically monitoring changes of the refractive index in the vicinity of the sensor surface (Figure 12).[296,297] General reviews on the subject of SPR analysis of carbohydrates and glycoconjugates are available.[298] Below, we summarize some of the research activities concerning carbohydrate SPR biosensor applications.

One of the detriments for the use of SPR in carbohydrate analysis has been whether effecient immobilization of the molecular recognition elements on the sensor chips is feasible. While a number of chemical processes have been introduced to immobilize proteins on the biochip surface,[299] similar procedures for carbohydrates have been limited. SPR sensors attained carbohydrate immobilization through the bridging biotin−avidin system.[300,301] Nonspecific interactions with avidin, however, become problematic in such arrangements.[283] Direct binding through covalent attachment of conjugated carbohydrates has been reported.[283] Ordered multilayers of heparin/albumin could serve as biocompatible films in SPR applications.[302] Neoglycoconjugates, consisting of synthesized molecules of glycosides and carrier molecules such as proteins or lipids, were used as affinity ligands for deposition on SPR sensor surfaces, although these molecules also exhibited relatively low affinities.[303] Recent studies attempted to construct stable and tightly bound carbohydrate layers through nanoscale coatings on solid surfaces.[169] Such systems could have potential uses in varied biosensor applications.

Numerous experiments designed for studying carbohydrate recognition using SPR biosensors have been reported. The technique was applied for analysis of carbohydrate−antibody recognition involving individual saccharide molecules[304] or oligosaccharide antigens on bacterial surfaces,[305] interactions between oligosaccharides and integral membrane proteins,[306] pattern receptor recognition by glucans in human monocytes,[307] substrate recognition by saccharide-digesting enzymes,[308] or the effect of clustering of glycosidic units.[309]

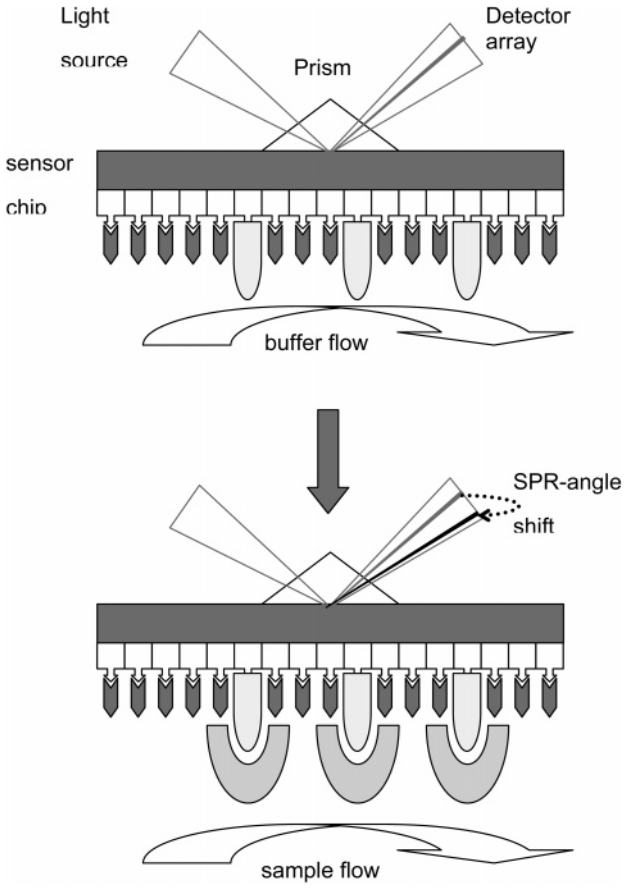

Figure 12. Schematic representation of a SPR biosensor. The ligand is immobilized on the sensor surface (above). When the analyte in a sample solution binds to the ligand, the refraction index of the surface is modified, resulting in a shift of the angle of refracted light.

SPR biosensor chips have been developed for capture and detection of vesicles containing glycolipids and other membrane-bound carbohydrate receptors.[163] This application required initial physical immobilization of the carbohydrate-containing vesicles, followed by addition of the complementary receptors, which were detected by the SPR signal. The method was demonstrated for detection of CT through vesicle-incorporated GM1 ganglioside.[163] This extension of the SPR technique is important because numerous carbohydrate species are bound or displayed on cell surfaces through hydrophobic residues. Such applications, however, necessitate efficient immobilization of the vesicles without destroying them, which could pose technical difficulties.

SPR-based biosensors have been used for detection of carbohydrate derivatives, such as glyocolipids and glycoproteins. The envelope glycoprotein gp41 of HIV was employed for studying antibody binding to the glycoprotein and the effect of serum on the recognition process.[310] Binding and kinetic profiles of the interaction between platelet glycoproteins and fibrinogen were evaluated by application of SPR.[311] Related experiments examined the occurrence and kinetic properties of biochemical reactions involving

carbohydrates, for example, the *in situ* analysis of dextran monolayer degradation by dextranase.[312]

Carbohydrate−lectin interactions have been frequently employed as a basis for SPR biosensor applications. These approaches were aided by the broad knowledge base regarding the pool of saccharide ligands attracted to various lectins. Examples of such SPR applications include steady-state and kinetic analyses of lectin binding to oligosaccharides and glycopeptides,[115,313] analysis of lectin interactions with C and O glycosides linked to a carboxymethyl dextran layer on the SPR sensor surface,[314] carbohydrate-binding activity and specificity of a lectin extracted from bulbs of spring crocus,[315] structural basis for the unusual carbohydrate-binding specificity of jacalin, the seed lectin from jack fruit (*Artocarpus integrifolia*),[316] and others. Lectin−glycolipid binding formed the basis of surface immobilization procedures in miniaturized SPR sensors.[317]

SPR was used for detection of carbohydrates in physiological solutions at high sensitivities.[318] A SPR sensor for heparin featured a surface that was coated either with protamine or polyethylene imine. Importantly, the degrees of heparin affinities were dependent upon the receptor species coating the surface in each case. The sensor performance was also found to be affected by incubation time, heparin dilution, and the presence of other components in the analyte solution. Nonspecific adsorption had to be additionally overcome by optimization of the experimental conditions, overall indicating that the intrinsic high sensitivity of the SPR technique could also pose problems for carbohydrate-binding analysis. Aside from detection of carbohydrates in varied solution environments, heparin and its derivatives have been employed as recognition elements in SPR biosensors. SPR was used to evaluate heparin binding to chemokines, a process believed to be central to chemokine functionality.[319] The relative degree of avidin binding to heparin and its derivatives was also evaluated using SPR biosensors.[320] Heparin and heparan sulfate were used as substrates for studying membrane interactions and host entry of HSV.[321] Another application employed heparin-modified gold surfaces for analysis of low-density lipoproteins (LDL).[322]

SPR has been additionally used for determination of glycosylation changes in proteins. SPR analysis of glycoproteins has been generally achieved through immobilization of the proteins on the sensor surface by using antibodies and identification of carbohydrate epitopes through binding of specific lectins.[323] It was also reported that modifications of the affinity between the biosensor-immobilized proteins in the cell-culture supernatant and added lectins allowed analysis of glycoprotein concentrations and changes in protein glycosylation.[323]

Evaluation of protein binding to surface-immobilized LPS was carried out using specially designed SPR biosensors, because of the importance of LPS constituents in affecting protein binding to varied cell surfaces.[324,325] Biosensor chip surfaces derivatized with different quantities of LPS were used for determination of peptide- and protein-binding constants.[326] Immobilization of LPS in these sensor chips was achieved thorough attachment of the biotinylated saccharide molecules to streptavidin-coated sensor surfaces. The protein affinities to LPS in such assays were evaluated through the changes in mass close to the sensor surface.

While most oligosaccharide immobilization techniques have been based on the avidin−biotin high-affinity system, other methods were reported. Catimel et al. described antibody detection by SPR, which was carried out through direct immobilization of gangliosides onto the sensor surface by hydrophobic interactions.[327] The advantage of this type of approach was the forestalling of chemical derivatization of the saccharide molecules or of the sensor surface, leading to simplification of biosensor construction. In a different modification of the SPR sensor chip, complete vesicles containing ganglioside GM1 were surface-immobilized, deriving affinity and kinetic information upon binding of CT.[163]

Other chemical methods were introduced to immobilize and display carbohydrates and glycoconjugates on SPR biosensor surfaces. Stein et al. reported on modifying a carboxymethyldextran surface to couple the lipid-anchored contact site A (csA) of a homophilic adhesion glycoprotein of the bacterium *Dictyostelium discoideum*.[328] The carboxy groups in the derivatized layer were modified to enable hydrophobic binding of the glycoprotein via its lipid anchor to the dextran matrix. Alternatively, the researchers employed covalent binding through a perfluorophenylazide-derived hydrophobic cross linker. Titration experiments verified that the bound csA molecules reacted with antibodies that recognize either the native or the denatured glycoprotein; thus, they most likely adopt a native state in the sensor surface environments.[328]

In addition to detection of carbohydrate-binding species, SPR has been used for studying various parameters contributing to such interactions. SPR has been applied, for example, to assess modulation by pH, divalent cations, and polyamines on the high-affinity binding of antibodies to polysialic acid (PSA) expressed on the vertebrate neural cell adhesion molecule (NCAM).[329] In such experiments, the sensitivity of the optical signal generated by the sensor response facilitated identification of slight changes in the binding events.

4. Concluding Remarks

The increasing awareness of the biological importance of oligosaccharide derivatives and growing interest in glycobiology applications have clearly become a major driving force toward development of new techniques for carbohydrate characterization. This review summarized the large body of recent experimental work dedicated to construction of biosensors and bioassays designed to detect and analyze carbohydrates and glycoconjugates, and sensors utilizing carbohydrates for detection of other soluble biomolecules.

The complexity and high variability of carbohydrate structures have often placed formidable barriers toward their practical applications; however, these properties might as well open new avenues to

biosensor applications specifically based on the differences among carbohydrate groups and their biological expression. Varied carbohydrate biosensor designs have been based on the molecular recognition and specific binding encountered between polysaccharides and other macromolecules, particularly proteins. Such molecular interactions, including carbohydrate−lectin, carbohydrate−toxin, or saccharide−enzyme affinities, play significant roles in diverse biosensor devices and bioassays, either those aiming to detect and/or analyze oligosaccharides or others that rely on embedded carbohydrates for detection of other biomolecules.

The diverse and proliferating literature on carbohydrate biosensors points to promising directions for future progress in the field. The increased synthetic capabilities and sophisticated biochemical techniques aiming to interfere with biosynthetic and cellular pathways responsible for carbohydrate production could have a major impact in the design of novel cellular-based biosensors, in the same way that genetic engineering has revolutionized genetic analysis and screening. Chemical routes for fine tuning the selectivity and targeting in biosensor design are another high-potential direction. Overall, understanding and harnessing the intrinsic complexity of carbohydrate structures is the underlying factor for development and utilization of oligosaccharide biosensor designs and applications.

5. Abbreviations

AFM	atomic force microscopy
b-SiA	bound sialic acid
CT	cholera toxin
con A	concanavalin A
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FPA	fluorescent polarization assay
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
HSV	herpes simplex virus
HPRG	histidine−proline-rich glycoprotein
HIV	human immunodeficiency virus
ISEFT	ion selective field effect transistor
LPS	lipopolysaccharides
MS	mass spectrometry
mAb	monoclonal antibody
PET	photoinduced energy transfer
PPD	polarized photometric detection
PDA	polydiacetylene
QCM	quartz crystal microbalance
SLeA	sialylated Lewis antigen
SPR	surface plasmon resonance
TFA	trifluoroacetic acid

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