Glyco-biosensors: Recent advances and applications for the detection of free and bound carbohydrates

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The field of biosensor development now encompasses several areas specifically geared toward the rapid and sensitive detection, identification, and quantification of target analytes. In contrast to the more mature research and development of nucleic acid and protein biosensors, the development of 'glyco-biosensors' for detecting carbohydrates and conjugates of carbohydrates (glycoconjugates) is at a relatively nascent stage. The application of glyco-biosensors aims to open novel analytical and diagnostic avenues, encompassing industrial bioprocesses, biomedical and clinical applications. This area of research has been greatly aided by advancement brought by interdisciplinary mergers of engineering, biology, chemistry and physical sciences and enabling the miniaturization of detection platforms. In this review, we briefly introduce the need for glyco-biosensors, discuss current analytical technologies, and examine advances in glyco-biosensor approaches aimed at the detection and/or quantification of glycoconjugates or carbohydrates derived from glycoconjugates since 2005.

Introduction

The biosensor field has, until recently, focused primarily on detecting nucleic acids, proteins and metabolites. This focus was appropriate because of the wealth of information validating the biomarker status of many of these classes of molecules for a variety of disease states and biological events. However, the original idea that genes and gene products (proteins) are the sole mechanisms responsible for phenotype has been slowly overturned by the discovery of many discrete roles for post-translational modifications (PTMs) of proteins which convey another layer of significant functional information.[1] At least 200 different forms of PTMs have been found on proteins, with glycosylation being one of the most abundant and important.[2–5]

Glycosylation involves the covalent attachment of carbohydrates (glycans) to specific amino acids within polypeptide chains of proteins during and after translation. Generally, carbohydrate components of glycoproteins fall into two main classes, *N*-linked oligosaccharides, which are attached to asparagine residues within the Asn-X-Ser/Thr motif where X is not proline, and *O*-linked oligosaccharides attached to serine or threonine residues (Fig. 1). Similar carbohydrate structures are also found attached to additional classes of molecules including lipids and metabolites. Unlike DNA replication, RNA transcription and protein translation processes, glycosylation is not

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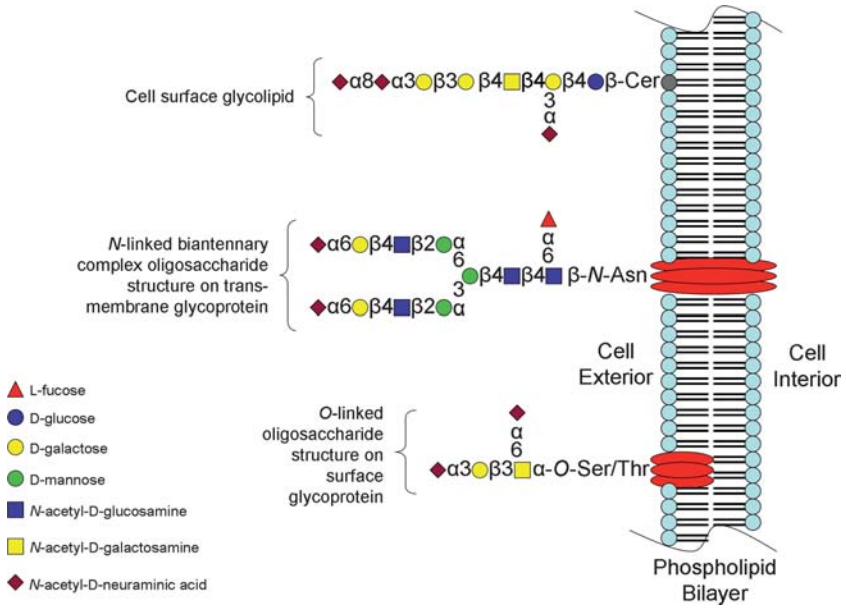

Fig. 1 Oligosaccharides are covalently-linked to glycoproteins and glycolipids attached to the cell membrane (depicted here) as well as to glycolipids of intra-cellular vesicles and secreted glycoproteins.

a template-dependent process. Instead, glycosylation and associated processes are indirectly controlled by the genome. Glycosylation modifications are the result of the activity of over 170 enzymes and other molecules in the cell.[6] Consequently, the carbohydrate structures linked to proteins, lipids and metabolites are indicative of the cellular microenvironment and its physiological state. Furthermore, the types of carbohydrate structures present as well as the extent of glycosylation on glycoconjugates can also vary in different organisms, tissues, cells, developmental and disease states. Considering that approximately half of all mammalian proteins and 80% of membrane proteins are believed to be glycosylated,[7,8] glycoconjugates and their carbohydrate components have enormous potential as diagnostic targets.

Carbohydrate-mediated interactions play a vital role in many physiological processes including fertilization, immune defense, cellular differentiation, cell–matrix interaction, cell–cell adhesion, and are known to be altered in disease states, such as viral replication, parasitic and pathogenic infection, cancer, and cardiovascular and neurological disorders. It has been estimated that human glycoproteins and glycolipids carry of the order of 3,000 distinct oligosaccharide structures, or glycan determinants, which are involved in binding to specific binding proteins.[9] Glyco-biosensors that enable the rapid detection of physiologically relevant glycosylation events (including these glycan determinants) with high sensitivity and specificity would be powerful additional tools for biological and clinical

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investigations, for early-stage disease diagnosis, and for prognostic monitoring during treatment.[10]

Biosensors for glycoconjugates will find application within the biopharmaceutical industry for on-line monitoring of biotherapeutic production and subsequent quality assurance.[11,12] The glycosylation profile of recombinant therapeutic glycoproteins is very important, affecting serum half-life, pharmacokinetic, pharmacodynamic, and immunogenic properties.[8,13] Correct and complete glycosylation, especially with respect to the type and distribution of sialic acids at the terminal positions of carbohydrate chains, is one of the most important benchmarks for effective recombinant protein drugs.[13] Rapid at-line or on-line estimation of the glycosylation status during bioreactor production of biopharmaceutical glycoconjugates would facilitate in-process decision making, improve product quality and have considerable economic impact.

Here, we review recent developments in the glyco-biosensor field, discuss promising approaches which have been reported, and identify some of the challenges ahead. We will focus on methods for analysis of the glycan moieties on or derived from glycoconjugates and exclude the analysis of free glucose. Glucose biosensors are the major success story of the biosensor field and have been extensively reviewed elsewhere.[14,15]

Background – Current methods of glycoconjugate analysis

Analysis of the carbohydrate component of glycoconjugates most commonly involves chemical and/or enzymatic release of the carbohydrates followed by application of liquid chromatography (LC), mass spectrometry (MS) or nuclear magnetic resonance (NMR) techniques, either alone or in combination, to determine the structures present. These well-established methods are the most powerful tools available for the structural analysis of glycoconjugates and are widely used in research settings.[5,16] However, they have serious limitations in point-of-care (POC) and other diagnostic settings (clinical, industrial or environmental), with respect to time, throughput, sample size, sample preparation, capital investment and requirement for specialised technical expertise. In situations where the purpose of the carbohydrate analysis is disease diagnosis, detection of infectious organisms or quality control of biomanufacturing samples, complete structural details are often not necessary. Instead, accurate determination of the terminal sugar residue(s) and their linkage arrangements is sufficient. This information can be obtained through the combined use of exo-glycosidases that clip specific sugars with specific linkages from the ends of the oligosaccharide chains and specific lectin binding assays. Specific binding assays (*e.g.* ELISA and variants) are widely used for analysis of glycoprotein terminal carbohydrate structures in biological samples. As a means to provide additional information, multiplexing of affinity-based assays is another possibility. Binding assays are unrivalled in terms of convenience and speed for measurements of biological molecules in complex samples and form the basis of most biosensors described for carbohydrates and glycoconjugates.

Glyco-biosensors – Technologies and current status

Biosensors are devices that use a biological recognition element to detect the target analyte and a transducer to convert the binding event into a quantifiable signal. While the term biosensor was often originally applied to large and expensive instrumentation, the current trend is towards relatively inexpensive point of care applications, with simplicity, compactness and portability being key attributes. Biosensors are classified on the basis of the biological signaling mechanism involved – enzyme activity or binding event[17,18] – or on the mechanism by which the signal is recorded – photodiodes measuring production or transmission of light, electrochemical sensors measuring current or voltage, piezoelectric sensors measuring mass or pressure, *etc.*[19–27]

A very comprehensive review of biosensor and biochemical technologies described for analysis of carbohydrate and involving carbohydrates as essential components was published in 2004.[28] This covered a broad range of approaches for analysis of carbohydrates and their interactions with biological systems, some of which, such as MS and NMR, still require a level of expertise and physical equipment prohibitive in terms of cost, ease of use and portability. Other glyco-analytical technologies reviewed, such as optical arrays and SPR, have seen marked advances in recent years leading resulting in easier to use, physically smaller instrumentation, which is potentially amenable to use outside of a traditional laboratory. The focus of this review is on those technologies that are poised to continue to evolve into fully self-contained, compact, portable devices for glyco-biosensor applications.

Carbohydrate biorecognition agents

Biorecognition agents used in biosensors generally fall into two classes, enzymes and binding or affinity agents. In the former, the activity of the enzyme on the analyte leads to an increase or decrease in concentration of some reactive species that can be measured. Apart from glucose biosensors, which depend on the activity of glucose oxidase, there have been very few developments reported of enzyme-based biosensors for carbohydrates found in glycoconjugates. A biosensor for sialic acid, based on sialic acid aldolase activity in conjunction with pyruvate oxidase and amperometric detection, was described. This sensor determined free sialic acid within 10 μM to 3.5 mM and, with addition of an appropriate sialidase, could be used to determine glycoconjugate-bound sialic acid levels.[29]

Lectins are by far the most widely used biorecognition agent for carbohydrates. Lectins are a group of highly diverse carbohydrate-binding proteins of non-immune origin which recognise and reversibly bind to specific free carbohydrates and those present on glycoconjugates without altering the structure of the carbohydrates or conjugated molecules.[30] They are found in most organisms, but plant and non-vertebrate lectins are most commonly used in glycobiological analysis. They have been used extensively for tissue staining (lectin histochemistry), lectin blotting, and in the lectin-based equivalents of the immunoassay, the enzyme-linked lectin assay (ELLA) or fluorescent-labeled lectin assay (FLLA). Over 500 natural lectins have been described.[30] A list of representative lectins and their binding preferences is provided in Table 1. Although some lectins can

Table 1 A representative list of characterized lectins useful for glyco-biosensor development

Abbreviation	Origin	Species	Common name	Major specificity
AAA	Animal	*Anguilla anguilla*	Fresh water eel lectin	α-Fuc
HPA	Animal	*Helix pomatia*	Garden snail lectin	α-GalNAc
LMA	Animal	*Limax flavus*	Slug lectin	Sialic acid
LPA	Animal	*Limulus polyphemus*	Horseshoe crab lectin	Sialic acid
ABL	Fungi	*Agaricus bisporus*	Edible mushroom lectin	Gal-β(1,3)-GalNAc, GlcNAc
Abrin	Plant	*Abrus precatorius*	Abrin, Jequirity bean lectin	Gal
AIA, Jacalin	Plant	*Artocarpus integrifolia*	Jack fruit lectin	Gal
APA	Plant	*Allium porrum*	Leek agglutinin	Man
BPA	Plant	*Bauhinia purpurea*	Camels foot tree lectin	GalNAc/Gal
CHA	Plant	*Cymbidium hybrid*	Orchid lectin	Man
ConA	Plant	*Canavalia ensiformis*	Jack bean lectin	Man, Glc, GlcNAc
DBA	Plant	*Dolichos biflorus*	Horse gram lectin	GalNAc
DSA	Plant	*Datura stramonium*	Jimson weed lectin	GlcNAc
ECA	Plant	*Erythrina cristagalli*	Cocks comb/coral tree lectin	Gal-β-(1, 4)-GlcNAc oligomers
EHA	Plant	*Epipactis helleborine*	Broad-leaved helleborine lectin	Man
Favin	Plant	*Vicia faba*	Broad bean lectin	Man, Glc
GNA	Plant	*Galanthus nivalis*	Snowdrop lectin	Man-α(1,3)-
GSL-I-B4	Plant	*Griffonia simplicifolia* (*aka Bandeiraea simplicifolia*)	Griffonia/Bandeiraea lectin-I	α-Gal
GSL-II	Plant	*Griffonia simplicifolia* (*aka Bandeiraea simplicifolia*)	Griffonia/Bandeiraea lectin-II	GlcNAc
HBA	Plant	*Hevea brasiliensis*	Rubber tree lectin	GlcNAc
HHA	Plant	*Hippeastrum hybrid*	Amaryllis agglutinin	Man-α(1,3)-Man-α(1,6)-
LCA	Plant	*Lens culinaris*	Lentil lectin	Man, Glc,GlcNAc
LOA	Plant	*Listera ovata*	Twayblade lectin	Man-α-(1,3)-
LTA	Plant	*Lotus tetragonolobus*	Lotus lectin	α-Fuc
MAA	Plant	*Maackia amurensis*	Maackia agglutinin	Sialic acid-α(2,3)-
MHA	Plant	*Myrianthus holstii*	Myrianthin	GlcNAc
ML-1	Plant	*Viscum album*	Mistletoe lectin	Gal/GalNAc
MPA	Plant	*Maclura pomifera*	Osage orange lectin	α-Gal
None	Plant	*Lathyrus odaratus*	Sweet pea lectin	Man, Glc, GlcNAc
NPA	Plant	*Narcissus pseudonarcissus*	Daffodil lectin	α(1,6)Man
PHA-E	Plant	*Phaseolus vulgaris*	Kidney bean erythroagglutinin	biantennary, bisecting GlcNAc,βGal/Gal-β(1,4)GlcNAc
PHA-L	Plant	*Phaseolus vulgaris*	Kidney bean leukoagglutinin	tri-tetraantennary βGal/Gal-β(1,4)-GlcNAc
PNA	Plant	*Arachis hypogaea*	Peanut lectin	Gal-β(1,3)-GalNAc
PSA	Plant	*Pisum sativum*	Pea lectin	Man, Fuc dependent
RCA	Plant	*Ricinus communis*	Castor bean lectin	Gal
RPbAI	Plant	*Robinia pseudoacacia*	Black locust lectin	Gal, GalNAc
SBA	Plant	*Glycine max*	Soy bean lectin	GalNAc
SJA	Plant	*Sophora japonica*	Pagoda tree lectin	βGalNAc
SNA-I	Plant	*Sambucus nigra*	Sambucus lectin-I	Sialic acid-α(2,6)-
SNA-II	Plant	*Sambucus nigra*	Sambucus lectin-II	Gal/GalNAc
STA	Plant	*Solanum tuberosum*	Potato lectin	GlcNAc oligomers
UDA	Plant	*Urtica dioica*	Stinging nettle lectin	GlcNAc oligomers
UEA-I	Plant	*Ulex europaeus*	Gorse lectin-I	α-Fuc
UEA-II	Plant	*Ulex europaeus*	Gorse lectin-II	Chitobiose
VVA-B4	Plant	*Vicia villosa*	Hairy vetch lectin	GalNAc
WFA	Plant	*Wisteria floribunda*	Japanese wisteria lectin	GalNAc/Sulfated GalNAc
WGA	Plant	*Triticum vulgaris*	Wheat germ agglutinin	NeuAc/GlcNAc

discriminate clearly between different structures, lectins tend to have broad specificities towards related carbohydrate structures. For this reason, lectins are routinely used in complementary combinations which include multiple lectins reported to have affinity for a single saccharide or class of saccharides.[31] Interaction of individual carbohydrates with lectins is characterised by relatively weak affinity binding of a single carbohydrate structure to the lectin receptor, but the binding is strongly enhanced by the multiple presentations of carbohydrates that occur in most of their biological targets.[32–34] Commercial lectins come from a variety of sources, and vary in their purity and properties depending on the source and vendor. To overcome this challenge, the expression of some lectins in prokaryotic recombinant systems has been described[35] and recombinant strategies have been used to tailor or enhance their binding characteristics by engineering.[36]

While antibodies are the primary binding agents used for analysis of proteins and many small molecules, anti-carbohydrate antibodies are much less widely used with none mentioned in glyco-biosensor reports in the last 5 years. This is because carbohydrates are generally accepted to be poor immunogens and resulting anti-carbohydrate antibodies to have low affinities. However, in a recent carbohydrate microarray survey of the binding specificity of 27 anti-carbohydrate antibodies currently in use, at least 10 showed high specificity towards their target, some with very high titres indicating probable high affinity

interactions as well.[37] These antibodies would be suitable candidates for use in biosensor platforms. Interestingly, despite the cross-reactivity displayed by the remainder of the antibodies surveyed, the antibodies were still found to be far more selective than most lectins.

The advent of recombinant antibody technology and phage display techniques holds considerable promise for the development of novel anti-carbohydrate antibodies. Naïve antibody single chain variable fragment (scFv) phage display libraries provide access to the entire repertoire of possible antibody specificities from any organism, bypassing the immunisation step, and further optimisation of selected scFvs is possible by expansion of the libraries using combinatorial approaches. Anti-carbohydrate antibodies generated using phage display include those targeted against non-reducing terminal mannose residues[38] the Tn antigen[39] and an scFv that recognises 3 consecutive Tn antigens[40] with a reported K_d of 10^{-7} M, which is high for carbohydrate-specific monovalent antibodies. A specially designed Fab (antigen binding region of antibody) library rich in antibodies that bind charged carbohydrates has also been described and has been used to generate binders for heparan sulfate and sulfated sialyl-Lewis X.[41] Use of genetic engineering approaches to generate an improved antibody directed against the blood group antigen, Lewis Y, in terms of both specificity and affinity has also been reported.[42]

Alternative sources of carbohydrate-binding agents include the carbohydrate-binding domains of carbohydrate-active enzymes. Here again, phage display technology enabled the convenient generation and screening of libraries of related sequences for the desired binding characteristics.[43,44] Phage display libraries of random peptides have also provided some carbohydrate binders. Peptides specifically binding to sialylgalactose were isolated from a random 15-mer library with micromolar affinity.[45] A more constrained peptide phage-displayed library using a helix-loop-helix approach was developed geared specifically for the identification of carbohydrate-binding peptides.[46] Peptides specifically binding the ganglioside, GM1, were isolated from this library having nanomolar affinities. Aptamers, RNA or DNA oligonucleotides that can specifically recognise almost any class of molecule, are another emerging class of specific binding molecules. Aptamers targeted against carbohydrates have been described, including *N*-acetylgalactosamine (GalNAc) or the serinyl- or threoninyl-linked monosaccharide (Tn antigen),[47] the oligosaccharide component on glycosylated fibrinogen,[48] and cellulose.[49] As well as being promising new sources of carbohydrate-binders or lectin mimics, phage display and aptamer technologies have also been used to generate carbohydrate mimics, lending themselves to use as suitable alternatives to expensive reagents in glyco-biosensor development.[50]

In addition to expansion of the carbohydrate affinity agents of biological origin, research is also being conducted to develop semi- and fully-synthetic agents which specifically recognise carbohydrate structures. To date, boron containing compounds have been used to enrich carbohydrate-containing molecules from larger pools through ester formation at diols present on component monosaccharides.[51,52] While these boronocompounds have been shown effective for the wholesale enrichment of carbohydrate-containing molecules, demonstration of highly selective identification of particular mono- or oligosaccharide species has not yet been reported. In efforts to improve this situation, Li and colleagues have combined the SELEX method with boron-enhanced DNA oligonucleotides to identify aptamers which bind selectively bind to glycoproteins.[48] In another strategy, a 'molecular tweezer' approach has been devised which incorporates two boronic acid groups reported to have highest stability when bound to fructose.[53] Very recently, a synthetic benzoboroxole-modified peptide structure was reported to selectively bind the tumour-associated T antigen (Gal-β-(1→3)-GalNAc).[54] Some success has been reported for selective recognition of carbohydrates presenting exclusively equatorial hydroxylation (*e.g.* glucose and substituted glucose species such as *N*-acetylglucosamine) through the use of lectin mimics not incorporating boron.[55–57] These synthetic lectins are designed around a 'temple' chemical architecture which is modeled on the hydrodynamic properties of natural glucose-binding carbohydrate recognition domains.[58] These approaches hint at the promise of new tools which may find use in future glyco-biosensor application.

Glyco-biosensor detection platforms

Lectin arrays

The application of microarray technology for rapid analysis of glycoconjugates and free glycans represents the most significant advance in the glyco-biosensor field in recent years. Microarray platforms have been employed in the investigation of binding interactions among nucleic acids and proteins for over a decade. These platforms were introduced for the interrogation of glycan-lectin binding events in 2002[59] and have seen more intensive use since 2005. The primary advantages of the array-based approach are that multiple interactions can be tested simultaneously with only minimal sample quantities required. Multiplex binding analysis is particularly important for deciphering the complex carbohydrate structures found in glycoconjugates, the full extent of which cannot be described by one or a small number of binding events. Instead, the pattern of binding to a series of binding agents with different binding specificities represents a profile, which is characteristic of a particular combination of linear and branched oligosaccharide structures present in the sample and allows comparisons between samples. Lectin microarrays are the most common form of array used for analysis of carbohydrate expression on purified glycoproteins,[60,61] glycoprotein pools,[62] bacterial and mammalian cell surfaces[63–65] and tissue sections,[66,67] with the number of individual lectins used ranging from approximately 20 up to approximately 100. Lectin microarrays are already commercially available under the Qproteome™ GlycoArray name and, in the case of the Tamm-Horsfall (Uromodulin) glycoprotein isolated from urine, the consistency of the resulting carbohydrate fingerprint with these arrays has been shown to be consistent with the known carbohydrate structures elucidated through more detailed analyses.[61] Lectins have also been used in conjunction with antibody arrays in a 'sandwich' format to examine the variation in carbohydrate structures on specific proteins.

For production of lectin microarrays, the lectins are either passively adsorbed to the surface or more frequently covalently linked to glass substrates using common microarray conjugation chemistries (*e.g.* EDC/NHS, epoxides, aldehydes). To retain maximal binding activity of the lectin and achieve good spot characteristics, it is important that the binding site is occupied during immobilization.[65] The coating density of lectins has been shown to affect the binding characteristics of multivalent carbohydrate dendrimers.[68] Appropriate inter-lectin distances were thought to encourage multivalent interactions. However, lectin coating concentrations are most often standardised at 0.5 to 1.0 mg/mL, or at concentrations that produce a specified signal in the assay. Lectins have also been immobilised in hydrogel matrices to simulate solution phase conditions, although care would have to be taken to ensure the carbohydrate nature of the hydrogel did not interfere with the binding activity of specific lectins.[69]

Fluorescence detection is the most common technology used in lectin microarray analysis, making use of the scanners and data acquisition programmes developed for nucleic acid and protein microarrays. The fluorophore is usually conjugated to the analyte directly or, for two-step protocols such as the antibody-lectin sandwich approach, the lectin is labeled. Where two heterogenous samples are to be compared, a ratiometric method was described, involving labeling of test and control samples with different fluorescent probes.[70] This approach should allow for variations in efficiency and consistency of labeling and improve quality control of lectin array experiments. An enhanced evanescent-field fluorescence detection method has been applied to a number of sample types.[63,71–74] Using an electromagnetic wave, termed the evanescent field, fluorophores are excited within a 100–200 nm range of the sensor surface.[71] The result is excitation of only fluorescently-tagged glycoconjugates which are bound to the lectins. This approach has the advantage that it eliminates the need for washing steps after application of the labeled analyte and therefore can detect even the very low affinity interactions that occur between lectins and their ligands. Sensitivity to below 1 nM (approx 50 ng/mL) has been reported.[73] Because washing is not necessary, there is also the possibility of real-time monitoring of binding events with this detection method. An interesting approach to reduce the volume of sample needed for a microarray experiment involved the use of a piezoelectric array printer, similar to that used for initial printing of lectins on the array, for application of labeled analyte instead of flooding the array with sample.[60] Sample volumes of less than 1 μl gave equivalent sensitivity to much greater volumes (100–450 μl).

A novel technique to work around labeling analytes directly is a hybrid of the non-covalently assembled lectin array in hydrogel matrix and competitive binding called bimolecular fluorescence quenching and recovery (BFQR).[69] Fluorescein-labeled lectins are incorporated into the hydrogel and specific ligands, coupled to dabcylic acid, which acts as a fluorescence quencher for fluorescein, are allowed to bind with their respective lectins. The test sample is incubated with the quenched lectin array to displace the quenching molecules. Positive binding events between analytes and lectins are measured as recovered fluorescence at each lectin position. A detection limit of 10 μM is claimed for a tetra-mannosyl structure binding to the lectin concanavalin A (ConA). This technique could be used with a small number of lectins displaying a distinctive profile for the target analyte, greatly simplifying the approach. A related approach was described for development of an assay for monitoring of maltose concentration in oat drinks. The assay involved Fluorescence/Förster resonance energy transfer (FRET), with the cyanine dyes Cy5 and Cy5.5 as donor acceptor pair. One dye was attached to an anti-maltose antibody and the other to BSA-maltotriitol neoglycoconjugate. The maltose analyte competed with the labeled maltose for binding to the antibody, which was associated with an increase in the fluorescence intensity ratio. The use of the assay for continuous monitoring of maltose concentration was made possible when both labeled components were retained within a semi-permeable capsule. Although only demonstrated for analysis of a single binding event, it is likely that the FRET phenomenon could also be applied to array-based assays.[75]

With regard to point-of-care applications of lectin array biosensors, the optical equipment required to 'read' microarrays is generally not simple to use nor compact enough for portability. While the majority of current research utilizing arrays relies on full-size diode laser slide scanners, advances in photonics are promising the miniaturization of these technologies for true portability.[76,77] The PortArray™ 5000 Microarray Reader is one such device.

Optical biosensors

As the current most popular tool for monitoring biological interactions, surface plasmon resonance (SPR) has been applied to the detection and measurement of lectin-glycoconjugate interactions, mainly for single binding events but more recently with the introduction of SPR imaging technology for array-based interaction analysis. The main advantage of SPR technology is that it enables real-time evaluation of interactions between biomolecules immobilised on the sensor chip surface and unlabelled solution phase molecules. Typical protocols involve the immobilization of the lectin on the sensor chip and capture of the glycoprotein analyte carrying the lectin-appropriate oligosaccharide structure. Alternatively, the glycoprotein analyte is captured by an immobilised antibody and its glycosylation then probed for interaction with different lectins.[78]

The signal generated in SPR biosensors is proportional to the mass of analyte bound onto the surface. Therefore, traditional SPR has limitations with respect to sensitivity towards lower molecular mass compounds, such as sugars.[79] As a result, recent efforts have been directed towards improvement of the sensitivity achievable with SPR for analysis of free sugars. Foley *et al.*[80] used a high-resolution method of differential SPR scanning, based on use of a quadrant photodiode and a control well to remove effects of refractive index change in the bulk solution and other noise effects, to detect binding of disaccharides to peanut agglutinin (PNA) immobilised on the sensor surface indirectly through anti-PNA antibodies.[80] The study demonstrated detection of the MW 383 Da tumor associated carbohydrate antigen (TACA), Gal-β-(1 → 3)-GalNAc (T-antigen), at concentrations down to 50 nM with no significant interference from the closely-related disaccharide, LacNAc (Gal-β-(1 → 4)-GlcNAc) up to concentrations exceeding 500 nM. The resolution of the system was also sufficient to allow kinetic parameters to be established.

The dissociation constant (K_d) calculated for LacNAc was 2–6 μM and it was 15–29 nM for the target disaccharide T-antigen, demonstrating the very high affinity of PNA for this disaccharide. Interestingly, the binding of sugars was associated with a decrease in response units rather than the more usual increase due apparently to the lower refractive index of sugar solutions compared to proteins.

A competitive approach using gold nanoparticles (20 nm diameter) coated with the target sugar as tracer was also shown to increase the sensitivity of SPR to low molecular weight sugars. Displacement of nanoparticle-labelled lactose bound onto immobilised *Ricinus communis* agglutinin (RCA_{120}) by free galactose enabled quantitative assay of galactose within the range 0.1–50 ppm.[81] This competitive approach has been used widely for SPR-based assays for small molecules.[82] The direct labeling of the low molecular weight glycan targets with organoplatinum (II) complexes was suggested in another study to increased signal.[83] Increased detection was reported but the need to label the analyte negates the major advantage of SPR as a label-free analytical platform. Also, affinity constant determinations were not possible using these labels. This method could possibly be used in competitive assay format. Four-fold improvements in sensitivity towards a glycoprotein analyte have also been noted when the sensor surface composition is changed from the usual gold/chromium to, for example, gold/zinc oxide.[84] The reported assay was based on an antibody directed towards the protein component of the glycoprotein but the same enhancement should apply to all binding pairs.

A recent focus within the SPR field has been the development of integrated, low cost SPR biosensors, which can meet the challenges of equal or improved sensitivity over existing instrumentation without compromising robustness. Progress in this field up to 2007 was reviewed by Hoa *et al.*[85] and is continuing, although no applications of these new approaches for glycoconjugate analysis have been reported. Multiplexing of SPR measurements is now also a possibility using SPR imaging (SPRi) technology.[67] Label-free binding of lectins to arrays of up to 40 different carbohydrates was monitored simultaneously using SPRi[86] confirming the suitability of this technology for monitoring interactions between lectins and glycans. Fluorescence however remains the most sensitive biosensing technique described for array-based glycan-lectin interactions to date.

Mechanical biosensors

Label-free acoustic biosensors detect changes in mass, elasticity, conductivity and dielectric properties from mechanical or electrical variations usually by using the piezoelectric effect. Quartz crystal microbalances (QCM) are the most common type of acoustic biosensor being used to study the interaction of biomolecules.[87–89] The resonant frequency of the QCM depends on the mass of material bound to the surface, allowing the real-time monitoring of adsorption/binding events. A review of QCM developments up to 2007 gives a number of examples of the application of this transducer technology in the analysis of carbohydrate-protein interactions.[90] Most frequently, the carbohydrate was immobilised on the sensor surface and attachment of the corresponding binding protein was studied because of the greater molecular mass of the latter. However, the study also reported evidence of a larger frequency change than expected when glucose bound to immobilised protein receptor, which was attributed to viscoelastic changes within the protein film.[91]

Since the 2007 review, the use of a polystyrene-coated quartz crystal microbalance for the determination of free mannose has been described.[88] Yeast mannans were immobilised on the crystal and, using a competitive format, mannose concentration was determined within the range 0.18 to 5.3 mM. Although not demonstrated, this approach could also be used for the determination of a mannose-containing glycoconjugate. The direct analysis of the glycoprotein (carboxypeptidase Y) was also demonstrated using Con A immobilised on the gold electrode of the quartz crystal.[87] An interesting application of QCM related to carbohydrates was its use in the panning of a phage-displayed random peptide library leading to the identification of a number of trimannoside-recognising peptide sequences.[92]

Determination of the bacterial content of water and food supplies is another area where simple, inexpensive and rapid biosensor methods would be especially welcome. A promising application of QCM, based on carbohydrate-lectin interactions, is its application for the rapid detection of bacteria. Use of an electrochemical QCM sensor with Con A immobilised on the surface allowed *E. coli* to be detected within the range 5.0×10^6 and 2.0×10^7 cfu per mL.[93] The detection limit dropped to 1×10^4 cfu per mL when free Con A was added to the bacterial suspension which led to an accumulation of Con A – *E. coli* conjugates in the form of multilayers at the electrode surface. Furthermore, the metabolic activity of the bound bacteria could also be monitored by amperometric measurement of hydrogen peroxide consumption by bacterial catalase on the gold-plated quartz crystal surface. Shen and colleagues[89] exploited the presence of both lectins and carbohydrates on the surface of bacteria to develop a more sensitive test for *E. coli*. The QCM sensor surface was coated with both Con A and mannose. Con A binds the O-antigen of the bacteria and mannose the mannose-binding lectin on their surface. Cooperative effects between the two binding events yielded an experimental detection limit of 7.5×10^2 cells per mL, a claimed four-fold increase in sensitivity over the mannose only sensor.[89] As far as the authors are aware, this detection limit is the lowest reported for protein-based binding assays for bacteria.

The related transducer technology, micro- and nano-electromechanical systems or cantilevers, have been well reviewed in recent years.[21,94–96] Compared to QCMs, they have potential for much greater sensitivity and are also suitable for simultaneous analysis of multiple binding events, because of their small size and ease of fabrication in array format. However, there are very few reports of the monitoring of binding events involving carbohydrates using this technology. The detection of HIV-1 envelope glycoprotein gp120 by microcantilever deflection was reported, although a sandwich immunoassay approach was used, which did not necessarily involve the carbohydrate moiety, and the detection limit was much higher (low μg/mL range) than the in vivo biologically relevant values.[97] This technology is still at an early stage of development and presents a number of challenges yet to be overcome, such as reproducibility, noise reduction, and optimisation of the coating procedure especially for cantilever arrays. It is the authors' opinion however, that this approach will

be seriously considered in the future as a platform for label-free, real-time lectin array studies.

Electrochemical biosensors

Early electrochemical biorecognition assays involved the labeling of one of the binding partners with an electroactive substance, or system that could generate an electroactive substance, *e.g.* an enzyme, following the molecular recognition event. While these early proof of concept approaches demonstrated that biosensors could be capable of achieving great sensitivity, substantial miniaturization, and inexpensive production, the need to label the analyte was and continues to be a considerable hindrance to realization of the potential of electrochemical methods in biosensor devices. As a result, recent trends have been directed towards the development of label-free electrochemical methods, including electrochemical impedance spectroscopy (EIS), originally used for characterization of all types of electrochemical sensors, and nanomaterial-based approaches.[98–101] While these technologies are still work in progress, there have been some reports of their application for the analysis of lectin-glycan interactions.

EIS was used to detect lectin-carbohydrate interactions on a chip based biosensor.[102] The plant lectins, SNA-I and PNA, were covalently attached to the gold sensor surface and impedimetric measurements demonstrated selective binding of the sialyl-T and T-antigen carbohydrate chains of sialyl- and asialo-glycoforms of bovine fetuin, respectively, with a sensitivity in the region of 10 pg/mL (150 fM). The related technique of square wave voltammetry is reported to show similar sensitivity but shorter analysis time than EIS.[103] A stripping voltammetry technique was used with metal nanoparticle enhancement and a competitive approach in a sensor for oligosaccharides.[104] PNA lectin was conjugated to a mixed self-assembled monolayer on the gold sensor chip surface. The assay involved competition between the target dissacharide, T-antigen, in the sample and monosachharide conjugated to CdS-nanoparticles. Following incubation, the remaining glyco-nanoparticles on the electrode were quantified by stripping voltammetry, giving a detection limit of approximately 0.1 μM. As well as being used for signal enhancement, nanoparticles have also been coated onto the electrode surface, so as to increase the surface area for immobilization of the lectin and, prior to detection of binding by EIS and voltammetry.[105]

Finally, in an interesting merging of technologies, energy transfer between lectin-coated quantum dots (QDs) immobilised on a glass slide and carbohydrate-coated gold nanoparticles (AuNPs), which leads to quenching of the photoluminescence of the QDs, was used in a competitive format for the detection of a glycoprotein. Displacement of the AuNPs by the glycoprotein increased photoluminescence in proportion to their concentration.[106]

Conclusion

It is clear that biosensor methods for detection of carbohydrates and their conjugates are closely following protein detection technologies and platforms. However, there are some specific considerations that remain to be addressed for glyco-biosensors. Firstly, the heterogeneity and wide diversity of glycan structures which may be attached to glycoconjugates and the relatively small amount available for analysis have presented significant analytical challenges to date.[10,70] Therefore, it is important that the analytical target, the specific glycan structure that needs to be monitored, is clearly defined, that the binding agent or agents used recognise the relevant components of this target and that there is an understanding of how binding is affected by other glycan components on the target analyte. Secondly, considerable efforts must be invested in and latest combinatorial technologies harnessed for the development of improved binding agents for carbohydrates, not only in terms of their purity, consistency and reproducibility, but also with regard to the analytical performance criteria of sensitivity and specificity. The relationship between the spatial arrangement of the sugar residues on the glycan chain of the glycoconjugate and the binding site conformation must also be understood. Finally, it seems most likely that multiple binding events and hence array technology will be necessary to generate clinically or industrially useful information on glycoconjugate structure. Hence, maturation of the glyco-biosensor field will be dependent on advances in array-based label-free detection technologies.

Summary

Although the glyco-biosensor approaches reviewed here have been shown to be successful only from a proof-of-concept perspective, advancing current technology platforms, creation of new platforms, along with automation and miniaturization, are promising to expand the future development of this area. As glycobiological information continues to be deconvoluted, the ability to tailor specific sensors to their end applications will become possible. There is a definite need for developing new effective analytical tools for glycobiology and advances in sensitivity, speed, simplicity, reliability and economy are paramount. Cost reduction will undoubtedly follow as these technologies are refined and adopted for a variety of settings. The availability of a suite of glyco-biosensors would bring about a seismic change in clinical analysis and point of care paradigms, in biopharmaceutical process monitoring and in basic biological/physiological investigations.

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