Glycoarrays—tools for determining protein–carbohydrate interactions and glycoenzyme specificity

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Carbohydrate arrays (glycoarrays) have recently emerged as a high-throughput tool for studying carbohydrate-binding proteins and carbohydrate-processing enzymes. A number of sophisticated array platforms that allow for qualitative and quantitative analysis of carbohydrate binding and modification on the array surface have been developed, including analysis by fluorescence spectroscopy, mass spectrometry and surface plasmon resonance spectroscopy. These platforms, together with examples of biologically-relevant applications are reviewed in this Feature Article.

Introduction

It is now widely accepted that carbohydrates are important partners in many biological recognition processes, and the characterisation of the "glycomes" (the set of carbohydrate–protein interactions) of cells, tissues and organisms has become one of the frontiers of post-genomic science. According to the SWISS-PROT protein database, more than 50% of all known proteins are estimated to be glycosylated.[1] In these glycoproteins, the carbohydrates participate in numerous biological events, such as protein folding, secretion and stability,[2,3] and biological functions, such as immunity[4] and cell signalling.[5,6] Many diseases have now been shown to be associated with abnormal glycosylation,[7] for example congenital disorders of glycosylation,[8,9] cancer,[10,11] diabetes[12] and neurodegenerative diseases.[13]

Currently, the major goals in glycomics are: firstly, the full characterisation of the set of carbohydrate structures present in a cell or organism, which is ultimately defined by the expression and specificity of carbohydrate-modifying enzymes, such as glycosyltransferases, glycosidases and transglycosidases. Secondly, these carbohydrates need to be tested individually against their carbohydrate-binding protein partners. Glycoscientists have, so far, relied heavily on complex time- and material-consuming biochemical tools to define protein–carbohydrate interactions, because the genetic tools successfully used in proteomics (such as knock-out mutants, iRNA, two hybrid systems and others) give limited information in glycomics. Array technology is particularly important in glycomics because it can dramatically increase the output of such biochemical data, and glycoarrays (arrays displaying carbohydrates) have found several applications (Fig. 1). Not only do arrays shorten the time for biochemical measurements, but glycoarrays also have the advantage of using less precious carbohydrate material because of the option of miniaturisation that is not possible in solution studies. Great strides have been made in the production of pure oligosaccharides and glycoconjugates, either by isolation from natural sources or by chemical[14–17] (including automated[18,19] and chemo-enzymatic[20–22]) synthesis. However,

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Josef Voglmeir graduated from the University of Applied Life Sciences in Vienna with a first class Diploma in Biotechnology. His interest in glycobiology began during his work as a full-time research assistant of Professor Iain B. Wilson on recombinant human and invertebrate glycosyltransferases in Vienna. He is currently performing research towards a PhD in biological chemistry under the supervision of Professor Flitsch. His main interest is in mammalian O-glycosylation and the role of post-translational modifications in hereditary diseases.

Sabine Flitsch graduated from University of Münster, Germany in 1982 with a first class Diploma in Chemistry. She received a DPhil from the University of Oxford under the supervision of Sir Jack E. Baldwin. She then obtained a DAAD Postdoctoral Fellowship at MIT to work with Professor H. Gobind Khorana until 1988, and has held academic positions at chemistry departments at the universities of Exeter, Oxford, Edinburgh and now Manchester, where she currently holds a Chair in Chemical Biology. Her research group is housed in the Manchester Interdisciplinary Biocentre (MIB) and has a strong focus in the glycosciences, in particular the involvement of enzymes, such as glycosyltransferases, in carbohydrate biosynthesis and their application in synthesis.

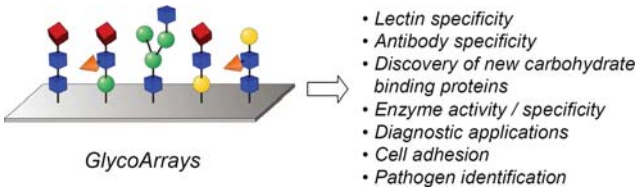


Fig. 1 Current applications for glycoarrays.

the quantities obtained are mostly in the order of milligrams and thus too small for extensive biochemical solution studies. On the other hand, glycoarrays only need very small amounts of material and thus provide a platform that, perhaps for the first time, allows the demands of glycobiologists for pure carbohydrates to be met by the capabilities of carbohydrate chemists.

Over recent years, the need for miniaturization and automated high throughput screening platforms has led to the development of several glycoarrays, onto which libraries of sugars are attached, either covalently or by physical adsorption. The display of sugar probes in an array format meets the requirements for multivalent presentation,[23–28] while the interrogation of such microarrays allows the fast, quantitative, systematic identification and characterisation of carbohydrate binding proteins (CBP), as well as glycan-processing enzymes (Fig. 1).[29–35]

Glycoarray fabrication

A number of different immobilisation strategies have been developed, which can be divided into two main classes, depending on the type of sugar immobilisation: by covalent attachment or by physical adsorption.

Covalent immobilization of carbohydrates on array surfaces

The chemical attachment of a glycan probe onto a surface requires its derivatization in a manner that is suitable for a chemical reaction with the functionalised solid support. Many strategies have been described so far, and these are listed in Table 1. Amongst them, amide bond formation between an amine-containing sugar and an activated ester-derivatized surface,[37–41] the use of a 1,3-dipolar cycloaddition between an azide and an alkyne[40,42–44] (so-called ''click chemistry''), and the chemoselective thiol–maleimide ligation[45–48] are the most widespread techniques for covalent immobilisation. Although covalent immobilisation offers highly stable arrays, the main limitation of this strategy is the need for the chemical modification of the sugar to introduce a linker, thus allowing its reaction with the surface. This modification is usually done by chemical manipulation, requiring complex multistep protection–glycosylation–deprotection sequences, with the need for often difficult purification. While this might not appear to be a major limitation, in the case of ''simple'' sugars (mono or disaccharides), it is a major challenge for glycoarrays of complex natural oligosaccharides as these are often available in tiny quantities.

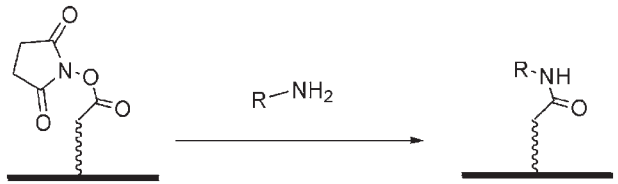
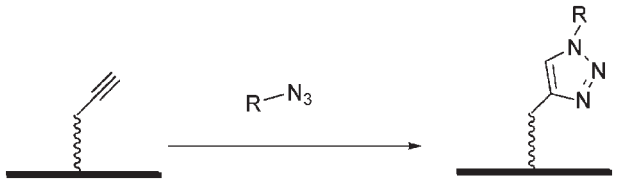
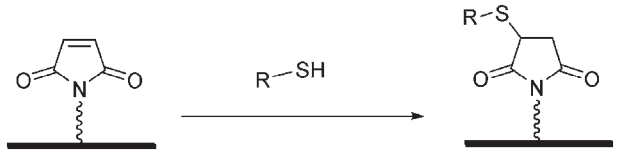
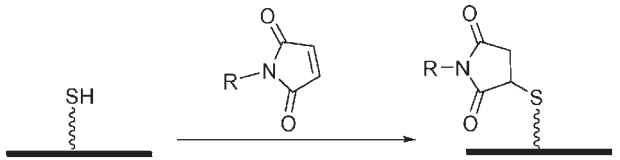
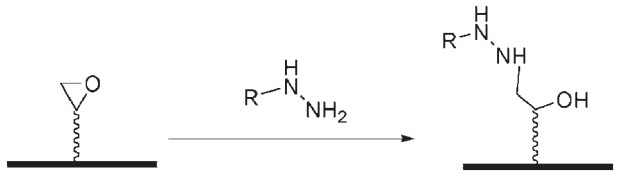
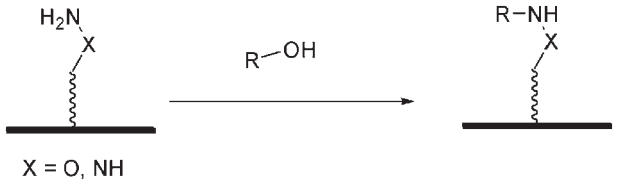
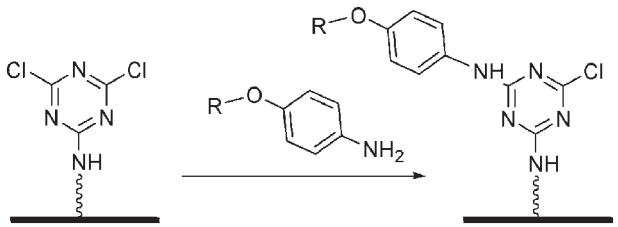
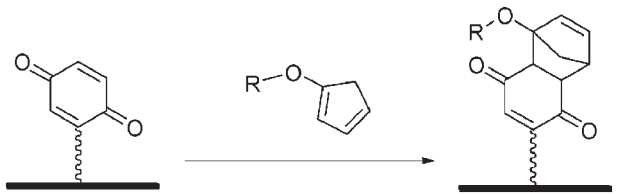
To overcome this limitation, the direct derivatization of unprotected glycans obtained from natural sources has been investigated by taking advantage of the reactivity of the masked aldehyde of the reducing end of an oligosaccharide (Fig. 2). Such chemoselective derivatization has been performed, either by imine formation,[49] by introducing an hydrazide[50,51] or an oxime[52] moiety (Fig. 2A) onto the sugar and subsequent immobilization, or by direct attachment of a reducing sugar onto a hydrazide- or aminooxime-modified surface.[53–55] Several methods for immobilising pectin oligosaccharides onto PEGA (poly(ethylene glycol)acrylate) resins have been described by Guillaumie *et al.*,[56] including reductive amination, oxime bond formation, hydrazide-based ligation and thiazolidine ring formation. Although this work was mainly accomplished for the structural analysis of pectin fragments on resin beads, the described methods can also be applied to generate arrays on solid surfaces. Glycosylamines can be prepared in a one-pot process starting from a range of unprotected sugars.[46,47,57] The anomeric amine can subsequently be used to introduce a linker (Fig. 2B). Aminations exclusively afford a β-anomer.

Inspired by a technology originally developed for DNA and protein arrays, Bovin *et al.* have developed carbohydrate arrays using the covalent attachment of 3-aminopropyl and 2-aminopyridinyl (2-AP) oligosaccharide derivatives onto polyacrylamide polymers.[58,59] 2-AP derivatives are widely used in the HPLC analysis of *N*-glycans and are therefore readily available in sufficient quantities (a few pmol of probe were necessary to construct the array). Three-dimensional presentation of the probes in a gel matrix allowed for a significant decrease in the detection limit of protein binding. However, virus and bacteria adhesion was limited by their low penetration into the gel. Photochemical immobilisation[60] has been achieved, either by using a surface-functionalized photoreactive layer that is able to covalently bind un-derivatized sugars,[61] or by introducing a photoreactive tag onto the sugar to allow site-specific photo-induced patterning of the surface.[52–64]

Non-covalent immobilisation

Immobilisation of polysaccharides, proteoglycans, glycoproteins and plant cell-wall extracts has been performed on black polystyrene slides through ionic interactions, hydrogen bonding and hydrophobic interactions.[65] Non-covalent passive adsorption of microbial polysaccharides has also been performed on a nitrocellulose-coated glass slide.[66] However, this method led to a random orientation and was limited to oligosaccharides large enough for tight adsorption onto the surface (typically 3.3–2000 kDa). For smaller oligosaccharides, conjugation with a carrier molecule (lipid, protein or polyacrylamide chain) was required to permit their immobilisation onto the same platform. All immobilised epitopes were shown to maintain their antigenic properties against a panel of specific antibodies. The non-covalent attachment of glycans has been very successfully performed by Feizi *et al.*, using neoglycolipids (NGL) that physically adsorb onto hydrophobic surfaces.[67] *O*- and *N*-glycans, fragments of glycosylaminoglycans, polysaccharides and synthetic glycans have been successfully arrayed using the NGL technology.[68–72] The advantage of non-covalent adsorption is that the sugar probes, while confined within microspots, still have the ability to move and rearrange as clusters for optimal protein binding.[73]

Table 1 Strategies for covalent immobilisation of sugars (R) on arrays

Immobilization	Ref.	Immobilization	Ref.
	37–41, 111		40, 42–44
	45, 46, 48		47, 103
	50, 51		53–55, 111
	139		101

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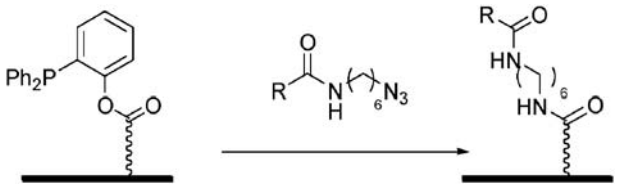
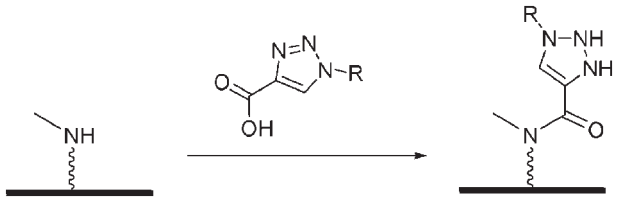
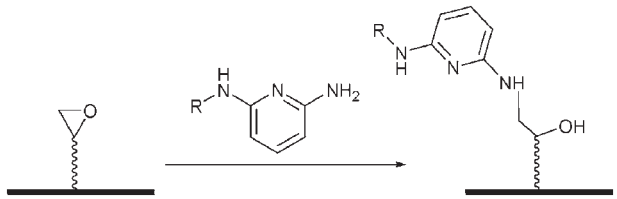
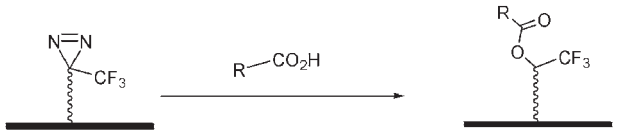
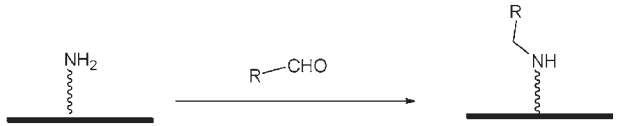
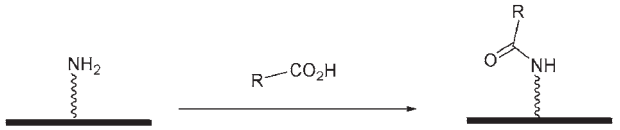
	36		104
	115		137
	113		113

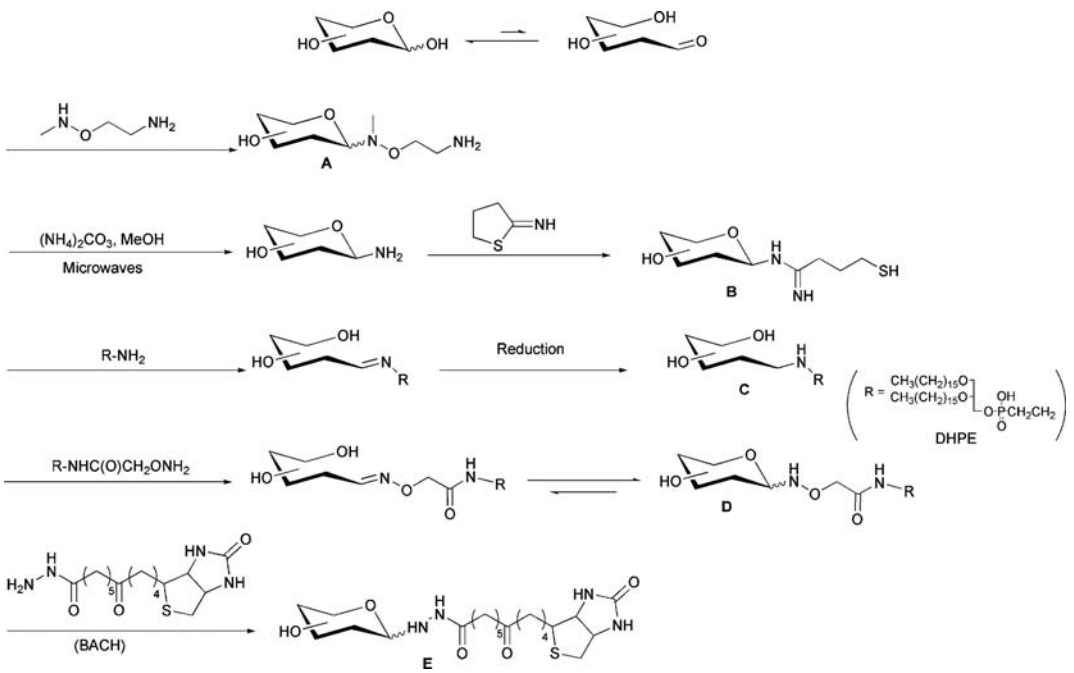


Fig. 2 Derivatization of a reducing glycan for covalent or non-covalent immobilisation.

Natural glycolipids (gangliosides or sphingoglycolipids) can be used directly for array preparation.[73] Alternatively, the lipid moiety has been introduced by reductive amination with an aliphatic primary amine,[74] olefin metathesis of an allyl glycoside[75] or by oxime-ligation.[76] Reductive amination with a lipid tail such as DHPE (1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine) produces a neoglycolipid with a ring-opened form of the monosaccharide at the reducing end (Fig. 2C), and is therefore restricted to large glycans, in which this structural modification does not hamper its biological recognition. In the case of shorter oligosaccharides and *N*-glycans, for which protein recognition may require an intact core, chemoselective oxime ligation with *N*-aminooxyacetyl-DHPE was developed to afford a ring-closed terminal unit (Fig. 2D).[76] A powerful ''deconvolution'' technique has been developed by Feizi and co-workers for the identification of NGL-glycan after microarray screening; mixtures of NGLs contained within a single spot of the array can be separated on TLC (thin-layer chromatography) plates. Protein binding and subsequent determination of the oligosaccharide substrate sequence by mass spectrometry can then be performed directly on the TLC plate.[77] Alternatively, the lipid tail has been introduced by reacting an isocyanate-containing hydrocarbon chain with various 2-aminoethyl glycosides, which can be used as common intermediates for preparing both covalently and non-covalently bound glycan arrays.[78] Interestingly, this conjugation can be performed directly on the microtiter plate at a microgram level. A C_{14} hydrocarbon chain length was sufficient to resist aqueous washing steps and biological assays. Similarly, NGLs were prepared by an azide–alkyne 1,3-dipolar cycloaddition, and were non-covalently bound to microtiter plates.[79]

A fluorous neoglycolipid library has been developed for selective immobilisation onto a fluorinated surface.[80] The short lipid tail necessary for immobilisation (C_8F_{17}) was also designed to allow automated solid-phase synthesis of the probes.

Specific adsorption through a non-covalent interaction has been achieved using the high affinity streptavidin/biotin complex.[81] Thus, a series of *N*-glycans were biotinylated at their anomeric asparagine residue and immobilised onto a 96-well microtiter plate coated with streptavidin to characterise their recognition by different lectins.[82] To illustrate this application, glycopeptide mixtures obtained from the tryptic digestion of glycoproteins were biotinylated and subsequently arrayed onto streptavidin-coated wells. Using horseradish peroxidase (HRP)-coupled lectins and colorimetric detection, the immobilised glycopeptides could be detected at the picomolar level.[83] Reagents commonly used to biotinylate glycans include biotinyl-L-3-(2-naphthyl)-alanine hydrazide (BNAH),[84] 6-biotinyl-aminocaproyl hydrazide (BACH, Fig. 2E),[85] 2-amino-6-amidobiotinyl pyridine (BAP)[86] and 4-(biotinamido)phenylacetylhydrazide BPH.[87] *N*-Glycans,[84] galactosyl ceramide analogues[88] and glycosylaminoglycan[89] derivatives were arrayed using these methodologies and probed for interactions with binding proteins and enzymes. It was also found that the background signal could be significantly reduced using plates coated with neutravidin, a deglycosylated form of streptavidin. Thus, black neutravidin 384-well plates constituted the basis of the Consortium for Functional Glycomics' (CFG) first generation of glycan array.[90] The Biacore surface plasmon resonance (SPR) microchip, used for the real-time monitoring of carbohydrate–protein interactions, also relies on neutravidin/biotin interactions.[91]

An original approach was used by Chevolot *et al.*[92] for the site-specific, non-covalent immobilisation of sugars onto DNA chips. To this end, the sugar probes were conjugated with a DNA strand by "click chemistry". Immobilisation was

performed through hybridization using a 52-well glass slide coated with either a complementary DNA sequence or a non-complementary one, as a control for non-specific adsorption. Relative surface densities were assessed by the introduction of a Cy3 fluorescent probe at the 5′-end of the DNA-glycoconjugate. A lectin-binding assay could be performed in a nanomolar range, either "on-chip" after hybridization or, alternatively, in solution, followed by hybridization of the carbohydrate–protein complex.

Glycopolymers were recently used to prepare carbohydrate chips of high density. Arraying of the glycosides relied on ionic interactions between an anionic polymer, carrying the carbohydrate epitopes, and a cationic coated surface.[93]

Factors affecting carbohydrate presentation and accessibility on array surfaces

The lectin recognition of immobilised ligands is highly dependent on the orientation of the probes and the length of the linker used to attach the molecules to the surface.[94] Passive adsorption of large oligosaccharides leads to a randomized orientation that might not reflect their natural orientations in living systems,[65,66] whereas covalent attachment and biotin/streptavidine or specific NGL adsorption appears to produce more uniformly oriented arrays. The distances between sugar residues (*i.e.* the density of the array) can be optimized to take advantage of the "cluster effect".[95–98] The density of the immobilised probes is also of critical importance for assaying enzyme activity. Thus, enzymatic glycosylation of an immobilised GlcNAc on an alkanethiol self-assembled monolayer (SAM)-coated gold surface was found to be optimal at a density of 70%. Higher densities led to a dramatically decreased yield, probably due to steric hindrance.[99]

The nature of the bond between the sugar moiety and the linker can also influence the mode of protein binding. For example, when glycosylamines are used to immobilise probes, the lectin concanavalin A (ConA), which has a high affinity for *O*-linked mannose, glucose and *N*-acetylglucosamine, is unable to recognize their *N*-linked analogues.[46]

The surface used to prepare the array should be compatible with the chemistry used to attach the probe, as well as the analytical techniques used to monitor the binding. In particular, fluorescence quenching on gold requires a careful choice of linker length to maximise the signal.[53]

Hydrophobic linkers, such as long aliphatic chains and aromatic- or charge-containing linkers, lead to non-specific protein adsorption, even when using passivation agents.[59] On the other hand, the use of SAMs of poly(ethyleneglycol)-terminated alkanethiols on a gold surface proved resistant to non-specific binding,[100] and are therefore more suitable for SPR and MALDI-ToF MS monitoring, where passivation of the surface would lead to a significant decrease in sensibility.

Analytical techniques used for glycoarray readout

Initial studies of glycoarrays used fluorescence detection. Lectin binding to glycoarrays was monitored using lectins coupled to a fluorescent probe (rhodamine,[101] indodicarbocyanine (Cy3 and Cy5)[50,53,54,65,92,102] or fluorescein isothiocyanate[103,104]). For enhanced sensitivity, an ELISA-type approach was used, where an antibody-conjugated lectin was recognized by a secondary antibody carrying the fluorescent probe.[53] Shao and Chin have used HRP-coupled lectins that can also provide fluorescent readout.[83]

More recently, advanced array platforms have been developed that allow a more detailed quantitative analysis of the binding constants and surface composition *in situ* on arrays. Surface plasmon resonance has emerged as a method of choice for analysing carbohydrate–protein interactions in a real time, label-free manner, allowing the measurement of association and dissociation constants on the array.[105] With the development of multi-channel instruments, allowing the independent SPR analysis of hundreds of spots in a single flow cell, SPR has been successfully applied for the measurement of lectin binding, in combination with carbohydrate microarrays (Fig. 3).[106,107]

Mrksich *et al.* have investigated the use of MALDI-ToF mass spectrometry directly on gold surfaces to characterize protein interactions and enzyme activity with a sugar or peptide that is covalently-immobilized on alkanethiol SAMs.[101,108] This analytical technique offers great experimental flexibility as it does not require the use of a labelled probe, fluorescent marker or radiolabel. Therefore, MS analysis is suitable for monitoring any transformation occurring on a

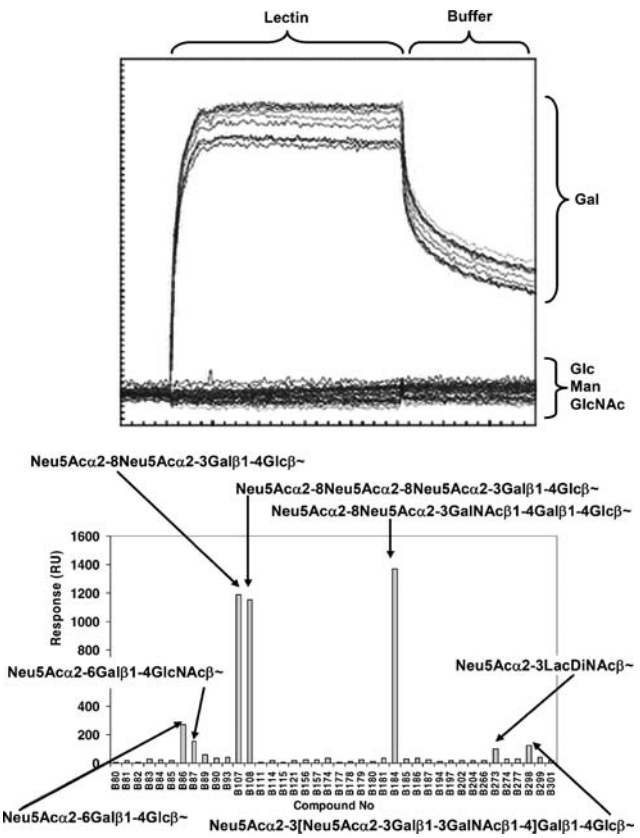


Fig. 3 Top panel: SPR traces of a monosaccharide array, showing the specificity of RCA_{120} lectin binding to its Gal ligand. Bottom panel: Binding profile of purified hSiglec7-Fc against sialylated glycan array.[107]

surface, and also allows multiplexing.[109] Recently, this MALDI-ToF MS method was used in an array format to probe the activity of various glycosyltransferases on peptide[110] and sugar[111] arrays.

Applications of glycoarrays

Carbohydrate–protein interactions. Originally referred to as hemagglutinins, due to their ability to agglutinate erythrocytes, lectins comprise a family of widely occurring proteins that bind specifically to glycan structures. Since their first description in the late 19th century, lectins have been found to be involved in many biological events, such as cellular recognition, lymphocyte mitosis, protection against pathogen infections, control of intracellular traffic of glycoproteins, *etc*., and they are now widely used in glycobiology, histochemistry and cytochemistry.[112] Lectins usually exhibit broad specificity towards monosaccharides, although some preferences have been noted and used to attempt their classification. Binding constants for monosaccharides are typically in the millimolar range, often requiring multivalent presentation of their ligands to achieve measurable association. In contrast, lectins often exhibit 1000-fold higher association constants for di-, tri- or tetrasaccharides compared to monosaccharides.[23] Initial efforts to characterize plant lectin binding using glycoarrays were performed in 1992 by using biotinylated glycans immobilized onto a 96-well microtiter plate coated with streptavidin. An array of twelve *N*-glycans was used to study the binding and inhibition properties of six plant lectins.[82,83]

Many more plant lectin-binding studies of oligosaccharides, as well as glycoproteins and protein extracts of cell cultures, immobilized on different platforms have been described over recent years, and these are listed in Table 2. The binding profile results have mainly been in agreement with known specificities from previously described solution studies. Recently, mammalian carbohydrate-binding proteins have been studied using glycoarrays. Carbohydrate arrays containing Lewis^x- and 3′-sialyl-Lewis^x-related structures unveiled the binding preferences of several human Siglecs, cell surface receptors that recognize sialic acids.[107,117] The glycan specificities of members of another class of cell surface receptors, the C-type lectin family, were also identified and compared using carbohydrate arrays.[71,118] In contrast to the C-type lectins of leukocytes, which were able to bind to analogues of Lewis^x-lacking sialic acid, the tested Siglecs displayed no detectable binding to these non-sialylated oligosaccharides. Furthermore, sulfatation of sialyl-Lewis^x structures was shown to have different effects on the binding of individual Siglecs. Similarly, using a carbohydrate microarray containing 200 glycans, Wilson and co-workers were able to determine different binding profiles for the various hemagglutinins, the main antigenic determinant of influenza responsible for binding to the receptors of the host cell. Screening studies revealed not only specificities for terminal α2,3- and/or α2,6- sialic acids, but also fine specificities for other glycan modifications, such as fucosylation or sulfatation.[119–121] Such a rapid screening technique for hemagglutinin mutants of human and avian viruses might be useful for characterizing emerging influenza viruses by analyzing binding profiles.

Although lectins are the main types of CBP studied due to their medical relevance, another class of protein has emerged as an invaluable tool for the determination of plant cell-wall architecture: the carbohydrate-binding modules (CBMs).[122] They are found in many modular carbohydrate-active enzymes, and bind with high specificities and high affinities to a wide range of polysaccharides. Their primary function is to increase the catalytic efficiency of glycosyl hydrolases against soluble and/or non-soluble substrates.[123] To date, 52 families of CBMs have been listed in the CAZy database (Carbohydrate Active enZyme, http://www.cazy.org/fam/acc_CBM.html). CBMs have been used as probes for *in situ*-analysis of plant cell-wall polysaccharides.[124,125] Recently, Moller *et al*. have developed a high-throughput mapping of cell-wall glycan occurrences, using specific monoclonal antibodies (mAbs) and CBMs in conjunction with microarrays.[126] To this end, polysaccharides were extracted from various tissues/organs of *Arabidopsis thaliana* and immobilised onto nitrocellulose membranes. The relative levels of 20 cell-wall glycan epitopes were then assessed by measuring the binding affinities of 20 mAbs and/or CBMs, thus providing a global snapshot of cell-wall composition. This method also offered the opportunity to reveal differences in cell-wall compositions between wild type and mutant plants, and furthermore was useful for analysing changes in cell-wall polymers in response to developmental, genetic or environmental changes.[127]

Glycosyltransferase specificities. Given that "glycoenzymes" such as the glycosidases and glycosyltransferases are responsible for the molecular make-up of the glycome, the definition of their specificity is a central task in glycomics. In addition, these enzymes are themselves useful tools for the generation of carbohydrate partners for binding studies. There has been great interest in using microarrays for studying glycoenzyme activity because it allows the specificity of the enzyme to be probed against a panel of immobilised potential substrates, and also expands the diversity of sugar arrays prepared by enzymatic means rather than chemical synthesis.[128] So far, a number of methods have been reported in proof-of-principle studies with a limited number of targets and/or enzymes. Park *et al*.[47] have used GlcNAc immobilised on glass slides by thiol–maleimide coupling to build a sialyl Le^x structure by three sequential enzymatic transformations: introduction of a 1,4-bound Gal residue on the GlcNAc with a bovine galactosyltransferase, 3′-sialylation by a α2,3-sialyltransferase and finally incorporation of the fucose moiety by a α1,3-fucosyltransferase. Each enzymatic coupling step was monitored by a fluorescent lectin, which binds to the newly incorporated sugar residue. The final Le^x structure was then detected by combination of an anti-sialyl Le^x antibody and a Cy5-anti-antibody. By using the same platform printed alternatively with GlcNAc and αFuc, the principle of enzyme specificity monitoring was also demonstrated: after incubation of the slide with GalT and UDP-Gal, fluorescence detection revealed that only the GlcNAc was converted to LacNAc. More recently, the same authors used a microarray of 20 different carbohydrates to monitor the specificity of bovine β1,4-GalT.[50] Substrates of the enzyme were detected by Cy3-RCA$_{120}$, a galactose-specific lectin. In this study, a greater affinity of the enzyme for βGlcNAc over αGlcNAc substrates was clearly highlighted.

Table 2 Plant lectins probed with glycoarrays

Lectin/glycan preference	Platform	Ref.
Con A (concanavalin A)	Biotinylated *N*-glycans immobilised on streptavidin-coated 96-well plates	82
Terminal mannose	Monosaccharides on SAMs-coated gold surfaces	101
	Maleimide-linked mono/disaccharides on thiol-derivatized glass sides	103
	Mannose on an amino-functionalized glass slide	113
	Non-derivatized saccharides on aminooxy-functionalized glass slides	114
	Biotinylated mono/di/oligosaccharides on a gold-surface	107
	Mono/disaccharides NGL adsorbed onto a 96-well plate	75
	2,6-Diaminopyrinyl saccharides on epoxy-activated glass slides	115
	Photogenerated arrays of mono/disaccharides	64
WGA (wheat germ agglutinin)	Biotinylated *N*-glycans immobilised on streptavidin-coated 96-well plates	82
Terminal *N*-acetylglucosamine	Maleimide-linked mono/disaccharides on thiol-derivatized glass sides	103
	para-Aminophenyl oligosaccharides covalently attached to 96-well plates	139
	Mono/disaccharides on amino-functionalized glass slides	113
	Biotinylated mono/di/oligosaccharides on a gold-surface	107
SNA (*Sambucus nigra* agglutinin)	Glycoproteins immobilised on a photoactivated surface	116
NeuAcα2,3Gal	Biotinylated *N*-glycans immobilised on streptavidin-coated 96-well plates	82
NeuAcα2,6Gal	2,6-Diaminopyrinyl saccharides on epoxy-activated glass slides	115
ECA (*Erythrina cristagalli* agglutinin)	Monosaccharides on SAMs-coated gold surfaces	101
Terminal galactose	Maleimide-linked mono/disaccharides on thiol-derivatized glass sides	103
	Non-derivatized saccharides on aminooxy-functionalized glass slides	114
	Mono/di/oligosaccharides covalently attached to a 96-well plate	44
LCA (*Lens culinaris* agglutinin)	Biotinylated *N*-glycans immobilised on streptavidin-coated 96-well plates	82
Terminal α-mannose		
DSA (*Datura stramoniun* agglutinin)	Biotinylated *N*-glycans immobilised on streptavidin-coated 96-well plates	82
Galβ1,4-GlcNAc, β1,4-GlcNAc	Glycoproteins immobilised on a photoactivated surface	116
E-PHA (*Phaseolus vulgaris* erytrhoagglutinin)	Biotinylated *N*-glycans immobilised on streptavidin-coated 96-well plates	82
Bisecting GlcNAc or a β1,6-linked branched		
BSI (*Bandeiraea simplicifolia* I)	Monosaccharides on SAMs-coated gold surfaces	101
Terminal α-D-galactosyl residues	Oligosaccharides covalently attached to a 96-well plate	139
UEA I (*Ulex europaeus* agglutinin I)	Monosaccharides on SAMs-coated gold surfaces	101
Terminal α1,2-linked fucose	Glycoproteins immobilised on a photoactivated surface	116
GNL (*Galanthus nivalis* lectin)	Monosaccharides on SAMs-coated gold surfaces	101
Terminal α1,3-linked mannose		
SBA (soybean agglutinin)	Photogenerated array of mono- and disaccharides	64
α-D-GalNAc		
BSII (*Bandeiraea simplicifolia* II)	Photogenerated array of mono- and disaccharides	64
Terminal α-D-Gal/α-D-GalNAc		
PNA (peanut agglutinin)	Photogenerated array of mono and disaccharides	64
Terminal α-D-Gal	Glycoproteins immobilised on a photoactivated surface	116
	Mono/disaccharides on an amino-functionalized glass slide	113
MAA (*Maackia amurensis* agglutinin)	Glycoproteins immobilised on a photoactivated surface	116
NeuAcα2,3Gal		
TPA (*Tetragonolobus purpureas* agglutinin)	Glycoproteins immobilized on a photoactivated surface	116
Terminal fucose	Non-derivatized saccharides linked on aminooxy-functionalized glass slides	114
	Mono/di/oligosaccharides covalently attached to a 96-well plate	44
	2,6-Diaminopyrinyl saccharides on epoxy-activated glass slides	115
RCA_{120} (*Ricinus communis* Agglutinin I)	Biotinylated mono/di/oligosaccharides on a gold surface	107
Terminal α-D-Gal/α-D-GalNAc	2,6-Diaminopyrinyl saccharides on epoxy-activated glass slides	115
RCA_{60} (*Ricinus communis* Agglutinin II)	Mono/disaccharides NGL adsorbed onto a 96-well plate	75
Galβ1,3GalNAc		
AAL (*Aleuria aurantia* lectin)	2,6-Diaminopyrinyl saccharides on epoxy-activated glass slides	115
L-fucose		

A chemoenzymatic synthesis of the sialyl Le^x epitope was also described by Fazio *et al.*[129] starting from a Le^x-NGL adsorbed onto microtiter plates, and using an α1,3-FucT and GDP-Fuc to introduce the fucose moiety. The same platform was later used to identify four potent fucosyltransferase inhibitors (K_i in the nanomolar range). Inhibition was monitored by observation of the transferred fucose on the carbohydrate array by the lectin *T. purpureas*.[130]

These studies heavily relied on lectin detection, which limits the range of enzymatic transformations that can be studied using glycoarrays. A more generic detection method of such enzyme-catalysed reactions is provided by on-chip mass spectrometry, which does not rely on labelling. Houseman and Mrksich have developed carbohydrate-modified surfaces based on SAMs of alkanethiols on gold surfaces and illustrated the importance of ligand density in the enzymatic glycosylation of an immobilised GlcNAc with β1,4-GalT, monitored by radiolabelled UDP-[^{14}C]galactose.[99]

Laurent *et al.* have used this method in an array format to explore the synthesis of mucin-type glycopeptide arrays on gold surfaces by the enzymatic glycosylation of immobilised peptides using a polypeptide GalNAc-transferase (ppGalNAcT2). Reactions were monitored by MALDI-ToF mass spectrometry and found to be quantitative for several substrates.[110] The same strategy was also used to assess the bovine β1,4-GalT specificity in an array format against a panel of

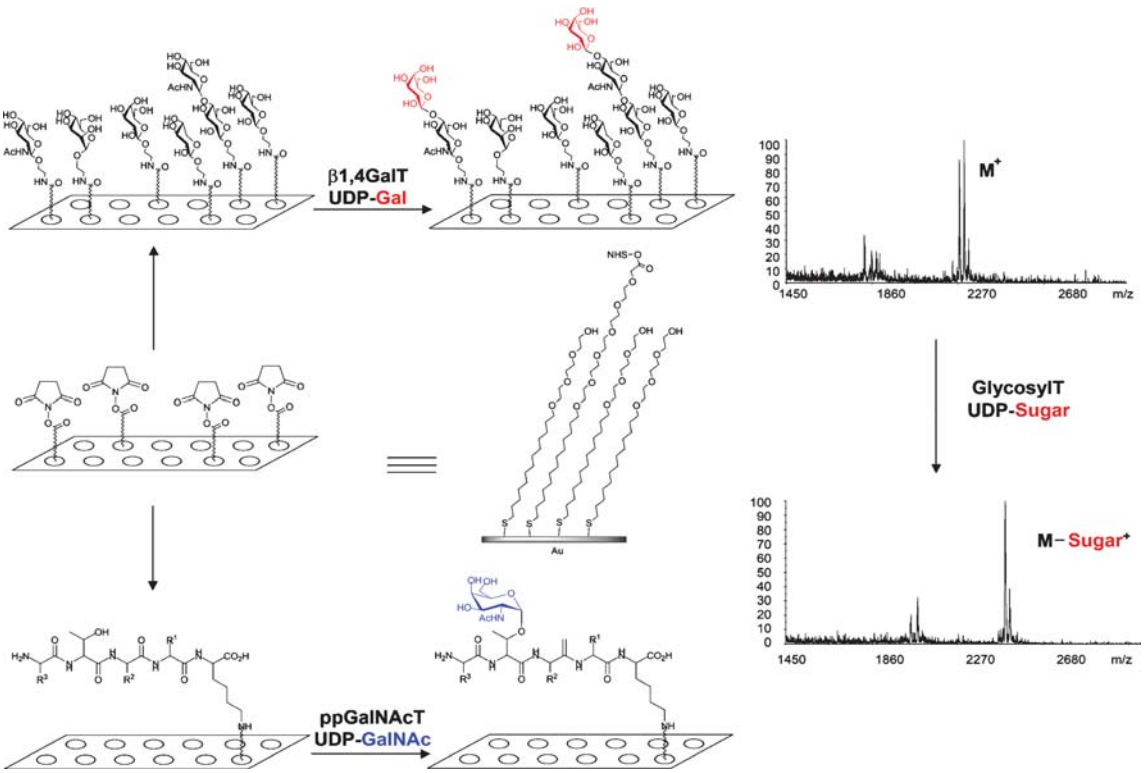


Fig. 4 A gold platform for monitoring glycosyltransferase activities by MALDI-ToF mass spectrometry.[110,111]

immobilised mono and disaccharides (Fig. 4).[111] This work showed both the use of substrate arrays to explore enzyme activity and specificity in a label-free manner using MALDI-ToF mass spectrometry, and the application of an enzymatic strategy to obtain arrays of otherwise synthetically challenging glycopeptides and oligosaccharides. Recently, Ban and Mrksich revealed the potential of *on-chip* oligosaccharide synthesis by a chemoenzymatic approach to expand the repertoire of glycan structures.[131] In this work, a hydroxyphenyl-terminated SAM of alkanethiols on gold was glycosylated using a suitably protected glycosyl trichloroacetimidate activated by TMSOTf. Selective removal of a levulinate group with hydrazine unmasked a hydroxyl function of the sugar, allowing another chemical glycosylation. An array of 24 disaccharides containing varied sugars and glycosidic linkages was thus prepared by using either Gal or Glc as the first residue and Gal, Glc or GlcNAc as the second residue. The array was then used to profile the substrate specificity of bovine β1,4-GalT, giving results in agreement with the known specificity of this enzyme; a selective glycosylation was observed for the GlcNAc-terminated disaccharides under standard enzyme assay conditions, with preference for the β-1,6 and β-1,4 linked disaccharide substrates, whereas the presence of lactalbumin modified the specificity and caused the enzyme to use glucose as a substrate. Seibel *et al.*[104] used a microarray approach on microtiter plates to identify new acceptor specificities of the non-Leloir glycosyltransferase R (GTFR) from *Steptococcus oralis*, using sucrose as a glucose donor. Interestingly, it was found that the GTFR was able to glycosylate not only maltose, but also immobilised primary alcohols to yield the corresponding α-glucoside.

Sialic acid-containing glycans are notoriously challenging to synthesize by chemical means.[132–135] Therefore, the use of sialyltransferases to introduce the sialic acid moiety onto carbohydrates has attracted considerable attention. A high-throughput screening of various recombinant sialyltransferase acceptor specificities was recently demonstrated by Blixt *et al.*; using a biotinylated CMP-NeuNAc as an activated donor for the sialyltransferases, the enzymatic reactions performed on the microarray were monitored with a fluorescein–streptavidin conjugate. Hence, previously known acceptor specificities were confirmed and additional specificities also discovered.[136] These findings can now be applied to the chemoenzymatic synthesis of sialylated oligosaccharide arrays.

Shipp *et al.* have developed a glycoarray to monitor plant cell-wall glycosyltransferase activities by phosphorimaging.[137] To this end, a panel of oligosaccharides and polysaccharides were first conjugated to poly-D-lysine by reductive amination, and then printed onto a glass slide coated with a thin film of Optodex (an aryldiazirine-containing polymer) by photochemical immobilisation. As a model enzyme, activity of the xyloglucan-fucosyltransferase AtFUT1, involved in xyloglucan biosynthesis, was investigated using a radiolabelled GDP-[^{14}C]Fuc. During the course of the study, it was shown that oriented anchoring of the oligosaccharides was required for optimal AtFUT1 activity.

Antibody specificity. The central role of glycans in development, carcinogenesis, cell adhesion and immunity has led to an urgent need for high-throughput analysis of carbohydrate–antibody binding. Using microarrays to analyse antibody specificity towards carbohydrate moieties, the amount of

material attached is magnitudes lower than for other methods of carbohydrate analysis, such as ELISAs and immunodot assays. A comparison of screening assays for mAbs against partially methyl-esterified lime pectins displayed on black polystyrene slides showed detection limits for microarrays of around 80 fg, compared with 5 pg for ELISAs and 10 ng for immunodot assays.[65] Another advantage over other methods is the screening of multiple antigens (compared with one or two with ELISA) at the initial stage of mAb production, where only a limited amount ($\sim$100 μl) of hybridoma supernatant is available.[138]

Willats *et al*. have developed a non-covalently bound glycan array to probe using a panel of mAbs with specificities for plant glycan epitopes (Fig. 5).[65] Similarly, Moller *et al*. have studied the binding of 30 anti-glycan mAbs that were specific for plant cell-wall extracts.[138] A panel of over 50 plant glycans, enzymatically modified *in situ* after immobilisation onto nitrocellulose membranes, was used to obtain data of recognition patterns. The probing of another glycan array in microtiter plate format with covalently-bound mono- and oligosaccharides with human immunoglobulins G (IgG) pools helped identify a novel antibody against β1,4glucose oligomers.[139] This anti-cellotriose antibody bound not only to β1,4glucose oligomers but also to crystalline and amorphous cellulose. The potential for investigating antigenic cross-reactivities was shown by the characterisation of anti-α(1,6)dextran antibodies on microarrays. An unexpected binding affinity to chondroitin sulfate B polysaccharide led to the discovery of a previously undescribed cellular marker.[66] Wang *et al*. also demonstrated that as little as 1 μl of serum specimen was enough to probe carbohydrate arrays displaying 48 distinct microbial pathogens, and identified the specificities of

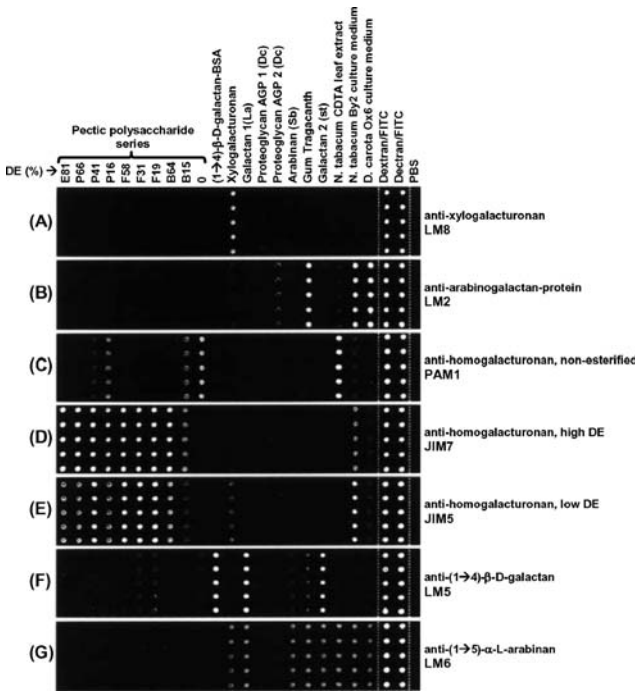


Fig. 5 Seven identical polysaccharide arrays (A to G) probed with specific mAbs. Binding was detected using Cy3-conjugated secondary antibodies.[65]

IgM and IgG antibodies of 20 individuals.[66] In another study, de Boer *et al*. covalently immobilised a fluorescent labelled glycan subset of *Schitsosoma mansoni* on a gold epoxide chip.[140] SPR analysis of serum antibodies clearly revealed differences between *S. mansoni* infected and non-endemic uninfected individuals, regarding antibody class and titer.

The interaction of antibodies with Globo H, a cell surface glycosphingolipid highly expressed in cancer cell lines, was studied by the immobilisation of several truncated and conjugated derivatives on a glycan array.[40] The screening illustrated that cancer patient sera had different specificities from monoclonal anti-Globo H antibodies, either due to their polyclonal nature or to the recognition of different antigen epitopes at different stages. Kaltgrad *et al*. compared the carbohydrate selectivity between the anti-Globo H antibody and polyvalent avian IgY antibodies, produced by immunisation with virus particles conjugated with blood group antigens (such as tri-LacNAc, sialyl Lewis^x, globo-H). Testing of the crude IgY samples on an array containing over 200 glycans revealed matching specificities to the monoclonal antibodies.[141] Carbohydrate arrays were also used to assess the binding of anti-Tn antibodies and lectins to the Tn antigen (αGalNAc linked to a Ser/Thr) and related epitopes. Gildersleeve and co-workers showed that these markers, which are extensively used in cancer diagnosis, could partly bind to other carbohydrate epitopes, and positive signals could be produced in the complete absence of the Tn epitope. The data obtained helped to explain the inconsistencies of previous work due to cross-reactive and promiscuous binding.[142] Recently, the same group screened the specificities of 27 anti-glycan antibodies on a microarray of 80 different glycans, including blood group, Lewis and other tumor-associated antigens. This work showed that many of the antibodies considered to be specific for their designated antigen could actually cross-react with other glycans, and that mis-interpretations could arise during the analysis of biological samples as a result.[143] The neoglycolipid technology developed by Feizi and co-workers was also successfully adopted for the binding studies of antibodies, revealing the potential of microarrays to probe the binding of monoclonal antibodies, antisera and growth factors on an array containing chondroitin sulfates, glucosaminoglycans and several Lewis^x-related structures.[72] Another example of using carbohydrate arrays was described by Blixt *et al*. by screening *Salmonella O*-antigen specific antibodies.[144] The work demonstrated the possibilities of such platforms as more rapid, precise and low cost alternative screening methods for human infections.

Cell adhesion. The use of microarrays to study whole-cell binding offers the advantage of multivalent presentation of the carbohydrate target in a manner that mimics interactions at cell–cell interfaces. Disney *et al*. designed an array of five monosaccharides, covalently printed onto glass slides, to assay *Escherichia coli* (ORN178) binding specificity.[41] Using bacteria cells that had been stained with a nucleic acid staining dye, specific binding to mannose was observed by fluorescence and bright-field microscopy, after incubation with either isolated bacteria or contaminated serum. Furthermore, bound bacteria could be harvested from the mannose-containing spots and

grown in Petri dishes for further antibiotic assays. Simultaneous screening of different mutant strains (ORN178 and ORN209) clearly highlighted altered carbohydrate binding affinities. Since pathogen virulence often correlates with carbohydrate binding, this experiment demonstrated the potential of glycoarrays in clinical applications for profiling pathogenicity and helping the design of strain-specific therapies, such as anti-adhesion compounds. An array of mono- and oligosaccharides on glass slides was also used to detect and quantify the adhesion of primary chicken hepatocytes expressing a GlcNAc-specific lectin and $CD4^+$ human T-cells specific for sialyl Lewis^x structures.[145]

Conclusion

Carbohydrate arrays are rapidly being adopted by glycoscientists as an important tool for studying carbohydrate–protein interactions and glycoenzyme specificities in a high-throughput fashion, both qualitatively and quantitatively. A number of platforms that allow for readout with different analytical methods have been developed, and are now starting to be used to address fundamental questions in the glycosciences and for diagnostic applications. Although there has been little direct systematic comparison between the different platforms, the binding results obtained so far in proof-of-principle studies appear to be in broad agreement with each other (with some discussed exceptions) and with previously reported solution studies. Thus, the choice of attachment chemistry and carbohydrate array platform will depend on the particular biological application and the required analysis. Some diagnostic applications of carbohydrate arrays in infection and cancer are already emerging.

The next step forward will be to populate carbohydrate arrays with more representative, or even complete, sets of carbohydrate structures of a cell or organism, beyond the currently available sets of a few hundred structures. Given that the more complex carbohydrates are generally not readily available, the challenge in this area of research will be the successful close collaboration of international interdisciplinary teams of highly specialised glycoscientists, ranging from carbohydrate synthetic and analytical chemists to cell biologists, and ultimately clinicians, who can use carbohydrate arrays for studying the role of protein–carbohydrate interactions in health and disease.

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