Carbohydrate microarrays as powerful tools in studies of carbohydrate-mediated biological processes

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Carbohydrate microarrays have become very powerful tools to elucidate the molecular basis of carbohydrate-recognition events in a high-throughput manner. This microarray technology has been applied in the rapid analysis of the binding properties of a variety of binding partners such as lectins, antibodies, mammalian cells, pathogens and viruses. In this feature article, methods for the preparation of carbohydrate microarrays and their applications in biological and biomedical research are described.

Introduction

Towards the end of the 20th century, Herculean efforts were made to determine the entire genome sequence of many species including the human. In the post-genomic era, functional studies of protein products of genes have received considerable attention by biological and biomedical researchers. It is generally accepted that genetic information flows from DNA to protein *via* mRNA. However, in many cases the information flow does not end with protein biosynthesis in the ribosome, but rather with protein glycosylation in the endoplasmic reticulum/Golgi apparatus, the most common of post-translational modifications. It is estimated that more than 50% of all proteins in higher organisms are modified with various glycans.[1] Since biological processes can not be completely understood without knowledge about the roles that glycans play, the functional study of glycans is one of the most important biological research areas in the post-genomic era.

Carbohydrates compose a large group of biomolecules with diverse structures and are found largely in the form of glycoconjugates inside or on the surface of cells. One major group of glycoconjugates are the glycoproteins in which glycans are linked to an asparagine (*N*-linked glycoproteins) or a serine/threonine side chain (*O*-linked glycoproteins) of the polypeptide backbone (Fig. 1). Proteoglycans differ from glycoproteins in that glycosaminoglycans (GAGs) are attached to a serine/threonine residue of the polypeptide scaffold *via* a xylose moiety. Another important group of glycoconjugates is the glycosphingolipid in which glycans are conjugated to ceramides. Glycans are also key components of glycophospholipid (GPI) anchors which attach proteins to the cell membranes.

These glycan substances participate in many important cellular processes, such as cell adhesion, signaling and trafficking, through their interactions with proteins.[2–4] In addition, the recognition of pathogenic glycans by host cell receptors induces immune responses to various pathogens, including yeast, bacteria and viruses.[5] Carbohydrate-mediated biomolecular interactions also play key roles in various pathological processes. For example, toxins, bacteria and viruses enter target cells by initial adhesion to host cells through interactions of the pathogenic proteins with host cell-surface glycans.[6] Tumor metastasis takes place through binding of *O*-linked glycans, often overexpressed on cancer cell surfaces, to selectins of host platelets, leukocytes or endothelial cells.[7] Leukocyte recruitment to sites of inflammation is mediated by initial selectin–sialyl Le^x interactions between the circulating leukocytes and the endothelial

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Sungjin Park received her BS in Chemistry from the Hankuk University of Foreign Studies. Her MS was awarded for studies in carbohydrate chemistry performed at Yonsei University under Injae Shin. She began her PhD with Professor Shin in 2005 and her current research interests include the synthesis of glycoconjugates, the fabrication of carbohydrate microarrays and their applications to the field of glycomics.

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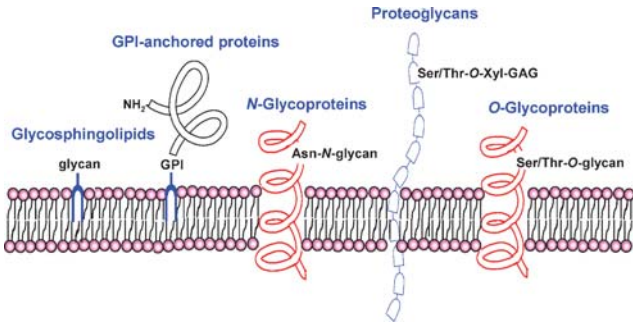

Fig. 1 Glycoconjugates found in cells.

cells.[8] As a consequence, understanding the molecular basis of glycan–protein interactions provides deep insights into carbohydrate-mediated biological processes which serve to drive the development of more effective therapeutic agents and diagnostic tools.

Conventional approaches, including the hemagglutination inhibition assay, enzyme-linked lectin assay, surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC), have been widely used over past decades to evaluate glycan–protein recognition events.[9–12] Although successfully applied in studies of biomolecular interactions, these conventional methods are labor intensive and they often require large amounts of carbohydrate samples. Therefore, a pressing demand existed for sensitive and rapid methods to elucidate the nature and consequence of glycan–protein interactions.

Beginning over a decade ago, microarray technologies, such as DNA and protein microarrays, have been developed as high-throughput analytic tools for genomics, transcriptomics and proteomics research (Fig. 2).[13–15] These technologies facilitate fast, quantitative and simultaneous analyses of a large number of biomolecular interactions. As a high-throughput analytic method for probing glycan–protein interactions, carbohydrate microarrays, which are composed of diverse glycans densely and orderly attached to a solid surface, were first described in 2002 by several research groups.[16–20] The carbohydrate microarray technology has advantage over most other conventional methods in that a large number of glycan–protein interactions can be analyzed simultaneously by using small amounts of carbohydrate samples. Another important feature of this technology is that glycans immobilized on solid surfaces display multivalent binding to proteins as a result of a cluster effect. Thus, proteins with low binding affinity to monovalent carbohydrates in solution can strongly interact with immobilized glycans. These beneficial aspects make glycan microarrays suitable for the rapid analysis of glycan–protein interactions for glycomics research.

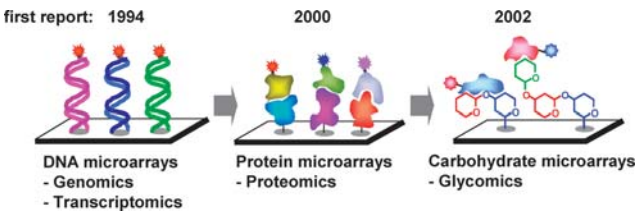

Fig. 2 Microarray-based technologies for applications to genomics, transcriptomics, proteomics and glycomics.

In this article, an overview of the immobilization methods that have been used for the preparation of carbohydrate microarrays is given. In addition, methods that have been utilized to detect binding events on carbohydrate microarrays are briefly discussed. Finally, various applications of the microarray technology in biological and biomedical research are presented. In contrast to conventional microtiter arrays which can be used to assess relatively small numbers of samples, tens of thousands of small quantity samples can be analyzed simultaneously in high-density microarray systems. Because of this, the high-density carbohydrate microarrays are the major focus of this article.

Immobilization chemistry

A variety of carbohydrate microarray formats that use different surfaces and immobilization methods have been developed over the past few years. In general, microarrays are constructed by attaching modified or unmodified glycans to appropriate solid surfaces.[21–26] A microscope glass slide is the most widely used surface material due to its easy manipulation and low price. Alternatively, gold and nitrocellulose membrane have also been employed as solid materials.

Carbohydrates used for immobilization are produced in either unmodified or functionalized forms by using chemical or chemoenzymatic synthesis, or are obtained from natural sources, such as glycoproteins and glycolipids. Since many efficient chemical methods, including one-pot oligosaccharide synthesis, combinatorial carbohydrate synthesis and automated oligosaccharide synthesis, have been developed, the chemical synthesis is more popular for the preparation of diverse carbohydrate probes.[27–29]

Immobilization methodologies can be broadly classified into two general strategies that involve either covalent or noncovalent attachment of glycans to the appropriate solid surfaces (Fig. 3). Noncovalent immobilization techniques rely on adsorption of free or functionalized glycans to underivatized or derivatized solid surfaces (Fig. 3(a) and (b)). The most straightforward procedure used for this purpose employs immobilization of free glycans on chemically underivatized surfaces. For example, microarrays containing polysaccharides, proteoglycans and neoglycoproteins have been prepared by printing unmodified substances on nitrocellulose-coated glass slides or oxidized black polystyrene slides (Fig. 4(a)).[17,20] In these processes, the glycans are noncovalently and site-nonspecifically adsorbed on solid surfaces. Owing to the nature of noncovalent attachment, the large glycans with large contact areas are efficiently adsorbed on the surface, whereas small glycans are only weakly attached to the solid surface and can be readily removed during washing steps.[17]

Other noncovalent methods have been exploited for polysaccharide immobilization. Free heparin polysaccharides with sulfate groups noncovalently bind *via* ionic interactions to surfaces possessing positively charged residues, such as poly-L-lysine (Fig. 4(b)).[30] In addition, chemically modified dextran polysaccharides have been noncovalently immobilized on the semicarbazide-derivatized glass slide in a size-dependent manner (Fig. 4(c)).[31]

The noncovalent immobilization methods described above are usually applicable to the construction of polysaccharide

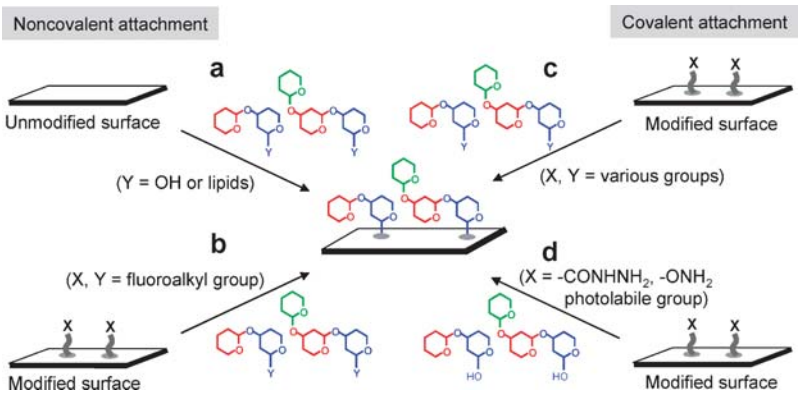

Fig. 3 Immobilization strategy for the construction of glycan microarrays. Noncovalent attachment methods: (a) attachment of unmodified polysaccharides or neoglycolipids to the underivatized surface and (b) attachment of fluoroalkylated sugars to the fluoroalkylated surface. Covalent attachment methods: (c) attachment of modified sugars to the derivatized surface and (d) attachment of unmodified sugars to the derivatized surface.

microarrays but they are not suitable for the preparation of microarrays containing simple carbohydrates and oligosaccharides owing to the poor retention of these glycans in washing steps. Interestingly, efficient noncovalent immobilization techniques have been developed for the fabrication of simple carbohydrates and oligosaccharides. Examples of this methodology are found in the site-specific, noncovalent attachment of lipid-conjugated glycans (termed neoglycolipids, NGLs) to nitrocellulose *via* hydrophobic interactions (Fig. 5(a))[18] or fluorous tag-conjugated glycans to the fluoroalkylated glass slides *via* fluorous-based interactions (Fig. 5(b)).[32] The lipid and fluorous tags are sufficient to retain immobilized glycans even after extensive washing. The required NGLs are obtained by reductive amination of oligosaccharides, generated by chemical or enzymatic methods, with an amino lipid (1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine). The fluorous-tagged glycans are typically prepared by coupling of glycosyl donors to fluorous alcohol followed by fluorous-based purification.

A much greater effort has been given to the construction of glycan microarrays using covalent attachment of sugars containing functional groups that selectively react with those present on the surface (Fig. 3(c)). This approach requires both chemically modified glycans and derivatized surfaces, and is suitable for the preparation of microarrays that contain simple carbohydrates and oligosaccharides. In these immobilization methods, the nature of linkers placed between glycans and the solid surface govern binding of proteins to immobilized glycans and is important to suppress nonspecific binding of proteins. In general, microarrays formed with hydrophilic linkers, such as oligo or poly(ethylene glycol), have better binding properties for proteins than do those generated using hydrophobic linkers. In addition, the lengths of tethers affect protein binding affinities to glycans on the surface. Glycans linked by shorter tethers interact with proteins less strongly than those connected by longer linkers, owing to the greater accessibility of proteins to the glycan ligands in the latter systems.

An early example of the application of this type of immobilization methodology was based on selective reaction

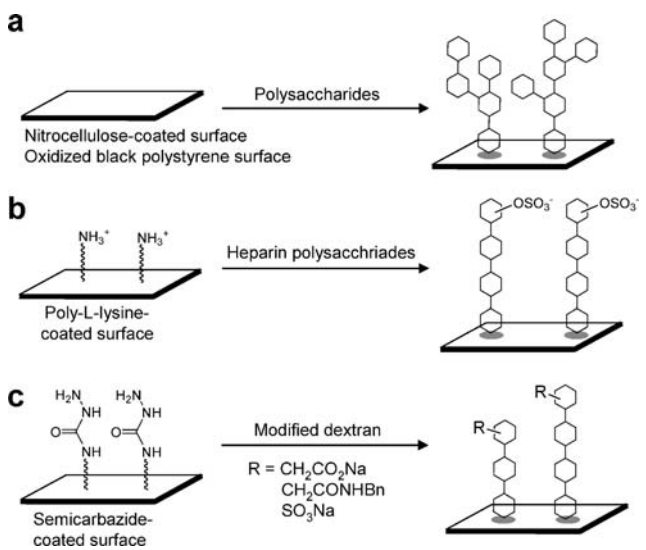

Fig. 4 Noncovalent immobilization of polysaccharides on the solid surface. (a) Attachment of free polysaccharides to the unmodified surface, (b) attachment of free heparin polysaccharides to the poly-L-lysine-coated surface and (c) attachment of modified dextrans to the semicarbazide-coated surface.

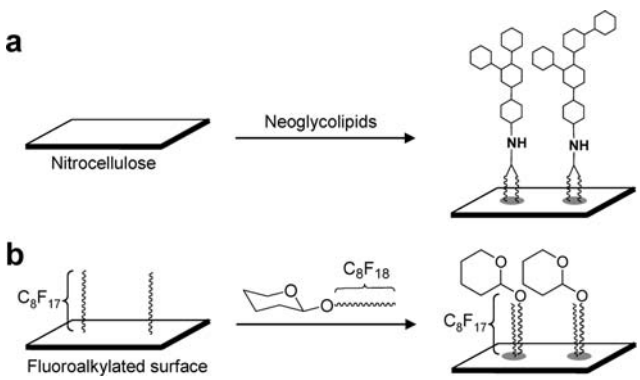

Fig. 5 Noncovalent immobilization of tail-conjugated glycans on the solid surface. (a) Attachment of lipid-conjugate glycans to nitrocellulose and (b) attachment of fluoroalkylated sugars to the fluoroalkylated surface.

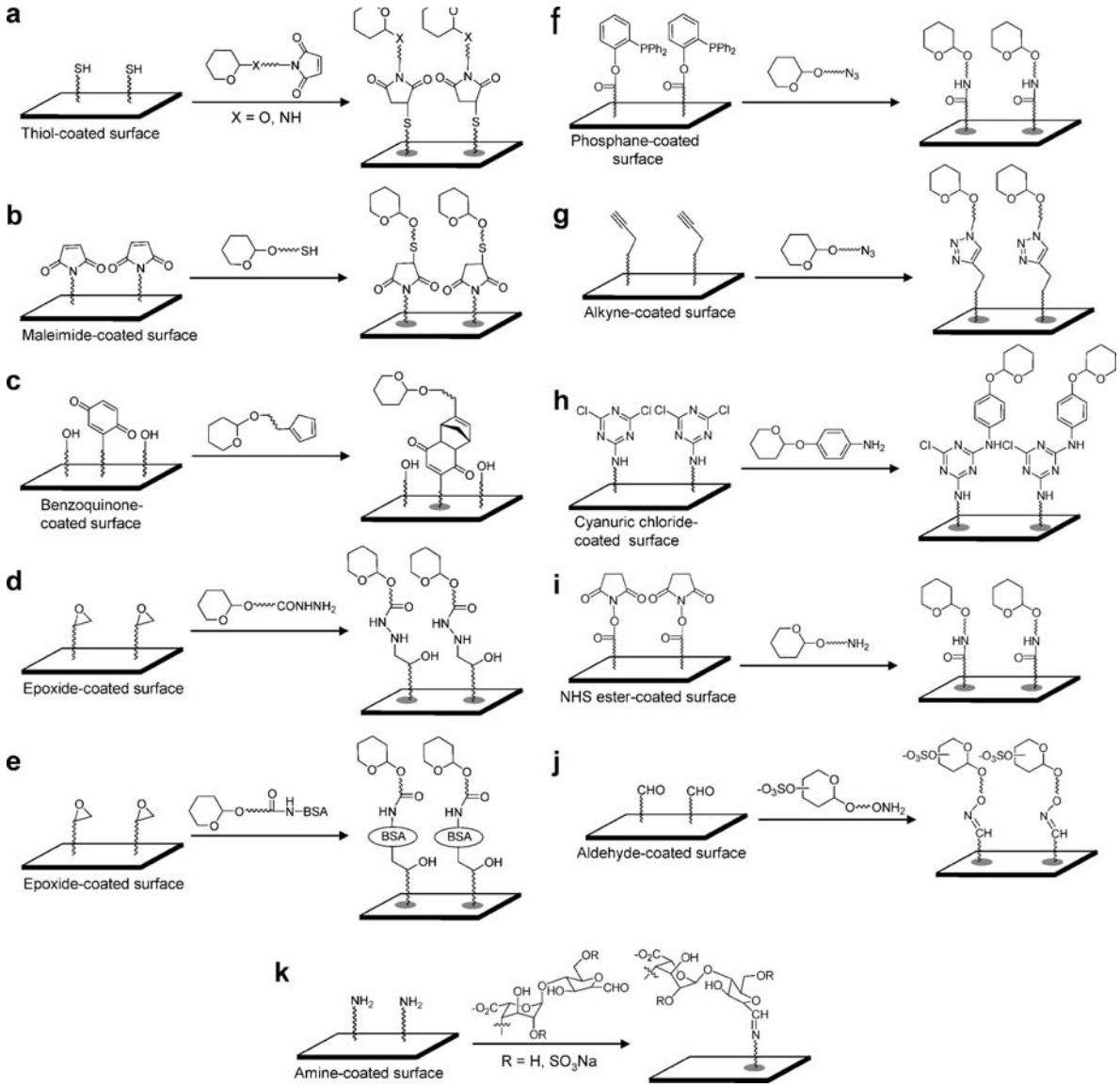

Fig. 6 Covalent and site-specific immobilization of functionalized glycans on the derivatized surface: (a) attachment of maleimide-linked sugars to the thiol-coated surface, (b) attachment of thiol-linked sugars to the maleimide-coated surface, (c) attachment of cyclopentadiene-linked sugars to the benzophenone-coated surface, (d) attachment of hydrazide-linked sugars to the epoxide-coated surface, (e) attachment of glycan-linked BSA to the epoxide-coated surface, (f) attachment of azide-linked carbohydrates to the phosphane-coated surface, (g) attachment of azide-linked glycans to the alkyne-coated surface, (h) attachment of *p*-aminophenyl-linked sugars to the cyanuric chloride-coated surface, (i) attachment of amine-linked sugars to the NHS ester-coated surface, (j) attachment of aminooxy-linked sugars to the aldehyde-coated surface and)k) attachment of aldehyde-linked sugars to the amine-coated surface.

between thiol and maleimide groups, a process which has been widely used to prepare various bioconjugates. In this process, maleimide-linked glycans are attached to thiol-coated glass slides (Fig. 6(a)).[16,33] In a reverse way, thiol-connected glycans were also immobilized on maleimide-derivatized glass slides, maleimide-derivatized self-assembled monolayers and the maleimide-conjugated bovine serum albumin (BSA)-coated surface (Fig. 6(b)).[34–36] The reaction between thiols and maleimides is suitable for the selective and efficient attachment of glycans to the surface. However, the fact that thiol-functionalized substances readily undergo air oxidation should be taken into account when this process is used for microarray construction.

Another early immobilization method involves covalent attachment of cyclopentadiene-conjugated glycans to the benzoquinone-coated surface *via* Diels–Alder reactions. A gold-coated glass slide is initially modified by immersing it into a mixture of alkanethiols with (1%) and without (99%) appended hydroquinone groups (Fig. 6(c)).[19] The distance between glycans immobilized on the surface can be controlled by adjusting the amount of alkanethiols which do not contain hydroquinone groups. Subsequent chemical or electrochemical oxidation converts the hydroquinone to the benzoquinone. Cyclopentadiene-conjugated glycans are then printed to attach to the benzoquinone-coated gold surface *via* the Diels–Alder reaction.

A variety of other ligation reactions have been used to prepare glycan microarrays. For example, hydrazide-conjugated carbohydrates can be covalently, selectively and efficiently attached to epoxide-derivatized surfaces (Fig. 6(d)).[37–39] Hydrazide-linked substances prepared on solid supports react more rapidly with epoxides on surfaces at pH 5 than do amine- and thiol-conjugated compounds. Epoxide-functionalized solid surfaces can also be used to prepare glycan microarrays *via* immobilization with glycan-conjugated BSA (Fig. 6(e)).[40] The chemoselective Staudinger reaction between azides and phosphanes has been applied to prepare glycan microarrays (Fig. 6(f)).[41a] Unlike with click chemistry which involves Cu(I)-catalyzed reaction between azides and alkynes (Fig. 6(g)),[41b] the Staudinger process does not require metal ions. Azide-conjugated substances, which are prepared by using a safety-catch linker strategy, are immobilized to the phosphane-coated surface. Amine-conjugated glycans have also been used for the efficient attachment to the cyanuric chloride-modified surface (Fig. 6(h))[42] or *N*-hydroxysuccinimide (NHS) ester-coated surface (Fig. 6(i)).[43,44] The ligation reactions between aldehydes and aminooxy or amino groups have been employed to prepare glycosaminoglycan microarrays. In these processes, synthetic chondroitin oligosaccharides linked by aminooxy groups or heparin oligosaccharides obtained by nitrous acid depolymerization of heparin are immobilized on respective aldehyde- or amine-coated surfaces (Fig. 6(j) and (k)).[45,46]

The covalent attachment of glycans on surfaces requires functional group-conjugated glycans, which are typically prepared by multistep reactions. To circumvent the labor-intensive and time-consuming nature of these synthetic routes, one-step procedures for the modification of free sugars with proper functional groups have been exploited. For instance, simple carbohydrates and oligosaccharides, when reacted with 2,6-diaminopyridine in the presence of sodium cyanoborohydride or *N*-methylaminooxy-containing bifunctional linkers, produce acyclic or cyclic adducts, respectively (Fig. 7).[47,48] The modified sugars appended by amine groups are then printed on the NHS ester-coated surface to bring about covalent immobilization.

Derivatization of various glycans by one-step or multistep sequences is a hurdle in the fabrication of microarrays containing diverse sugars. In order to avoid the requirement for modified glycans, covalent immobilization strategies that are applicable to unmodified glycans have been developed (Fig. 3(d)). One approach involves the covalent attachment of unmodified sugars to the surface derivatized with a photolabile group in a site-nonspecific manner (Fig. 8(a)).[49] In this procedure, photolabile aryltrifluoromethyldiazirine groups coated on the surface are converted to reactive carbenes by UV irradiation. The carbene intermediates react with sugars in their vicinity to form covalent bonds. A major drawback of this technique is the nonspecific attachment of glycans to the surface owing to the unselective nature of the carbene reactions.

A more ideal method for construction of carbohydrate microarrays is the site-specific, covalent attachment of unmodified sugars irrespective of their size to the proper surface. One example of this involves immobilization of free carbohydrates, including simple carbohydrates, oligosaccharides and polysaccharides, on aminooxy- or hydrazide-derivatized surfaces (Fig. 8(b)).[50–52] Reducing sugar moieties of glycans

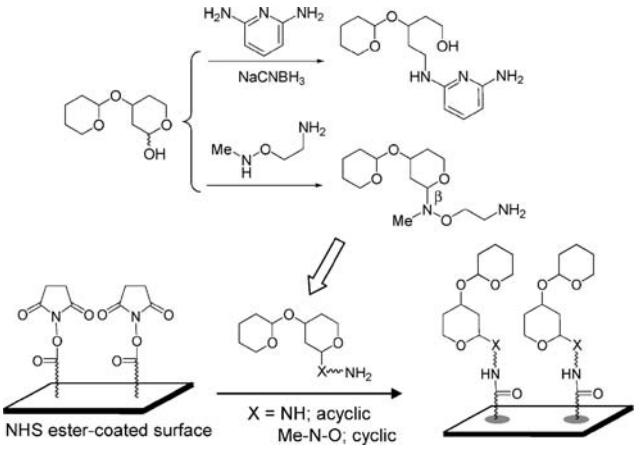

Fig. 7 Immobilization of glycans obtained by one-step reactions on the NHS ester-derivatized surface.

bind to hydrazide groups on the surface to form cyclic structures with β-configurations at their anomeric positions.[50] In contrast, acyclic modifications are formed when an aminooxy surface is used. The hydrazide-based immobilization procedure has been found to be more efficient for microarray preparation than the method employing aminooxy surfaces. This founding may be due to the different nature (cyclic in hydrazide surfaces verse acyclic in aminooxy surfaces) of linkages of the anomeric position. Importantly, immobilization techniques that rely on free carbohydrates can be used even by biologists who lack organic synthesis experience.

To date, the most extensively developed types of carbohydrate microarray have two-dimensional (2-D) formats. Alternatively, 3-D hydrogel carbohydrate microarrays have been constructed by immobilizing amine-conjugated glycans in gel drops on the unmodified surface *via* photo-induced radical polymerization (Fig. 9).[53] In this process, a gel solution (methacrylamide, *N*,*N*′-methylene bisacrylamide and glycerol), mixed with amine-conjugated sugars, is printed onto the silane-treated glass slide and then irradiated to promote polymerization-mediated immobilization. One advantage of the 3-D microarray format over its 2-D counterpart is that it has higher densities of sugars on the surface and thus shows higher signal intensities upon probing with binding partners.

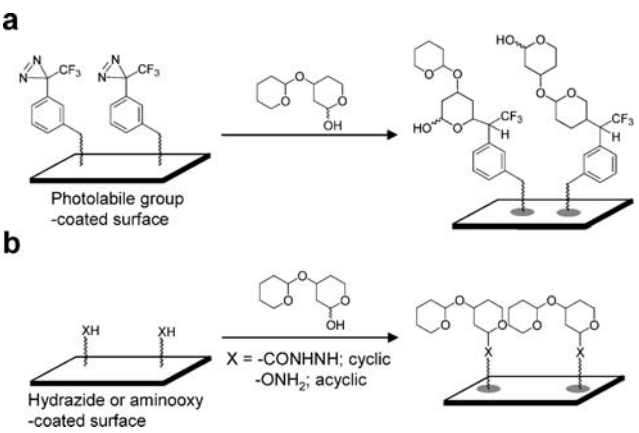

Fig. 8 Immobilization of unmodified glycans on (a) diazirine-derivatized surface and (b) hydrazide- or aminooxy-derivatized surface.

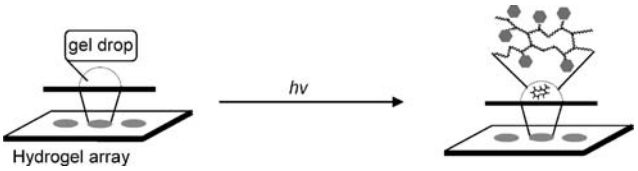

Fig. 9 Construction of 3-D hydrogel carbohydrate microarrays by photo-induced polymerization-mediated immobilization of amine-conjugated glycans in gel drops.

Detection methods

Another important component associated with useful carbohydrate microarray systems is detection of binding of proteins, cells and other biomaterials to glycans immobilized on surfaces. Fluorescence-based methods are the most widely used for this purpose because of their high sensitivity and throughput as well as the availability of fluorescence detectors such as a high-resolution microarray scanner (Fig. 10(a)). Any surface material, such as glass, gold and membrane, is compatible with this detection method. In this procedure, carbohydrate microarrays are incubated with fluorophore-labeled proteins. The fluorescence intensities of spots, which are proportional to the amount of bound proteins, are quantitatively determined by using a microarray scanner. It should be noted that labeling of proteins with fluorescent dyes (*e.g.* fluorescein, Cy3 or Cy5) often causes protein denaturation and/or interference with their binding to glycan ligands. Nonetheless, this detection technique has been applied successfully in many cases.

Alternatively, secondary reagents (usually antibody) that specifically recognize primary proteins bound to glycans can be used to evaluate protein binding. In this procedure, the microarrays are initially incubated with unlabeled protein and subsequently treated with the secondary reagent, labeled with a fluorescent dye or enzyme. The use of secondary reagents for detection avoids denaturation of the primary protein promoted by direct protein labeling. With the enzyme-labeled secondary protein, the fluorescent signal can be amplified to enhance sensitivity by using a substrate that generates a fluorescent product. However, owing to their limited availability, the use of secondary reagents is not applicable to all systems. Also, whole cells bound to glycans on the surface can be fluorescently detected by labeling them with cell-permeable dyes prior to incubation or after binding to the glycans.[54,55]

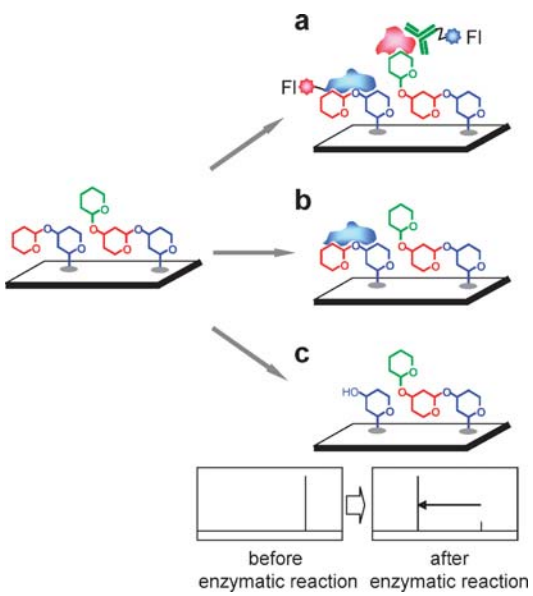

Fig. 10 Detection methods: (a) fluorescence method, (b) SPR imaging method and (c) MS method (Fl: fluorescent dye).

Label-free detection methods have been developed to overcome problems caused by protein denaturation or the limited availability of secondary reagents. Although conventional SPR spectroscopy does not require labeled proteins, it can not be applied to characterize protein–carbohydrate interactions in a high-throughput manner. Recently, a SPR imaging technology has been developed for rapid analysis of biomolecular interactions.[56] This method has been utilized in conjunction with carbohydrate microarrays to evaluate recognition events between carbohydrates and proteins (Fig. 10(b)).[57,58] A gold surface is required since gold is suitable for monitoring surface plasmon resonance phenomena. In this technique, SH-functionalized glycans are directly attached to the surface *via* the Au–S linkage or other functionalized glycans are indirectly attached to the gold surface *via* chemoselective ligation reactions.

Another label-free detection method based on mass spectrometry has been developed. This technique can be utilized to detect the modification of carbohydrates on glycan microarrays but it is inappropriate for detection of bound proteins on surfaces. For example, the results of enzymatic reactions promoted by carbohydrate-processing enzymes on carbohydrate microarrays are readily characterized by using MALDI-TOF MS (Fig. 10(c)).[59,60] This technique can also be applied to determine the time-dependence of enzymatic glycosylation.

Applications

Since their advent in 2002,[16–20] applications of carbohydrate microarrays to biological and biomedical research have been rapidly expanded (Fig. 11). Most extensive use of this technology has been in the high-throughput analysis of the binding properties of proteins, such as plant and animal lectins, antibodies, cytokines, chemokines and growth factors.

Plant lectins have been utilized as biological research tools and diagnostic agents. Since information about the binding specificities of these proteins is important, they have been extensively investigated by using conventional solution-based methods, such as the hemagglutination inhibition assay.[61,62] To evaluate the detailed binding patterns for plant lectins, microarrays containing a number of glycans have been probed with a variety of plant lectins (Fig. 12). The results obtained by using these microarrays have provided information about the detailed binding properties of these proteins.[19,33,43,63] Since a large number of carbohydrates can be probed by using the microarray technique, new glycan–lectin interactions were also identified. Recently, plant lectins are mostly used to validate the correct preparation of glycan microarrays since their binding properties have been extensively investigated by using this microarray technology.

Animal lectins are more interesting since (1) their interactions with glycans play a variety of important roles in

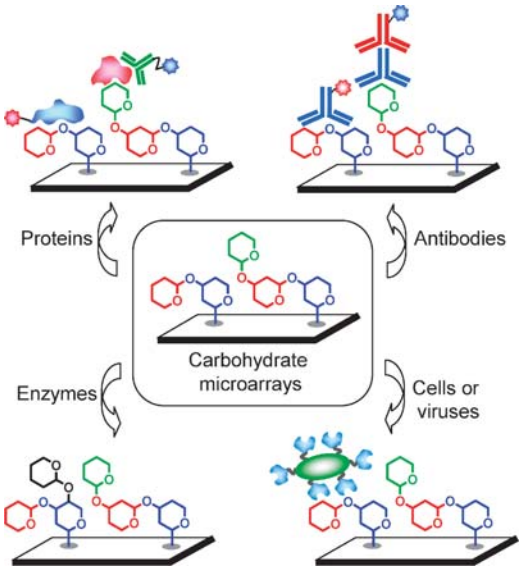

Fig. 11 Applications of carbohydrate microarrays for biological and biomedical research.

biological processes, and (2) information about their glycan binding specificities can be used to develop novel therapeutic agents. The binding patterns of various animal lectins have been evaluated by using carbohydrate microarrays. One example involves the analysis of binding preferences of DC-SIGN and DC-SIGNR (DC-SIGN related), members of the C-type lectins (Ca^{2+}-dependent lectins). DC-SIGN, mainly expressed on dendritic cells, is involved in pathogenesis of viruses and innate immunity. DC-SIGNR, a closely related receptor found on endothelial cells, is also likely to be involved in pathogen infection. The results of microarray experiments show that both receptors interact with high mannose oligosaccharides.[64,65] In addition, DC-SIGN, but not DC-SIGNR, also recognizes fucose-containing sugars such as Le^a, Le^b, Le^x and Le^y. The results of DC-SIGN recognizing mannose- and fucose-containing glycans support the proposal that it binds to pathogens with high mannose glycans (*e.g.* HIV, hepatitis C virus, Ebola virus, *M. tuberculosis* and *Leishmania* parasites) as well as with fucose-containing glycans (*e.g.* schistomes and *H. pylori*).

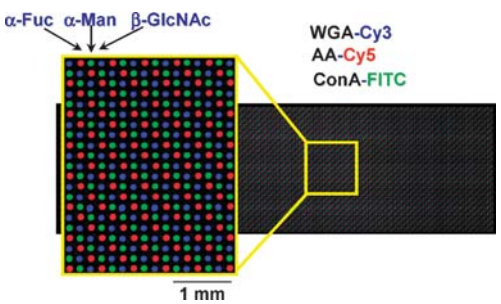

Fig. 12 Fluorescence image of 12 000 microspots (60 × 120) consisting of α-Fuc, α-Man and β-GlcNAc probed with a mixture of Cy3-wheat germ agglutinin (WGA), Cy5-*A. aurantia* (AA) and FITC-ConA.

The binding properties of another antigen-presenting cell receptor, langerin (C-type lectin), were also examined by using glycan microarrays.[65] This receptor is expressed on Langerhans cells and is involved in innate and acquired immunity. The results of microarray experiments show that langerin binds to sulfated Le^x sequences with the sulfate group at position 6 of galactose but it rarely interacts with mannosylated glycans unlike DC-SIGN and DC-SIGNR.

The microarray technology has been also used to profile other C-type animal lectins. The scavenger receptor C-type lectin (SRCL), an endothelial receptor involved in innate immunity, interacts with Le^x-containing glycans.[66] However, this lectin does not bind to sialylated or sulfated forms of these sugars. The microarray studies also demonstrate that the primary binding site of SRCL is a galactose rather than a fucose moiety. Dectin-1, a C-type lectin-like receptor expressed on leukocytes, binds to 1,3-linked glucose oligomers.[67] However, Dectin-2 recognizes high-mannose structures with greatest recognition of $Man_9GlcNAc_2 > Man_8GlcNAc_2$ and to a lesser extent $Man_7GlcNAc_2$.[68]

Siglecs, another type of the animal lectin, are a subset of the immunoglobulin gene superfamily. Glycan microarrays have been used to characterize binding preferences of Siglecs, including Siglec-2, Siglec-F and Siglec-8. Siglec-2 selectively recognizes glycans with Neu5Acα2,6Galβ1,4GlcNAc epitope.[43] Interestingly, 6-sulfation of the GlcNAc moiety in these sequences enhances the recognition by this protein. Mouse Siglec-F, an eosinophil surface receptor, binds to the 6′-sulfo-sialyl Le^x with the highest affinity and related glycans such as sialyl Le^x and 6-sulfo-sialyl Le^x with a much lower binding affinity.[69] This observation suggests that sulfation of galactose residue is important for Siglec-F binding. Human Siglec-8 interacts with 6'-sulfo-sialyl Le^x but rarely binds to non-sulfated sialyl Le^x and 6-sulfo-sialyl Le^x like mouse Siglec-F.[70] Galectins, β-galactoside-binding animal lectins, were also profiled by using carbohydrate microarrays.[43,71,72] These lectins recognize terminal and internal galactose moieties.

In addition, glycan microarrays have been employed to investigate cross-reactivity of anti-glycan antibodies. Profiling of glycan-antibody interactions by using carbohydrate microarrays demonstrates that some monoclonal antibodies, which are considered to be specific for their designated glycan antigens, cross-react with other glycan epitopes.[17,73–75] This finding suggests that the results obtained from the use of anti-glycan antibodies should be carefully analyzed and interpreted.

Glycosaminoglycans (GAGs), such as chondroitin sulfate, heparin/heparan sulfate, keratan sulfate, dermatan sulfate and hyaluronan, are a large class of polysaccharides that consist of disaccharide repeating units. These glycans play various roles in physiological processes, including homeostasis, cancer metastasis, cell growth, cell migration and development, through their interactions with proteins. It has been reported that GAGs interact with a host of proteins, including growth factors, proteases, cytokines, chemokines, and cell adhesion molecules.[76] However, little is known about the GAG motifs that these proteins recognize. Glycan microarrays containing synthetic chondroitin sulfate and heparin oligosaccharides

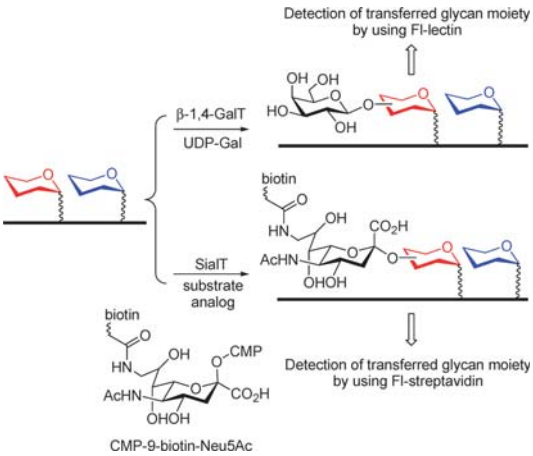

Fig. 13 Analysis of the acceptor specificities of glycosyltransferases by using carbohydrate microarrays.

have been applied to rapidly evaluate binding properties of growth factors, cytokines and chemokines, and for profiling the sulfation specificities of GAG interactions with proteins.[77–80] It has been demonstrated that specific sulfation motifs in glycans are essential for binding to proteins.

Another interesting application of glycan microarrays involves the rapid analysis of the acceptor specificities and catalytic activities of glycosyltransferases. The substrate specificities of galactosyl- (GalT) and sialyltransferases (SialT) have been evaluated by treating glycan microarrays with the enzymes in the presence of either natural glycosyl donor (UDP-Gal)[38] or biotinylated glycosyl donor (CMP-9-biotin-Neu5Ac)[81] and subsequent detection of the transferred sugar moiety by using fluorophore-labeled RCA_{120} or streptavidin, respectively (Fig. 13). In addition, the relative rates of glycosylation of glycans by glycosyltransferases on the microarrays have also been determined by measuring time-dependent glycosylation of sugars immobilized on the surface by the enzymes.[38] Moreover, complex oligosaccharides have been prepared from simple carbohydrates on carbohydrate microarrays by using glycosyltransferases. For example, sialyl Le^x tetrasaccharide was prepared by consecutive treatment of immobilized GlcNAc with β-1,4-galctosyltransferase/UDP-Gal, α-2,3-sialyltransferase/CMP-NeuAc and α-1,3-fucosyltransferase/GDP-Fuc (Fig. 14). Probing the enzyme-treated sugars with the anti-sialyl Le^x antibody shows that sialyl Le^x is successfully synthesized from GlcNAc in these processes.[33] The results of these studies demonstrate that carbohydrate microarrays can be utilized to characterize novel carbohydrate-processing enzymes. Furthermore, this microarray technology can potentially play an important role in drug discovery programs for the development of novel inhibitors for carbohydrate-processing enzymes.[82]

Glycan microarrays can be also applied to determine binding affinities of proteins to sugars. In initial studies in this area, IC_{50} values of soluble inhibitors for protein binding to carbohydrates immobilized on the surface were determined. For this purpose, microarrays containing specific carbohydrates were treated with

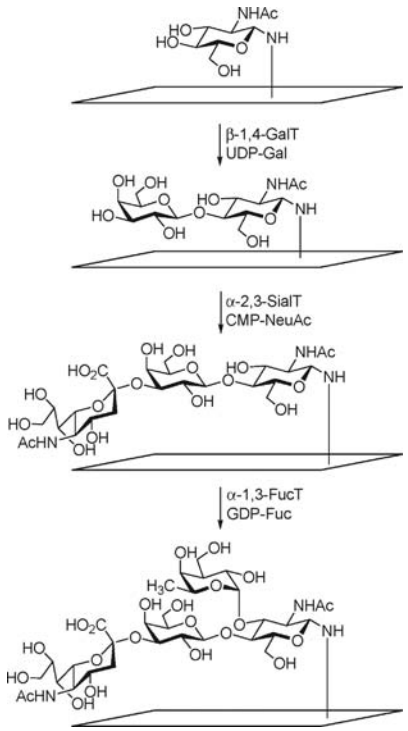

Fig. 14 Enzymatic synthesis of sialyl Le^x from GlcNAc on the microarrays by using three glycosyltransferases consecutively.

a series of pre-incubated mixtures of the fluorescent dye-labeled protein and an inhibitor. The IC_{50} values of soluble inhibitors were then determined by measuring fluorescence intensities of bound proteins on the microarrays.[19,33] More recently, various K_d values (dissociation constants) between proteins and surface-immobilized glycans were measured from a single experiment, in which microarrays containing several glycans were probed with various concentrations of labeled proteins.[38,83] Dissociation constants for surface-bound sugars with proteins were then determined by measuring fluorescence intensities of bound proteins on the microarrays. The K_d values obtained from these experiments were found to be similar to those obtained in SPR experiments.

Glycan-mediated recognition of mammalian cells has been investigated by using carbohydrate microarrays. For example, sugar interactions of primary chicken hepatocytes, that constitutively express C-type GlcNAc-binding lectin on the cell surface, were examined. Microarray experiments demonstrate that these cells adhere to nonreducing terminal GlcNAc but do not bind to nonreducing terminal Gal and GalNAc.[54] This technology was also applied to evaluate glycan-binding properties of human $CD4^+$ T-cells.[54] These cells were found to adhere to sialyl Le^x, perhaps *via* cell surface L-selectin, but rarely interact with the nonfucosylated form of this glycan.

Carbohydrate microarrays serve as an attractive platform for diagnostic applications since glycans are involved in many pathological processes. Examples of diagnostic applications include the detection of antibodies raised against pathogenic glycans. Most pathogens express specific immunogenic glycans on their surfaces and pathogen-infected humans provide antibodies that interact with the pathogenic glycans. These antibodies can be detected by utilizing carbohydrate microarrays

that contain pathogenic glycans. For instance, the microbial polysaccharide or *Salmonella* O-antigen microarrays were created for the diagnosis of pathogen infection using human serum samples.[17,84–86]

Further diagnostic applications of glycan microarrays include the detection and study of binding properties of intact pathogens. For example, carbohydrate microarrays were incubated with stained *E. coli* bacteria that express a mannose-binding protein (Fim H) on the surface. The bacteria selectively recognize mannose residues among various carbohydrates but the strain lacking Fim H does not bind to these glycans.[50,55] In addition, the binding properties of *Helicobacter pylori* were also characterized by incubating glycoconjugate arrays with labeled bacteria since some strains of these bacteria express SabA (sialic acid-binding adhesin) and BabA (Le^b-binding adhesin).[87] In addition, the detailed binding profiles of isolated bacterial proteins have been investigated by using carbohydrate microarrays.[88] Cyanovirin-*N* (CVN) and scytovirin inhibit the initial step of HIV-1 entry into host cells by binding to high-mannose glycans on the HIV-1 envelope glycoprotein gp120. Analysis of the carbohydrate binding profiles of these cyanobacterial proteins with glycan microarrays shows that Manα1,2Man linkages are necessary for carbohydrate recognition by CVN. However, scytovirin binds to Manα1,2Man linked to α1,6Man moiety, indicating that this protein has a more restricted binding preference than CVN.

Glycan microarrays have been also utilized to analyze the binding properties of viral proteins and intact viruses. The surface glycoprotein hemagglutinin (HA) on influenza A viruses binds to glycans with terminal sialic acids. In general, human influenza viruses preferentially adhere to the Neu5Acα2,6Gal residues on epithelial cells of the lungs and upper respiratory tract. However, avian influenza viruses are specific for Neu5Acα2,3Gal residues on intestinal epithelial cells. To provide detailed profiles of the glycan specificities of several types of influenza viruses (human and avian H1 and H3 viruses, and 1918 H1N1 pandemic strains), binding properties of the hemagglutinins have been evaluated by using glycan microarrays. The results of these experiments show that human and avian hemagglutinins preferentially bind to Neu5Acα2,6Gal and Neu5Acα2,3Gal residues, respectively, although subtle differences in hemagglutinin specificities occur when the terminal trisaccharides undergo fucosylation, sulfation and sialylation.[89,90] Glycan arrays have been also applied to evaluate the specificities of the intact influenza virus. This virus adheres to Neu5Acα2,3Gal and Neu5Acα2,6Gal containing oligosaccharides on the arrays.[43]

Carbohydrate microarrays containing the tumor antigen Globo H and its fragments have been prepared in order to analyze two monoclonal antibodies (MBr1 and VK-9) against the Globo H epitope and the serum from breast cancer patients.[91] The microarray studies show that two monoclonal antibodies recognize the terminal tetrasaccharide (Fucα1,2-Galβ1,3GalNAcβ1,3Gal) with the same binding affinity as Globo H and that the terminal fucose moiety in the Globo H epitope is essential for binding of these antibodies. However, antibodies in the serum from breast cancer patients interact equally well with both the full sequence Globo H and the pentasaccharide lacking the terminal fucose residue. The difference between the binding properties of two monoclonal antibodies and the serum is probably due to the polyclonal nature of the antibodies in the serum and/or the presence of various antibodies generated at different stages. The carbohydrate microarray technology has also been applied to compare serum antibody levels between Hodgkin's lymphoma patients and a health control.[92] The findings show that several carbohydrates have a differential antibody response in patients compared to control sera.

Conclusions

As described above, carbohydrate microarrays, prepared by various immobilization techniques, can be applied for studies of a wide variety of carbohydrate-mediated biological processes. In addition, they can be used as powerful diagnostic tools to detect pathogens, viruses and disease-related glycan specific antibodies from sera. As a result, carbohydrate microarrays have become an indispensable technology for glycomics research in a manner like DNA microarrays are for genomics research. Sustained advances in this area will make this microarray technology a more general and practicable platform for studies of functions of glycans.

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