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Chapter 16

Carbohydrate Microarrays as Essential Tools of Postgenomic Medicine

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I. Introduction

Carbohydrates, like nucleic acids and proteins, are essential biological molecules carrying important biological information. Carbohydrates are prominently displayed on the surface of cell membranes and expressed by virtually all secretory proteins in bodily fluids. This is achieved by the events of posttranslational protein modification called glycosylation. Importantly, expression of cellular glycans, in the form of either glycoproteins or glycolipids, is differentially regulated. Cell display of precise complex carbohydrates is characteristically associated with the stages or steps of embryonic development, cell differentiation, as well as transformation of normal cells to abnormally differentiated tumor or cancer cells (12,22,23,28). Sugar moieties are also abundantly expressed on the outer surfaces of the majority of viral, bacterial, protozoan, and fungal pathogens. Many sugar structures are pathogen specific, which makes them important molecular targets for pathogen recognition, diagnosis of infectious diseases, and vaccine development (16,20,29,40,48,65).

Exploring the biological information content in carbohydrates is one of the current focuses of postgenomic research and technology development. Biophysical,
biochemical, and immunological methods have proven very valuable in studying carbohydrate–carbohydrate and carbohydrate–protein interactions. For example, X-ray crystallographic and NMR spectroscopic techniques have been employed to determine binding modes between carbohydrates and proteins. Surface plasmon resonance spectroscopy and isothermal titration calorimetry (ITC) can provide information on the binding affinities of carbohydrates to proteins. Many well-established immunochemical methods have been applied to determine the specificity and cross-reactivity of carbohydrate–antibody and carbohydrate–lectin interactions. These classical approaches were, however, designed to monitor carbohydrate-based molecular recognition on a one-by-one basis and have limited analytical power or throughput in practical applications.

A pressing need is, thus, the establishment of high-throughput technologies to enable the large-scale, multiplex analysis of carbohydrates and their cellular receptors. These include especially the characterization of immunological properties of carbohydrates that are important for medical applications of carbohydrate antigens and interactions of carbohydrates with other biomolecules or intact cells that play key roles in establishing comprehensive biological functions of essentially all existing living organisms. In parallel with developing microarray-based high-throughput technologies for nucleic acids (5,15,49) and proteins (37,38,54), significant progress has been made in developing carbohydrate microarrays (1,21,25,32,46,64,68,70).

In this chapter, we attempt to illustrate a few examples, with a focus on infectious diseases, to discuss the medical application of carbohydrate microarrays. We also discuss the principles for construction of various platforms of carbohydrate microarrays. This information may be helpful in selecting the proper technologies to address biomedical questions related to carbohydrates.

II. Carbohydrate Microarrays as Essential Tools in the Postgenomics Era

In the past few years, a number of experimental approaches have been applied to construct carbohydrate microarrays (1,21,25,32,46,64,68,70). In spite of their technological differences, these carbohydrate microarrays are all solid-phase binding assays for carbohydrates and their interactions with other biological molecules. They share a number of common characteristics and technical advantages. First, they contain the capacity to display a large panel of carbohydrates in a limited chip space. Second, each carbohydrate is spotted in an amount that is drastically smaller than that required for a conventional molecular or immunological assay. Thus, the bioarray platform makes an effective use of carbohydrate substances. Third, they have high detection sensitivity. The microarray-based assays have higher detection sensitivity than most conventional molecular and immunological assays. This was attributed to the fact that the binding of a molecule in solution phase to an immobilized microspot of ligand in the solid phase has minimal reduction of the molar
concentration of the molecule in solution (19). Therefore, in a microarray assay, it is much easier to have a binding equilibrium take place and result in a high sensitivity.

Carbohydrate microarrays constructed by various methods may differ in their technical features and suitability for a given practical application. Some platforms may be applied complementarily to solve biological questions. The method of nitrocellulose-based immobilization of carbohydrate-containing macromolecules, including polysaccharides, glycoproteins, and glycolipids, is suitable for the high-throughput construction of carbohydrate antigen microarrays (25,62,63,67,68). This platform of carbohydrate microarrays is readily applicable for the large-scale immunological characterization of carbohydrate antigens and anti-carbohydrate antibodies. It is also useful for the initial screening of carbohydrate-binding proteins, such as those newly identified by the human genome project with preserved carbohydrate-binding domains and are predicted to have carbohydrate-binding properties. However, the detection specificity of this carbohydrate microarray would be at the level of a carbohydrate antigen, not a glycoepitope, if the native carbohydrate antigens were spotted. This is owing to the fact that many carbohydrate antigens display multiple antigenic determinants or glycoepitopes. Examining the finer details of the binding properties would require the use of microarrays of defined oligosaccharide sequences. Oligosaccharide array-based binding assays can be applied, in combination with saccharide competition assays, to decipher precise saccharide components of a specific antigenic determinant or glycoepitope (25,64).

We present here a few examples of medical applications of carbohydrate microarray technologies. The studies summarized below involve the use of carbohydrate microarrays to study emerging infectious agents, including SARS-CoV, Influenza virus A, and Bacillus anthracis (B. anthracis). This progress highlights the potential of the relatively nascent carbohydrate microarray technologies in exploring the mysteries of life shrouded in the structure of carbohydrates. The areas that require carbohydrate microarrays are far beyond infectious diseases in medicine.

A. Recognition of Autoimmunogenic Reactivity of SARS-CoV

Severe acute respiratory syndrome (SARS) is an emerging infectious disease that became a global fear in 2003. A previously unrecognized coronavirus, SARS-CoV, is responsible for the epidemic spread of SARS (24,35). In an effort to understand the immunogenic properties of carbohydrate structures expressed by the virus, a carbohydrate microarray printed on nitrocellulose-coated microglass chips was applied (68). This study involves three steps of experimental investigation. The first step is to perform a carbohydrate microarray characterization of the antibody responses to the virus. The second step focuses on identification of lectins and/or antibodies that are specific for the glycoepitopes that are recognized by the pathogen-elicited antibodies. This provides specific structural probes to enable the
third step of investigation, that is, to probe the glycoepitopes of the pathogens using specific lectins or antibodies identified by steps 1 and 2.

The first step, microarray analysis, revealed that immunization of horses with a preparation of inactivated SARS-CoV induced antibodies specific for an abundant human glycoprotein asialo-orosomucoid (ASOR). Since the horse antisera has no reactivity toward agalacto-orosomucoid (AGOR), which lacks galactose in the upper stream nonreducing ends, the glycoepitopes with terminal galactose may contribute significantly to the antigenic reactivity of SARS-CoV. This glycan array finding gave an important lead in terms of identifying appropriate immunological probes to further characterize the glycoepitopes expressed by SARS-CoV. A microarray containing a panel of galactose-containing complex carbohydrates was created to scan for immunological probes specific for the ASOR-glycans. The lectin PHA-L was shown to be highly specific for the spotted ASOR preparation. The latter is known to be specific for the glycoepitopes that are composed of tri-antennary Gal\(\beta_1\)–4GlcNAc (Tri-II) or multiantennary Gal\(\beta_1\)–4GlcNAc (m-II) (see Fig. 1 for an asialo-Tri-II structure of N-glycans) (72). With this specific probe, the authors characterized SARS-CoV-infected and -uninfected cells. PHA-L was found to stain cells infected with SARS-CoV and this reactivity could be inhibited by ASOR but not AGOR. The authors concluded, therefore, that the glycoepitopes Tri-II or m-II of ASOR are highly expressed by SARS-CoV-infected cells.

These observations raise important questions about whether autoimmune responses are in fact elicited by SARS-CoV infection in human and other animal species and whether such autoimmunity contributes to SARS pathogenesis. ASOR is an abundant human serum glycoprotein and the ASOR-type complex carbohydrates are also expressed by other host glycoproteins (13,43). Thus, the human immune system is generally nonresponsive to these “self” carbohydrate structures. However, when similar sugar moieties were expressed by a viral glycoprotein, their cluster configuration could differ significantly from those displayed by a cellular glycan, and in this manner, it generates a novel “nonself” antigenic structure. Much remains to be learned regarding the specificity and cross-reactivity of the carbohydrate-mediated molecular recognition and its role in the “self/nonself” immune discrimination.

B. Deciphering the Sugar Codes for Selective Viral Entry of Host Cells (Influenza A)

Glycan arrays have also been applied to study the interaction between the Influenza virus A and its cellular receptors (51–53). This virus recognizes specific saccharides on the host’s epithelial cells and utilizes these cellular glycans as receptors to initiate an infection. An antigenic protein on the virus’ coat, hemagglutinin (HA), recognizes sialic acid-terminated glycans (see Fig. 1 for sialic acid-terminated glycans in the Tri-II sugar chain configuration). In addition, HA can distinguish between different kinds of sialic acid–galactose linkages. For example, HA variants adapted to humans recognize an \(\alpha 2–6\) linkage whereas strains specific for
birds recognize an \(\alpha 2-3\) linkage. The specificity of a variety of HAs toward carbohydrates containing sialic acid residues attached via \(\alpha 2-3\), \(\alpha 2-6\), and \(\alpha 2-8\) linkages was screened using a glycan array. The assay showed that specific mutations control the specificity of a given HA that selectively binds to a given linkage. The microarray could also be used to probe the effect of charge, size, sulfation, fucosylation, and sialylation on the binding specificity. The binding specificity between two previous pandemic strains of HA, 18NY and 18SC, could be distinguished by the microarray since the 18NY recognizes both \(\alpha 2-6\) and \(\alpha 2-3\) linkages whereas 18SC recognizes only \(\alpha 2-6\) linkages. Only two mutations are required to make the avian strain sufficiently virulent toward humans. This study illustrates an example that glycan array analysis, in conjunction with mutation studies, helps in understanding and predicting how pathogenic strains can become virulent toward humans.

Figure 1  Characteristic carbohydrate moieties serve as markers for biological recognition. N-Glycan type II chains (Galβ1 →4GlcNAc) in the tri-antennary cluster configurations with (Tri-II) and without sialic acid terminal residues (asialo-Tri-II). The asialo-Tri-II sugar moieties but not the Tri-II structures are specifically targeted by a horse-neutralization antibody of SARS-CoV (68).
C. Identification of Immunogenic Sugar Moieties of *B. anthracis* Exosporium

Recent effort in hunting for the highly specific immunogenic sugar moiety of *B. anthracis* further demonstrates the potential of glycan array technologies in biomarker identification (64). *B. anthracis*, the etiological agent of anthrax infection, is a gram-positive, rod-shaped bacterium. The most lethal form of human anthrax is the pulmonary infection caused by inhaled spores. In view of the risk of *B. anthracis* spores as a biological weapon of mass destruction (WMD) (69), it is necessary to achieve the capacity for the rapid and specific detection of *B. anthracis* spores in various conditions (41,71). It is also important to develop new vaccines to block the anthrax infection at its initial stage before spore germination takes place (11,60). In this context, identification of highly specific immunogenic targets that are displayed on the outermost surfaces of *B. anthracis* spores is of utmost importance.

A photogenerated oligosaccharide array was introduced to facilitate identification and characterization of anthrax spore-specific immunogenic carbohydrate moieties (64). In essence, the authors utilized a photoactive surface on glass substrates for covalent immobilization and micropatterning of carbohydrates (7). They then applied this glycan array to probe specific antibodies that were elicited by immunizations using anthrax spores. The authors assumed that if *B. anthracis* spores express potent immunogenic carbohydrate moieties, immunization with the spores would elicit antibodies specific for these sugar structures. Such antibody reactivities would then be detected by glycan arrays that display the corresponding sugar structures. A schematic overview of this biomarker identification strategy is shown in Fig. 2.

This investigation demonstrates that IgG antibodies elicited by the native antigens of the *B. anthracis* spore recognize synthetic anthrose-containing sugar moieties. The saccharide-binding reactivities correlate directly with the sizes of the saccharides displayed by the glycan arrays. The terminal anthrose monosaccharide is marginally reactive and the anthrose-containing tetrasaccharides highly reactive, regardless of their anomeric configuration. However, the smaller saccharide units, including the anthrose mono-, di-, and trisaccharides, are potent inhibitors of the specific antibody reactivities to the tetrasaccharides displayed either by the photogenerated glycan arrays or by a bovine serum albumin (BSA) conjugate on an enzyme-linked immunosorbent assay (ELISA) microtiter plate. It was, thus, concluded that the anthrose-containing tetrasaccharide is immunogenic in its native configuration as displayed by the exosporium BcLA glycoprotein and its terminal trisaccharide unit is essential for the constitution of a highly specific antigenic determinant.

Since the anthrose-containing carbohydrate moiety is displayed on the outermost surfaces of *B. anthracis* spores (14,55) and its expression is highly specific for the spore of *B. anthracis* (14), this unique tetrasaccharide is likely an important immunological target. Its applications may include identification of the presence of *B. anthracis* spores, surveillance and diagnosis of anthrax infection, and development of novel vaccines targeting the *B. anthracis* spore. In general, this glycan
array-based biomarker hunting strategy is likely applicable for exploring the immunogenic carbohydrate moieties of other microbial pathogens.

### III. Progress in Developing Complementary Platforms of Carbohydrate Microarrays

In order to make the best use of available technologies of carbohydrate microarrays, it is important to conceptually understand the design and chemical principles of different carbohydrate microarray platforms. Different platforms may be technically complementary and can be applied in combination in addressing biomedical questions. For this purpose, we outline below a number of carbohydrate array platforms based on the chemical principle of array construction. These include technologies that directly utilize underivatized carbohydrates in microarray construction, technologies that require chemical modification of carbohydrates...
before microarray fabrication, methods of noncovalent immobilization of carbohydrates, and methods of covalent coupling of saccharides on array substrates. There are also technologies that are designed to display saccharides in defined orientations or specific cluster configurations in order to resemble the native configuration of functional carbohydrate ligands.

A. Carbohydrate Microarrays Fabricated by Using Underivatized Carbohydrates

The use of underivatized saccharides for microarray construction has the unique advantage of preserving the native structures of the carbohydrate molecules. It requires, however, a ready-to-use microarray surface with appropriate surface chemistry that can be directly used to fabricate comprehensive carbohydrate microarrays with underivatized carbohydrates from a wide range of sources. Methods include noncovalent binding of underivatized carbohydrate probes on a chip by passive adsorption and methods for covalently immobilizing underivatized carbohydrates on a slide surface by appropriate chemical-linking techniques.

1. Nonsite-Specific and Noncovalent Immobilization of Underivatized Carbohydrates in Microarrays

Noncovalent adsorption of native carbohydrate probes on a substrate surface is the simplest way to prepare carbohydrate microarrays. This method relies on the formation of a variety of noncovalent interactions between the surface and the arrayed carbohydrates. In addition to its simplicity and high-throughput characteristics in array construction, these approaches may be favorable in supporting the preservation of the native structure of spotted carbohydrate antigens since there is no need to modify the carbohydrates before microarray application. However, given that the saccharides are noncovalently immobilized on an array substrate, the efficiency of immobilization must be verified for each spotted carbohydrate.

Wang et al. (67) described a method of noncovalent immobilization of unmodified carbohydrates for construction of carbohydrate microarrays. They applied robotic microarray spotters to array the carbohydrates onto nitrocellulose-coated glass slides without any chemical modification. After air-drying at room temperature, the spotted arrays are ready for application. A wide range of carbohydrate antigens, including polysaccharides, glycoproteins, proteoglycans, and semisynthetic glycoconjugates, were tested by spotting them on the substrate and then probed with specific antibodies and lectins to verify the epitopes preserved in the carbohydrate microarrays.

The investigators showed that the nitrocellulose-coated glass chip is a ready-to-use substrate for carbohydrate microarrays by proving the fact that (i) carbohydrate-containing macromolecules of various structural configuration can be immobilized on a nitrocellulose-coated glass slide without chemical conjugation; (ii) the immobilized carbohydrate antigens are able to preserve their immunological properties and solvent accessibility; (iii) the system reaches the sensitivity, specificity, and capacity to detect a broad range of antibody specificities in clinical
specimens; and (iv) this technology allows highly sensitive detection, as compared with other existing technologies, of the broad range of carbohydrate–lectin/antibody interactions. This strategy takes advantage of the existing cDNA microarray system, utilizing a spotter and a scanner for an efficient production and application of carbohydrate microarrays. In addition to the carbohydrate microarray application, this bioarray platform has been extended to spot microarrays of proteins (ProtoArray®, Invitrogen, CA) and cell lysates (8), as well as lectins for glycan profiling analysis (Procognia, United Kingdom).

However, this substrate is unlikely applicable for the immobilization of unmodified mono- and oligosaccharides. Using fluorescein-labeled preparations of α(1,6)dextran, ranging from 20 to 2000 kDa, and inulin of 3.3 kDa, the authors investigated whether the size and molecular weight (MW) of saccharides influence their surface immobilization. They found that the efficiency of immobilization was dependent on the molecular mass of the spotted carbohydrates; the larger molecules were better retained than the smaller molecules. The reduced capacity of surface immobilization of smaller saccharide chains is likely owing to the fact that saccharide immobilization is based on passive interactions between spotted saccharides and the nitrocellulose-coated glass slides.

A practical way to compensate this weakness was described, which involves the use of glycoconjugates, either oligosaccharide–protein conjugates (67,68) or neoglycolipid (NGL) conjugates (25), for construction of the epitope-defined microarrays. In order to examine whether desired glycoepitopes or antigenic determinants are preserved after immobilization, the authors stained the microarrays using well-characterized monoclonal antidextran antibodies. These include antibodies bearing either the groove-type or the cavity-type antibody-combining sites (10,65). The former recognizes the internal linear chain of α(1,6)dextran; while the latter is specific for the terminal nonreducing end structure of the polysaccharide. Results of this analysis confirmed that the desired glycoepitopes were well preserved by the spotted polysaccharide α(1,6)dextrans and by oligosaccharide–protein conjugates, that is, isomaltotriose (IM3) and isomaltotriosepentaose (IM7) coupled to BSA (67). Recently, Feizi’s group further demonstrated the use of this platform for the construction of NGL-based oligosaccharide microarrays (25).

The nitrocellulose polymer substrate was a fully nitrated derivative of cellulose, in which the free hydroxyl groups are substituted by nitro groups, and is thus hydrophobic in nature. Researchers have shown that the immobilization of proteins on nitrocellulose surfaces relies on hydrophobic interactions. However, polysaccharides, being rich in hydroxyl groups, are hydrophilic in nature (42,61). The molecular forces for the carbohydrate–nitrocellulose interaction remain to be characterized, but it has been suggested that the three-dimensional (3D) microporous configuration of the nitrocellulose on the slides and the macropolymer characteristics of polysaccharides play important roles for the stable immobilization of many polysaccharides on the nitrocellulose surface. The polysaccharide molecules immobilized onto the nitrocellulose film are in a nonsite-specific format (Fig. 3).

A surface-modified polystyrene substrate provides another type of polymer surface that can be directly used to prepare carbohydrate microarrays through
the noncovalent immobilization of underivatized polysaccharides. The polystyrene substrate is produced by injection moulding of black polystyrene and the surface is modified by oxidation. This type of slide is commercially available from Nunc Roskilde, Denmark (http://www.nuncbrand.com/). Willats et al. of University of Leeds, United Kingdom, showed the applicability of this slide surface to produce comprehensive microarrays of polysaccharides, glycoproteins, proteoglycans, and cell extracts (70). These carbohydrate microarrays were directly fabricated by applying 50 pL of polysaccharide solution per spot on the black styrene substrate with a pitch of 375 \( \mu \text{M} \). Probing these arrays using previously characterized monoclonal antibodies and a phage-derived antibody, the predicted patterns of antibody binding were observed.

2. Covalent Immobilization of Underivatized Carbohydrates in Microarrays

Covalent attachment is often preferred over other types of immobilization modules, such as those based on noncovalent bonds, including van der Waals forces, hydrogen bonds, hydrophobic forces, and ionic bonds in aqueous solutions, and various affinity-based binding reactions. Covalent bond formation provides a more stable linkage between the carbohydrate and the array substrate. Since the coupling efficiencies of the carbohydrate moieties are more readily controlled, the immobilization reproducibility is likely independent of the differences in the structures of carbohydrate probes.

A number of investigators have put effort in developing general, simple, and efficient array substrates that can be applied to a range of unprotected and unmodified oligosaccharides and polysaccharides. Four types of surface-functionalized substrates and related chemical-linking techniques have been reported to date for fabrication of carbohydrate microarrays through covalent immobilization of underivatized carbohydrates irrespective of the carbohydrate size.

Zhou et al. reported the slide surface containing aminooxy– groups as a platform for immobilizing an array of oligosaccharides through the formation of an oxime bond with the carbonyl group at the reducing end of a given carbohydrate (73). The use of aminooxyacetil-terminated self-assembled monolayers for the immobilization of carbohydrates takes advantage of the oxime formation reaction between a highly reactive amine group of the nucleophilic aminooxyacetil group and the carbonyl group at the reducing end of suitable carbohydrates via irreversible condensation.
Aminooxyacetyl-functionalized glass slides were prepared in four steps starting with a (3-glycidyloxypropyl)trimethoxysilane (GPTS)-functionalized glass slide. The synthesis of the functionalized glass slide is presented in Figure 4. The glycidyl group of the GPTS monolayer was treated with diamino-poly(ethylene glycol) (PEG), resulting in a PEG monolayer end-functionalized with an amine. The amine groups were then coupled to the carboxyl groups of an N-Boc-Aoa-OH that was activated with a hydroxyl succimide group. Free aminooxyacetyl groups were then obtained upon treatment of the glass slide with HCl/acetic acid in order to remove the Boc– group. The aminooxyacetyl groups on the slide surface reacted with formyl groups at the reducing ends of the oligosaccharides to form oxime bonds. In contrast to reductive amination, the sugar structure was preserved after coupling; equilibrium between the closed-ring and the open-ring forms might occur at the surface of the support. This chemical-linking technique reported requires only a few modification steps on the surface, allowing for the functional chips to be created in a timely manner making it an attractive method for preparing carbohydrate microarrays in individual laboratories.

Figure 4 Chemical procedure for the preparation of aminooxyacetyl-functionalized glass slides and the immobilization of underivatized oligosaccharides. The aminooxyacetyl groups react selectively with the carbonyl group at the reducing end of carbohydrates via an irreversible condensation while the penta(ethylene glycol) groups serve as spacer arms and prevent the nonspecific adsorption of protein to the monolayer.

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The poly(ethylene glycol) layer on the glass slides provides essentially complete resistance to unwanted protein adsorption and other nonspecific interactions at the surface and ensures that only specific interactions between soluble proteins and immobilized ligands occur. The poly(ethylene glycol) containing carbohydrate...
microarrays showed lower background signal even without commonly used blocking procedures such as treating the substrate with bovine serum albumin or other blocking proteins to passivate the surface. This excellent control over unwanted adsorption has also been reported with monolayers presenting oligo(ethylene glycol) groups (9,30). The poly(ethylene glycol) also functions as a spacer between the carbohydrate and the substrate, which is expected to increase the accessibility of proteins to the binding site of the carbohydrates.

Since the carbohydrates are chemically linked to the aminooxy-functionalized substrate surface through the reducing end, the carbohydrate moieties are immobilized in a well-defined orientation (i.e., site-specific immobilization). The main advantages of an aminooxy-functionalized substrate for the fabrication of carbohydrate microarrays include (i) the ease of formation of oxime bonding under mild conditions between the underivatized carbohydrates and the slide surface; (ii) good stability of the oxime bonding under a wide range of pH; (iii) the monosaccharides are in a ring-closed format which will not affect protein binding in an irrelevant manner, allowing for a more accurate evaluation of the protein-binding function of the carbohydrate.

To demonstrate the utility of this chemistry for the immobilization of carbohydrates and the use of the arrayed carbohydrates for parallel determination of protein–carbohydrate interactions, Zhou et al. (73) printed 10 oligosaccharides on the aminooxyacetyl-functionalized glass slide. The arrayed substrates were kept in a humidified chamber at room temperature overnight, washed with water, and dried. These conditions permitted near quantitative immobilization using minimal quantities of carbohydrate conjugates. After incubation and washing away the unbound oligosaccharide, the remaining aminooxyacetyl groups on the substrate were inactivated by treatment of the glass slides with succinic anhydride [10 mM in dimethylformamide (DMF)] overnight followed by rinsing with DMF to remove physically adsorbed succinic anhydride.

To investigate the carbohydrate–protein-binding properties of the fabricated carbohydrate microarrays, identical arrays were treated separately with three biotin-labeled lectins [with a concentration of 2 μM in phosphate-buffered saline/tween (PBST)] for 2 h, and then washed with PBST. Detection of the bound analyte was subsequently achieved by incubating the microarray with Cy3-streptavidin at a final concentration of 5 μg/ml, and then imaged with a confocal array scanner. Figure 5 shows the results of the analyte characterization on the carbohydrate microarray. As expected, the oligosaccharides were found to bind to their specific lectin proteins. For example, the carbohydrate array probed with Con A showed significant fluorescence intensity in the spots arrayed with mannose, glucose, and N-acetylglucosamine (GlcNAc) (Fig. 5A). Analysis of the fluorescence intensity further reveals that the binding of Con A to the oligosaccharides is in the order of mannose >> glucose >> GlcNAc. The affinity binding difference of the arrayed oligosaccharides is consistent with solution-phase assays. A weak signal was obtained in the spots that were arrayed with maltooligosaccharide which has 4–10 units of α-glucose. This could be the result of an inefficient immobilization of the sugar on the substrate due to the reducing activity of the formyl groups of the
maltooligosaccharide. In addition, no signal was observed in the spots arrayed with methyl-α-mannoside in which the C1 position was substituted with a methyl group. This result suggests that methyl-α-mannoside could not be immobilized on the glass surface. Probing the microarrays with the two other lectins also gave the expected results: *Lotus tetragonolobus* bound to the spots presenting α-fucose (Fig. 5B), whereas *Erythrina cristagalli* bound only to spots presenting lactose and galactose (Fig. 5C). Nonspecific adsorption was not observed on the spots arrayed with cellobiose and rhamnose and essentially no fluorescence was obtained from surfaces without carbohydrates. The weak signal obtained from the glucose spots when the microarray was probed with *L. tetragonolobus* may be caused by the weak cross-reaction of the lectin *L. tetragonolobus*. However, this weak cross-interaction that gave less than 8% of signal compared with the specific interaction would not affect the rapid determination of the presence or absence of specific carbohydrate epitopes.

Overall, these results demonstrated that the binding of lectins with the prepared carbohydrate microarrays are specific and multiple-analytic characterization can be achieved on the aminooxyacetyl-functionalized slide with good selectivity. Furthermore, periodate oxidation of the immobilized oligosaccharides with NaIO₄ resulted in the loss of lectin binding. These experiments verify that the fabricated carbohydrate microarray is well suited for the selective identification of carbohydrate-binding proteins.

Figure 6 shows the dose–response curves of lectins applied to the fabricated oligosaccharide microarrays. It was apparent that an increase in lectin concentration resulted in a corresponding increase in the fluorescence emitted from the arrayed spots, and saturation of affinities was obtained at high concentration. The calculated limit of detection (LOD, the concentration which gives a fluorescent signal higher than the background + three standard deviation units) was determined to be approximately 0.008 μg/ml for Con A, which is lower than the microtiter plate assay developed by Hatakeyama.
Carbohydrate arrays have the characteristics required for quantitative assays of multiple protein–carbohydrate interactions with minimal quantities of reagents. To assess the utilization of carbohydrate microarrays fabricated on aminooxyacetyl-functionalized slides for quantitative assays, α-methyl mannose was applied to inhibit Con A binding to the immobilized mannose spots. A series of mixtures containing biotin-labeled Con A (2 μM in PBST) and α-methyl mannose (0–4 mM in PBST) was applied on the microarray surface and incubated for 1 h at 25°C. The substrates were rinsed with PBST, stained with 5 μg/ml of Cy3-streptavidin, and analyzed with a fluorescence scanner to quantify the amount of bound Con A on the spots of mannose and glucose. The amount of lectin that bound to the chips for each concentration of soluble ligand (i.e., α-methyl mannose) is shown in Fig. 7. The IC50 (concentration of inhibitor required to prevent 50% of lectin binding to array spot) was determined using α-methyl mannose as an inhibitor of Con A binding to glucose and mannose. The results verified that the microarray spots of mannose (IC50 = 60 μM) competed more effectively with the soluble carbohydrate for Con A than that of glucose (IC50 = 23 μM). The relative binding affinities of these carbohydrates for Con A is consistent with those obtained in previous studies (31).

In a similar method, Lee and Shin (36) have developed hydrazide-coated glass slides to immobilize a wide range of carbohydrates including mono-, di-, and oligosaccharides in a simple, efficient, and chemoselective fashion. Preliminary protein-binding experiments show that carbohydrate microarrays prepared by this method are suitable for the high-throughput analysis of carbohydrate–protein interactions.

The advantages of the above two methods for covalent immobilization of underivatized carbohydrates rely on the ease of formation and on the good
stability of the oxime linkage and hydrazide linkage for oligosaccharides up to pH 9. However, reactions of aminooxy or hydrazide groups with free carbohydrates are slow when the carbohydrate MW increases because of the reducing activity of the aldehyde group of the carbohydrates. To improve the immobilization of larger carbohydrates, such as polysaccharides, on the aminooxy- and hydrazide-functionalized slide substrates, Zhou et al. have been investigating the utilization of microwave radiation energy to facilitate the fabrication of carbohydrate microarrays on a 3D polymer film bearing aminooxy- and hydrazide-functional groups (Zhou X., personal communication). Using microwave radiation to facilitate the reaction, oligosaccharides and polysaccharides can be covalently linked on the aminooxy- and hydrazide-functionalized surface within minutes.

Carroll et al. developed a method for covalent immobilization of underivatized mono-, oligo-, and polysaccharides onto glass substrates functionalized with self-assembled photoactive phthalimide chromophores (7). Upon exposure to UV light, the photoactive aromatic carbonyls presumably react with the C–H groups of the sugars by hydrogen abstraction followed by radical recombination to form a covalent bond (Fig. 8). Immobilization of unmodified carbohydrates by this approach was demonstrated to be much less dependent on the MWs of the spotted carbohydrates compared to a nitrocellulose-coated slide. Furthermore, for oligosaccharides the grafting efficiency was shown to be much higher than nitrocellulose. However, the method of photocoupling, which is expected to target any CH– group on the sugar rings with varying specificity depending on the structure of the ring, may interfere with the protein-binding specificity of monosaccharides. Wang et al. recently used this approach to generate a glycan array containing a large panel of synthetic carbohydrates and characterized their antigenic reactivities with pathogen-specific antibodies (64). As described in Section I, this investigation led to the discovery of a highly specific carbohydrate moiety of B. anthracis spores.
Another type of photoactive microarray platform, based on dextran-coated glass slides (PhotoChips from CSEM, Switzerland) was reported by Sprenger’s group (2). The dextran-based polymer OptoDex is functionalized with aryl-trifluoromethyl-diazirine groups. On illumination, aryl-trifluoromethyl-diazirine groups form reactive carbenes, which can undergo a variety of reactions with a vicinal molecule that result in covalent bond formation including insertion into $\sigma$ and $\pi$ bonds, addition of a nucleophile or electrophile and hydrogen abstraction. The authors have demonstrated that this substrate immobilizes polysaccharides and glycoligands. However, since the aryl-trifluoromethyl-diazirine-functionalized surface can react with any type of biomolecule, this type of array substrate is not suitable for preparing carbohydrate microarrays with unpurified carbohydrate extractions from cells or plants. Precaution must be made when applying this platform for serological studies since antidextran natural antibodies are frequently detected in human circulation (33,67).

The above slide surfaces and linking techniques provide the feasibility to fabricate microarrays of carbohydrates by using underivatized carbohydrate moieties. These methods are especially useful when working with complex oligosaccharides isolated from natural sources and when derivatized carbohydrates are not available. In many cases, the glycoepitopes contained in these microarrays are reactive toward appropriate antibodies, lectins, or other carbohydrate-binding partners of defined carbohydrate-binding specificities. However, these methods of saccharide immobilization are not expected to be site specific. Instead, the carbohydrates are attached without control of the orientation of saccharide display. A given saccharide spotted may, thus, present a glycoepitope in a spectrum of different configurations, although one or a few might be predominant. Thus, in order to further characterize the fine specificity of carbohydrate binding, especially the orientation effect of epitope display, one may want to explore the technologies described in the subsequent sections.
B. Carbohydrate Microarrays Fabricated by Using Derivatized Carbohydrates

Derivatized carbohydrates, termed glycoligands, are carbohydrate moieties with functional tags prepared by chemical modification. Glycoligands provide more flexibility in the selection of array substrates and chemical-linking techniques for carbohydrate microarrays. Most importantly, the use of glycoligands in combination with properly functionalized surfaces allows for the site-specific immobilization of carbohydrates onto the substrates. With these technical features, it is possible to construct carbohydrate microarrays with control over the ways of presentation of carbohydrate moieties for molecular recognition. These characteristics are important for achieving the specificity or selectivity of carbohydrate–protein interactions that play important roles in cell–cell communication, signaling, and modulation of immune responses (12,39,65). Microarray presentation of the native configurations of glycoepitopes is likely a challenging issue that requires substantial and relatively long-term collaborative efforts by carbohydrate researchers and microarray experts.

Specific technical considerations in exploring this approach may include (i) the feasibility of preparing carbohydrate derivatives; (ii) the spacer between the glycoligands and the slide surface should provide optimal presentation of glycans and prevent nonspecific binding of proteins; (iii) the suitability of materials comprising the chip, for example, a functionalized glass slide versus a metallic surface; and (iv) the availability of tagged carbohydrate ligands for desired chip substrates.

The Consortium for Functional Glycomics (www.functionalglycomics.org) (3) has provided remarkable support to this field, building a library of about 200 synthetic glycoligands, which represent the most typical terminations and core fragments of mammal glycoproteins and glycolipids. A similar set of biotinylated oligosaccharides (~180 in total) are also available in the Consortium (www.functionalglycomics.org) (4). The number of described and well-characterized 2-amino-pyridine derivatives of N-glycans (56–58) reaches several hundred; many of these derivatives are commercially available. With the advances being made in chemical and chemoenzymatic syntheses, increasing numbers of carbohydrate derivatives of known oligosaccharide sequences will become available for fabrication of carbohydrate microarrays.

1. Noncovalent Immobilization of Derivatized Carbohydrates in Microarrays

Because of the small molecular size and hydrophilic nature, most oligosaccharides cannot be directly immobilized onto nitrocellulose or black polystyrene surfaces for microarray applications. The oligosaccharide probe can be modified with a tag or coupled to a larger carrier molecule for noncovalent immobilization. A research group led by Feizi has developed oligosaccharide microarrays by noncovalently immobilizing NGLs on nitrocellulose (25,44). The oligosaccharides were obtained by chemical or enzymatic methods by using glycoproteins, glycolipids, proteoglycans, polysaccharides, or whole organs, or from chemical synthesis. The chemical derivatives of the oligosaccharides were synthesized by reductive
amination of the oligosaccharides to the amino phospholipid 1,2-dihexadecyl-
sn-glycero-3-phosphoethanolamine or its anthracene-containing fluorescent ana-
logue. The immobilization efficiency of the NGLs on nitrocellulose was found
to be high irrespective of the size of carbohydrates. The carbohydrate-binding
proteins were investigated with known monoclonal antibodies, the E- and
L-selectins, a chemokine (RANTES), and a cytokine. Binding was detected by
colorimetric ELISA-type methods. It was shown that carbohydrate-binding pro-
teins could single out their ligands, not only in arrays of homogeneous, structur-
ally defined oligosaccharides but also in an array of heterogeneous O-glycan
fractions derived from brain glycoproteins. The unique feature of this carbohy-
drate microarray technology is that deconvolution strategies are included with
mass spectrometry for further determining the sequences of ligand-positive
components within mixtures.

Wong’s group developed a method for fabricating oligosaccharide arrays,
which is a noncovalent but site-specific immobilization. In essence, they applied
aliphatic derivatives of monosaccharides and oligosaccharides onto a polystyrene
96-well microtiter plate (6). They found that the carbohydrates were efficiently
immobilized when the saturated hydrocarbon chain was between 13 and 15 car-
bons in length. Several di- to hexasaccharides containing terminal galactose, glu-
cose, and/or fucose residues were chemically modified with a C_{14}-saturated
hydrocarbon chain. Figure 9 illustrates the attachment of the modified carbohy-
drates to microtiter plate surfaces. All the sugars were stable after repeated wash-
ings and elicited the predicted binding signals with the lectins Ricinus B chain, Con
A, and Tetragonolobus purpurea.

In addition, Wong and colleagues (6,21) reported that azide-derivatized forms
of galactose and several azide-derivatized neutral and sialic acid containing di- to
tetrasaccharides were immobilized onto aliphatic alkyne-coated plastic microtiter
plate surfaces. These saccharides were immobilized on the surfaces by a 1,3-dipolar
cycloaddition reaction between the azide and alkyne groups (Fig. 10). The noncova-
lent attachment also allowed convenient characterization of the lipid-linked pro-
ducts by mass spectrometry, as well as the detection of lectin binding. Using
Guanosine diphosphate (GDP)-fucose and α-1,3-fucosyltransferase, fucosylation of
sialyl-N-acetyllactosamine was carried out within the wells, showing that the surface
is well suited for the high-throughput identification of enzyme inhibitors.

2. Covalent Immobilization of Derivatized Carbohydrates in Microarrays

Several other types of carbohydrate derivatives have been used for the fabrication
of carbohydrate microarrays. Thiolated carbohydrate derivatives were immobi-
лизирован на гетерогенной самосборной мономолекулярной пленке, что предstawляют метиленовый конечный и OH конечный функционализованные пента(этиленовый гликолов) цепи на стеклянные слайды (31,47). Метиленовая группа предоставляет необходимую функциональность, что реагирует с тиоловыми конечными гликолигандами, в то время как пента(этиленовый гликолов) цепи предотвращают неспецифическое адсорбирование белка на поверхность. Пента(этиленовый гликолов) цепь также служит как разрывитель за счет уменьшения стерического препятствия на протеиновое взаимодействие с углеводами на поверхности.
Figure 9  Carbohydrate microplate arrays prepared by the noncovalent immobilization of aliphatic alkyne-derivatized carbohydrates to microtiter plate surfaces. Carbohydrates can then be screened against a variety of biologically important substrates such as lectins and RNA.

Figure 10  Carbohydrate microplate arrays prepared by the noncovalent immobilization of azide-derivatized carbohydrates to microtiter plates via a 1,3-dipolar cycloaddition reaction between alkynes and azides. Carbohydrates displaying terminal azides can be captured on microtiter plate surfaces through a terminal alkyne attached to a long, aliphatic tether and screened directly on the microtiter plate surface.
In contrast to the above approach, Shin’s group prepared carbohydrate microarrays by covalent immobilization of maleimide-derivatized carbohydrates to thiol-functionalized glass slides (45,46) (see Fig. 11). Lectin-binding experiments showed that carbohydrates with different structural features selectively bound to the corresponding lectins with relative binding affinities that correlated with those obtained from solution-based assays. The author also demonstrated the fabrication of carbohydrate microarrays that contained more diverse carbohydrate probes. Enzymatic glycosylations on glass slides were consecutively performed to generate carbohydrate microarrays that contained the complex oligosaccharide, sialyl Le^x.

Mrksich and coworkers (32) reported a chemical strategy for preparing carbohydrate arrays by the Diels–Alder-mediated immobilization of cyclopentadiene-derivatized carbohydrates to self-assembled monolayers that present benzoquinone and penta(ethylene glycol) groups (Fig. 12). Modification of the gold surface was initiated by immersing gold-coated glass slides into a mixture of alkanethiols with (1%) and without (99%) appended hydroquinone groups to produce self-assembled monolayers of hydroquinone and penta(ethylene glycol) groups. Chemical or electrochemical oxidation was then performed to convert hydroquinone to benzoquinone groups. Finally, the cyclopentadiene-derivatized monosaccharides were covalently immobilized on the gold surface through the Diels–Alder reaction. This reaction was found to be highly efficient and selective for the immobilization of carbohydrates on the surface. Carbohydrate arrays presenting 10 monosaccharides were then evaluated by profiling the binding specificities of several lectins. These arrays were also used to determine the inhibitory concentrations of soluble carbohydrates for lectins and to characterize the substrate specificity of β-1,4-galactosyltransferase.

Blixt et al. (3) constructed a diverse glycan microarray by using standard robotic microarray printing technology to couple amine-derivatized glycoligands to an N-hydroxysuccinimide (NHS)-functionalized slide. The array comprises 200 synthetic and natural glycan sequences representing major glycan structures of glycoproteins and glycolipids. This array uses commercially available amine-reactive
NHS-functionalized glass slides, which allow rapid covalent coupling of amine-functionalized glycans or glycoconjugates. The fabricated glycan microarray has shown utility for profiling the specificity of a diverse range of glycan-binding proteins, including C-type lectins, siglecs, galectins, anti-carbohydrate antibodies, lectins from plants and microbes, and intact viruses.

A microarray substrate for covalent immobilization of aminophenyl-derivatized carbohydrates is commercially available (GlycoChip, Glycominds, Lod, Israel). Schwarz et al. reported the application of this substrate to fabricate oligosaccharide microarrays by using \( p \)-aminophenyl-derivatized carbohydrates via a cyanurichloride-activated linker (50). This approach allows the covalent attachment of glycans containing a terminal aliphatic amine by forming an amide bond under aqueous conditions at room temperature. The fabricated oligosaccharide microarray was used to analyze the glycan-binding antibody repertoire in a pool of affinity-purified IgG collected from a healthy human population. In addition, a novel anti-cellulose antibody was detected that binds specifically to \( \beta4 \)-linked saccharides with a preference for glucopyranose over galactopyranose residues with the oligosaccharide microarray.

The group led by Waldmann has prepared carbohydrate microarrays by using Staudinger reactions between phosphane-functionalized glass slides and azide-derivatized carbohydrate moieties (34). The glass slide surface was first functionalized with polyamidoamine (PAMAM) dendrimers bearing 64-aminofunctional groups with the purpose of maximizing potential reactive sites on the surface. The amino groups of the PAMAM-modified slide were then converted to terminal carboxylic acid groups by reacting with glutaric anhydride. The carboxylic acid of the dendrimer film was finally converted to a phosphane group by reacting with the 2-(diphenylphosphinyl)phenol. The phosphane group has a high reactive efficiency to azide-derivatized molecules. The azide-derivatized carbohydrate moieties were prepared by solid-phase
synthesis using a safety-catch linker strategy. The azide-derivatized carbohydrates were found to be efficiently immobilized onto the phosphane-functionalized slide surface. A spot volume of a 0.25 nl sample arrayed on the phosphane-functionalized slide surface produced a spot size of 400 μM in diameter. A mannose-containing carbohydrate microarray was fabricated on this substrate. Carbohydrate–protein interactions were evaluated by incubating with Alexa647-labeled Con A. This shows that the immobilization of azide-derivatized carbohydrates via the Staudinger reaction is highly efficient and can be employed to detect biomolecular interactions.

Bovin’s group reported a method principally different from all described above. Saccharides were immobilized inside droplets of a porous polymeric gel (26). Immobilization of amino-derivatized oligosaccharides was achieved by the formation of a covalent bond between the amino group and the growing polymer chain during photo-initiated polymerization in the presence of a cross-linking agent. The authors have demonstrated that a hydrogel carbohydrate microarray contains three different classes of glycomolecules, which are as follows: (i) oligosaccharide derivatives bearing a primary amino group, (ii) oligosaccharide–polyacrylamide conjugates bearing allyl groups, and (iii) oligosaccharide derivatives bearing 2-aminopyridine groups. All of the three types of oligosaccharide derivatives are readily subjected to covalent attachment in the same conditions during the radical process of gel formation. For hydrogel microarray manufacturing, the gel-forming monomers, that is, methacrylamide, methylenebisacrylamide, and oligosaccharide derivatives are printed onto hydrophobized glass and irradiated with UV light. The double bond of methylenebisacrylamide readily reacts with the amino group of the oligosaccharide derivatives at pH 10.5 giving rise to a Michael addition product. After polymerization, an array of individual 3D approximately 1-nl gel drops, 150 μm in diameter, and 25 μm in height was formed. The authors have shown that the 3D hydrogel provides high sensitivity in probing proteins due to the large amount of carbohydrates immobilized in the 3D hydrogel spots.

3. Affinity Immobilization of Derivatized Carbohydrates

Biotin-derivatized carbohydrates can be immobilized on a streptavidin-coated substrate through the affinity interaction of the streptavidin–biotin pair to create carbohydrate microarrays. Biotin-derivatized carbohydrates include carbohydrate ligands that are biotinylated via a short aliphatic spacer or at the peptide part of glycopeptides. Several commercially available streptavidin-coated microwell plates can be applied when biotin-derivatized carbohydrates are available, such as the streptavidin-coated 384-well plate with a well volume 25 μl (4,18) and a streptavidin-coated 192-spot slide format (27). The first was designed to be in maximal proximity to the traditional immunochromatographic assay using commercial streptavidin-coated black 384-well plates.

IV. Concluding Remarks

A number of carbohydrate microarray platforms have reached or are very close to the stage of the current nucleic acid-based microarrays that are readily available
for practical uses. Technical issues that require immediate attention may include but are not limited to optimization of existing technologies for array construction, quality control, and technical standardization in both microarray production and application, establishment of specialized bioinformatic tools to handle the massive amount of carbohydrate microarray data, and to effectively extract diagnostic or research information from each microarray assay. Taking care of these issues would facilitate biological and medical applications of carbohydrate microarrays.

Exploring the repertoires of glycoepitopes and their receptors represents a long-term goal of carbohydrate research. How big is the repertoire of glycoepitopes? Addressing this question is one of the most important topics in the postgenomics era. It was estimated that there are about 500 endogenous glycoepitopes in mammals (17). However, this estimation did not consider the repertoires of the “hybrid” structures that are generated by protein posttranslational modification, including both N- and O-glycosylation. Furthermore, the conformational diversity of carbohydrates and microheterogeneity of carbohydrate chains substantially increases the repertoire of carbohydrate-based antigenic determinants or glycoepitopes (39,63,66). Considering carbohydrate structures of the microbial world, which are directly relevant to medicine, the sizes and diversity of the repertoires of glycoepitopes are unpredictable. Establishment of high-throughput platforms of carbohydrate microarrays provides powerful means to facilitate the identification and characterization of carbohydrate-based pathogen signatures and other biomarkers.

Joint effort by academic and industrial sectors is highly recommended to direct the establishment of libraries of monoclonal antibodies, lectins, and other carbohydrate-binding proteins. These biomolecules are critical for defining glycoepitopes and are useful for detection of glycoepitopes in living organisms. Thus, using specific immunological probes to characterize glycoepitopes is equally important to the structural determination of glycoepitopes. Similar effort has been successfully made for protein-based biomarkers. A notable example is the establishment of a large collection of monoclonal antibodies for cell differentiation antigens (CD antigens). Availability of specific probes for CD antigens, in combination with the state-of-the-art technologies of flow cytometry (Hi-D FACS) (59), has revolutionized research in cellular biology and immunology and medical applications of CD antigens, especially in the clinical diagnosis of leukemia and other human diseases. Exploring the repertoires of glycoepitopes and their cellular receptors, with the aid of carbohydrate microarray technologies and specific immunological probes, represents one of the highly active areas of postgenomics research that may last for a few decades and likely accompanied with a fruitful outcome.

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