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Abstract

Carbohydrates, like nucleic acids and proteins, are essential biological molecules. Owing to their intrinsic physicochemical properties, carbohydrates are capable of generating structural diversity in a multitude of ways and are prominently displayed on the surfaces of cell membranes or on the exposed regions of macromolecules. Recent studies highlight that carbohydrate moieties are critical for molecular recognition, cell-cell interactions, and cell signaling in many physiological and pathological processes, and for biocommunication between microbes and host species. Modern carbohydrate microarrays emerged in 2002 and brought in new high-throughput tools for “glyco code” exploration. In this section, some basic concepts of sugar chain diversity, glyco-epitope recognition, and the evolving area of glyco-epitomics and biomarker discovery are discussed.

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Two complementary technologies, carbohydrate antigen arrays and photogenerated glyco-chips, serve as models to illustrate how to apply carbohydrate microarrays to address biomedical questions.

Keywords

Antibodies • Carbohydrates • Carbohydrate microarrays • Glycans • Glyco-epitopes • Glycoconjugates • Glycome • Glycomics • Lectins • Oligosaccharides • Polysaccharides

Abbreviations

Ab	Antibody
Ag	Antigen
AGOR	Agalacto-orosomuroid
ANOVA	Analysis of variance
ASOR	Asialo-orosomuroid
BSA	Bovine serum albumin
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (also known as CD209)
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
GBP	Glycan-binding protein
Gn	GlcNAc
GNA	<i>Galanthus nivalis</i> lectin
Hi-D FACS	High-dimensional fluorescence-activated cell sorting
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KLH	Keyhole-limpet hemocyanin
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
mAbs	Monoclonal antibodies
Man9	Man9Gn2Asn moiety
m-II	Multivalent type II chains
Neu5Ac	<i>N</i> -acetylneuraminic acid
OR	Orosomuroid
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
P-Man	Yeast phosphomannan Y-2448
PHA-L	<i>Phaseolus vulgaris</i> -L lectin
RAR	Relative antibody reactivity
SARS CoV	Severe acute respiratory syndrome-associated coronavirus
SNA	<i>Sambucus nigra</i> I agglutinin
Stpv	Streptavidin
TI	T-independent

Tn	GalNAc α 1→Ser/Thr
Tri/m-Gn	Tri-antennary and multivalent GlcNAc cores
Tri/m-II	Tri-antennary and multivalent type II chains
Tri-II	Tri-antennary type II (Gal β 1→4GlcNAc) chains

1 Introduction

Cellular carbohydrates are present in multiple structural configurations, including mono-, oligo-, and polysaccharides, as well as various glycan-hybrid molecules. The latter include, but are not limited to, glycolipids, glycoproteins, proteoglycans, and glycosaminoglycans. Unlike proteins, which are composed of amino acids that are connected solely by one possible peptide bond, carbohydrates utilize many possible glycosidic linkages so as to extensively diversify their structures. For example, two amino acid residues, such as two alanines, can produce only one possible dipeptide; however, two molecules of glucose have the potential to generate 11 different disaccharides. A trimer of any of the nine common sugar residues of the human body theoretically can give rise to 119,736 different structural isomers; this is in striking contrast to the maximal construction of 8,000 tripeptides using 20 different amino acid residues. Theoretically, sugar chain structures can have unlimited variation.

In human and virtually all animal species, cell display of specific complex carbohydrates is characteristically associated with the stages or steps of embryonic development, cell differentiation, and transformation of normal cells to abnormally differentiated tumor or cancer cells (Feizi 1982; Hakomori 1985; Crocker and Feizi 1996; Focarelli et al. 2001). In plants, a highly complex set of polysaccharides are associated with structural proteins and lignin to form cell walls (Avci et al. 2012). Even in a given tissue or cell, cell wall layers and domains may have very different carbohydrate structures and express different glyco-epitopes (Albersheim et al. 2010; Avci et al. 2012). Many microbial organisms also carry unique glycosylation systems and produce specific sugar signatures for almost every microorganism in the living world (Dochez and Avery 1917; Heidelberger and Avery 1923; Ezzell et al. 1990; Robbins and Schneerson 1990; Mond et al. 1995; Wang and Kabat 1996). The term “glycome” has been recently introduced to cover the universe of carbohydrate moieties in all living organisms.

Importantly, multiple carbohydrate recognition systems are present in living species. For example, there are numerous anti-glycan antibodies produced by human and other animal species that play key roles in protecting a host from microbial infections (Behring and Kitasato 1890; Wang and Kabat 1998; Lucas et al. 2008) and families of lectin-like glycan-binding proteins (GBPs) that are evolved for carbohydrate-mediated cell-cell communication (Drickamer 1988; Sharon 2007; Varki 2009). The well-known GBPs that are associated with cell signaling and immunomodulation are the receptors of the innate immune system, such as Dectin-1 that recognizes fungal β -glucans (Brown et al. 2002), mannose

receptor that recognizes carbohydrate moieties on multiple pathogens and is involved in the clearance of inflammatory molecules in vivo (Gruden-Movsesijan and Milosavljevic Lj 2006), and DC-SIGN (dendritic cell-specific ICAM-3-grabbing non-integrin) that selectively detects viral glycoproteins, such as HIV-1 gp120 glycoprotein (Curtis et al. 1992). The interaction between HIV-1 gp120 and DC-SIGN plays an important role in the CD4-independent association of HIV with human cells (Curtis et al. 1992). Thus, carbohydrates are suitable for storing biological signals in forms that are identifiable by other biological systems.

In the immunological and glycobiological literature, “glyco-epitope” is often used to specify the carbohydrate moiety that is recognized by an antibody or by a GBP. The antibody-binding glyco-epitopes are also classified as B cell epitopes or antigenic determinants. The term “glyco-epitome” was recently introduced to describe the entire repertoire of glyco-epitopes, including the B cell epitopes and those that are recognized by GBPs. Differing from the term “glycome,” which covers all the existing carbohydrate molecules in living organisms, glyco-epitome refers to a unique subset of carbohydrates that serves as the sugar signatures for molecular recognition and biosignal transmission. “Glyco-epitomics” is, thus, an area of glycomics research focusing on identifying, characterizing, and understanding the carbohydrate moieties that serve for multiple levels of biocommunication.

It is noteworthy that glyco-epitope characterization requires not only carbohydrate structural analysis but also immunological studies. The structural aspects of glyco-epitomics focus on the elucidation of the glycan structures that display glyco-epitopes. This research area has been substantially enhanced by the development of advanced profiling and structural characterization strategies. Notably, these include high-resolution chromatography methods coupled with exoglycosidase digestions (Campbell et al. 2008; Royle et al. 2008), modern mass spectrometry (Babu et al. 2009; Goldberg et al. 2009; North et al. 2009) and nuclear magnetic resonance spectroscopy analyses (Petrescu et al. 1997; Wormald et al. 2002; Petrescu et al. 2006) of carbohydrates and state-of-the-art methods of glycan structural modeling (Woods and Tessier 2010; Jo et al. 2013).

However, availability of carbohydrate structural information alone is not sufficient in defining a glyco-epitope unless its specific binding by an antibody or a GBP is also demonstrated immunochemically and/or cryptographically. For example, chemical determination of a tetrasaccharide that decorates the spore of *Bacillus anthracis* appears to be an important discovery in microbial glycomics (Daubenspeck et al. 2004). Based on past knowledge of immunogenic carbohydrate moieties, this structural glycomics progress may suggest that this unique sugar moiety may have potential in an immunological application (Saksena et al. 2005, 2007). However, whether such a carbohydrate moiety preserves a B cell epitope or a potent antigenic determinant must be determined immunologically, including at least demonstration of its antibody-binding specificity and capacity in eliciting immune responses in vivo (Wang et al. 2007). It was the integrated structural and immunological investigation of glyco-epitopes (Wang et al. 2007; Lucas et al. 2008) that has revealed anthrose tetrasaccharides as key immunological targets of *B. anthracis*.

2 Classification of Carbohydrate Microarrays

Four research articles about carbohydrate microarrays first appeared in the scientific literature in 2002 (Borman 2002, 2012). These include polysaccharide and glycoconjugate microarrays, reported by Denong Wang's group at Columbia University's Genome Center (now at SRI International, CA, USA) (Kiessling and Cairo 2002; Wang et al. 2002); monosaccharide chips, by Milan Mrksich and coworkers at the University of Chicago (Houseman and Mrksich 2002); arrays of natural and synthetic neoglycolipids, by Ten Feizi's group at Imperial College Faculty of Medicine, Harrow, UK (Fukui et al. 2002); and arrays of synthetic oligosaccharides in microtiter plates, by a Scripps Research Institute group led by Chi-Huey Wong (Bryan et al. 2002). A specialized book, "Carbohydrate microarrays, Methods and Protocols (Humana Press)," recently edited by Dr. Yann Chevolut of Université de Lyon in France (Chevolut 2012), provides a comprehensive summary of the emerging technologies for construction of carbohydrate microarrays.

2.1 Spotting Carbohydrates with Different Structural Characteristics

Given the various structural characteristics of carbohydrates displayed on chips, carbohydrate microarrays are classified as monosaccharide chips (Houseman and Mrksich 2002; Park and Shin 2002), oligosaccharide arrays (Bryan et al. 2002; Fukui et al. 2002; Blixt et al. 2004; Wang et al. 2007), and microarrays of carbohydrate-containing macromolecules (Wang et al. 2002; Willats et al. 2002). The latter includes polysaccharides and various glycoconjugates. These different sugar chips or arrays were developed to accommodate multipurpose applications in carbohydrate research. For example, mono- and disaccharide microarrays are suitable for screening and characterizing novel carbohydrate-binding proteins or carbohydrate-catalyzing enzymes and for identifying novel inhibitors of carbohydrate-protein interactions.

A large class of carbohydrate-binding proteins, called lectins, was initially classified by their binding specificities to monosaccharides and recently by disaccharides. However, there are lectins and many antibodies with anti-carbohydrate reactivities that bind to larger and more complex carbohydrate ligands or antigenic determinants. Mono- and disaccharide sugar chips are not sufficient for investigations involving such molecular targets. Oligosaccharide, polysaccharide, and glycoconjugate microarrays fill this gap by displaying carbohydrates of complex structures or longer sugar chains on the chips.

2.2 Strategies for Carbohydrate Immobilization on a Chip

Based on the technologies that are applied to immobilize carbohydrates on bioarray substrates, the various methods to construct carbohydrate microarrays can be classified as distinct technological platforms. These include technologies that directly utilize underivatized carbohydrates in microarray construction,

technologies that require chemical modification of carbohydrates before microarray fabrication, methods of non-covalent immobilization of carbohydrates, and methods of covalent coupling of saccharides on array substrates.

The use of underivatized saccharides for microarray construction has the 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 currently in use include non-covalent binding of underivatized carbohydrate antigens by passive adsorption on a chip, such as nitrocellulose-coated glass slides (Wang et al. 2002) or black polystyrene surfaces (Willats et al. 2002), and methods for covalently immobilizing underivatized carbohydrates on a slide surface by appropriate chemical linking techniques (Angeloni et al. 2005; Lee and Shin 2005; Carroll et al. 2006; Zhou and Zhou 2006; Wang et al. 2007; Zhou et al. 2009, 2012).

Carbohydrate microarrays can also be fabricated by using derivatized carbohydrates. Due to their small molecular size and hydrophilic nature, most oligosaccharides cannot be directly immobilized onto nitrocellulose or black polystyrene surfaces for microarray applications. However, an oligosaccharide probe can be modified with a tag or coupled to a larger carrier molecule for non-covalent immobilization. Methods include non-covalent immobilization of derivatized carbohydrates on microarray chips (Fukui et al. 2002; Palma et al. 2006) or on enzyme-linked immunosorbent assay (ELISA) microtiter plates (Bryan et al. 2002) and covalent immobilization of derivatized carbohydrates on microarray chips. The latter includes, but are not limited to, the popular Consortium for Functional Glycomics (CFG) printed glycan arrays (Blixt et al. 2004; Bochner et al. 2005) and various technologies of notable technical features that were developed independently (Houseman and Mrksich 2002; Park and Shin 2002; Galanina et al. 2003; Kohn et al. 2003; Park et al. 2004; Parthasarathy et al. 2006; Gerland et al. 2012a, b; Morvan et al. 2012; Goudot et al. 2013).

Affinity immobilization is another class of approaches for coupling derivatized carbohydrates to solid surfaces. For example, 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 (Guo et al. 2004; Bochner et al. 2005; Dyukova et al. 2006). DNA-directed immobilization (DDI) is another practical strategy for immobilization of oligonucleotide glycomimetic conjugates on a chip surface for the preparation of carbohydrate microarrays (Gerland et al. 2012a, b; Morvan et al. 2012; Goudot et al. 2013).

2.3 Common Technical Features of Carbohydrate Microarrays

Despite technical differences among different platforms of carbohydrate microarrays, they are all solid-phase binding assays and share a number of common

characteristics and technical advantages. For instance, they contain the capacity to display a large panel of carbohydrates in a limited chip space, they are high-throughput quantitative assays, they make an effective use of carbohydrate substances that are often difficult or cost-inefficient to synthesize, and, as discussed below, they are highly sensitive in monitoring carbohydrate-anti-carbohydrate interactions in multiplex manners.

In a carbohydrate microarray, each carbohydrate is spotted in an amount that is drastically smaller than that required for a conventional molecular or immunological assay. This technical feature ensures a condition in which the binding of a molecule in solution phase to an immobilized microspot of ligand on the microarray substrate has minimal reduction of the molar concentration of the molecule in solution (Ekins et al. 1990). Thus, microarray-based assays are intrinsically optimized for binding equilibrium to take place, which is the basis for this class of hypersensitive binding assays (Stoll et al. 2002). Carbohydrate microarrays have higher detection sensitivity than most conventional carbohydrate analytical tools, such as carbohydrate-specific ELISA and the glycolipid-based “Eastern blot” assays that were developed in the 1980s by a number of early researchers in this field (Wood and Kabat 1981; Tang et al. 1985). Historically, this situation is very similar to the relationship between conventional blotting methods for nucleic acids or proteins, such as Southern, Northern, and Western blots, and nucleic acid-based or protein/peptide-based microarrays.

2.4 Complementary Platforms of Carbohydrate Microarrays

Carbohydrate microarrays constructed by various methods may differ in their technical characteristics and suitability for a given practical application. Some platforms may be applied complementarily to solve a practical question. For example, 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 (Wang et al. 2002, 2005; Wang and Lu 2004) to support the large-scale immunological characterization of carbohydrate antigens and anti-carbohydrate antibodies. However, the detection specificity of this carbohydrate microarray would be at the level of a carbohydrate antigen, not a glyco-epitope, if the native carbohydrate antigens were spotted. This is owing to the fact that many carbohydrate antigens display multiple antigenic determinants or glyco-epitopes (Cisar et al. 1975; Wang and Kabat 1998; Wang 2004). 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 glyco-epitope (Fukui et al. 2002; Blixt et al. 2004; Wang et al. 2007; Zhou et al. 2009). These technical features of carbohydrate microarrays are further discussed in two models, i.e., a carbohydrate antigen microarray platform and a technology of photogenerated oligosaccharide microarrays in subsequent sections.

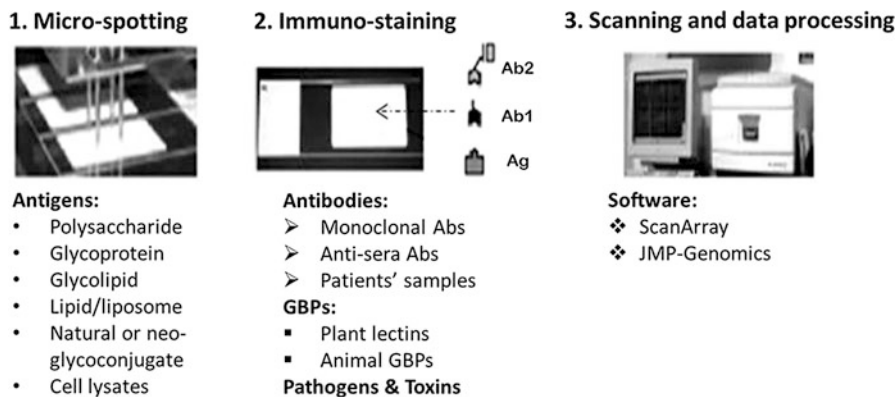


Fig. 1 A high-throughput platform for carbohydrate-based microarrays. A high-precision robot designed to produce cDNA microarrays is utilized to spot carbohydrate antigens of various structural configurations onto a nitrocellulose-coated glass slide. The microspotting capacity of this system is approximately 20,000 spots per chip. The antibody- or GBP-stained slides were then scanned for fluorescent signals with a microarray scanner that was developed for cDNA microarrays. Toxins or viral pathogens can also be applied on this platform of carbohydrate microarrays to probe potential glycan-receptors of viruses or toxins

3 A Versatile Carbohydrate Antigen Microarray Technology

A practical approach for construction of carbohydrate microarrays is to print carbohydrate antigens onto nitrocellulose-coated glass slides. This was the first reported method for high-throughput production of carbohydrate microarrays (Borman 2002; Kiessling and Cairo 2002; Wang et al. 2002). Using this technology, carbohydrate-containing macromolecules of diverse structures, including polysaccharides, natural glycoconjugates, and mono- and oligosaccharides coupled to carrier molecules, can be stably immobilized on a glass chip without chemical modification. This approach was subsequently extended to production of lipids/glycolipid and liposome microarrays (Fukui et al. 2002; Wang et al. 2005, 2014). Recently, this approach has been applied to produce integrated protein, lipid, and carbohydrate microarrays (Wang et al. 2014). Owing to the technical simplicity of this approach, anyone who has access to a standard cDNA microarray facility would be able to explore this technology for his or her own research interests (Fig. 1).

3.1 Preparing Carbohydrate Antigens for Microarray Printing

Soluble antigen preparations are generally applicable for construction of microarrays in this platform. Except certain antigens that require special solutions, proteins and carbohydrates for spotting are dissolved in phosphate-buffered saline (PBS; pH 7.4) and saline (0.9 % NaCl), respectively. Liposomes of various compositions, including *homo*- and *hetero*-liposomes, are suitable for printing on this

substrate (Wang et al. 2014). The former were produced via a single lipid preparation, e.g., phosphatidylcholine (PTC), cerebroside, and sulfatide. The latter contained two different lipid molecules with PTC as the support to display other lipid/glycolipids in desired ratios or epitope densities. For example, the *hetero*-liposome of sulfatide is composed of sulfatide and PTC at a ratio of 1:10 (wt/wt), i.e., 0.2 mg sulfatide and 2.0 mg PTC per ml of liposome suspension in saline. Methods employing sonication and extrusion (mechanical energy) to produce liposomes for microarray production were similarly described by a number of investigators (Wang et al. 2005; Palma et al. 2012).

3.2 Printing Carbohydrate Microarrays Using Conventional Microspotting Devices

Microarray printers that were designed for DNA or protein microarrays, such as PixSys 5500C (Cartesian Technologies, Irvine, CA), are suitable for spotting carbohydrates onto glass slides pre-coated with nitrocellulose polymer (FAST Slides; Schleicher and Schuell, Keene, NH) (Wang 2012). We often spot antigens in triplicate with spot sizes of 150 μm and at 375 μm intervals, center to center. The printed microarrays are air-dried and stored at either room temperature or 4 $^{\circ}\text{C}$ before application.

3.3 Staining and Scanning Microarrays

Immediately before use, the printed microarrays were rinsed with PBS, pH 7.4, with 0.05 % (vol/vol) Tween 20 and then blocked by incubating the slides in 1 % (wt/vol) bovine serum albumin (BSA) in PBS containing 0.05 % (wt/vol) NaN_3 at room temperature (RT) for 30 min. They were then incubated at RT with antibodies at an indicated titration in 1 % (wt/vol) BSA in PBS containing 0.05 % (wt/vol) NaN_3 and 0.05 % (vol/vol) Tween 20. The secondary antibodies or streptavidin conjugates applied for microarray staining are specified in the figure legends. The stained slides were rinsed five times with PBS with 0.05 % (vol/vol) Tween 20, air-dried at room temperature, and then scanned for fluorescent signals using a ScanArray 5000A microarray scanner (PerkinElmer Life Science) following the manufacturer's manual.

3.4 Producing Customized Arrays for Defined Purposes

Large-scale “repertory” microarrays containing thousands of microspots or larger are powerful means for discovering unexpected molecular targets. For example, microarray scanning of autoantibody responses allows one to “fish out” potential autoantigens in the glycome in autoimmune diseases. Customized, smaller scale carbohydrate microarrays containing a few dozen antigens are, however, suitable

for more defined purposes, such as antibody fine-specificity mapping, differential diagnosis among a number of known infectious diseases, measurement of autoantibodies for known targets in autoimmune diseases, etc. Fig. 2 shows a sub-array design where each chemically modified microglass slide contains eight separated sub-arrays. The microarray capacity is ~ 400 microspots per sub-array. A single slide is, thus, designed to enable eight microarray assays. Similar sub-array designs with various array capacities are commercially available (Schleicher and Schuell, Keene, NH; ArrayIt, Sunnyvale, CA).

- Each microglass slide contains twelve sub-arrays of identical content. There is a chip space for about 400 microspots per sub-array, with spot sizes of approximately $200\ \mu\text{m}$ and at $300\text{-}\mu\text{m}$ intervals, center to center. A single slide is, therefore, designed to enable 12 detections. This design is typically for printing four 96-well plates of antigen preparations ($96 \times 4 = 384$).
- Repeats and dilutions: Our team usually prints carbohydrate antigens at the initial concentration of $0.1\text{--}0.5\ \mu\text{g}/\mu\text{l}$. The absolute amount of antigens printed on the chip substrate is in the range of $0.1\text{--}0.25\ \text{ng}$ per microspot for the highest concentration. They are further diluted at 1:3, 1:9, and 1:27, or as specified in each experiment. A given concentration of each preparation is repeated at least three times to allow statistical analysis of detection of identical preparations at given antigen concentrations.
- Positive controls and standard curves: Fluorescent conjugates, such as BSA conjugates of FITC, Cy5, Cy3, or other dyes, are routinely applied for microarray printing to provide positive markers for each fluorescent channel. These markers are helpful for scanning calibration, alignment of microarray spots during data-capturing, the subsequent microarray data normalization, and cross-chip scaling of microarray detection. For serological studies, antibodies

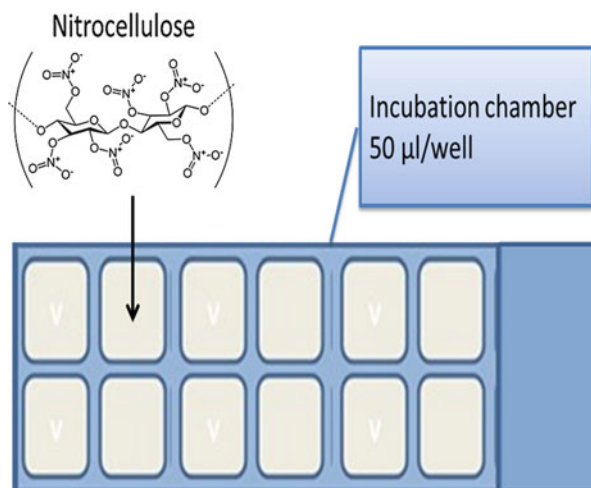


Fig. 2 Illustration of the 12-chamber sub-arrays

of IgG, IgA, and IgM isotypes of corresponding species are spotted to produce standard curves in microarray format. These curves serve as reference standards for quantifying antibody signals of a specific IgH chain isotype that are captured by spotted carbohydrate antigens.

3.5 Technical Notes of Nitrocellulose-Based Bioarray Substrate

A number of nitrocellulose-coated glass slides with different technical characteristics are commercially available. Given the structural diversity of carbohydrate antigens, examining each antigen preparation to determine the efficacy of its immobilization in a given type of substrate and the surface display of the desired glyco-epitopes in a microarray assay is essential. A practical approach is to incubate the printed microarrays with antibodies, receptors, or lectins known to react with the printed substance.

Figure 3 is an example of such analysis where lectin *Galanthus nivalis agglutinin* (GNA) and antibody 2G12 were applied to examine specific glyco-epitopes on the spotted microarrays. Inspection of both microarray images (Fig. 3a) and the quantitative datasets show that the GNA-epitopes were presented by three glycoconjugates (Fig. 3c), i.e., Man9-cluster (4[#]), M9_2G12-cluster (3[#]), and Man5-9 RB (1[#]). In contrast, 2G12-glyco-epitopes were preserved only by one of the three, i.e., M9_2G12-cluster (3[#]), on this microarray substrate (Fig. 3d). This carbohydrate microarray analysis demonstrates, therefore, an example that the same sugar chain may generate different glyco-epitopes when the sugar moiety is presented in different cluster configurations. In this case, the Man9GlcNAc2Asn moiety was coupled to the protein carriers in either (Man9GlcNAc2Asn) n- or [(Man9GlcNAc2Asn)₄] n-configurations (Fig. 3b). The latter but not the former preserves well the 2G12-defined broadly HIV-1 neutralizing epitope.

4 A Photogenerated Glyco-Chip Technology

Carroll and colleagues (2006; Wang et al. 2007; Carroll and Wang 2012) developed a photochemical method to covalently immobilize carbohydrates on chips. As illustrated in Fig. 4, the method employs a self-assembled monolayer to present photoactive phthalimide chromophores at the air-monolayer interface. Upon exposure to UV radiation, the phthalimide end-groups graft to surface-adsorbed carbohydrates to form a covalent bond. The amount of surface-grafted carbohydrate is enhanced when carbohydrate surface interactions are increased by the incorporation of amine-terminated molecules into the monolayer. One of the important applications of this technology is to identify immunogenic sugar moieties of microbial pathogens by screening the corresponding antisera obtained from vaccinated or infected subjects.

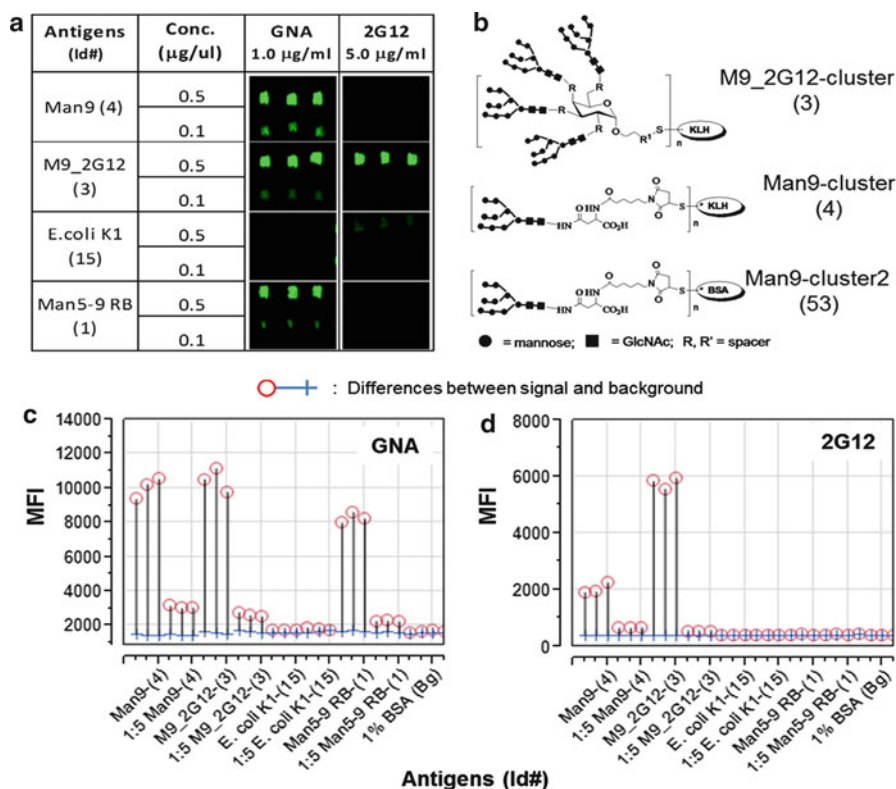


Fig. 3 A carbohydrate microarray analysis of oligomannosyl antigens for expression of the 2G12-like and GNA-like glyco-epitopes. The mannose-cluster-containing microarrays were stained with 2G12 (5 μg/ml) and a biotinylated GNA (1.0 μg/ml), respectively. The former was revealed by Alexa⁶⁴⁷-tagged Goat anti-human IgG Fc-specific antibodies at 2 μg/ml and then developed with Streptavidin-Cy5 conjugate at 2 μg/ml. (a) Shows microarray images stained with either GNA or 2G12. (b) Illustrates the cluster configuration of the three Man9-conjugates. Microarray detection signals are shown as the mean fluorescent intensities (MFIs) of each microspot as captured by the ScanArray 5000A for the arrays stained with GNA (c) and 2G12 (d), respectively. Results were compared using overlay plots of the MFIs of staining signal (red circles) versus those of local backgrounds surrounding the antigen microarrays (blue +) (c, d) (Adapted from Wang et al. (2014))

4.1 Synthesis of Photoactive Compounds

The phthalimide chromophore used in the photogenerated glycan-chip was modified with a silane derivative in order to form a stable bond to glass. A 3.3 mmol portion of 11-bromoundecanetrimethoxysilane (Gelest) was added to a solution of an equimolar amount of potassium phthalimide (Aldrich) in 60 mL of anhydrous DMF (Aldrich). The solution was stirred overnight at room temperature (RT) under argon. Chloroform (50 mL) was added. The solution was transferred to a separatory flask containing 50 mL of H₂O. The aqueous layer was separated and then extracted

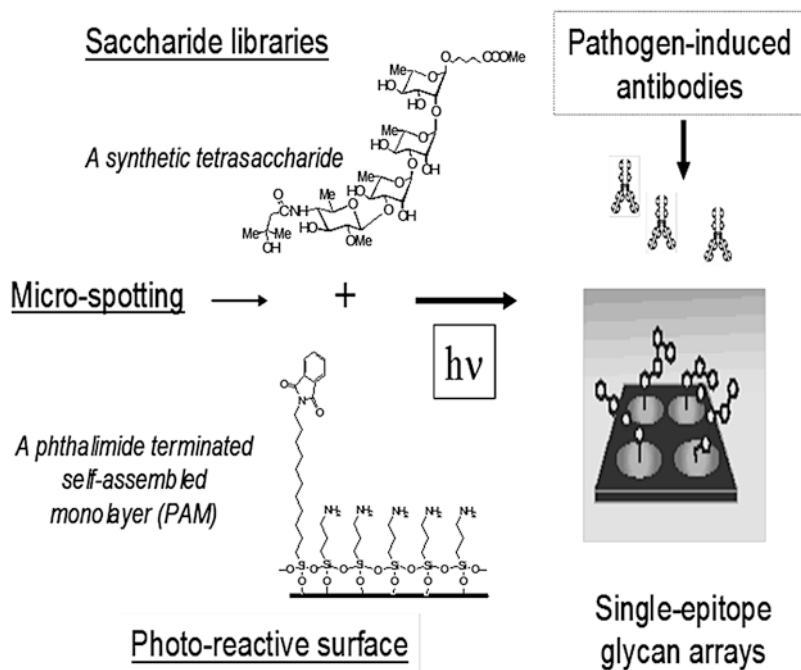


Fig. 4 Photogenerated glycan arrays for rapid identification of pathogen-specific immunogenic sugar moieties. Saccharide preparations were dissolved in saline (0.9 % NaCl) at a given concentration and spotted using a high-precision robot (PixSys 5500C, Cartesian Technologies, Irvine, CA) onto the phthalimide amine (PAM)-coated slides. The printed PAM slides were subjected to UV irradiation (300 nm) for 1 h to activate the photocoupling of carbohydrates to the surface. Pathogen-specific antisera were then applied on the glycan arrays to identify potential immunogenic sugar moieties of given pathogens (Adapted from Wang et al. (2007))

with two 20 mL portions of chloroform. The combined chloroform extract was washed with several 20 mL portions of H_2O . The chloroform was removed by rotoevaporation, and residual DMF was removed on a high vacuum line to give a pale yellow liquid (0.99 g, 72 % yield). The compound was used without further purification. Note that, for self-assembly experiments, residual DMF was not removed. 1H NMR: ($CDCl_3$) δ 7.82 (m, 2H), 7.69 (m, 2H), 3.66 (t, J 7 Hz, 2H), 3.55 (s, 9H), 1.44–1.15 (m, 18H), 0.71–0.51 (m, 2H). LRMS-FAB+ (m/z): (M-H) 420.2 (experimental), 420.2 (calculated); (M-OCH₃) 390.1 (experimental), 390.2 (calculated).

After synthesis of the phthalimide-silane, PAM was prepared by immersing a clean glass slide into a toluene solution containing 1 mM of the phthalimide-silane and 5 mM of aminopropyltrimethoxysilane (Gelest). The H_2O contact angle of the resulting surface was $72 \pm 1^\circ$. Note that the glass slide was cleaned with a 7:3 mixture of H_2SO_4 : 30 % H_2O_2 . Extreme caution should be used when preparing and using such a solution, which can react violently and explosively if mixed with other chemicals.

4.2 Photocoupling of Carbohydrates onto Chips

Microarray spotting is performed as outlined in the above section. After spotting of carbohydrates, the PAM slides were air-dried and placed in a quartz tube. The sealed tube was subsequently purged with argon or nitrogen before irradiation. UV irradiation was conducted by placing the quartz tube under a desktop lamp containing a 300 nm Rayonet bulb for 1 h. Precaution was made to avoid skin and eye contact with the radiation during the irradiation process.

4.3 Probing Immunogenic Sugar Moieties Using Photogenerated Glyco-Chips

The photogenerated glycan arrays were applied to probe the potential immunogenic sugar moieties of *Bacillus anthracis* spores (Wang et al. 2007). The rationale was that if *B. anthracis* spores expressed immunogenic carbohydrate structures, the spore antigen-immunized or *B. anthracis*-infected animals would be possible to mount antibody responses to these carbohydrates. This assumption was made on the basis of the fact that the host immune system is able to recognize subtle changes in sugar structures, especially those that are exposed on the surfaces of microbial pathogens that are foreign components of the mammalian hosts.

Figure 5 below is an example of photo-chip characterization of the rabbit antisera elicited by *B. anthracis* spores. The photo-chips used were spotted with a large panel of saccharide structures, including synthetic fragments and derivatives of the anthrose-containing tetrasaccharide side chain of the *B. anthracis* exosporium and a number of control carbohydrate antigens. Antibody staining was performed in the presence or absence of saccharide inhibitors. Images (a–f) display a portion of the stained glycan arrays: (a) no saccharide inhibitor; (b) anthrose; (c) D-glucose; (d) α -anthrose trisaccharide; (e) α -anthrose tetrasaccharide; (f) β -anthrose tetrasaccharide. The locations of surface-bound anthrose-containing saccharides that are recognized by the antibody in the absence of inhibitor are highlighted by colored boxes: *White*, β -anthrose-trisaccharide; *Brown*, β -anthrose-tetrasaccharide; *Yellow*, α -anthrose-tetrasaccharide. Microarray data sets are available upon request.

This analysis confirmed that a tetrasaccharide of BclA glycoprotein bears a dominant antigenic determinant, which is composed of a terminal anthrose residue and three adjacent L-rhamnoses. The terminal trisaccharide unit is essential for the constitution of a highly specific antigenic determinant. Given the fact that this carbohydrate moiety is displayed on the outermost surfaces of *B. anthracis* spores and its expression is highly specific for the spore of *B. anthracis*, the anthrose-containing tetrasaccharide can be considered 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.

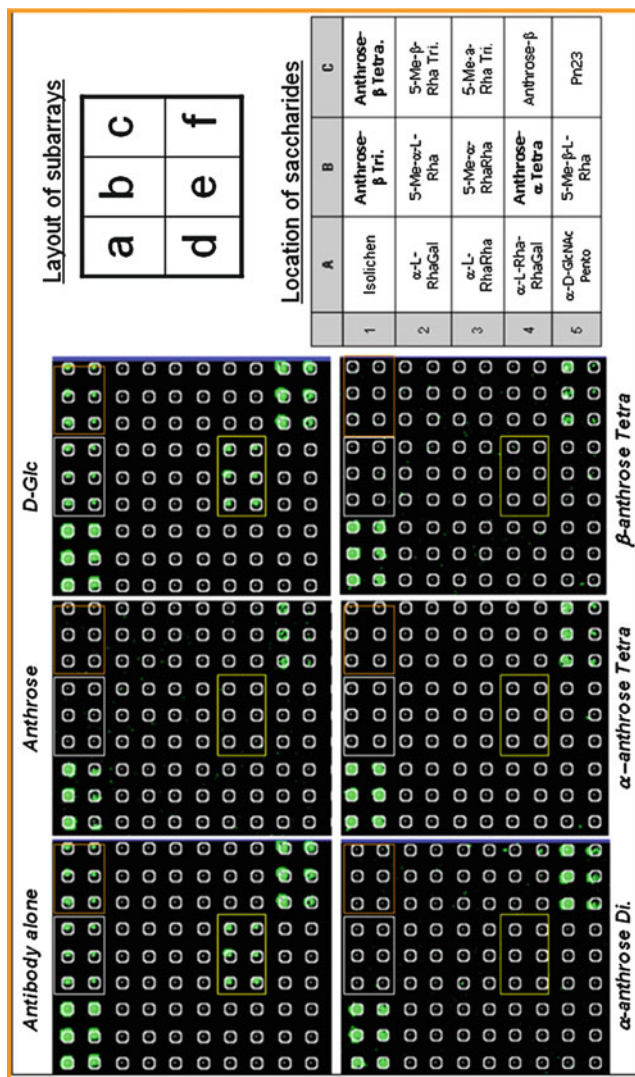


Fig. 5 Photogenerated glycan arrays recognize immunogenic sugar moieties of the *B. anthracis* spore. A panel of 35 mono-, oligo-, and polysaccharides were photocoupled to the surface of derivatized glass slides. After incubating the glycan arrays with rabbit anti-*B. anthracis* spore polyclonal IgG antibodies (Abcam, Cambridge, UK) in the absence or presence of saccharide inhibitors, the bound rabbit IgG was revealed by a tagged anti-rabbit IgG antibody (Adapted from Wang et al. (2007))

4.4 Technical Notes for Photogenerated Glyco-Chips

A unique technical advantage of this method is the ability to produce epitope-specific glycan arrays using unmodified mono- and oligosaccharides. The quality of the surfaces obtained depends in part on cleanliness. Ideally, water with a resistivity of 18.2 M Ω -cm and total organic contaminant of less than five parts per billion should be used; however it is still possible to prepare the surfaces after rinsing with water of lower quality. Self-assembly of trimethoxysilanes can be enhanced by adding small amounts of water and acid. Such treatment was not used for the mixed surface described above but may possibly enhance the reaction times or surface coverage obtained. If the surface is allowed to dry upon removal from self-assembly solution, the aminosilane may polymerize, leaving white deposits that are difficult to remove.

5 Carbohydrate Microarray Data Processing and Statistical Analysis

Although specialized bioinformatics tools for carbohydrate microarrays are yet to be developed, some software packages developed for cDNA microarrays are applicable for carbohydrate microarrays. This is owing to the fact that these different microarray assays are commonly based on the laser fluorescence detection systems regardless of the contents of the spotted microarrays. A number of advanced microarray software packages are currently available. These include, but are not limited to, Significance Analysis of Microarrays (SAM, <http://www-stat.stanford.edu/~tibs/SAM/>), Prediction Analysis for Microarrays (PAM <http://www-stat.stanford.edu/~tibs/PAM/index.html>), and JMP Genomics (www.jmp.com/genomics).

5.1 Presenting Microarray Raw Datasets and Microarray Images

A straight forward way to present the raw results of a carbohydrate microarray assay is to illustrate the microarray raw datasets and/or images. For example, carbohydrate researchers often present microarray raw datasets with or without background subtraction for the carbohydrate-binding profiles of monoclonal antibodies (Palma et al. 2011), lectins (Blixt et al. 2004), or viruses (Childs et al. 2009). Figure 3 above illustrates two examples of binding profile presentations based on microarray raw data.

In this experiment, the mannose-cluster-containing microarrays were stained with mAb 2G12 (5 μ g/ml) and a biotinylated GNA (1.0 μ g/ml), respectively. Figure 3a shows microarray images and Fig. 3b illustrates the cluster configuration of the three Man9-conjugates. Microarray detections are shown as the mean fluorescent intensities (MFIs) of each microspot as captured by the ScanArray 5000A for the arrays stained with GNA (Fig. 3c) and 2G12 (Fig. 3d), respectively.

Results were compared using overlay plots of the MFIs of the antigen-binding signals (*red circles*) versus those of local background signals surrounding the antigen microarrays (*blue +*) (Fig. 3c, d). Analysis of such microarray raw data in association with visual inspection of the microarray image provides an initial evaluation of reproducibility and variation of this antigen microarray technology.

5.2 Statistical Tests of Carbohydrate Microarray Detection

In order to identify disease-associated biomarkers, such as antigen-specific antibodies with diagnostic and/or prognostic values, we compare microarray detection between a disease group and a normal control group or to more generally between multiple groups. Proper statistical analysis is essential for making generalizable conclusions. Typical steps include quality control, normalization, and statistical model fitting. In our general practice, carbohydrate array datasets are preprocessed and statistically analyzed using JMP Genomics software from SAS Institute.

Antigen-specific antibody reactivities are represented as microarray scores, which are the log₂ transformed values normalized by some method. We have explored several different normalization methods, including mean and/or variance centering, loess, and quantile normalization, and found the interquartile range (IQR) method to provide an appropriate degree of standardization (based on distribution and correlation plots) without overly correcting the data. The IQR method sets the 25th and 75th percentiles of the microarray distributions to be equal.

After normalization, we utilize an antigen-by-antigen ANOVA model to obtain statistically significant differences. Data from triplicate spots for each antigen are included in the ANOVA model for that antigen. A cutoff to detect significant differences is determined by applying a multiple testing correction to statistical results from the ANOVA model. These procedures are further discussed below in an example of clinical sample analysis.

5.3 Clinical Sample Analysis Using Customized Carbohydrate Microarrays

Here we present an example to illustrate general statistical approaches to carbohydrate microarray analysis of clinical samples. In this case, customized autoantigen microarrays were applied to characterize the cerebrospinal fluid (CSF) of multiple sclerosis (MS) patients. MS is a complex neurological disorder in which an adaptive autoimmune response is thought to target myelin sheath in the central nervous system. We created a microarray displaying a panel of 32 carbohydrate and lipid antigens to examine MS-associated autoantibody responses.

A technical challenge to this study is the fact that the total Ig concentrations in the CSF of MS patients are higher than those in the CSF of other neurological diseases (OND) subjects, which reflects one of the hallmarks of MS

(Kabat et al. 1948, 1951; Steinman 1996; Genain et al. 1999; Raine et al. 1999; Hueber et al. 2002). However, it causes difficulty in identifying the disease-specific autoantigens and autoantibodies. In our microarray analyses, we reconfirmed this observation. Figure 6a, b show the overlay plots of antibody profiles of the two groups. The colored needles that link the pairs of group mean values provide a global comparison of the antibody profiles between the MS group (*red circles*) and the OND group (*blue crosses*). This comparison reveals global differences in antibody profiles between the two groups. Specifically, the microarray scores of CSF-antibody activities in the MS group are generally higher than those seen in the CSF of OND subjects. These include not only anti-lipid antibodies (*Right, 26–44[#]*), as previously reported (Ho et al. 2012), but also anti-carbohydrate antibodies (*Left, 1–22[#]*).

We further examined whether there is any selective enrichment of antigen-specific antibodies in the CSF of MS patients. We reasoned that identifying such antibodies might provide clues to pinpoint key autoimmunogenic targets of MS. For this purpose, we introduced an approach to establish RAR scores for microarray signals and then sought targets that capture the antibody signal with higher RAR scores in MS patients. Specifically, we normalized the microarray datasets by setting their IQR to be identical using the JMP Genomics software package. This statistical operation effectively “quenches” the variation seen between subjects that are due to variable antibody concentrations in the CSF. The two groups illustrate similar Ig-RAR profiles for both IgG and IgM antibody activities (Fig. 6c, d). However, a number of probes show higher IgG-RAR scores in the MS group than in the OND controls. These include two Man9-clusters (3[#], and 4[#]), three glucose polysaccharides, dextran N279 (8[#]), B1299S (9[#]), B1355S (10[#]), and a Bacto-Agar (20 °C, extracted) antigen (13[#]) (Fig. 6c).

In Fig. 7, an antigen-by-antigen analysis of variance (ANOVA) model was applied to obtain statistically significant differences between groups in comparison. Results are graphically presented as a volcano plot (Zink et al. 2013) for a global comparison of all RAR scores between the groups in comparison (Fig. 7a) and as one-way analysis scatterplots for selected targets (Fig. 7b, c). In the volcano plot, each dot represents a statistically weighted and quantified difference between MS and OND groups. The x-axis is the normalized difference (\log_2 scale) and the y-axis uses $-\log_{10}$ (*p*-value) for the difference. Spots above the red-dashed line represent signatures that differ significantly between the groups after a multiple testing correction.

For the one-way scatterplots in Fig. 7b, c, each data point represents the mean of triplicate determinations. The means of the points are shown as horizontal green bars and standard deviations as green diamonds around the mean value. The comparison circles for the Student’s “t”-test appear to the right of the mean diamonds to illustrate the significance of the differences among the means. These circles allow visual inspection of the statistical significance of the differences. The more the circles intersect, the less significant their difference, and vice versa.

Of the 126 antibody signatures captured in this assay (Fig. 6c, d), two were above the cutoff line [$-\log_{10}$ (*p*-value) = 2.5] as highly significant

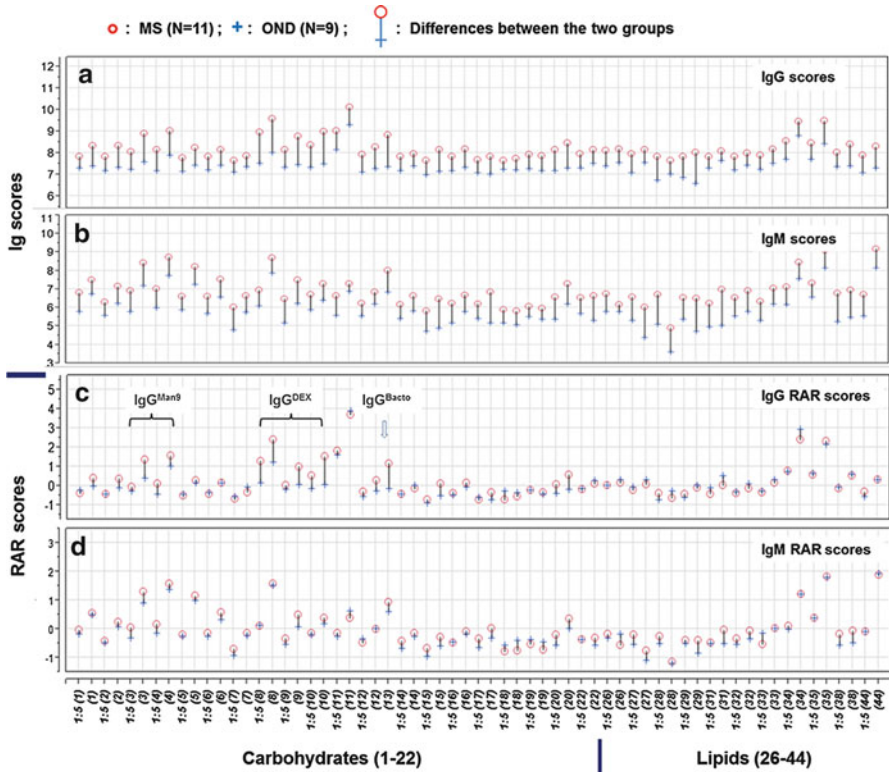


Fig. 6 Integrated lipid/carbohydrate arrays recognize globally elevated antigen-specific antibodies in the CSF of MS patients as compared to those detected in the OND CSF. CSF samples from 11 MS (10 RRMS and 1 SPMS) and 9 OND subjects were characterized using microarrays spotted with 12 lipid and 20 carbohydrate antigens. Each preparation was spotted in triplicate at 2–4 dilutions. As illustrated in the overlay plots (a–d), this microarray supports detection of 126 unique antibody signatures. These included 63 IgG (a, c) and 63 IgM (b, d) signatures. Anti-human IgG or IgM secondary antibodies were used to reveal the antigen-specific antibodies detected by these microarrays. The captured IgG were stained with an anti-human IgG antibody conjugated with Cy3 at 2 $\mu\text{g/ml}$ and the captured IgM in the same array revealed by a biotinylated anti-human IgM secondary antibody at 2 $\mu\text{g/ml}$ and developed with Streptavidin-Cy5 conjugate at 2 $\mu\text{g/ml}$. Microarray datasets in (a) and (b) were illustrated as microarray scores. In (c) and (d) microarray datasets were further processed using JMP Genomics to produce RAR* scores. The results are presented as overlay plots of the mean microarray scores for each group. The colored needles that link the group mean values of each pair of scores provide a global comparison of the antibody profiles of MS (red circles) and OND (blue crosses) groups (Adapted from Wang et al. (2014)). *RAR Score: RAR Relative Antibody Reactivity, the value of log2 transformed and IQR-standardized microarray value (mean-background). For each antigen in a given concentration, the mean value of triplicate array detections was calculated

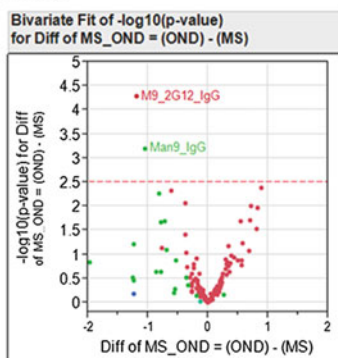
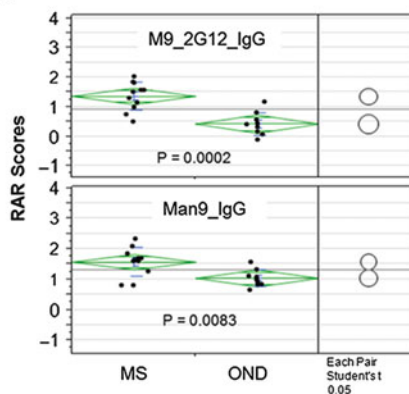
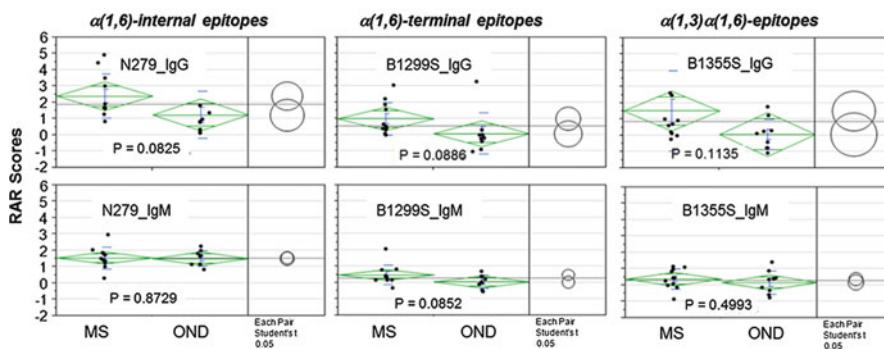
a Volcano Plot**b** Anti-Man9 clusters**c** Anti-dextrans (glucose polysaccharides)

Fig. 7 MS-associated autoantibodies in CSF samples target high-mannose clusters, the cores of N-glycans. **(a)** is a volcano plot analysis of the RAR scores of all antibody signatures. In the volcano plot, each point represents a biologically unique feature captured by the microarray, i.e., a normalized difference for a specific antibody signature ($RAR^{MS} - RAR^{OND}$). The x-axis is the normalized difference (log₂ scale); the y-axis is the $-\log_{10}(p\text{-value})$, which weights the levels of significance of a difference. Points above the *red-dashed line* (cutoff level 2) represent signatures that differ significantly between the groups based on the Bonferroni test. Two signatures, M9_2G12_IgG and Man9_IgG, were identified by this critical statistical test as highly significant markers. In **(b)** and **(c)** one-way analysis was performed to compare group means among the selected carbohydrate antigens listed in each panel. Each point in the panels represents the mean value (RAR score) of triplicate array detections of a subject (Adapted from Wang et al. (2014))

differentiators between MS and OND. These were M9_2G12_IgG and Man9_IgG. Results of the one-way ANOVA of the two Man9 clusters are shown in Fig. 7b. All other antibody signatures had $-\log_{10} p$ -values below the cutoff line (Fig. 7a), including four signatures that were variably higher in the MS group than in the OND group. These were N279_IgG ($P = 0.0825$), B1299S_IgG ($P = 0.0886$), B1355S_IgG ($P = 0.1135$), and Bacto_Agar_IgG ($P = 0.021$). This microarray analysis identified, therefore, oligomannoses as potential immunological targets

for further investigation. Overall, JMP Genomics is a valuable tool for statistical analysis of carbohydrate microarray data.

6 Conclusion/Prospects

Modern carbohydrate microarrays emerged in 2002 (Borman 2002, 2012; Kiessling and Cairo 2002; Wang and Collins 2013) and introduced new glycomics tools to decipher the biological information content in the glycome. These technologies are especially useful in exploring the repertoire of glyco-epitomes. A number of carbohydrate microarray platforms have now reached or are very close to the technical stage of the current nucleic acid-based or protein-based microarrays that are readily available for practical uses. Technical issues that require further improvement 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, and establishment of specialized bioinformatics tools to handle the massive amount of carbohydrate microarray data and to effectively extract diagnostic or research information from each microarray assay.

Nevertheless, exploring the repertoires of glyco-epitopes represents a long-term goal of glycomics research. It was estimated that the human glycome contains 10,000–20,000 minimal epitopes for glycan-binding proteins (Cummings 2009). In considering the repertoires of the “hybrid” structures that are generated by protein posttranslational modification, including both *N*- and *O*-glycosylation, the repertoires of carbohydrate-related antigenic structures can be much larger. Furthermore, the conformational diversity of carbohydrates and microheterogeneity of carbohydrate chains substantially increases the repertoire of carbohydrate-based antigenic determinants or glyco-epitopes (Wang and Kabat 1996; Wang 2014). Including carbohydrate structures of the microbial world, which are directly relevant to medicine, the sizes and diversity of the repertoires of glyco-epitopes are unpredictable.

Further development of carbohydrate microarrays requires libraries of carbohydrate antigens, including purified natural antigens and synthetic glycoconjugates, as well as anti-glycan mAbs, lectins, and other glycan-binding proteins. Naturally purified carbohydrate antigens have the advantage of preserving the native antigenic structures and often offer highly sensitive detection of antigen-specific antibodies. Availability of synthetic oligosaccharides and glycoconjugates is, however, critical for epitope determination and fine-specificity studies of carbohydrate-anti-carbohydrate interactions.

Anti-glycan mAbs and GBPs of known carbohydrate-binding specificities are required to characterize glyco-epitopes that are presented by carbohydrate microarrays. Thus, collaborative efforts by both academic and industrial sectors are required to facilitate the establishment of large collections of glycan-targeting probes. This situation is similar to established flow cytometry technology and services. Availability of specific antibody probes for CD antigens, in combination with the state-of-the-art technologies of flow cytometry (high-dimensional

fluorescence-activated cell sorting, or Hi-D FACS) (Tung et al. 2004), 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 carbohydrate-based biomarkers and targeting agents, with the aid of carbohydrate microarray technologies and other high-throughput omics tools, may represent one of the highly active areas of postgenomics research and technology development in future years.

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