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Glycan microarrays for decoding the glycome

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Abstract

In the last decade glycan microarrays have revolutionized the analysis of the specificity of glycan binding proteins, providing information that simultaneously illuminates the biology mediated by them and decodes the information content of the glycome. Numerous methods have emerged for arraying glycans in a ‘chip’ format, and glycan libraries have been assembled that address the diversity of the human glycome. Such arrays have been successfully used for analysis of glycan binding proteins that mediate mammalian biology, host-pathogen interactions, immune recognition of glycans relevant to vaccine production and cancer antigens. This review covers the development of glycan microarrays and applications that have provided insights into the roles of mammalian and microbial glycan binding proteins.

Keywords

Carbohydrate; Glycan-Binding Protein; Glycan; Microarray; Glycomics; Lectin

Introduction

Glycans decorate the surfaces of all living cells, comprising highly diverse structures that mediate a wealth of biology through glycan binding proteins (GBPs) that recognize them as ligands (1–7). The development of glycan microarrays at the turn of the 21st century filled a critical need for high-throughput methods to systematically array glycan libraries and identify the specificities of GBPs to enable investigations into their biological roles. Since their introduction, publications on the design of glycan microarrays and their biological applications have grown exponentially. The advances in glycan array technology have paralleled those of complementary technologies used for interrogation of glycan structure and function, including chemical glycobiology (8), lectin microarrays (9, 10), and analytical glycomics (11, 12), which together have led to rapid advances in decoding the information content of the glycome. This review covers progress and challenges in the development of glycan microarray platforms, the generation of diverse glycan libraries, and the impact of glycan microarrays in understanding the specificities and biological roles of glycan binding proteins. Limited space has necessitated selection of representative studies and omission of many important contributions for which the reader is referred to other recent reviews on this subject (13–16).

Development of Glycan Microarrays: Historical perspective

Glycan microarrays were introduced with fanfare in 2002 with publications by several independent groups aiming to systematically array this class of biological molecules. Two reports demonstrated robotically printed arrays of diverse glycan libraries (17, 18), and many other laboratories demonstrated various approaches for immobilization of glycans in printed slide or multi-well plate formats (19–23). These achievements were a natural extension of the successful development of printed arrays of other classes of biomolecules,

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including DNA arrays in 1995 (24), and recombinant protein arrays a few years later (25, 26). Just as large scale DNA printed arrays were preceded by the development of spotted arrays of DNA using low throughput manual methods, elegant methods for glycan immobilization and detection were developed years earlier. Thin layer chromatography had been used for decades for resolution of mixtures of glycolipids with subsequent detection by antibodies and other glycan binding proteins (27). In the 1980s, this method was generalized to other classes of glycans by Feizi and colleagues who chemically attached lipid linkers to the reducing end of native glycans creating "neoglycolipids", allowing analysis of the specificity of glycan binding proteins by immobilization to various surfaces prior to the advent of printed glycan microarrays (17, 28, 29).

In the eight years following the first reports of large-scale glycan microarrays, there has been an explosion of interest for developing glycan libraries, efficient methods of immobilization of glycans on array surfaces, and applications for analysis of glycan binding protein specificity. Although focused arrays of various microorganism glycans have been assembled (18, 30–32), the major focus to date has been arrays of mammalian glycans. In this review, we will cover the challenges in the development of diverse glycan libraries, approaches for immobilization of glycans on array surfaces, and applications that have yielded valuable insights into the biology of glycan binding proteins.

Assembly of Glycan Microarray Libraries

The utility of a glycan microarray depends upon a match between the glycan library and its application. Examples of libraries covering various classes of glycans reported to date are summarized in Table 1. The largest are libraries that attempt to cover the diversity of glycans expressed on mammalian glycoproteins and glycolipids. These are intended for use in analysis of the specificity of numerous glycan binding proteins whose function involves binding to these structures (Table 1a). Other more focused libraries represent a set of related glycans from one class or glycans substituted with unnatural substituents that are typically assembled for analysis of the detailed specificity of glycan binding proteins of related function (Table 1b).

All these libraries are comprised of synthetic glycans, glycans isolated from natural sources, or both. As discussed in sections below, regardless of their source, assembled libraries need to have a common functional group or property that allows them to be immobilized/arrayed on a compatible surface. Currently, a significant restriction in the size of glycan libraries is the difficulty in both the synthesis of carbohydrates and the isolation of natural glycans in pure from. Yet, despite these obstacles, the libraries assembled to date have yielded a wealth of biologically important information.

Covering the diversity of the human glycome

As alluded to above, a major strategic issue for design of glycan arrays is how to construct a library to cover the structural diversity needed for the biological problem of interest. Focused libraries designed to address a particular question are typically limited to a manageable size. But what about a library meant to cover the diversity of the glycome? Where does one start? A practical approach embraces the fact that the majority of glycan binding proteins have binding pockets that accommodate just a few monosaccharide residues.

This is put into better context by considering the information content of glycans. The glycome is indirectly tied to the genome through the specificity of the glycosyltransferases that carry out the non-template mediated biosynthesis of glycans. By regulating the expression of glycosyltransferases, a cell can produce glycan structures that are distinct from

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those of neighboring cell types. Within each cell, the glycans produced are a highly heterogeneous nested set of related structures resulting, from alternative branching patterns, incomplete glycosylation, and 'post-glycosylational' sulfation and acetylation. The sum of these variations produces an enormous number of biosynthetically permissible glycan structures. The size of the cellular glycome is a matter of debate, but is estimated to be in excess of 100,000–500,000 glycan structures (33). Yet, the information content relevant for the majority of glycan binding proteins is comprised within a very finite number of structural variations that occur at the 'tips' of complex glycan chains (e.g. N-linked and O-linked glycans or glycolipids), or are functional epitopes of small linear segments of a glycan polymer (e.g. proteoglycan or bacterial polysaccharide).

Consider the classes of glycans that comprise the ligands of glycan binding proteins, such as the major classes of mammalian glycans illustrated in Figure 1. For N-linked and O-linked glycans of glycoproteins and glycans of glycolipids, the core structures highlighted in grey are common to virtually all cell types. A major aspect of structural variation from cell to cell occurs in the pattern of terminal sugars (e.g. sialic acid, fucose) and sulfation or acetylation at the non-reducing end of the glycoprotein glycan chains. Although a survey of the types of structure variation in mammalian glycans is beyond the scope of this review, the number of unique glycan structures that comprise the terminal sequences of glycoprotein and glycolipid glycans has been estimated to be between 500–3500 (34, 35). Similarly, for polymers like the proteoglycan heparan sulfate, the structural variation present in a stretch of 8 monosaccharides is approximately 4100 (34). With current synthetic methodologies, production of libraries of this size is feasible. As will be discussed below, libraries of 500–600 glycans already capture a major portion of the information content of glycans that are used as ligands by glycan binding proteins, and have the potential to cover the majority of glycan binding determinants in the foreseeable future.

Glycans Isolated from natural sources

The first printed glycan microarrays (17, 18) used libraries of glycans isolated from natural sources, and this class of carbohydrates continues to be featured as components of diverse glycan libraries (Table 1). Sources of natural glycans include milk oligosaccharides, glycans released from glycoproteins and glycolipids by endoglycosidases or chemical hydrolysis, proteoglycans (GAGs) and fragments obtained by enzymatic or chemical degradation, and polysaccharides from bacteria and plants (1, 17, 36, 37). In principle, natural glycans comprise the full diversity of glycans that exist in the glycome. However, a major challenge with natural glycans is the isolation of pure compounds and verification of their structure. Another strategic issue for natural glycans is the mode of attachment to the array. Although polysaccharides can be directly immobilized by adsorption (18, 23), polysaccharide fragments and glycans released from glycoproteins and glycolipids typically require derivatization for immobilization onto the array surface.

Glycans released from glycoproteins and glycolipids contain a reducing sugar (Figure 2) that can be used for introducing a functional group via a variety of efficient microscale conjugation strategies (Figure 3). This monosaccharide is in equilibrium between a closed ring hemiacetal and an open chain, aldehyde-containing form, which can serve as an electrophilic group for a chemoselective reaction with a number of nucleophilic amine, hydrazide, or oxyamine containing reagents (Figure 3). Although reductive amination has been widely used in the past, it has the disadvantage of yielding an unnatural ring-opened product that eliminates key structural information encoded by the reducing terminal monosaccharide. For this reason, several groups have explored alternative hydrazide, aminoxy, and N-alkylaminoxy linkers for conjugation to the reducing end aldehyde. These methods differ in the degree to which they give ring-opened and ring-closed products, with unsubstituted aminoxy linkers giving a predominantly ring-opened form (38), where as

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hydrazide (39) and N-alkylaminoxy (40) linkers give predominantly a ring-closed β-glycoside. Another approach to the generation of stable, ring-closed β-glycosides derived from natural sources relies on glycosylamine formation and trapping of this species by an acid chloride (41).

Several groups have employed a strategy involving attachment of an aglycone to a mixture of natural glycans prior to purification. In this case the aglycone both facilitates purification and provides a means for subsequent immobilization to the glycan array support. For example, in the "neoglycolipid" approach of Feizi and colleagues, an amine (17) or hydroxylamine (38) containing phospholipid is conjugated to a mixture of natural glycans, which allows separation into individual glycoforms by HPTLC and subsequent adsorption onto the nitrocellulose array. Similarly, Cummings and coworkers coupled a fluorescent, di-amine containing-linker, by reductive amination (37, 42). The resulting fluorescent and hydrophobic aglycone facilitates detection and purification by multidimensional HPLC. One amine is tied up in a glycosidic linkage, and the remaining primary amine allows for immobilization on various reactive surfaces.

Glycans produced by chemical and chemo-enzymatic synthesis

Synthesis of glycans by chemical or chemoenzymatic approaches presents a viable alternative to isolation of natural glycans. While great strides have been made in the synthesis of carbohydrates over the last 20 years (43), there are still no systematic methods for routine synthesis of glycans of defined sequence like those available for DNA and proteins. This is due, in part, to the myriad of products that can be obtained by linking two monosaccharides via alternative hydroxyl groups on the ring in either alpha or beta anomeric linkage, and moreover, the potential for branching in each residue of an oligosaccharide. In chemical synthesis, the similar reactivity of the hydroxyl groups on the sugar ring requires complex blocking strategies with different protecting groups on the glycosyl acceptor, and a suitable leaving group at the anomeric position of the glycosyl donor (Figure 2). Although enzymatic synthesis provides an alternative, relying on the specificity of the enzymes to form the desired glycosidic linkage, the limited availability of glycosyltransferases continues to impede the generality of this approach. The need for libraries with diverse glycan structures has stimulated the development of systematic strategies for chemical, enzymatic and combined chemo-enzymatic synthesis of glycans (44–46).

With an aim to automate the synthesis of complex carbohydrates, a number of sophisticated chemical methodologies have been developed for rapid generation of glycan libraries. The approach pioneered by Seeberger and colleagues utilizes solid-phase synthesis to carry out the iterative glycosylation and deprotection steps (45). They have utilized this method, along with more traditional solution-phase synthesis, to create libraries of heparin sulfate glycans, GPI-anchors, and high-mannose oligosaccharides (47–50). Another elegant technology is the Optimer-based one-pot, solution-phase oligosaccharide synthesis method (44). In this system, a computer program is used to select appropriate glycosyl donor and acceptor building blocks such that when added sequentially to a reaction vessel the desired oligosaccharide will be assembled. Wong and colleagues have used this method to create a library of Globo-H related oligosaccharides (51) as well as high-mannose structures (52, 53) for the creation of a number of custom arrays. Although automated methods are gaining a foothold, both still require specialized training in carbohydrate synthesis and a relatively large number of building blocks which are not trivial to assemble, and at present, are not commercially available.

Chemoenzymatic synthesis has also been used by a number of labs to generate glycan libraries (46, 54, 55). In general, a chemically synthesized mono- or oligosaccharide, with a linker containing an appropriate functional group, is elaborated upon using an appropriate

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glycosyltransferase and nucleotide sugar. This process can be repeated iteratively for elongation to the final product (Figure 2). An inherent benefit of this approach is that the numerous protection and deprotection steps, which are required to afford the appropriate regio- and stereoselectivity in chemical synthesis, are instead installed by the natural specificity and selectivity of the glycosyltransferase. This is especially a benefit for difficult to synthesize linkages such as sialosides. A major limitation, however, is the availability of the glycosyltransferases for synthesis of the desired linkage. Nonetheless, the Consortium for Functional Glycomics (CFG) assembled a collection of over 30 glycosyltransferases, which allowed expansion of their library by over 250 glycans. Because some glycosyltransferases are tolerant of substituents on the donor sugar, the chemoenzymatic approach is not limited to natural structures. For instance, one-pot, multiple enzyme systems, have been applied for the generation of unnatural sialoside analogs for inhibitor screening of siglecs (55, 56).

In practice, production of glycans by synthetic approaches can be combined with isolation of natural glycans to generate diverse libraries. This strategy entails derivatizing glycans from both sources with the same group used for immobilization. Notably, the two largest libraries assembled to date, that of the CFG (~500 glycans) and the neoglycolipid library of Feizi and coworkers (~600 glycans) have used this principle, using amino terminated linkers and lipids, respectively, as the basis for immobilization to the array surface.

Array Fabrication and Immobilization Methods

Ultimately, the production of a glycan microarray requires that the functionality installed in a glycan library be matched with an appropriate method for immobilization to the array surface. Methodologies reported to date include array chemistries and robotic instrumentation developed for DNA and protein microarrays, as well as novel methodologies developed specifically for arraying glycans. These immobilization and fabrication methods can be broadly categorized by the covalent or non-covalent nature of the interaction between the glycan library and the array surface as shown in Figure 4. As these are discussed, the appropriate entry in Figure 4 will be highlighted to guide the reader through the broad encompassing, but not encyclopedic account of the chemistries and methodologies employed in modern glycan arrays.

Non-covalent immobilization technologies

Non-covalent methods for immobilization of polysaccharides were amongst the first reported glycan arrays in 2002. Utilizing nitrocellulose (18) and oxidized polystyrene (23) it was shown that polysaccharides, as well as proteoglycans and neoglycoproteins, could be arrayed in microchip format (**A**). Despite the random orientation of glycans on the array, it is notable that in both studies the carbohydrates retained their antigenicity when screened with different antibodies. Electrostatic interactions have also allowed for the fabrication of polysaccharide arrays of various GAGs (57) as well as chemically modified, negatively charged dextrans to amine-based slides (58) (**B**). Although powerful techniques, an inherent limitation of these is the large molecular weight of glycan required for efficient immobilization.

Lower molecular weight oligosaccharides can also be arrayed by hydrophobic adsorption, however, they require a long-chain alkyl linker (**C**). As discussed above, Feizi and colleagues had demonstrated that neoglycolipids could be adsorbed to TLC and 96-well plates for glycan binding protein interrogation in the 1980s, and this was elegantly expanded to a PVDF microarray format in 2002 (17). Since then, this expanding neoglycolipid library has been successfully implemented in a nitrocellulose chip-based format (28). In similar work, Bryan et al. showed that long-chain alkyl glycosides ranging from mono to

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pentasaccharides could be efficiently immobilized onto polystyrene microtiter plates and detected by various lectins (19). In analogy to the hydrophobic forces governing the above adsorption techniques, Pohl and coworkers developed carbohydrate microarrays based on the selective immobilization of fluorous (C_8F_{17}) tagged mono-saccharides to similarly coated (C_8F_{17}) glass slides (59) (**D**).

As in other fields, the "tried-and-true" biotin-streptavidin interaction has found great utility in glycobiology (**E**). The initial glycan array developed by the CFG, for instance, was a streptavidin 384-well plate assay with a library of some ~200 biotinylated glycans (60, 61). Although glycopolymers have also been arrayed on streptavidin (62, 63), printing of monovalent biotinylated glycans onto streptavidin microarray slides has not been successfully demonstrated.

A newly emerging technology in the development of non-covalent glycan microarray platforms is a DNA-directed immobilization strategy (**F**). In this approach a synthetic carbohydrate is covalently attached to an oligonucleotide probe, while a complimentary DNA sequence immobilized on the chip is used to array the glycoconjugate for detection by a lectin (64). Alternatively, a mixture of oligonucleotide-glycans can be incubated with a lectin followed by addition of this solution onto the DNA-chip which allows binding to be quantified by the fluorescence and the identify of the glycan to be determined by the DNA barcode on the chip (65).

Covalent immobilization technologies

The majority of covalent methods for arraying glycans have employed robust thiol and amine chemistries (Figure 4). Early work by Shin et al. reported the use of maleimide functionalized glycans and thiol-derivatized slides for the immobilization of a library of sugars (22, 66) (**G**). The reverse combination has also been utilized (67), for instance to array a collection of high-mannose glycans to study the binding epitope of cyanovirin N (50), as well as in a custom GPI glycan array to study antibody responses to plasmodium (49, 68) (**H**). Disulfide exchange reactions have been used to fabricate carbohydrate SPR chips (69), and more recently, a novel rewritable glycochip (70) (**I**). Direct immobilization of thiol terminated heparin fragments onto gold surfaces has also been reported for SPR, but not directly used for microarray studies (71) (**J**).

Probably the most widely used glycan microarray technology at present is based upon amine chemistry. The only commercially available glycan chip (Glycominds) utilizes amine/ cyanuric chloride coupling chemistry (72) (**M**), while the robust technology developed by Blixt et al. with amine-terminated glycans and commercially available NHS-ester activated slides has served as the platform for the CFG for years (54) (**N**). Similar methodology has been utilized by a number of groups to array heparin glycans (47, 48), Globo-H related structures (51, 73), and sialosides (74). Cummings and colleagues have employed this method as well, but have also shown that epoxide ring opening by amine terminated sugars may be slightly more effective for immobilization of their naturally derived glycan libraries (37, 41, 42, 75) **(O)**. Finally, although glycoproteins and neoglycoproteins can be immobilized efficiently by noncovalent techniques, they may also be covalently attached to the array surface by amine or thiol chemistries (76) (**O**).

Condensation reactions between amines, hydrazides, and oxyamines with aldehydes have also found their place. Elegant examples of direct immobilizaton of free reducing glycans with all of the above slide coated surfaces have been reported (39, 77) (**P**). Moreover, the reverse combination has also been used to gain insight into the importance of sulfation regioselectivity on chondroitan sulfate and GAG-binding protein interactions (78, 79) (**Q**).

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Aside form thiol and amine chemistries, cycloaddition reactions also served as the basis of some of the first glycan arrays. Work by Houseman et al. showed that a library of monosaccharides could be attached to a self-assembled monolayer via Diels-Alder chemistry (21) (**K**). In the same year, another cycloaddition, the increasingly utilized “click” reaction, was also employed to array a diverse set of of di, tri-, and tetrasaccharides in microtiter plates (20) (**L**). Although, not broadly used for arraying DNA and peptides, a number of notable publications utilizing click chemistry to array glycans (80, 81), aminoglycosides (82), and glycopolymers (83) have emerged. Moreover, the more recently developed method of microcontact printing has been used to array glycans via both of the above chemistries (81, 83, 84).

Most of the above immobilization methods require linkered glycans with distinct reactive groups. An alternative method for the covalent attachment of underivatized glycans utilizes array surfaces with photoreactive groups. Commercially available aryl-trifluoromethyldiazirine dextran modified slides have been used to immobilize plant xyloglucans (85) as well as various bacterial polysaccharides, mammalian glycoproteins, and even whole cell extracts (86) (**R**). Photoreactive surfaces have also been generated from SAMs by attachment of phthalamido groups for the covalent immobilization of dextrans (87) and bacterial polysaccharides (31) (**S**). The opposite combination has also been demonstrated whereby mono- and disaccharide perfluoroarylazide glycosides were immobilized onto PEG-ylated surfaces and successfully detected with a panel of lectins (88) (**T**).

With this nearly overwhelming number of glycan array methodologies, a remaining challenge for the field resides in comparison of glycan binding data across array platforms, since it is possible that different immobilization chemistries may influence the results obtained amongst various groups. On the other hand, although many array formats have been described, few have moved beyond proof-of-principle to allow such comparisons. Based on the substantive results and biological insights that have stemmed from this technology to date, further refinements will only increase the quality of these already robust platforms.

Decoding the Glycome: Biological Applications of Glycan Microarrays

Glycan binding proteins (GBPs) mediate diverse biological roles that range from cell-cell recognition, cell trafficking, glycoprotein targeting, and host-pathogen interactions (1–5). Since the advent of glycan microarrays, they have been used to survey the specificity of a highly diverse set of GBPs including various families of mammalian GBPs, viruses and recombinant viral GBPs, bacterial adhesins and toxins, carbohydrate specific antibodies, and plant lectins. In the sections below, we briefly discuss the strategies used to analyze GBPs on glycan microarrays, and then provide examples from each category where microarray data has yielded novel information on GBP specificity that led to insights into the underlying biology that it mediates.

Exploiting multivalency for analysis of GBPs on glycan microarrays

Most GBPs exhibit low intrinsic affinity for their ligands (e. g. Kd values of 1 μM - 1 mM), and achieve a biological effect through multivalent interactions (13, 89). Due to the low avidity, it is not intuitively obvious that glycan arrays would have broad utility in the analysis of GBP ligand specificity since final washing steps could elute any bound GBP. In practice, however, this has not turned out to be a major problem. The work-flow used for assessing binding to glycan microarrays is fairly standard, and has been extensively reviewed (13, 15, 16, 54). In brief, a solution of GBP is applied to the array, either directly labeled or complexed with a detection agent, followed by washing steps, imaging by a

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fluorescence scanner, and processing of the data. In many cases the combination of the natural valency and affinity of the GBP is sufficient to allow fluorescently labeled protein to remain bound to the array, as illustrated for a virus and multivalent GBP in Figure 5. In other cases, additional valency is needed. This can be supplied by pre-complexing with an antibody that binds to the protein or a peptide sequence (e.g. anti-his tag) incorporated into a recombinantly produced GBP (35, 54, 90). Another solution to the problem is to avoid washing the slide altogether. This is possible using a slide scanner based on real time evanescent-field fluorescence that detects labeled protein bound to the printed glycan from beneath the slide (91).

Most glycan arrays are printed at a glycan concentration intended to achieve maximal density of glycans. Investigators have come to rely on the observation that signal intensity for different glycans reflects their relative affinity for the GBP. In general, if many ligands are detected, varying the concentration of the GBP applied to the array can distinguish between high affinity and low affinity ligands. At high concentrations, the differences between these ligands are minimized due to saturation of signal during scanning. As the concentration of the GBP is decreased, only high affinity ligands are detected (92).

Although less work has been done varying glycan densities, it is clear that such factors are also important in interpretation of glycan array data (13, 93, 94). In one case, the C-type lectin, mannose binding protein, did not bind to a glycan array even though the array contained putative ligands and the protein was active in other assays, suggesting that the array did not achieve sufficient density to support multivalent binding (13). In another, binding of a plant lectin, conconavalin A showed equivalent binding to high and low affinity mannose containing ligands when printed at high density, but bound only to the high affinity glycan printed at reduced density (94). Such examples document the need for more systematic investigations into glycan presentation in different array formats.

Mammalian Glycan Binding Proteins

The largest glycan microarray libraries assembled to date (Table 1a) focus on the diversity of the mammalian glycome (13, 14, 95), and are thus well suited for analysis of the specificity of mammalian GBPs. It is estimated that there are ~70 mammalian GBPs, of which the majority are in the C-type lectin, siglec and galectin families (1, 5, 13, 96, 97). Members of each of these families have been studied on glycan microarrays, revealing highly diverse specificities for GBPs within and between each family (Table 2A) resulting in significant biological insights.

C-type lectins—The C-type lectins are the largest family of glycan binding proteins. They are prominently expressed in various white blood cells of the immune system and have become increasingly recognized as pattern recognition receptors (PRRs) that mediate innate and adaptive immune responses to pathogens (3, 5). Several of them, including DC-SIGN, SIGNR related proteins (SIGN-R1–7), langerin, scavenger receptor, and Dectin-1 have been assessed for their specificity on glycan microarrays (see examples in Table 2A) (54, 60, 92, 98–104). Human DC-SIGN recognizes two distinct classes of glycan ligands, high mannose N-linked glycans, and fucose containing Lewis × glycans (54, 60, 100, 102). Structure analysis has revealed that both types of ligands bind to the conserved carbohydrate recognition domain, with mannose or fucose coordinating with the bound calcium (13, 60). While the mannose binding specificity is well documented to play a role in the interaction with viral pathogens such as HIV, the fucose containing ligands may play a role in interactions with pathogens displaying Lewis × glycans (e.g. *H. pylori*) or on self cells involved in other known functions of DC-SIGN such as cell trafficking and antigen presentation to T cells (105). Of the murine SIGN related proteins, the ligand specificity of

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SIGN-R3 is similar to that of DC-SIGN, suggesting that it may a functional ortholog of DC-SIGN in mice (100, 106).

Several of the myeloid C-type lectins exhibited specificities involving calcium dependent binding of galactose, not mannose or fucose. Like DC-SIGN, the scavenger receptor binds Lewis × containing glycans, but it does so with primary binding to galactose instead of fucose (5, 13, 98). Human macrophage galactose lectin (MGL) exhibits strong specificity for N-acetylgalactosamine/galactose. Mouse has two homologs (MGL1 and MGL2), which bind Lewis × related structures, and galactose/N-acetylgalactosamine terminated glycans, respectively, suggesting that MGL2 is the murine ortholog of the human MGL (101).

Dectin-1 is unique in that it binds β1–4 glucans with high specificity (104). Since mammals do not produce β1–4 glucans (these are made by fungal pathogens), this is truly an example of pattern recognition of non-self. Dectin-1 is also unique in that it is a ‘C-type-like’ lectin whose binding to glucans is calcium independent, leaving open the possibility that other members of the ‘C-type like’ lectin subgroup (~50) may also recognize carbohydrate-based ligands.

One of the best understood C-type lectin subfamiles is the selectins (E-, P- and L-, selectin), which are variously expressed in lymphocytes, neutrophils, platelets and endothelial cells, and mediate trafficking of leukocytes to sites of inflammation and lymphoid tissues. Although the detailed ligand specificities of these GBPs to sialyl-Lewis-X related glycans had been documented before the advent of glycan microarrays, analysis on the glycan array of the CFG has revealed binding to a diverse set of glycans comprising sialylated and sulfated analogs of Lewis × (Galβ1–4(Fucα1–3)GlcNAc) and Lewis A (Galβ1–3(Fucα1–4)GlcNAc), which can be accessed from the CFG database (107).

Another endothelial cell C-type lectin is LSECTin, which has been implicated in the pathogenesis of Ebola virus infection, and exhibits a unique specificity for glycans with the terminal GlcNAcβ1–2Man sequence (92). It is notable that GlcNAcβ1–2Man terminated glycans presumed to be present on Ebola virus, and the high mannose glycans on HIV virus recognized by DC-SIGN, are both biosynthetic intermediates of complex type N-linked glycans that are not commonly found on cell surfaces. Thus, in a sense, these host-produced glycans are detected as ‘non-self’.

Siglecs—The siglecs are a sub-family of the immunoglobulin super family that have in common a N-terminal Ig domain that recognizes sialic acid containing glycans as ligands. Most of them are expressed in one or more white blood cell types, and participate in cell signaling through regulatory motifs found in the cytoplasmic domains (4). Prior to the advent of glycan microarrays, the siglec family had been demonstrated to exhibit differential specificity for the various sialoside sequences found in mammalian glycans on glycoproteins and glycolipids. Microarray analysis, however, allowed simultaneous screening against ~ 100 different sialosides sequences and has revealed that several siglecs preferentially bind glycans with both sulfate and sialic acid (54, 61, 108–110). Human Siglec-8 and the murine paralog, Siglec-F, were found to specifically bind to 6′-sulfo-Sialyl-Lewis × (61, 107, 110), with a Kd estimated to be in the range of 1–2 μM (61), representing one of the highest affinity interactions of any siglec for a sialoside ligand. Subsequent analysis by Rapoport et al (109) and Bochner (107) show that Siglec-8 binds with equivalent avidity to the non-fucosylated structure 6′-sulfo-3′-sialyl-LacNAc (NeuAcα2–3[6S]Galβ1–4GlcNAc). These findings are anticipated to be relevant to the natural ligands of Siglec-8/F, however, their existence on eosinophils or cells that interact with eosinophils has not yet been established.

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High avidity binding of the human B cell specific siglec CD22/Siglec-2 to 6-sulfo-6′-sialyl-LacNAc (NeuAcα2–6Galβ1–4[6S]GlcNAc) was first detected on a glycan microarray (54). However, its significance as a natural ligand of CD22 was not appreciated until Kimura et al. demonstrated that an antibody that specifically recognizes this sequence (KN343) binds to naïve human B cells, and abrogated binding of recombinant CD22-Fc chimera (111). Germinal centers of human lymph nodes were negative for KN343, suggesting that the ligands of CD22 are down-regulated upon B cell activation (111).

Galectins—As the name implies, the galectins are a family of homologous proteins that contain carbohydrate recognition domains, which recognize galactose-containing glycans. They comprise 15 members and predominately exist as non-covalent or covalent dimers, with identical CRDs (‘prototype subgroup’; galectins-1, 2, 5, 7, 10, 11, 13–15) or two non-homologous CRDs (tandem sub-group; galectins-4, 6, 8, 9, 12). The exception is galectin-3, which exists as a monomer with a C-terminal extension that allows multimerization upon binding ligand (97). While their functions are only beginning to be revealed, they are differentially expressed in most mammalian cell types, and have well documented roles in both adaptive and innate immune functions (3, 97, 112). While the galectins, like the siglecs, were extensively studied for their specificity prior to the advent of glycan microarrays (113, 114), microarray analysis has provided new information that allows discrimination of their specificities and provides important biological insights (37, 54, 91, 115–117). For example, galectins-1, -2, -3, -10 and -14 exhibit differential specificity for LacNAc ligands substituted with blood group, fucose, and sialic acid substituents, and only galectin-3 recognizes internal galactose in poly-N-acetyllactosamine sequences (37, 116, 117). Each CRD of the tandem repeat galectins-4, -6 and -8 exhibit different specificities, with the C-terminal domain binding blood group A and B structures and the N-terminal domain having a broader specificity, and in the case of galectin-8, very high affinity for α2–3 linked sialylated glycans (112, 115, 118). The blood group specificity of the C-terminal domain of intestinal galectins -4 and -8 mediates *in vivo* killing of bacteria bearing lipopolysaccharides with blood group B structures, suggesting an innate immune function of these galectins in the gut (112).

Other mammalian lectins—There are numerous mammalian GBPs outside these three major families, and new GBPs continue to be discovered. Notable results from analysis on glycan arrays (Table 2A) include: M-Ficolin, a soluble serum protein involved in innate immunity shown to bind sialylated glycans(119); and malectin, an ER protein whose function was unknown until demonstration of its binding to a $Glc_3Man_9GlcNAc_2$- N-linked glycan, suggesting that it was involved in the processing of N-linked glycans intermediates in the biosynthetic pathway(120).

Microbial binding proteins

Microbial pathogens were known to recognize glycans as receptors on mammalian host cells long before the discovery of mammalian glycan binding proteins and the advent of glycan microarrays (6, 7). It is currently believed that glycan mediated host-pathogen interactions have exerted evolutionary pressure on their hosts, and account in part for the species specific differences in the glycome repetoire of mammals (121). In recent years glycan microarrays have become a standard method for investigating the specificity of novel microbial GBPs, and many well-studied microbial GBPs are being re-evaluated on glycan microarrays revealing new insights into their biology, as illustrated with selected examples below.

Influenza viruses were demonstrated to bind sialic acids over 60 years ago (7), and have been shown to exhibit specificity based on species of origin with human and avian viruses preferentially recognizing sialosides with NeuAcα2–6Gal and NeuAcα2–3Gal linkages,

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respectively (90). This paradigm was confirmed upon analysis of either recombinant hemagglutinin or influenza virus on glycan microarrays (54, 122), but it was soon evident that individual isolates differed in their fine specificity for natural sialosides sequences that contained α2–3 and α2–6 linked sialic acids (107, 122–127). In addition to influenza A viruses, glycan arrays have been used to assess the specificity of influenza B (124) and parainfluenza (128) viruses. With the vast amount of new information on virus specificity, it has become evident that little is yet known about the glycan sequences expressed on human airway epithelium and analytical glycomic approaches, in conjunction with glycan microarrays, will be undoubtedly necessary to understand the adaptation of new pandemic viruses to the glycan repertoire of human hosts (123, 125–127).

The glycan specificity of viruses with protein capsids, including parvovirus (129), adenovirus (130), JC virus and natural mutants (107), and the polyoma related murine virus, SV40 (131, 132), have also been analyzed on glycan arrays. Interestingly, SV40 exhibits remarkable specificity for the pentasaccharide from ganglioside GM1 (Table 2B), with highest specificity for NeuGc vs NeuAc as the sialic acid (131, 132). This likely stems from the simian species origin of this virus, as this form of sialic acid is not produced by humans, but is found in all other non-primate mammalian species.

Bacterial adhesins and toxins have also been subjected to carbohydrate array analysis. The FedF adhesion from the enterotoxigenic *E. coli* (133), the PA-IL lectin of the pathogenic *Pseudomonas aeruginosa* (134), the adhesin of *C. jejeuni* (135), and the soluble BC2L-C lectin from *B. cenocepacia* (136) have all yielded array data which may aid a deeper understanding of the roles of these adhesins in the interaction of these organisms with mammalian epithelium. In an elegant study, comparision of the cyanobacterium lectin, cyanovirin (*N. ellipsosporum*) with two homologous fungal cyanovirins showed that all three bound high mannose N-glycans, but differed in their fine specificity (137).

Similarly, examination of the glycan specificity of several toxins has also provided new biological insights. Analysis of the different CRDs of *C. difficile* toxin (138), *C. tetani* tetanus neurotoxin (139), and pertussis toxin (140) helped understand their contribution to the overall receptor specificity of the intact toxins. In a remarkable finding, the subtilase cytotoxin secreted by Shiga toxigenic *E. coli*, which causes severe gastrointestinal disease in humans, was found to bind only to glycans containing NeuGc (141). The results suggest that human susceptibility to this toxin is a consequence of a diet of red meat, which provides a source of NeuGc that is absorbed by the host cells and incorporated into the cell surface glycans that then serve as receptors for the toxin.

Glycan specific antibodies: reagents, vaccines and biomarker discovery

The antigenicity of carbohydrates has been recognized for nearly a century and underlies the basis of human blood group specificity and antigenic markers of embryonic stem cell differentiation. In recent years, microarrays have been increasingly used to characterize the specificity of glycan specific antibodies used as reagents (107), and to compare the antibody repertoire in normal serum with serum from animals immunized with glycan based vaccines, or serum of patients with various diseases to identify glycan-specific biomarkers.

Since the discover of Heidelberger and Avery in 1923 that pneumococcas serotype was based on antibody recognition of the capsular polysaccharide (142), nine carbohydrate-based vaccines have been approved and over a dozen others are in clinical trials (143). Some of the earliest vaccine studies using glycan arrays involved mapping the epitope of the broadly neutralizing HIV antibody 2G12, which recognizes a cluster of high-mannose N-linked glycans on the gp120 coat protein (53, 80, 144). By understanding the molecular basis of 2G12 recognition it was hoped that “reverse vaccinology” could be used to guide the

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synthesis of a carbohydrate-based HIV vaccine. Various glycoconjugates containing high mannose glycans have been used in attempts to elicit a 2G12-like response (145, 146). In analyzing sera from animals after immunization, glycan microarrays have been extremely useful tools. Although to date not successful, the above works have shown that the immune system can distinguish high mannose glycans displayed in different biological contexts.

Another intense carbohydrate-based vaccine effort impacted by glycan arrays focuses on the anthrax-producing bacterium, *Bacillus anthracis*. The BclA glycoprotein on the spore surface contains a unique tetrasaccharide, which terminates with a previously unidentified monosaccharide, aptly named, anthrose. In work by Wang and colleagues, the immune response of rabbits immunized with anthrax spores was evaluated by a custom array of anthrose containing synthetic oligosaccharides (31). What was clearly evident was that anthrose was extremely immunogenic and a promising candidate for vaccine development. This has since been followed up in more synthetic detail by Oberli et al. with an array of 16 carbohydrates including anthrose analogs as well as native structures (32).

The GPI-anchors of Plasmodium and the glycolipid Globo-H are also validated carbohydrate antigens with vaccines currently in clinical trials for malaria and breast cancer, respectively (143). Indeed, it has been demonstrated that a synthetic hexasaccharide-KLH conjugate is extremely effective in preventing malarial infection in a murine model (147). An array of various GPI-anchors has since been used to better understand the anti-malarial humoral response to these immunogenic plasmodium glycoconjugates (49, 68). Moreover, a custom array of Globo-H related oligosaccharides has been developed by Wong and colleagues to study the specificity of various anti-Globo-H antibodies (51) as well to validate Globo-H as a biomarker for breast cancer (73).

The fact that many cancers show aberrant glycosylation also has implications for glycan-based vaccine design and, along with it, the use of glycan microarrays in their development. Although a number of vaccines for the Tn-antigen are already in clinical trials for breast and prostate cancers, there have been discrepancies in the literature on the utility of the Tn-antigen as a biomarker. Gildersleeve and coworkers have since utilized a neoglycoprotein-based array to further dissect these inconsistencies and to trace it back to cross reactivity of the various antibodies with other glycan structures (148). To prevent these sorts of problems from stalling further carbohydrate-biomarker discovery, the same group has since developed exquisitely specific antibodies utilizing glycan array technology to rapidly screen hybridomas. One of these, an anti GalNAcαGal antibody, has since been used as a tool to predict the survival rate of cervical cancer patients (149).

A glycopeptide array of MUC1 related structures has been developed by Wandall and Blixt to study the antigenicity of this tumor specific antigen in cancer patients. They observe high IgG titers to unique glycoforms of MUC1 glycopeptides in various cancers that are completely absent in healthy individuals (150). Westerlind and colleagues have also generated a MUC1 glycopeptide array for similar studies (151). These initial results suggest that this technology will become an important tool for unraveling the “glycopeptidome”.

Plant lectins

Since the discovery of plant lectins over a century ago (7), numerous plant lectins have been identified and demonstrated to have highly diverse specificities for glycan ligands. They are widely recognized as important tools for glycobiology research (9, 10), and lectins with novel specificities continue to be identified and characterized. Glycan microarrays have now become a principle tool for defining the detailed specificities of plant lectins. The CFG has analyzed the specificity of ~100 plant lectins contributed by investigators or obtained from commercial sources (107). Although a thorough review of this important body of work is

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beyond the scope of this review, the CFG databases and website provide an excellent repository of the accumulated data and resulting publications (107).

Glycosyltransferases and other glycan modifying enzymes

Several early reports demonstrated that glycosyltransferases could utilize immobilized glycans as acceptors (20, 21). Subsequently, a number of groups have used microarray technology to assess the specificity of glycosyltransferases. Both the Mrksich (152) and Park (153) groups examined the specificity of bovine β1,4 GalT with libraries of ~20 unique structures. In the former case, the array was constructed on SAMs, allowing quantitative analysis of the products by on-chip MS. This technology has since been used to assess the specificity of a polypeptide GalNAcT (154) to initiate the synthesis of O-linked carbohydrate chains on an array of peptides immobilized on gold SAMs.

Utilizing the CFG glycan array, Blixt et al. surveyed the specificity of four mammalian sialyltransferases using a novel chemical probe, CMP-NeuAc with a biotin substitution at the C-9 of the sialic acid (155), while in another report, researchers created a catalytically inactive N-acetylgalactosaminidase from *S. pneumoniae* and evaluated its binding to infer its substrate specificity (156).

Plant polysaccharide arrays have also been used to look at carbohydrate processing enzymes (85, 157), including the screening of recombinant mutants of a pectin-methylesterase for activity (158). Such examples illustrate how rapidly investigators are utilizing array technology to assess details of enzyme specificity that would otherwise be difficult to obtain without a substantial time investment.

Conclusions

In a short time, glycan microarrays moved from proof of principle to being a powerful tool for glycobiology research. Despite the documented utility of glycan microarrays, progress to date represents only a beginning. Although the glycome is generally acknowledged to be larger than the genome, and even the proteome, the size of mammalian glycan array libraries still pales in comparison to the libraries that have been assembled for DNA and protein microarrays. Similarly, pathogen glycan arrays constructed to date represent only a small fraction of microorganisms that have pathogenic or commensal interactions with mammalian hosts. Growth of these libraries will require advances in technologies for the synthesis of glycans, isolation and characterization of natural glycans, cooperation of a community network, or a combination of all of these. As the glycan libraries grow and use of these arrays expand, databases that archive and compare results, such as those of the CFG, will become increasingly important.

What is abundantly clear, however, is that glycan microarrays have already had a tremendous impact on the glycobiology field. The accumulating data in databases (107) will continue to propel research into the natural ligands of GBPs, and the biological roles of GBP-ligand interactions. The progress to date will fuel advancements in the synthesis and isolation of glycans, and provide motivation for the assembly of glycan arrays that far exceed the size of those currently available. This will ensure that the use of arrays to probe the biology of glycan binding proteins will continue at a rapid pace.

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Acronyms

GBP	glycan binding protein
GPI	glycophosphatidylinositol
SAM	self-assembled monolayer
GAG	glycosaminoglycan
NHS	N-hydroxysuccinimide
SPR	surface plasmon resonance
CFG	Consortium for Functional Glycomics
CRD	carbohydrate recognition domain
Sialyl Lewis X	NeuAcα2, 6Galβ1, 4[Fucα1,3]GlcNAc
HPTLC or TLC	High Performance Thin-Layer Chromatography or Thin Layer Chromotography

Terms/Definitions (up to 10)

Glycan	A general term to describe monosaccharides, oligosaccharides, or polysaccharides, which may be found as free entities or as constituents of glycoproteins, glycolipids, or other glycoconjugates
Reducing End	This is the end of a sugar chain that is attached to a protein or lipid, which upon release as the free glycoside, the terminal monosaccharide has a closed ring form in equilibrium with an open-chain form containing an aldehyde or ketone functional group
Aglycone	Any non-sugar residue that is glycosidically linked to a monosaccharide or oligosaccharide. Most commonly, this is the linker that is used to array a glycan library to the array surface
Anomeric Position/ Carbon	The carbon atom in a closed ring monosaccharide that forms an acetal/ ketal when glycosidically linked, or a hemiacetal/ketal when not glycosidically linked. For this report the anomeric carbon is C1 for all monosaccharides except sialic acids, which is C2
Glycosyl Donor and Acceptor	In synthetic and chemoenzymatic synthesis, the glycosyl acceptor is the sugar residue containing a nucleophilic group, typically a hydroxyl, which attacks the glycosyl donor, a sugar residue with a leaving group at the anomeric position, to create a glycosidic linkage
Sialoside	Any sialic acid containing glycan
High Mannose	A class of N-glycan structures containing only mannose residues attached to the common N-glycan core structure
Glycoside	A mono- or oligosaccharide which is anomerically linked to an aglycone
N-linked/O-linked glycan	Two common classes of glycoprotein glycans that are attached to the polypeptide via the primary amide of Asn residues (N-linked) or the hydroxyl side chains of serine or threonine (O-linked)
Lectin	A carbohydrate-binding protein that is not an antibody

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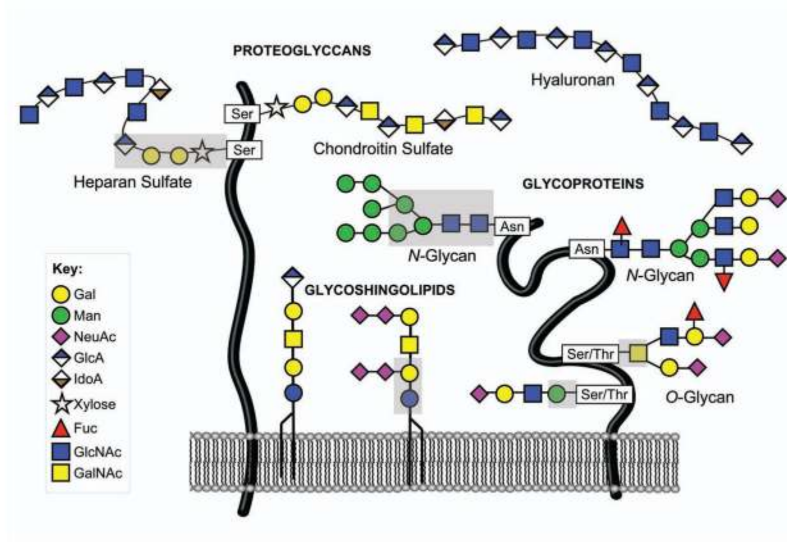

Figure 1. Major classes of mammalian glycans

The major classes of mammalian glycans are depicted with the common core structures of each class boxed in grey. Sequences at the tips of the glycan chains are representative structural variations found in mammalian cells. Adapted from Reference 1 (1).

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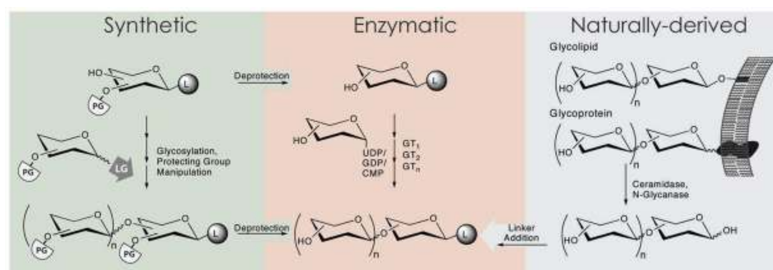

Figure 2. Generation of Glycan Libraries

Common methods for generation of carbohydrate libraries for glycan arrays are shown. As shorthand, PG = protecting group, LG = leaving group, and GT = glycosyltransferase.

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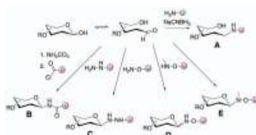

Figure 3. Reducing End Conjugation

Chemical methods for attachment of linkers to the reducing end aldehyde are shown along with the stereochemistry of the products generated by (A) reductive amination, (B) glycosylamine formation and trapping, (C) hydrazide chemistry, and (D) oxyamine or (E) N-alkyl oxyamine condensations. For (D) a ring-opened product is also formed as described in the text.

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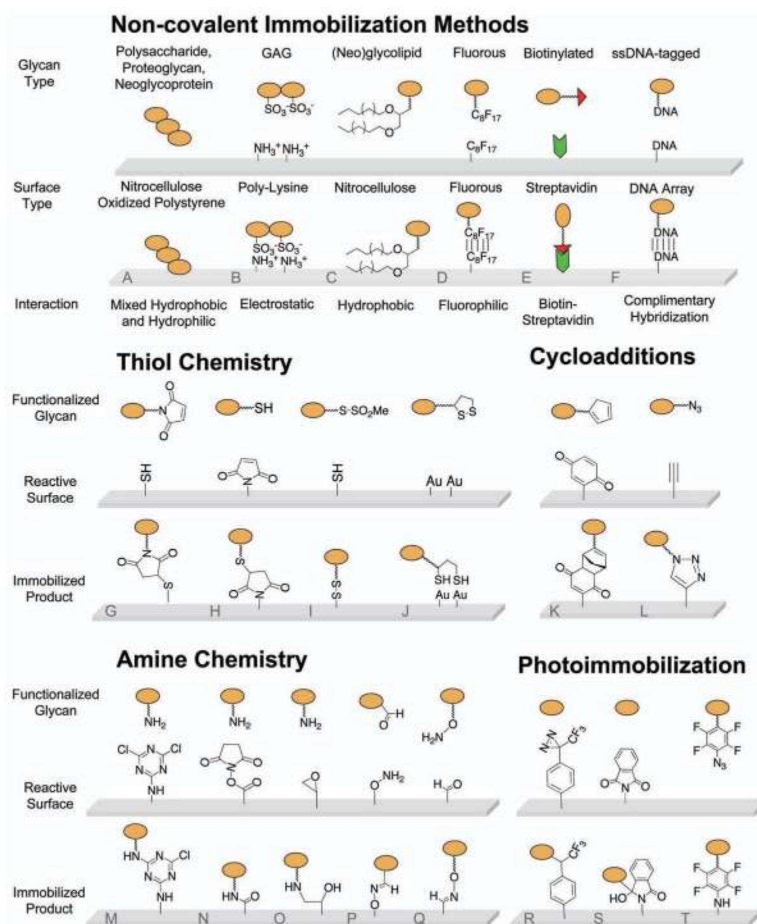

Figure 4. Methods for Immobilization of Glycans to Array Surfaces

Lettering corresponds to examples described in the accompanying text and the immobilization method used for arraying the various glycan libraries shown in Tables 1a/b.

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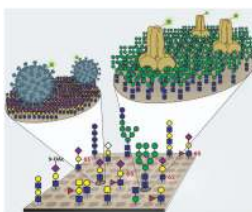

Figure 5. Multivalent Binding Interactions on a Glycan Array Surface

Two representative examples, viral hemagglutinin (left) and DC-SIGN (right) are shown to illustrate the multivalency afforded by both the GBP and the array surface, leading to a stable interaction and subsequent detection.

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Table 1a

Diverse libraries for screening the specificity of glycan binding proteins

Glycan Type	Size	Research Group	Synthetic	Naturally Derived	Immobilization Method[a]	References
Mammalian	500	CFG	+	+	N	(107)
	600	Feizi	+	+	C	(14)
	200	Bovin	+	−	N	(159)
	200	Cummings	−	+	N	(37)
	200	Gildersleeve	+	+	O	(149)
Bacterial	96	CFG	−	+	N	(107)
	48	Wang	−	+	A	(18)

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Table 1b

Glycan libraries for custom applications

Glycan Type	Subtype	Size	Application	Synthetic	Naturally Derived	Immobilization Method[a]	References
High Mannose	Phosphorylated	26	P-type Lectins	−	+	N	(160)
	Synthetic	7	2G12 Mapping	+	−	N/H	(53, 144)
	Natural	19	2G12 Mapping	−	+	C	(146)
Sialosides	Native	24	Influenza HA	+	−	N	(74)
	9-Modified	45	Siglec Inhibitors	+	−	N	(56)
	9/5-Modified	72	Siglec Inhibitors	+	−	E	(55)
GAGs	Heparin Sulfate	12	GAG-BPs	+	−	N	(48)
	Heparins	6	GAG-BPs	−	+	P[b]	(77)
	Chondroitan Sulfate	5	GAG-BPs	+	−	Q	(78, 79)
	Various	13	GAG-BPs	−	+	B	(57)
Bacterial	B. anthracis glycans	33	Serum Abs	+	+	S	(31)
	B. anthracis glycans	16	Abs	−	+	H	(32)
	Salmonella O-antigens	15	Serum Abs	+	+	N	(30)
Misc.	GPIs	7	Serum Abs	+	−	H	(49)
	Globo-H Related	8	Serum Abs	+	−	N	(73)
	Plant Glycans	50	Abs	−	+	A	(161)

[a]The lettering shown corresponds to the immobilization method used to array the glycan library with details of each shown in Figure 4.

[b]In this report amine surface is used to give the condensation product.

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Table 2A

Glycan microarrays reveal diverse glycan ligand specificities of GBPs.

Glycan Structure	Mammalian Glycan Binding Protein[a]													
	Galectins			C-type Lectins					Siglecs				Other	
	Gal1	Gal3	Gal8	DC- SIGN	LSECT	MGL	L- SEL	DECT	Hu- CD22	Mur CD22-	Sig-8/F	Sig-9	MLCTN	FCLN
1	+++	++	+	−	−	−	−	−	−	−	−	−	−	−
2	−	+++	+++	−	−	−	−	−	−	−	−	−	−	−
3	−	+	+++	−	−	−	−	−	−	−	−	−	−	−
4	−	−	−	+++	−	−	−	−	−	−	−	−	−	−
5	−	−	−	+++	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	+++	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	+++	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	+++	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	+++	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	+++	±	−	−	−	−
11	−	−	−	−	−	−	−	−	++	+++	−	−	−	−
12	−	−	−	−	−	−	−	−	−	−	+++	−	−	−
13	−	−	−	−	−	−	−	−	−	−	−	+++	−	−
14	−	−	−	−	−	−	−	−	−	−	−	−	+++	−
15	−	−	−	−	−	−	−	−	−	−	−	−	−	+++

[a]Examples of the specificities of GBPs from the galectin (91, 113, 115, 116), C-type lectin (13, 60, 91, 92, 98, 99, 101–103), siglec (4, 54, 61, 91, 108, 110) and other families (119, 120) are shown. (Abbreviations: Gal, galectin; LSECT, LSECTIN; L-SEL, L-selectin; Sig, Siglec; MLCTN, malectin; FCLN, M-Ficolin).

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Table 2B

Ligand specificities of microbial binding proteins revealed by glycan arrays.

Glycan Structure	Virus[a]			Bacteria[a]					
	Enveloped		Capsid	Adhesins			Toxins		
	hInfA	avInfA	SV40	PA-IL	BC2L-C	CNV	TcdA	TeNT-R	SubB
16	+++	±		−	−	−	−	−	−
3	±	+++		−	−	−	−	−	−
17	−	−	+++	−	−	−	−	−	−
18	−	−	−	+++	−	−	+++	−	−
19	−	−	−		+++	−	−	−	−
5	−	−	−	−	−	+++	−	−	−
20	−	−	−	−		−	−	+++	−
21	−	−	−	−	−		−	−	+++

[a]Examples of the specificities of microbial GBP from enveloped (90) and capsid (131, 132) viruses, and bacterial adhesins (134, 136, 137) and toxins (138, 139, 141). (Abbreviations: human, h; avian, a; influenza, inf; cyanovirin, CNV; *C. difficile* toxin A, TcdA; Tetanus neurotoxin, TeNT-R; Subtilase cytotoxin B, SubB)