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Carbohydrate arrays as tools for the glycomics revolution

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Determining the roles of carbohydrates in cell biology is hindered by the tremendous efforts that must be taken either to synthesize carbohydrates chemically or to isolate them from natural sources. Several platforms have been developed to study glycobiology. These platforms include the covalent and non-covalent immobilization of carbohydrates on microtiter plates, nitrocellulose membranes, coated slides, glass slides, gold surfaces and microspheres. Each platform has both positive and negative attributes for studying carbohydrate biology, such as flexibility and the amount of material required for study. Here we discuss these techniques with the goal of developing a 'gold standard' in the field of glycomics.

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▼ Scientific tools have led to a renaissance in our understanding of the physical world. DNA microarrays [1], nucleotide sequencing [2], automated DNA synthesis [3,4], polymerase chain reaction (PCR) [5], recombinant DNA techniques [6], NMR [7] and computational methods [8,9] all have an important place in the tool kit of modern scientists. These tools have facilitated discoveries in fields as diverse as medicine, structural biology, nanotechnology and physics. By contrast, they have been applied only recently to the study of carbohydrates, and this delay has greatly hindered the emerging field of glycomics.

Carbohydrates are known to have essential roles in diverse biological processes including host–pathogen interactions [10], signal transduction [11], inflammation [12] and development [13]. Carbohydrates with medicinal uses include heparan, which is the most widely used anticoagulant [14], antibiotics [15] and vaccines [16]. Uncovering the contributions of carbohydrates to cell biology would greatly facilitate advancements in science and medicine; however, such discoveries are hindered by the large investments that must be made to procure carbohydrates of sufficient purity and quantity for biological testing. Several reasons underlie the difficulties involved in obtaining carbohydrates; for example, carbohydrate synthesis is not template-driven and the isolation of carbohydrates from natural sources, although possible, yields only limited quantities of material that is often microheterogeneous. Chemical synthesis and cell biology can aid in procuring defined chemical materials, but non-specialists do not use these experimental routes routinely and molecular biology techniques can be used to transfer glycosylation pathways to bacteria to enable the genetic manipulation of glycan structures. In addition, the automated synthesis of carbohydrates can be achieved, but the synthetic efforts required to get the desired carbohydrates are not trivial [17–19]. Thus, to use efficiently the limited quantities of carbohydrate that are available, sensitive screening techniques for studying carbohydrates are urgently needed.

In this review, we focus on tools that have been developed to investigate the roles of carbohydrates in cell biology and medicine, placing particular emphasis on several high-throughput screening (HTS) techniques for probing carbohydrate–protein interactions. The hallmark of these techniques is the immobilization of carbohydrates on a surface.

Platforms for HTS in glycobiology

In HTS, proteins are incubated with a carbohydrate-containing surface, unbound protein is washed away and the binding ensemble is determined. Binding is detected either through the fluorescence of a bound labeled protein or colorimetrically by enzyme-linked immunosorbent assay (ELISA) techniques. For studying ligand binding, the presentation of carbohydrates on a surface is advantageous because interactions in biological systems often occur on surfaces and are multivalent in nature [20].

1741-8372/04/$ – see front matter PII: S1741-8372(04)02443-0

Such HTS techniques have several important advantages: first, the carbohydrates that interact with proteins can be defined precisely and the specificities of these interactions determined; second, these techniques can facilitate the rapid identification of new compounds that disrupt important protein–carbohydrate interactions or inhibit enzymatic reactions; and last, biologists and chemists armed with these tools are empowered to probe biological pathways to elucidate previously unappreciated roles of carbohydrates.

In the broadest sense, two methods are used to immobilize carbohydrates on surfaces: non-covalent and covalent attachment. Here, the advantages of these methods are discussed, along with specific examples of the different platforms.

Non-covalent immobilization

Unmodified carbohydrates can be immobilized non-specifically on nitrocellulose-coated glass slides [21]. Wang *et al.* [21] have used this technique to immobilize several different oligosaccharides of high molecular weight (>20 kDa). However, the loading of oligosaccharides on these slides is affected by oligosaccharide length. For example, when dextrans of 2000 kDa and 20 kDa were applied to nitrocellulose slides at the same concentration, two times greater loading was observed for the 2000 kDa dextran after the slides were washed. This difference is the result of the greater affinity that longer oligosaccharides have for nitrocellulose.

Wang *et al.* [21] also used an arraying robot coupled with this platform to construct high-density arrays displaying a series of 48 polysaccharides, glycosaminoglycans, glycoprotein and semisynthetic glycoconjugates. These arrays were then probed for their ability to bind anti-dextran, human IgM and human IgG antibodies. The screening technique was found to be extremely sensitive, because antibody specificities could be detected by incubating a chip with as little as 1 ml of a serum sample [21]. A limit of this approach is that small oligosaccharides or monosaccharides cannot be loaded on nitrocellulose surfaces unless they are modified by coupling to a protein such as bovine serum albumin [21].

Glycolipids or neoglycolipids containing a lipid tail on reducing end sugars have been non-covalently immobilized on nitrocellulose membranes, as demonstrated by the Feizi group (Figure 1a) [22]. This technique has enabled the construction of arrays of carbohydrates obtained from various sources including glycoproteins, glycolipids, proteoglycans, whole organs and chemically synthesized saccharides (containing 2–20 monomers). Thus, addition of the lipid group enables the immobilization of small carbohydrates. However, the resulting neoglycolipids have an open sugar on the reducing end, which limits application to polysaccharides (Figure 1a) [23].

Arrays of heterogeneous oligosaccharides have been made and successfully probed with proteins known to bind to a carbohydrate present in the oligosaccharide mixture [23]. This suggests the arrays could be used to identify new carbohydrate–protein interactions by screening proteins for binding to mixtures of saccharides. Once mixtures are identified that bind to a protein, the components can be isolated and tested further for binding. At present, this approach seems particularly advantageous because it focuses efforts towards purifying mixtures that have a binding affinity of interest. For example, new carbohydrate–protein interactions have been identified through analysis of the binding of sulfated oligosaccharides by antiserum and the binding of an antibody to chondroitin sulfate [23].

Lipid-bearing saccharides have been immobilized in the wells of polystyrene microtiter plates in work undertaken by the Wong group (Figure 1a) [24–26]. The main challenge in developing this method was the identification of a linker system and washing conditions that did not affect the immobilization of saccharides in the wells. A systematic study of the retention of lipid-bearing galactose conjugates was undertaken to tackle this problem. The results showed that C_{13}–C_{15} linked carbohydrates were retained on the plates when the wash buffers did not contain detergents or solvents other than water [24]. Further experiments using a 1,3-dipolar cycloaddition reaction between a carbohydrate bearing an azido group and an alkyne-bearing C_{14} linker afforded lipid-bearing saccharides that could be immobilized on the plates (Figure 1a) [26].

An advantage of the microtiter plate approach is that it can be coupled with ELISA assays to probe the carbohydrate-binding specificities or glycosyltransferase modification experiments to characterize further the carbohydrates immobilized, or it can be used to screen for glycosyltransferase inhibitors [26]. Specific examples of the use of microtiter plates include chemoenzymatic synthesis of Lewis X, a mediator of inflammation that is often found in the sera of individuals with cancer, and studies of an inhibitor of a α-1,3-fucosyltransferase.

Covalent immobilization

The covalent immobilization of compounds on glass slides has been used for various applications. This method has been used to construct high-density DNA arrays to probe RNA expression levels [1]. Combinatorial chemistry techniques have also been inspired by this approach. Chemical libraries have been spatially arrayed on glass slides to facilitate screening of the library for members that bind tightly

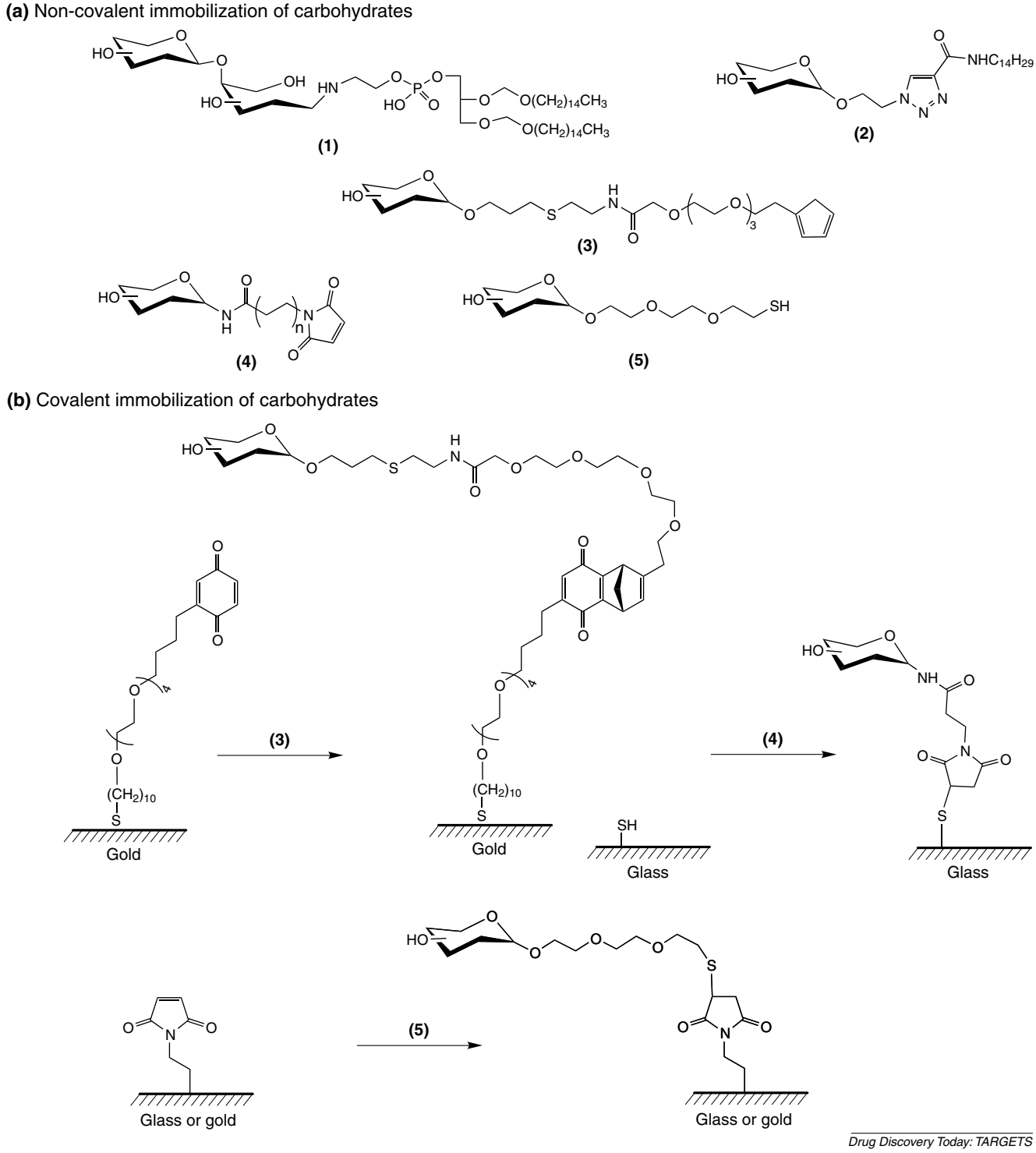

Figure 1. Non-covalent and covalent immobilization of carbohydrates. **(a)** Carbohydrate structures that have been immobilized on various surfaces to facilitate the high-throughput screening of carbohydrate–protein interactions. Non-covalent immobilization techniques include the neoglycolipid (**1**) method used by the Feizi group [22] for immobilization on nitrocellulose membranes, and lipidated carbohydrates (**2**) immobilized on polystyrene microtiter plates and formed via a 1,3 dipolar cycloaddition from an azide-bearing sugar and an alkyne-bearing linker [26]. Covalent immobilization techniques include cyclopentadiene-modified carbohydrates (**3**) immobilized on benoquinone-functionalized gold surfaces [30], maleimide-functionalized carbohydrates (**4**) immobilized on thiol-coated glass slides [31], and thiol-functionalized carbohydrates (**5**) immobilized on maleimide-coated surfaces [33] or encoded microspheres [32]. **(b)** Covalent immobilization of carbohydrates on various surfaces. The numbers on the arrows correspond to the compounds in (a) that are used to create the carbohydrate functionalized surface.

to proteins [27–29]. For carbohydrate arrays, three different research groups have exploited covalent attachment chemistries.

Houseman and Mrksich [30] have used Diels–Alder chemistry to immobilize carbohydrates bearing a cyclopentadiene group attached through a polyethylene glycol (PEG) linker to a monolayer presenting benzoquinone groups on a gold surface (Figure 1). These gold surfaces are resistant to non-specific protein absorption owing to their PEG nature, and several different monosaccharides have been immobilized by this method. The binding of monosaccharides displayed on the arrays to commercially available lectins was found to correlate well with previous measurements. For example, the mannose- and glucose-specific lectin concanavalin A (ConA) bound only to mannose, α-glucose and α-*N*-acetylglucosamine displayed on the arrays and did not bind to any of the other seven carbohydrates immobilized on this surface.

The inhibition of lectin binding was also probed [30]. ConA binding was measured in the presence of varying concentrations of α-methyl mannose. The results showed that different concentrations of α-methyl mannose were required to compete off lectin binding, and the competition depended on the carbohydrate–lectin interaction. For example, mannose, α-glucose and α-N-acetylglucosamine required 55, 23 and 8 mM of α-methyl mannose, respectively, to inhibit 50% of ConA binding [30]. Glycosyltransferase specificities were also probed. The detection of enzymatic elaboration was completed by probing arrays with lectins before and after their modification by β-1,4-galactosyltransferase. The differences in lectin-binding profiles were then used to monitor enzymatic transformations [30].

Park and Shin [31] have attached maleimide-linked carbohydrates to glass slides that present thiol groups (Figure 1). They showed that high-density arrays with over 12,000 spots on a single glass slide could be constructed by an automated arraying robot, and that each spot could be probed individually for binding to a carbohydrate lectin. This example illustrates the number of compounds that can be screened in parallel by this approach. Furthermore, the arraying and analysis of binding could be completed by using standard techniques for the construction of DNA microarrays.

Seeberger *et al*. [32,33] immobilized carbohydrates that incorporated a PEG tail that terminated with a thiol functionality on the reducing end (Figure 1) [32,33]. The PEG tail has also been used to eliminate non-specific binding in a manner analogous to the Diels–Alder immobilization technique [30] described earlier. Carbohydrates of lengths ranging from monosaccharides to nonasaccharides were chemically immobilized on maleimide-activated surfaces. The carbohydrates chosen for arraying were mannosides with different lengths and linkage stereochemistry (Figure 2) that were based on the *N*-linked oligosaccharides present on the cell membranes of many pathogens, including HIV [34], ebola [35] and some coronaviruses [36]. In particular, these methods have been used to probe the interactions of high mannosides to the HIV-neutralizing enzyme cyanovirin N (CVN). This protein was isolated from cyanobacterium and binds to high mannose residues displayed on the surface of HIV [37]. Binding of CVN to HIV inhibits viral entry in both *in vitro* and *in vivo* models and this protein is currently undergoing testing as an anti-HIV prophylactic [38,39].

Two other methods are available for constructing high-density arrays containing complex carbohydrates [32,33]. In the first method, encoded fiber-optic microspheres, each containing a unique covalently attached carbohydrate structure, are incubated with fluorescently labeled ConA and CVN [32]. To aid in their visualization, the beads are internally encoded with fluorescent dyes that represent the carbohydrate immobilized on the bead. Binding is determined by an increase in the fluorescence signal, which is monitored by imaging the arrays under a fluorescence microscope [32]. A standard DNA arraying robot has been used to develop a second method in which thiol-terminated carbohydrates are arrayed on maleimide-functionalized glass slides (Figure 2) [33]. High-density arrays, with several hundred spots per slide, have been constructed. The results from both of these screening techniques show that ConA binds only to mannose displayed on the surface and that CVN binds to mannosides containing part of the D1 arm present on *N*-linked glycoproteins (Figure 2) [32,33].

Surface plasmon resonance spectroscopy

Immobilized carbohydrates are also used for surface plasmon resonance (SPR) spectroscopy experiments, which provide valuable insights into the binding of analytes to ligands in real time and can measure both low- and high-affinity interactions. In SPR experiments, a solution containing an analyte is washed over a gold surface and binding constants are determined from the change in the refractive index of the surface that occurs when analytes bind. Because interactions are measured without the need for labeling, any influence of a label on the experimental results can be excluded.

SPR imaging studies have been used to determine the interaction of the carbohydrate-binding proteins ConA and jacalin with mannose and galactose, respectively [40]. In this case, thiol-modified carbohydrates were immobilized inside poly(dimethylsiloxane) microchannels on a gold

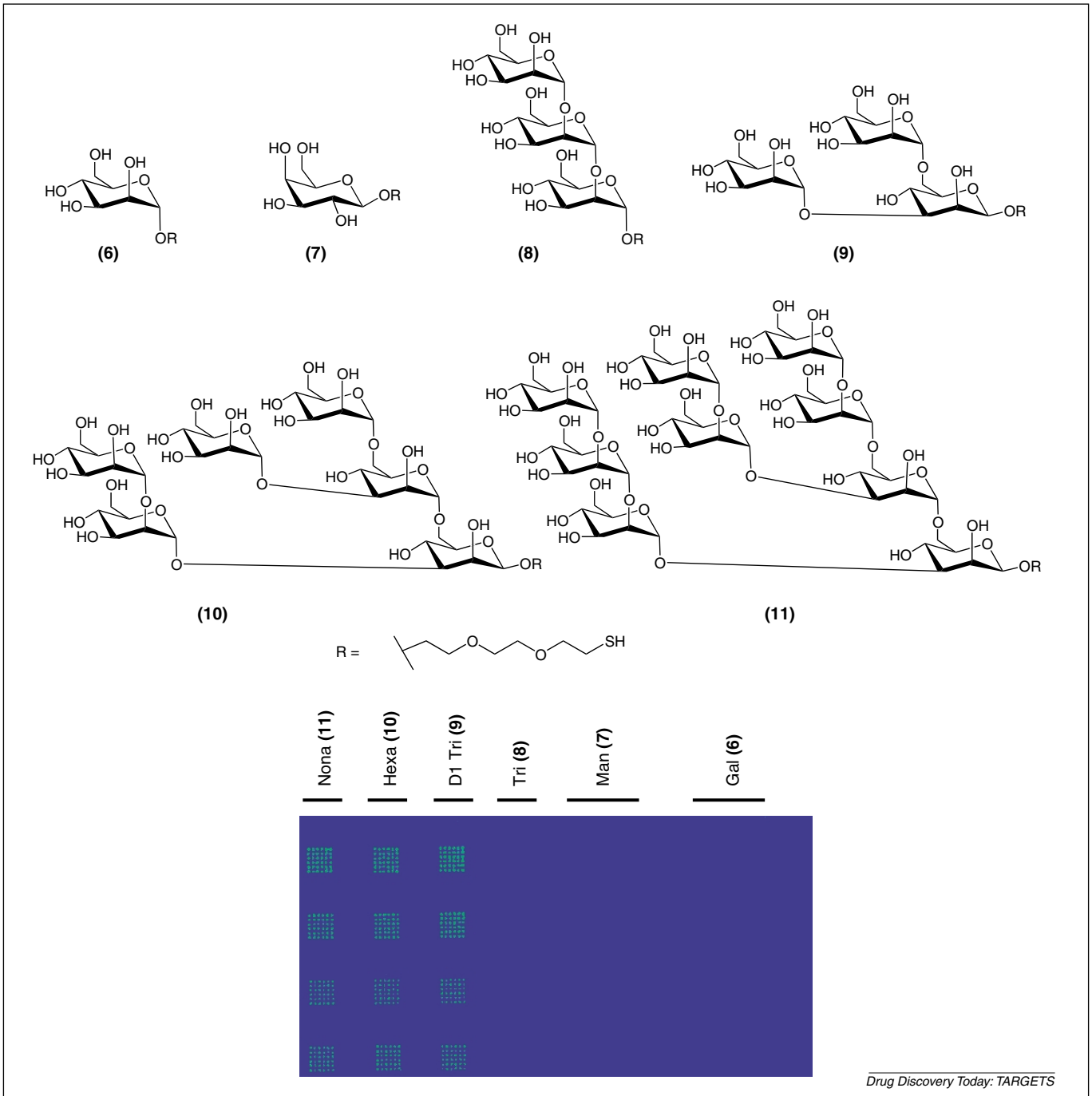

Figure 2. A carbohydrate microarray. This array was designed to screen for interactions between carbohydrates and CVN by using carbohydrates (**6–11**) and the corresponding immobilization chemistry shown in Figure 1 (reaction step 5). Compounds were spotted on glass surfaces by using standard robotic and scanning equipment that is used for DNA gene chips. A sample slide for probing the carbohydrate-binding specificities of CVN is shown. Each carbohydrate is spotted with a diameter of approximately 100–200 μm. Thus, several thousand different carbohydrates can be placed on a single slide and screened in parallel for protein binding. Data are from Ratner *et al.* [33]. Abbreviations: CVN, cyanovirin N; D1 tri, D1 trisaccharide; Gal, galactose; Hexa, hexasaccharide; Man, mannose; Nona, nonasaccharide; Tri, trisaccharide.

film, and the adsorption coefficients for proteins binding to the carbohydrate-modified surfaces and the solution equilibrium constants of the protein–carbohydrate interactions were measured. The results further show that mannose binds to ConA much more tightly than it binds to the galactose-specific lectin jacalin [40].

SPR experiments have been also carried out by Ratner *et al.* [33], who investigated interactions between a series

of high mannose structures and CVN (Figure 2). Carbohydrates were immobilized on self-assembled gold monolayers, which provide the advantage that the density of carbohydrates displayed on these monolayers can be controlled and thus used to present carbohydrates in a homogeneous environment. Ratner *et al.* [33] used this SPR approach to investigate binding of CVN to the mannosides, as well as the inhibition of this binding event [33].

Expanding the scope of SPR investigations to arrays of several hundred saccharides would provide an extremely powerful technique in glycomics, because structure–activity relationships could be assessed quickly. Such results might facilitate the discovery of inhibitors of carbohydrate–lectin interactions and would serve as a basis on which to improve the design of new inhibitors.

A comparison of different HTS platforms

A discussion of the features of different HTS platforms is important for the advancement of glycomics and will facilitate the development of a 'gold standard' screening technique. All non-covalent immobilization strategies have a disadvantage in that the buffers and washing conditions must be carefully chosen so that the carbohydrates remain bound to the surface. The buffers are limited to those that do not include detergents or non-aqueous solvents, which are often a component of enzyme buffers [24]. However, this limitation does not apply to covalent immobilization techniques where the bonds formed between the carbohydrate and the slides are stable under various conditions [30–33].

The main advantage of the nitrocellulose immobilization technique used by Wang *et al.* [21] is that the carbohydrates are not modified before immobilization. The chief drawback is that only large compounds or ones that are conjugated to proteins, such as bovine serum albumin, are bound by this surface. However, small neoglycolipids (containing 2–20 monomers) can be immobilized on nitrocellulose [22]. Only oligosaccharides and saccharides in which the reducing end sugar is not involved in binding can be studied by this approach [23].

Immobilization on microtiter plates requires a lipid that must be added by synthetic methods [24–26]. The advantage of this method is that each well can contain a different carbohydrate and, by using ELISA techniques, enzymatic reactions can be colorimetrically monitored and inhibitors of the reactions discovered directly. This process contrasts with methods that use glass slides, in which lead inhibitors are often identified by their tight binding to the target. However, binding data obtained from arrays can lead to red herrings because potential inhibitors could bind tightly to a position on the protein that does not affect catalysis.

The disadvantage of the microtiter approach relative to methods that use arraying robotics is the large quantities of carbohydrate that are placed in each well (m moles) [26]. Because the amount of material required is greater than what can be typically obtained from natural sources, this approach is limited to chemically synthesized carbohydrates. Arrays constructed on glass slides use significantly less material, and a single spot suitable for detecting binding can contain as little as picomoles of carbohydrate [31,33,41,42]. Thus, the sensitive approach of arraying carbohydrates on glass slides by a robot can be used even when carbohydrates are in short supply or have been isolated from natural sources. The number of parallel screens performed by a microtiter assay is limited by the number of wells on a single plate, which is typically 96. Arrays can be constructed such that the density of different carbohydrates on a single slide is greater than 10,000 [31]. Furthermore, construction and analysis of the arrays can be completed by using standard equipment that is commonly available for DNA arrays at most institutions.

Applications of carbohydrate arrays in medical science

A major application of the tools described above is to the discovery of compounds that have potential for treating disease. However, thus far, most of the work has used model systems to investigate lectin–carbohydrate interactions. Below we describe the relatively few examples of these techniques that have applications in medicine, but once these technologies become a routine part of our tool kit they will facilitate even more exploration of medicinal relevance.

Aminoglycoside antibiotic arrays have been constructed to study the interactions of antibiotics with therapeutic targets and enzymes associated with antibiotic resistance [41,42]. There is an urgent need for the discovery of new antibiotics owing to increased antibiotic resistance; furthermore, there are few screening techniques for evaluating interactions between therapeutic targets and enzymes encoding antibiotic resistance in parallel. Aminoglycoside antibiotics have been non-specifically immobilized on slides containing succinimide esters by immobilization via amino groups that are present on these antibiotics [41,42]. With these arrays, binding to both the target RNA, which is an oligonucleotide mimic of the binding site for the aminoglycoside antibiotic in the ribosome, and to the enzymes encoding antibiotic resistance can be achieved with minimal material (picomoles). Aminoglycoside derivatives that have their amino groups replaced with guanidino groups or guanidinoglycosides have been discovered that inhibit resistance enzymes and are not substrates for these

enzymes. By using these screens for testing combinatorial libraries, the discovery of improved antibiotics is likely to be accelerated.

Carbohydrate arrays have been also used to characterize the carbohydrates that specifically bind to HIV-neutralizing proteins [33]. The antibody 2G12 has been shown to be effective at neutralizing the virus, although the particular carbohydrate structures that this protein binds to are not specifically known [43,44]. An in-depth investigation of the binding of this protein to mannosides has been undertaken with high mannose arrays [45]. The results show that the structure of the oligomannoses affects binding and that mannosides that resemble the nonasaccharide most closely show the strongest binding. Information from these studies will lay the foundation for development of carbohydrate-based HIV vaccines.

Future directions

Historically, the study of carbohydrates has been challenging because their isolation from natural sources yields only a little material that is often heterogeneous. Recently, new methods have been developed for the chemical synthesis of defined carbohydrates [17,19]. However, at present, only experts use these routes and not all oligosaccharides are accessible. To facilitate discoveries in the emerging field of glycomics, sensitive screening techniques for probing carbohydrate–protein interactions have been developed, ranging from the non-covalent immobilization of carbohydrates on nitrocellulose or polystyrene microtiter plates, to the covalent immobilization of carbohydrates on encoded microspheres, gold surfaces or glass slides.

We favor an approach in which compounds are covalently attached to glass slides because minimal material is used (both carbohydrate and protein) and the construction and analysis of slides can be achieved with standard DNA gene chip instrumentation. Because the complexity of carbohydrates far exceeds that of proteins or nucleic acids, it is probable that these tools will aid the discovery of a diverse set of roles for carbohydrates in cell biology that were previously unimaginable. Information from these glycomics studies can rival or even exceed the information that has been recently uncovered in the field of genomics.

Acknowledgements

This work was supported by the Roche Research Foundation [http://www.research-foundation.org (postdoctoral fellowship to Matthew D. Disney)], GlaxoSmithKline [http://www.gsk.com (scholar award to Peter H. Seeberger)], the Alfred P. Sloan Foundation [http://www.sloan.org (fellowship to Peter H. Seeberger)] and Merck [http://www.merck.com/ (academic development award to Peter H. Seeberger)].

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