Tools for Glycomics: Mapping Interactions of Carbohydrates in Biological Systems

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The emerging field of glycomics has been challenged by difficulties associated with studying complex carbohydrates and glycoconjugates. Advances in the development of synthetic tools for glycobiology are poised to overcome some of these challenges and accelerate progress towards our understanding of the roles of carbohydrates in biology. Carbohydrate microarrays, fluorescent neoglycoconjugate probes, and aminoglycoside antibiotic microarrays are among the many new tools becoming available to glycobiologists.

1. Introduction

Progress in genomic and proteomic research has elevated these fields to the forefront of scientific and biomedical research. These scientific endeavors have been facilitated by the myriad of modern laboratory techniques at the disposal of today's researcher. Automated synthesis of nucleic acids and peptides, rapid DNA and peptide sequencing, gene expression profile analysis by using cDNA microarrays, protein-expression systems, siRNA (small interfering RNA) gene silencing, and knockout organisms are widely used to elucidate the role of genes and proteins in biological systems. Until now, a complementary set of biophysical tools has remained out of reach to the growing discipline of glycomics and this void has greatly hindered the emergence of this field.

Analogous to proteomics and genomics, glycomics explores the role of carbohydrates in biological processes.[1] This includes carbohydrate–carbohydrate, carbohydrate–protein, and carbohydrate–nucleic acid interactions (see Figure 1). Carbohydrates, in the form of glycopeptides, glycolipids, glycosaminoglycans, proteoglyans, or other glycoconjugates have long been known to participate in a plethora of biological processes. These include viral entry,[2] signal transduction,[3] inflammation,[4] cell–cell interactions,[5] bacteria–host interactions,[6] fertility, and development.[7,8] Rapid advances in the field of glycomics, however, have been hindered by the complexity of the biomolecules involved. Due to their frequent branching and linkage diversity, oligosaccharides have greater structural complexity than nucleic acids and proteins.[9] Furthermore, the difficulty in isolating, characterizing, and synthesizing complex oligosaccharides has been a significant challenge to progress in the field.

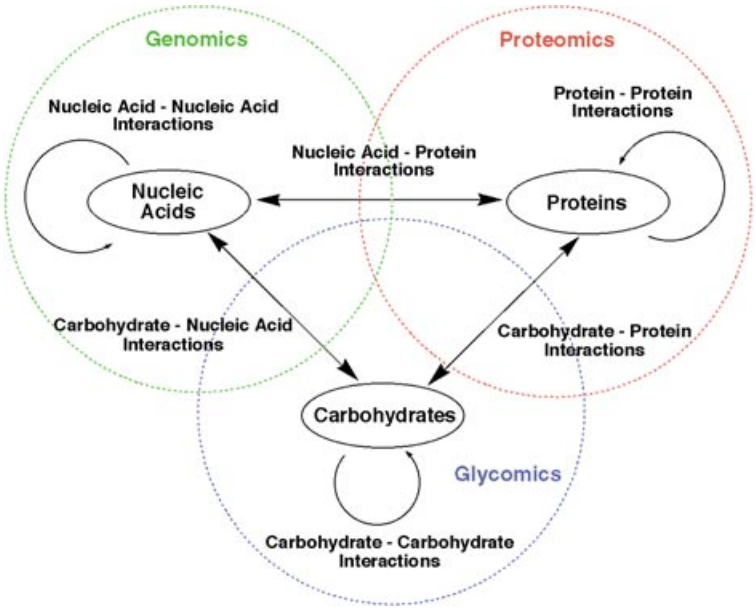

Figure 1. *Biopolymer interactions. (Reproduced from ref. [1c] with permission of the Royal Society of Chemistry.)*

Recent chemical advances, such as improved synthetic methods, including the development of an automated solid-phase synthesizer,[10] and methods for enzymatic synthesis,[11] have opened new and exciting possibilities in obtaining pure, chemically defined carbohydrates. At the same time, the field has seen growing interest in the development of carbohydrate microarrays,[12] and neoglycoconjugates[13] to facilitate otherwise laborious biological studies. By unifying synthetic advan-

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ces and new biochemical tools, it is now possible to expand the tool-chest available to the glycomics researcher. This Concepts paper illustrates the potential of some of these emerging technologies.

At present a number of synthetically derived carbohydrate tools are becoming available to glycobiologists. These tools include: monovalent fluorescent conjugates, neoglycoproteins, multivalent quantum-dot conjugates, affinity-tagged saccharides, derivatized magnetic particle and latex microspheres, sepharose affinity resins, carbohydrate microarrays, and surface-plasmon resonance to probe carbohydrate–protein interactions (Figure 2).

This variety of glycoconjugate tools is made possible by a number of viable linking chemistries that involve the reducing end of carbohydrates. Amine-containing linkers permit conjugation with amine-reactive substrates, including activated esters. In addition, carboxy-terminated reducing-end chemistries are amenable to coupling to amine-presenting molecules. Maleimide-derivatized linkers permit conjugation to thiol-presenting structures. Thiol-presenting linkers may be coupled to maleimide- and iodoacetyl-containing structures.

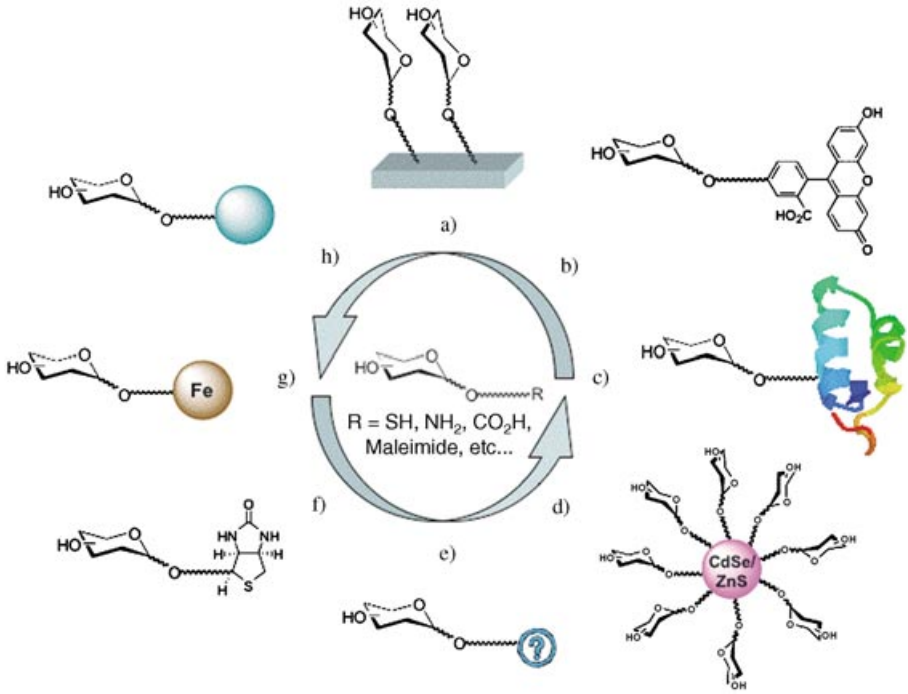
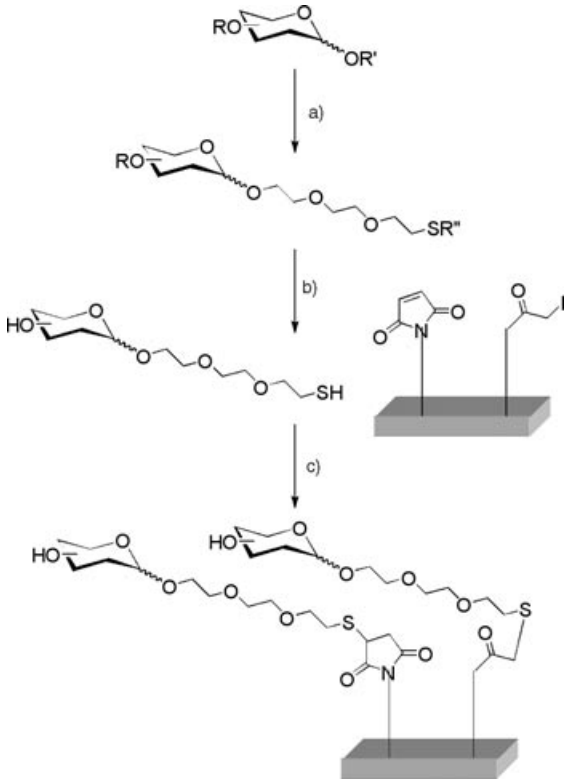


Figure 2. *Tools for glycobiology: a) modified surfaces for microarrays and SPR, b) monovalent fluorescent conjugates, c) neoglycoproteins and carbohydrate vaccines, d) multivalent quantum dot conjugates, e) future neoglycoconjugates, f) affinity tag (biotin, etc.) conjugates, g) magnetic particle conjugates, h) latex microsphere and sepharose affinity resin conjugates.*

Our laboratory has developed a single linking chemistry for the purposes of streamlining the development process of new tools in glycobiology. A 2-(2-(2-mercaptoethoxy)ethoxy)ethanol linker was selected for the preparation of neoglycoconjugates.[14] This linker chemistry was selected based on its compatibility with existing synthetic methods, the ease of temporarily masking the thiol functionality with a protecting group, and the reliability of thiol-based conjugation chemistries—in particular, thiol–maleimide and thiol–iodoacetyl couplings (Scheme 1). The orthogonal reactivity of a terminal thiol to the functional groups presented by carbohydrates allows for defined covalent immobilization of oligosaccharides to a functionalized surface, creating a cell-surface-like environment on the chip.[15]



Scheme 1. *2-(2-(2-mercaptoethoxy)ethoxy)ethanol as a linker for preparing neoglycoconjugates. a) Linker synthetically incorporated into reducing end of mono or oligosaccharide. b) All protecting groups removed from carbohydrate and thiol. c) Reduced thiol coupled to maleimide or iodoacetyl-functionalized structure.*

2. Carbohydrate Microarrays

There is great interest in developing microarray-based methods for probing the roles of nucleic acids, proteins, and carbohydrates in biology. The chip-based format offers many advantages over conventional methods. These include the ability to screen several thousand binding events in parallel and the fact that a minimal amount of analyte and ligand are required for study—making the most of precious synthetic or naturally procured materials. Many methods for preparing carbohydrate microarrays have been described to date: nitrocellulose-coated

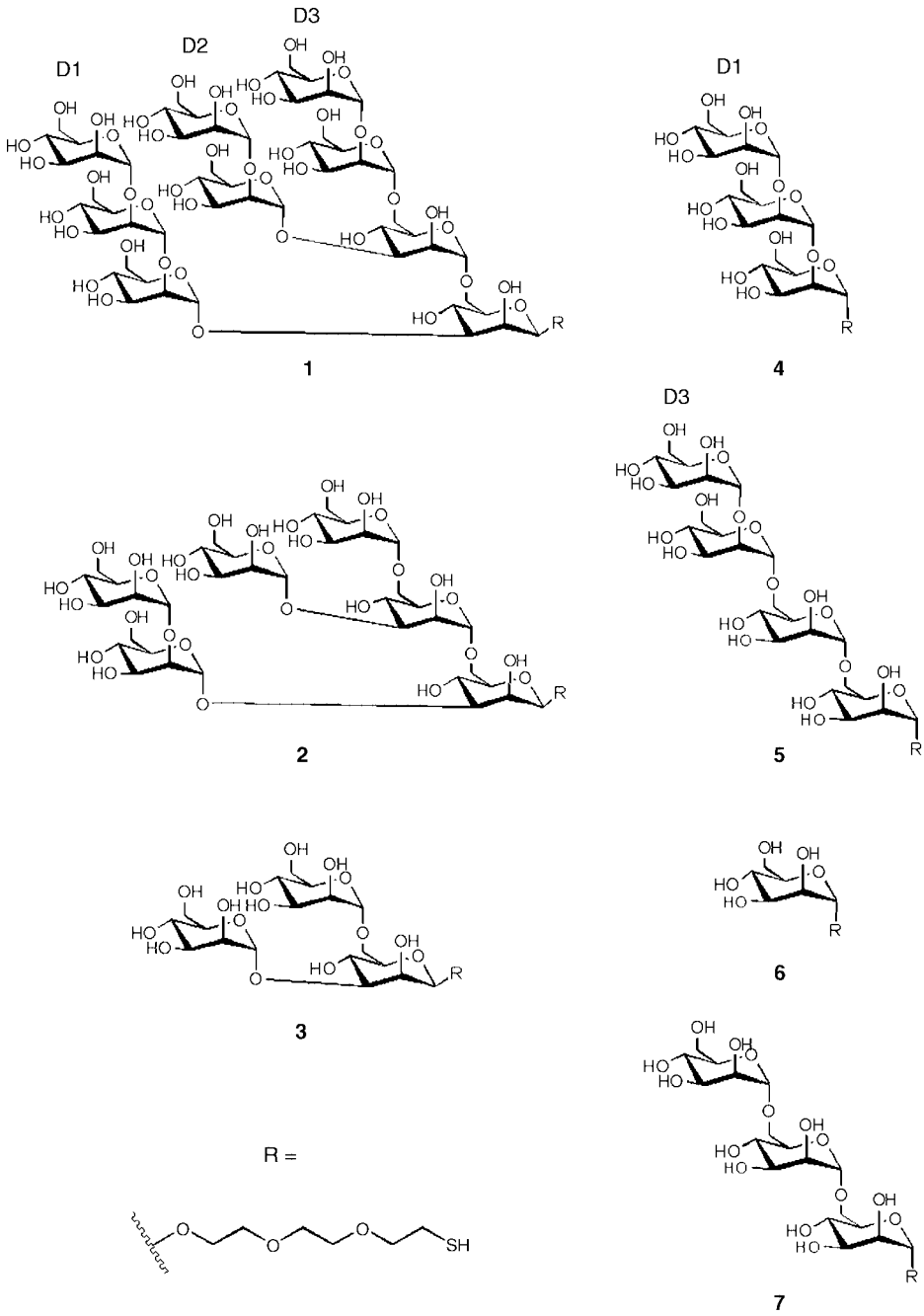
slides for noncovalent immobilization of microbial polysaccharides and neoglycolipid-modified oligosaccharides;[16] polystyrene microtiter plates for presenting lipid-bearing carbohydrates;[17] self-assembled monolayers modified by Diels–Alder-mediated coupling of cyclopentadiene-derivatized saccharides;[18] thiol-derivatized glass slides modified with maleimide-functionalized oligosaccharides;[19] and thiol-functionalized carbohydrates immobilized on maleimide-derived gold and glass slides.[14,20]

We adopted two surface chemistries for the preparation of our carbohydrate microarrays. Both involve maleimide functionalization of glass slides to form a stable bond between the slides and thiol-containing synthetic oligosaccharides. In one case, BSA-derivatized aldehyde glass slides were functionalized with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) to present a maleimide reactive surface.[14] Alternatively, amine-derivatized Corning GAPS II slides were directly modified with SMCC prior to incubation with thiol-presenting saccharides.[21] Microarrays were printed at high density by using standard DNA microarray robotic printers. These two methods of surface functionalization offer different advantages. BSA-derivatized slides present a relatively low density of immobilized oligosaccharide and excellent resistance to nonspecific binding of proteins to the surface. The GAPS II slides permit high-density immobilization of oligosaccharides, permitting examination of carbohydrate clusters at the surface, and present the carbohydrate in a peptide-free context.

These immobilization chemistries were developed, in part, to address the limitations inherent in existing methods for preparing carbohydrate microarrays. For instance, the microtiter method requires relatively large quantities of oligosaccharide and does not offer the same degree of high throughput available to robotically printed glass microarrays. In addition, the reliance upon noncovalent, hydrophobic interactions to anchor carbohydrates to the microtiter wells places considerable limitations on the stringency of washes one may employ; the use of detergents to reduce nonspecific interactions invariably leads to loss of carbohydrate from the microtiter wells. Nitrocellulose-based immobilization is limited to large polysaccharides or lipid-modified sugars. More sophisticated synthetic methods for immobilization have limited applications in the preparation of large oligosaccharides due to the sensitivity of the complex chemistries.

2.1 High-mannose microarrays

To establish the viability of the carbohydrate microarray, a panel of mannose-containing oligosaccharides was prepared (Scheme 2). These structures were selected based on their rele-



Scheme 2. *Synthetic substructures of the triantennary N-linked mannoside, including thiol-containing linker for immobilization and conjugation chemistry. Reprinted from ref. [21] with permission from Elsevier.*

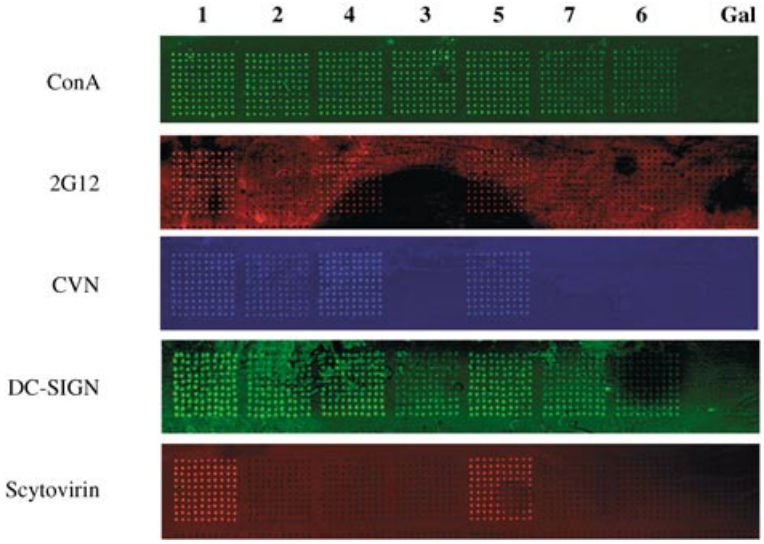


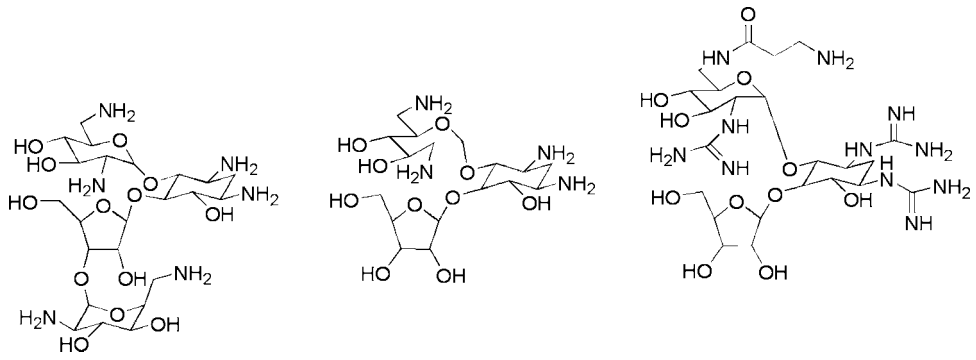
Figure 3. *Carbohydrate microarrays containing synthetic mannans **1**–**7** and galactose, printed at 2 mM. False-color image of incubations with fluorescently labeled ConA, 2G12, CVN, DC-SIGN, and Scytovirin.*[21]

vance to the glycans found decorating viral-surface envelope glycoproteins of HIV. Specifically, the arrays are composed of a series of closely related structural determinants of $(Man)_9$-$(GlcNAc)_2$. By using these arrays, precise profiles of the carbohydrate-binding capacity of a series of gp120 binding proteins (DC-SIGN, 2G12, Cyanovirin-N, and Scytovirin) was determined (Figure 3).

By presenting the various structural determinants of an important glycan on a single array, multiple proteins can be screened to determine their binding profiles. Figure 4 illustrates the carbohydrate-binding profiles of two potent HIV-inactivating proteins isolated from cyanobacterium, Cyanovirin-N (CVN)[22] and Scytovirin.[23] The results clearly illustrate that these two proteins recognize different structural motifs within the high-mannose series of structures arrayed. The ability to obtain this result in a single experiment saves a significant amount of time compared with conventional methods.

2.2 Antibiotic microarrays

Aminoglycosides are carbohydrate antibiotics that contain amino sugars and are composed of two to five monomers (Scheme 3). Clinically, these compounds are used as broad-spectrum antibiotics against a variety of therapeutically important bacteria. Aminoglycosides exhibit their antibacterial effect by binding bacterial ribosomes and inhibiting protein synthesis. The most common binding site for this class of drugs is the A-site in the small ribosomal subunit, or 30S, portion of the bacterial ribosome. The therapeutic efficacy of aminoglycosides, however, has decreased recently due to antibiotic resistance. Resistance to aminoglycosides can be acquired either through the transfer of plasmid DNA or from over expression of endogenous enzymes. Several mechanisms cause resistance



Scheme 3. *Examples of aminoglycoside antibiotics and derivatives thereof. Left, the aminoglycoside neomycin; middle, ribostamycin; right, a guanidinylated derivative of ribostamycin that was found to inhibit AAC(2′)- and AAC(6′)-catalyzed acylation of several clinically important antibiotics.*[31]

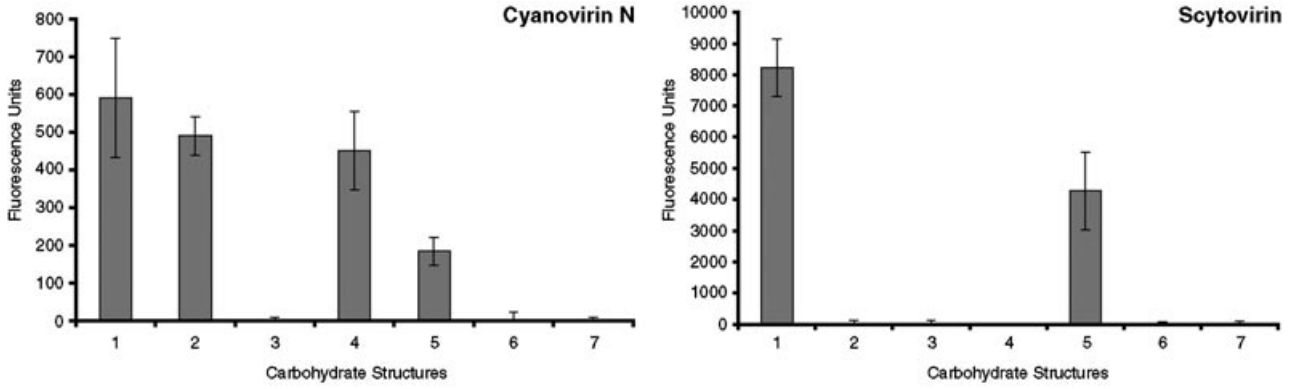


Figure 4. *Comparison of the binding profiles of fluorescently labeled Cyanovirin-N and Scytovirin, incubated with synthetic mannans **1**–**7**. Reprinted from ref. [21] with permission from Elsevier.*

including decreased uptake into cells, mutation of the target, binding to proteins, and covalent modification of the drug by enzymes.[24] Enzymatic modification is the most common aminoglycoside-resistance mechanism. The result of aminoglycoside modification is a large decrease in binding affinity to the therapeutic target.[25] In recent years, the incidence of resistant bacteria has increased. In order to combat the growing threat that bacteria pose to human safety, new antibiotics must be identified. To facilitate the discovery of such compounds, high-throughput methods to identify compounds that weakly bind to resistance- and toxicity-causing proteins and strongly bind to therapeutic targets have been developed by using the microarray techniques described herein.

2.2.1 Antibiotic microarrays to interrogate interactions to therapeutic targets and resistance-causing enzymes: Aminoglycoside microarrays were constructed by random covalent immobilization of the antibiotics onto amine-reactive glass slides by using a DNA arraying robot. This approach provides a versatile platform for probing the interactions of these compounds with a variety of targets. Arrays were probed with an RNA mimic of the bacterial and human A-sites (Figure 5).[26] These two different RNA sequences were used to establish this microarray method as a screen not only for tight binding to RNA but also specific binding. Results from these studies showed that the antibiotic amikacin binds the tightest to both the bacterial and the human A-site mimics. These results do not exactly correlate with in-solution measurements of aminoglycoside binding affinity due to the nonspecific immobilization of the compounds. Arrays were also incubated with two types of acetyltransferase (AAC) resistance enzymes, a AAC(6′) from *Salmonella enterica* and a AAC(2′) from *Mycobacterium tuberculosis*.[27,28] Binding of these enzymes to the aminoglycosides correlated well with a previous calorimetric study of binding affinity.

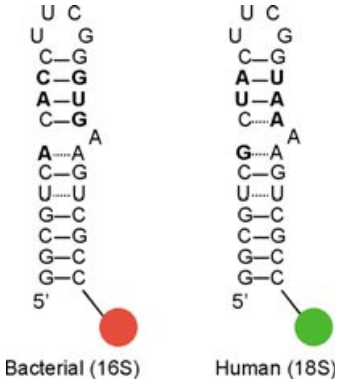


Figure 5. *The oligonucleotide mimics of rRNA A-sites that were incubated with the aminoglycoside arrays. The bacterial oligonucleotide has been shown to be the binding site for some aminoglycosides in the ribosome. The human oligonucleotide has been tested for aminoglycoside binding by using MS experiments. Each oligonucleotide was fluorescently labeled; the bacterial RNA is labeled with TAMARA and the human with fluorescein.*

2.2.2 Antibiotic microarrays to facilitate discovery of inhibitors of resistance-causing enzymes: A library of aminoglycoside mimetics was arrayed onto glass slides in order to find inhibitors of resistance. Guanidinoglycosides were chosen because: 1) they are easily synthesized from aminoglycosides;[29] 2) the increased charge that guanidinoglycosides have relative to aminoglycosides might allow for tighter binding to the negatively charged aminoglycoside binding pocket in these enzymes;[30] 3) the difference in pK_a between a guanidinino group and an amino group (12.5 versus 8.8, respectively) suggests that the guanidinoglycosides may not be substrates for these enzymes. Screening this library revealed that each of the guanidinoglycosides exhibited higher affinity for the resistance enzymes than the corresponding aminoglycosides. Guanidinoglycosides were tested by using a spectrometric assay for their ability to serve as substrates for AAC(2′) and AAC(6′).[27,28] The results demonstrate that guanidinoglycosides are not substrates and inhibit acylation of several clinically important aminoglycosides.[31] Information from these studies will allow the development of new antibiotics that evade resistance.

2.3 Hybrid carbohydrate/glycoprotein microarrays

A hybrid carbohydrate/glycoprotein microarray was developed to rapidly determine the contribution of protein–protein interactions in addition to carbohydrate–protein interactions in binding events. By arraying both the glycoprotein and the carbohydrate it displays, binding determinants can be rapidly identified. To develop these screens, a GAPS II glass slide was modified at the surface by using two chemistries: on one side maleimide and on the other an NHS-activated ester. The carbohydrates and glycoproteins were printed on the maleimide and NHS-activated ester sides of a single chip, respectively. Hybridization with a carbohydrate-binding protein established whether the peptide context is required for binding. Figure 6 shows two incubations that make use of a hybrid array. In the case of Cyanovirin-N, both free carbohydrate and gp120 are bound. In contrast to a crude plant extract known to contain a high-mannose binding protein, free carbohydrate is not bound in the absence of the glycoprotein; this strongly suggests that either protein–protein contacts are required for glycan recognition or that protein-conformation-dependent presentation of the high-mannose glycans influences recognition by this carbohydrate-binding protein.

2.4 Microsphere arrays to detect protein–carbohydrate interactions

In contrast to the microarray systems described above, a system developed in collaboration with the Walt laboratory uses optically addressable, internally encoded microspheres to define the position and structure of a series of carbohydrates.[32] While solid-phase carbohydrate libraries have been employed previously,[33] miniaturization of the assay, combined with fluorescently encoded microspheres, allows for rapid screening while requiring amounts of material comparable to or less than what is required by microarrays. To detect binding, the immobilized microsphere array is incubated with a fluorophore-labeled carbohydrate-binding protein. The binding profile is determined by measuring the fluorescence of beads that

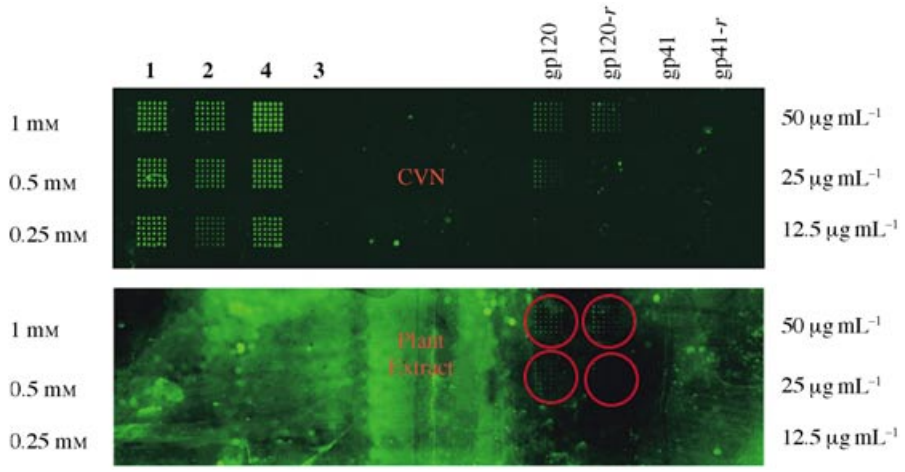


Figure 6. *Carbohydrate/glycoprotein hybrid microarrays containing synthetic mannans **1**–**4**, gp120, and gp41, incubated against fluorescently labeled CVN and biotinylated crude plant extract.*

emit at both the wavelength of an internal code, which is used as a marker for the carbohydrate displayed on a microsphere (an entrapped fluorescent dye), and the labeled protein. Fluorescence colocalization indicates a binding event. Using this system, we examined the binding profiles of Concanavalin A and Cyanovirin-N (Figure 7).

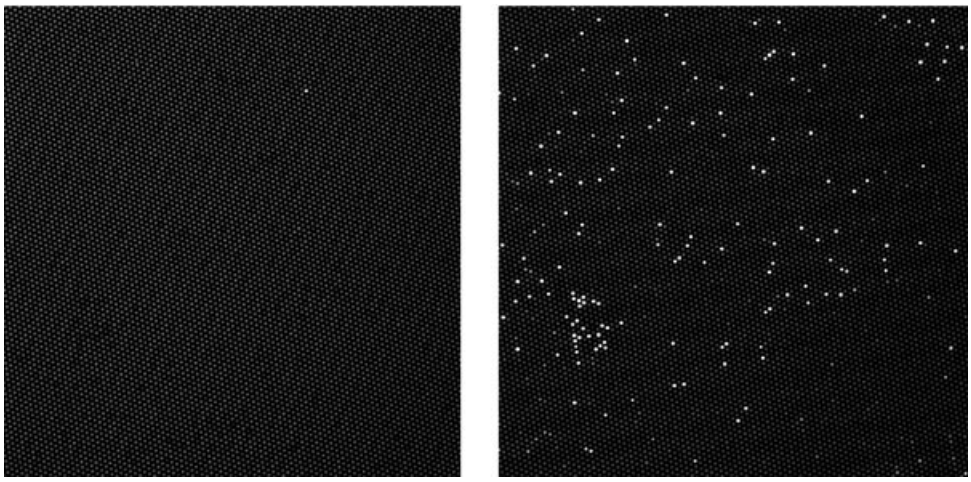


Figure 7. *Internally encoded, randomly oriented microsphere arrays bearing structures **1**–**4** and **6** (Scheme 2) and incubated with BODIPY-labeled Cyanovirin-N. Specific binding events were observed by detecting BODIPY emission at 520 nm. Left: Fluorescence at 520 nm prior to BODIPY-CVN incubation; right: fluorescence at 520 nm after BODIPY-CVN incubation. (Taken from ref. [32].)*

3. Surface Plasmon Resonance (SPR) to Study Protein–Carbohydrate Interactions

Immobilized carbohydrates are also used for SPR experiments to provide valuable insight into the binding of analytes to ligands in real time and to allow for both low- and high-affinity interactions to be measured.[20] In these experiments, a solution containing an analyte is washed over a surface. Binding is measured by the change in the refractive index of the surface upon accumulation of analytes. Since interactions are measured without the need for labeling, any influence of a label on the experimental results can be excluded.

SPR was completed by using self-assembled monolayers (SAMs), which offer extensive control over the density of immobilized carbohydrate at the surface. By controlling the ratio of homogenously displayed maleimide in the SAM, it is possible to determine the concentration of thiol-modified oligosaccharide immobilized on the surface. Utilizing these precisely characterized SAMs in concert with SPR, we established that complex synthetic carbohydrates can be used for detailed studies characterizing the activity of carbohydrate-binding proteins. To demonstrate the potential of such a system, monolayers of linear trimannoside **4** were used to explore the activity of CVN (Figure 8).[14] This study illustrates how the density of immobilized saccharide affects the amount of bound lectin. Additionally, the platform was used for testing inhibitors of CVN binding of **4**.

4. Fluorescent Carbohydrate Conjugates as Probes for Cell Biology

While the microarray format yields a plethora of information regarding protein–carbohydrate interactions, such arrays may not be appropriate tools for studying cell-surface receptors with presumed carbohydrate-binding activity (i.e., lectins). One limitation of the arrays is the requirement for purified receptor. Furthermore, due to the high density of immobilized oligosaccharide, observation of binding to the surface is restricted to clustered or multivalent arrays of carbohydrate. While it may be possible to immobilize carbohydrate at densities sufficiently low to approximate monovalent presentation of oligosaccharide, the microarray format is not ideal for examining monovalent protein–carbohydrate interactions.

To define the influence of oligosaccharide clustering on recognition by cell-surface lectins, we have generated monovalent and multivalent fluorescent probes for applications in cell biology. These probes serve as reporters to enable an investigator

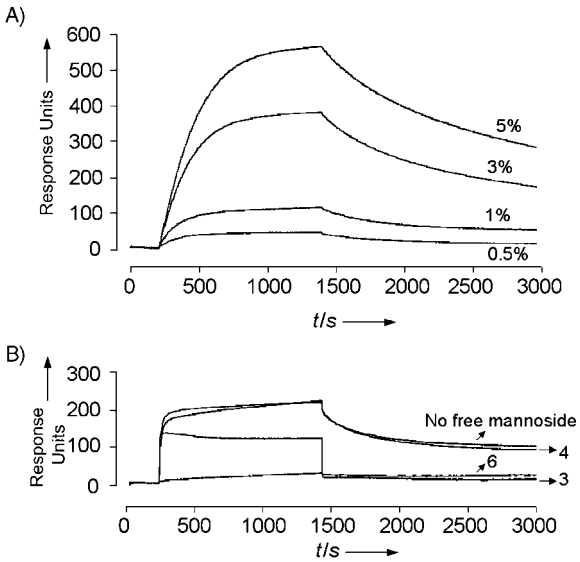


Figure 8. *A) SPR experiments that show the real-time binding of CVN to a self-assembled monolayer presenting linear trimannoside **4**. CVN (0.1 mM) in PBS buffer was applied to monolayers presenting the trimannoside at surface densities ranging from 0.5% to 5%. B) Soluble Man_9 **1** (0.02 mM), linear trimannoside **4** (0.2 mM), and branched trimannoside **3** (0.2 mM) were used to test for inhibition of association of CVN to the a monolayer presenting **3** at 1% density. (Taken from ref. [14].)*

to track receptor–carbohydrate interactions by fluorescence microscopy and flow cytometry.

4.1 Monovalent oligosaccharide–fluorophore constructs for receptor studies

Most lectins have an increased affinity for a carbohydrate ligand that is proportional to the valency of the interactions.[34] Traditionally, this phenomenon has been investigated by solid-phase assays that test the ability of carbohydrates to displace radioactively labeled lectin from binding to a high affinity ligand (e.g., 10^{-8} M).[35] Cell-based assays developed on the same principle of inhibitory concentrations have been employed as well. In these experiments, cells are incubated with fluorophore-labeled neoglycoproteins, as the high-affinity ligand, then with potential unlabelled ligands, and the amount cell-associated fluorescence is measured by flow cytometry.

To establish a more direct method of detecting oligosaccharide–receptor interactions, we have generated monovalent oligosaccharide–fluorophore conjugates (Figure 9A). These conjugates can be used to observe the approximate affinity of a cell-surface lectin for the monomeric oligosaccharide in solution. For example, we have used a panel of high-mannose oligosaccharide–fluorescein conjugates to follow the concentration-dependent binding and endocytosis of complex mannans by the dendritic cell lectin DC-SIGN (E.W.A., *unpublished results*; Figure 9B) in DC-SIGN-transfected HeLa cells and monocyte-derived dendritic cells, which express a high level of endogenous DC-SIGN.

4.2 Multivalent oligosaccharide platforms for cell biology

To enable direct assessments of the effect of multivalency on oligosaccharide binding to cell-surface lectins, we explored the use of semiconductor nanocrystal (quantum dot)-based systems as platforms to present multiple oligosaccharide monomers (>100) on a single particle. Early nanoparticle-based multivalent platforms for evaluating multivalent oligosaccharide interactions were based on carbohydrate-modified gold nanoparticles.[36] The success of these nanoparticle studies led us to

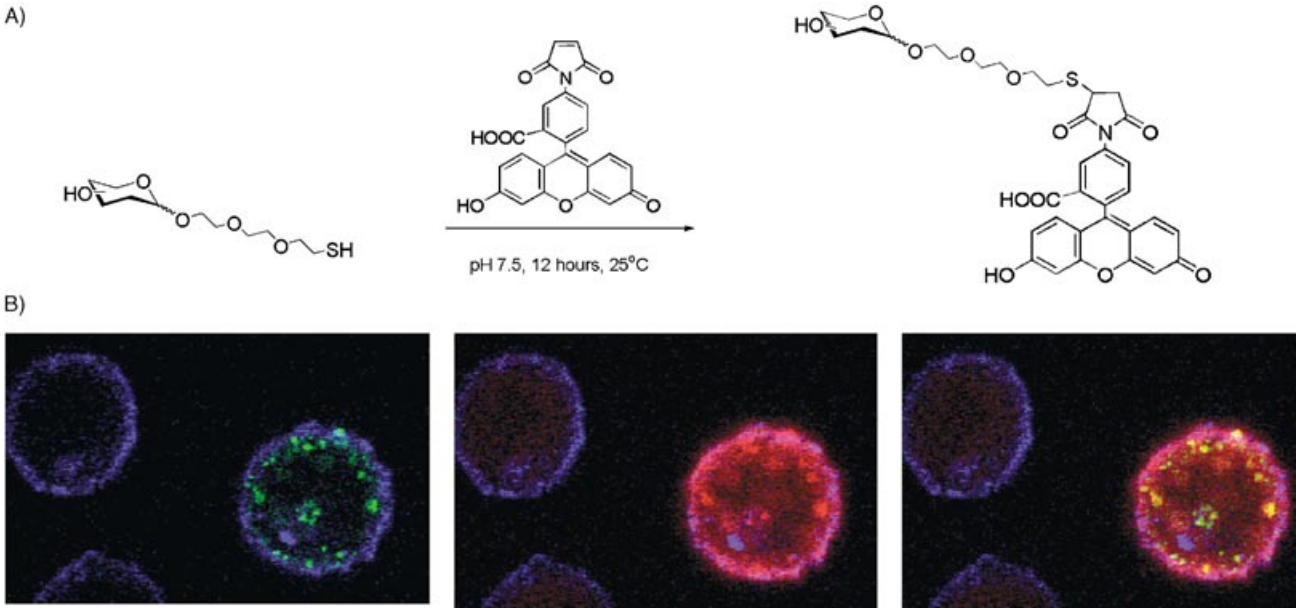


Figure 9. *DC-SIGN-mediated endocytosis of monovalent oligosaccharide–fluorescein conjugates. A) Maleimide conjugation chemistry employed to generate monovalent carbohydrate–fluorophore conjugates to study endocytosis. By using this simple, aqueous chemistry, carbohydrate–fluorescein conjugates were prepared for structures **1**–**4** and **6** (Scheme 2) and used in studies of DC-SIGN-mediated carbohydrate endocytosis. B) Transiently transfected HeLa cells expressing DC-SIGN endocytose **1**-fluorescein. Left: confocal microscopy image of **1**-fluorescein (green) and phalloidin staining (blue), middle: phycoerythrin-labeled anti-DC-SIGN antibody staining of DC-SIGN's subcellular localization, right: merge of the first two panels showing colocalization of internalized oligosaccharide and DC-SIGN.*

believe that quantum dots could enhance the utility of the nanoparticle platform. Given the high quantum yields in aqueous systems and unique photophysical properties (e.g., their lack of excitation-induced photobleaching, their extremely narrow, nonoverlapping emission spectra, and the ability to achieve multiple wavelength emissions following excitation from a single excitation source), quantum dot–carbohydrate conjugates will become a powerful tool in studying the cell biology of cell-surface lectins.[37]

We have developed a conjugation scheme using our linking chemistry to control the number of carbohydrates per quantum dot and methods to monitor the efficiency of conjugation (Scheme 4). By using quantum dots bearing different amounts of saccharide, we are trying to further elucidate carbohydrate recognition by DC-SIGN and other mammalian lectins.

5. Carbohydrate-Affinity Screening

Synthetic tools can facilitate the isolation and purification of carbohydrate-binding proteins from crude mixtures or biological extracts. Latex beads, magnetic particles, and agarose or sepharose resins modified to display a specific oligosaccharide can be used for affinity-based purification of carbohydrate binding partners. Investigators have traditionally employed monosaccharide-derivatized matrices to identify and isolate carbohydrate-binding proteins. While these matrices facilitate the isolation process, little information is gleaned regarding the true structural specificity of the isolated protein. Matrices displaying more complex oligosaccharides will enable the simultaneous isolation of carbohydrate-binding proteins and determination their structural specificity.

6. Outlook

Historically, the study of carbohydrates in biology has been a significant challenge. The isolation of carbohydrates and glycoconjugates from natural sources is tedious, frequently yields heterogeneous products, and produces little material. Based on advances in synthetic chemistry, sensitive screening techniques for probing carbohydrate–protein interactions are being developed to facilitate discoveries in the emerging field of glycomics. Currently, access to this expanding set of tools remains limited, primarily due to the specialization required for preparing the synthetic oligosaccharides. Developments like the automated solid-phase oligosaccharide synthesizer are likely to greatly expand access to these synthetically based advances. Until synthetic means are more widely available to the nonexpert, progress in the field is dependent on cross-discipline collaboration between glycobiologists and chemists with the synthetic capacity to generate structures of interest.

With these new tools at the disposal of the glycobiologist, it is likely that previously unimagined roles for carbohydrates in cell biology will be discovered. These stand to be exciting years ahead, as revealing these new roles for complex glycans will illuminate fundamental cellular processes. In conjunction with genetic methods, biophysical tools of the kind described in this Concepts paper will aid the growth of glycomics into a mature field, equal to genomics and proteomics.

Acknowledgements

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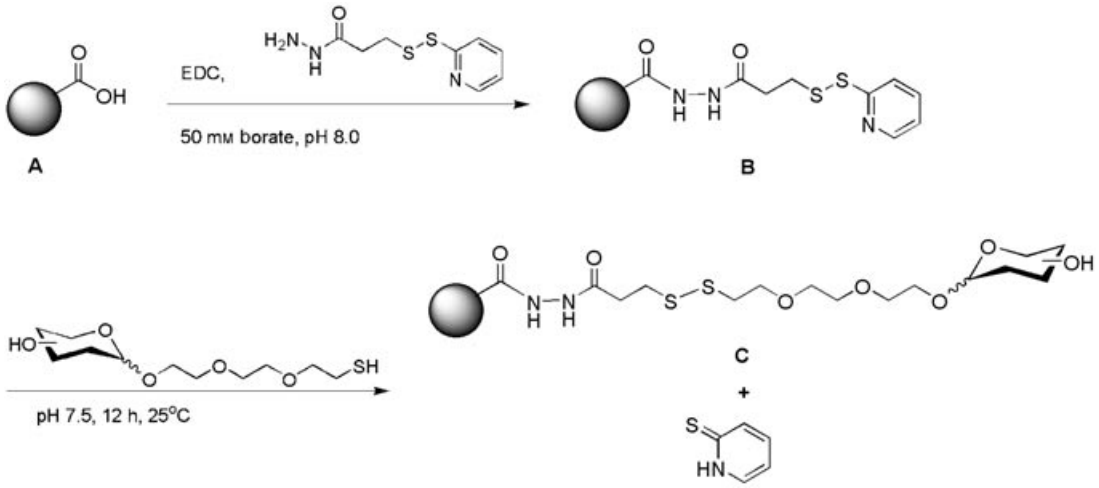

Scheme 4. *Modification of carboxylate-functionalized semiconductor quantum dots enables facile conjugation of thiol-bearing saccharides. The COOH-functionalized quantum dots (**A**) are treated in buffered solution with water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in the presence of 3-(2-pyridyldithio)propionyl hydrazide (PDPH) to generate a thiol-reactive nanoparticle (**B**). Coincubation of **B** with thiol-functionalized saccharides results in disulfide exchange between the quantum dot-anchored PDPH and the solution-phase saccharide to yield carbohydrate–quantum dot conjugates (**C**) and liberated pyridine-2-thione. After purification of the carbohydrate–quantum dot conjugates away from liberated pyridine-2-thione, one can determine the amount of pyridine-2-thione in solution spectrophotometrically (UV/Vis at 343 nm) and thereby determine the number of saccharides per particle.*

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