Chapter 9
Photo-Generation of Carbohydrate Microarrays

Gregory T. Carroll, Denong Wang, Nicholas J. Turro, and Jeffrey T. Koberstein

Abstract The unparalleled structural diversity of carbohydrates among biological molecules has been recognized for decades. Recent studies have highlighted carbohydrate signaling roles in many important biological processes, such as fertilization, embryonic development, cell differentiation and cell–cell communication, blood coagulation, inflammation, chemotaxis, as well as host recognition and immune responses to microbial pathogens. In this chapter, we summarize recent progress in the establishment of carbohydrate-based microarrays and the application of these technologies in exploring the biological information content in carbohydrates. A newly established photochemical platform of carbohydrate microarrays serves as a model for a focused discussion.

9.1 Introduction

The human genome project has shown that about 30,000 genes are available for constructing the human proteome. However, the number of genes revealed by whole genome sequencing does not set the upper limit of the repertoire of proteins. Protein posttranslational modifications, especially glycsylation, further diversify the available repertoire of functional proteins in a living organism. Protein glycosylation results in attachment of carbohydrate moieties at certain sites of a newly synthesized protein by either *N*-glycosylation or *O*-glycosylation. About 50% of the proteins in eukaryotes

G.T. Carroll
Department of Chemistry, Columbia University, 3000 Broadway, MC 3157, 10027, New York, NY

D. Wang (✉)
Stanford Tumor Glycome Laboratory, Department of Genetics, Stanford University School of Medicine, Beckman Center B006, 94305, Stanford, CA
e-mail: dwang1@stanford.edu

N.J. Turro and J.T. Koberstein
Department of Chemical Engineering, Columbia University, 500 West 120th Street, 10027, New York, NY 10027

K. Dill et al. (eds.), *Microarrays: Preparation, Microfluidics, Detection Methods, and Biological Applications*,

are found to be glycosylated [1]. Virtually all mammalian cells and micro-organisms are decorated with characteristic carbohydrate moieties. Exploring the biological information content in carbohydrates represents one of the current focuses of postgenomic research and technology development.

Carbohydrates are structurally suitable for the generation of diversity. Analogous to nucleotides and amino acids, monosaccharides are the building blocks of sugar chains. As in DNA and proteins, a carbohydrate's structural diversity comes from the composition of its repeat units and the sequence of these units. In mammals, there are nine common monosaccharide repeat units. A unique source of diversity to carbohydrates is the possibility to form branched structures because linkages can occur at multiple locations on a monosaccharide. As discussed below, the location of the linkage can be an important parameter in molecular recognition. For example, the flu virus recognizes the same monosaccharide units in both humans and birds; however, strains specific for birds recognize a linkage at a different location than those specific for humans (see below). In addition, the configuration at the anomeric position is another source of diversity.

Monosaccharides are linked together by forming glycosidic bonds between an anomeric OH on a monosaccharide and any OH (or other reactive functional group) on another monosaccharide. A pyranoside (six-membered ring monosaccharide) can bond to another pyranoside at five different positions with each linkage being in an α or β orientation. The potential to create differential linkages allows monosaccharides to form a more diverse array of oligomers in comparison to oligonucleotides and amino acids. For example, any fully hydroxylated pyranoside can form 11 possible disaccharides. A given amino acid can form only one dipeptide. A trimer of the nine common sugar residues found in mammals could potentially give rise to 119,736 different biological "expressions." A trimer of the 20 amino acids or four oligonucleotides can form only 8000 and 64 "expressions" in comparison. It should be noted that bacteria contain over 100 types of monosaccharides, often containing functional groups not found in mammalian monosaccharides, making the potential number of sugar structures found in nature well beyond the number found in mammals.

The "marriage" of carbohydrates with other biomolecules produces a large repertoire of carbohydrate-containing biomolecules with a variety of hybrid structures, called glycoconjugates. The two major classes of glycoconjugates, glycolipids and glycoproteins, are present in many living organisms. Each of these can be further subdivided. Glycoproteins bearing amino sugars, called glycosaminoglycans, are categorized as proteoglycans. Peptidoglycans are glycosaminoglycans cross-linked by peptides. Glycopeptides are oligosaccharides bound to oligopeptides and are either degradation products or are chemically synthesized. Glycosphingolipids are glycolipids in which an oligosaccharide is covalently attached to the lipid sphinganine. Lipopolysaccharides are glycolipids in which the sugar is a polymeric carbohydrate derivative.

Appending carbohydrates to lipids and proteins serves various roles. It allows carbohydrates to be anchored to the surface of biological membranes and can change the physical properties of the molecules to which they are conjugated. For example,

carbohydrates attached to glycoproteins in deep-sea fish are thought to disrupt the formation of ice crystals allowing the fish to survive at temperatures below 0° C.

In addition, glycoconjugation can endow a molecule with a saccharide molecular marker. For example, sialic acid residues found on an erythrocyte *N*-linked glycoprotein indicates whether the blood cell is "new" or "old" [2, 3] as depicted in Fig. 9.1a. If the glycoprotein loses its sialic acid residue, a galactose residue is exposed and recognized by a liver surface protein called the asialoglycoprotein receptor (*asialo* meaning "without sialic acid"). When enough galactose residues are exposed, multiple interactions bind the "old" erythrocyte to the liver. The "old" erythrocyte is then absorbed into the liver for degradation. However, the native

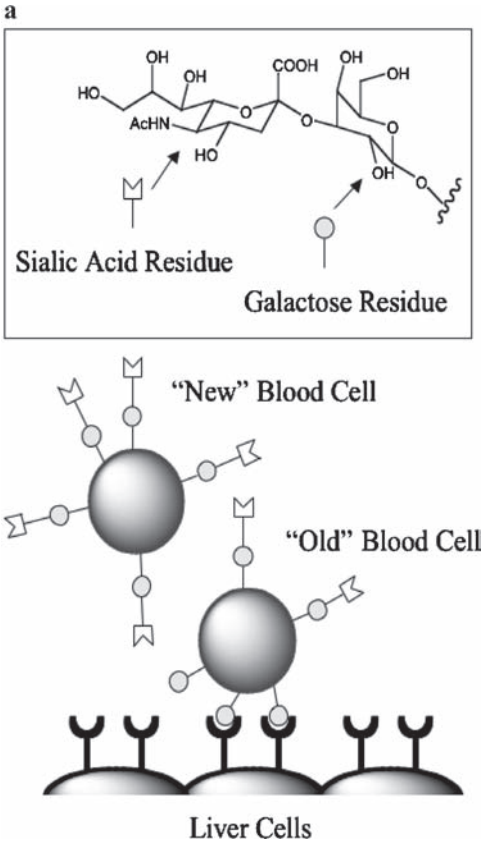

Fig. 9.1 Characteristic carbohydrate moieties serve as markers for biological recognition. (a) Sialic acid residues found on erythrocytes provide a molecular marker to indicate whether the cell is "new" or "old" [3]. Loss of sialic acid exposes a galactose residue that is recognized by the asialoglycoprotein receptor in mammalian liver [4]. When multiple galactose residues are exposed multivalent interactions result in a tight binding to the liver cells

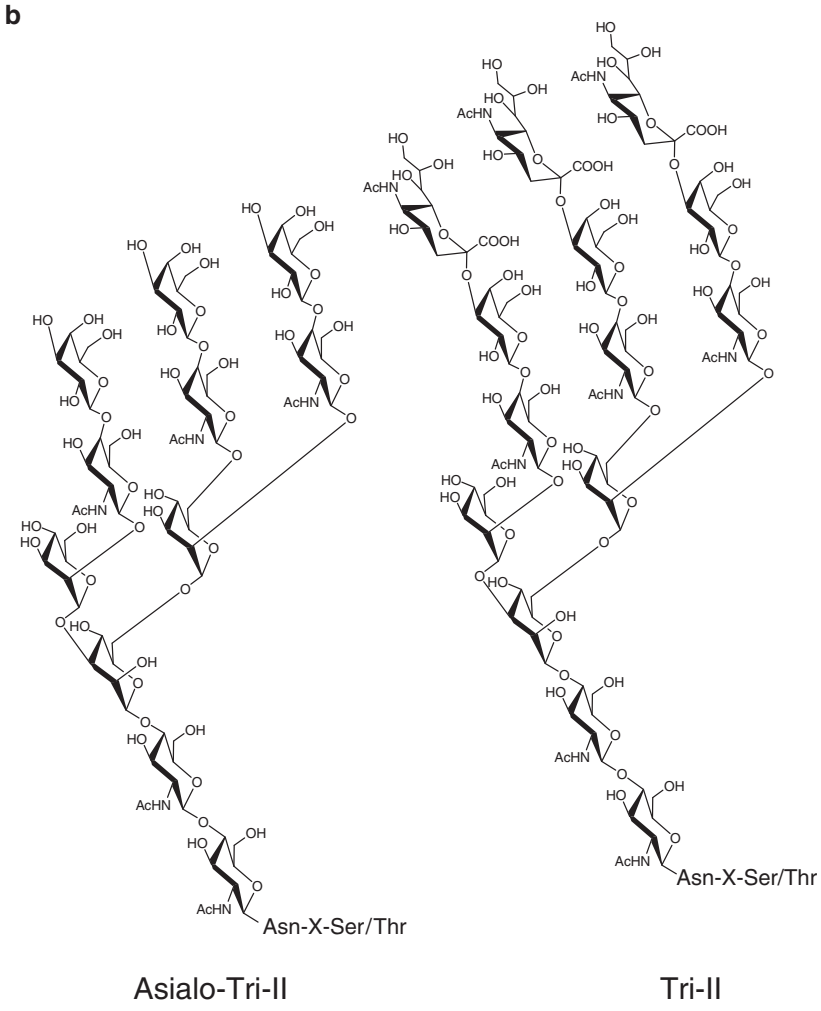

Fig. 9.1 (continued) (b) *N*-glycan type II chains [Galβ(1 → 4)GlcNAc] in the triantennary cluster configurations with (Tri-II) and without sialic acid terminal residues (asialo-Tri-II). The asialo-Tri-II sugar moieties but not the Tri-II structures are specifically targeted by a horse-neutralization antibody of SARS-CoV [5]

structures of cellular *N*-glycans are much more complex than the schematics in Fig. 9.1a. The sugar moieties recognized by the liver asialoglycoprotein receptor are in triantennary and multiantennary configurations without sialic acid caps [4]; Fig. 9.1b).

Recent studies have demonstrated that carbohydrate-mediated molecular recognition plays key roles in many important biological processes. These include cellular

events, such as fertilization [6, 7], embryonic development [8], and cell differentiation and cell–cell communication [9–11]. In addition, important molecular processes such as blood coagulation [12, 13], inflammation [3, 14], and chemotaxis [15], as well as host recognition and immune responses to microbial pathogens [16–18] are mediated or regulated by carbohydrate biosignals.

The carbohydrate-mediated processes mentioned above rely on the presence of appropriate biological molecules that are able to "decode" the biosignals of carbohydrate moieties. There are at least two classes of biological molecules in living organisms that have evolved to play such roles. They are known as anticarbohydrate antibodies produced by immune cells (B lymphocytes; [18]) and lectins of nonimmune origins [19].

The carbohydrate moieties that accommodate binding and are complementary to the combining-sites of lectins and/or antibodies are termed glycoepitopes. It was estimated that there are about 500 endogenous glycoepitopes in mammals [20]. However, this estimation did not consider the repertoires of the "hybrid" structures that are generated by protein posttranslational modification, including both *N*- and *O*-glycosylation. Furthermore, the conformational diversity of carbohydrates substantially increases the repertoire of carbohydrate-based antigenic determinants or glycoepitopes [21–23].

9.2 Carbohydrate Microarrays as Essential Tools in the Postgenomics Era

To meet the technical challenges posed by the structural diversity and functional complexity of carbohydrates, substantial efforts have been made to establish different platforms of carbohydrate microarrays [24–31]. The following examples demonstrate that these relatively nascent technologies have been utilized to explore the mysteries of life shrouded in the structure of carbohydrates.

A carbohydrate microarray printed on nitrocellulose-coated glass slides has been applied to explore the potential immunogenic sugar moieties expressed by a previously unrecognized viral pathogen, SARS-CoV [5]. The strategy established in the SARS virus investigation is likely applicable for other microbial pathogens. It involves three steps of experimental investigation. In the first step, a carbohydrate microarray is used to characterize antibody responses to an infectious agent or antigen preparation in order to recognize the disease- or pathogen-associated anticarbohydrate antibody specificities. The second step focuses on identifying lectins and/or antibodies that are specific for the glycoepitopes that are recognized by the pathogen-elicited antibodies. This provides specific structural probes to enable the third step of investigation, that is, to identify the glycoepitopes in the candidate pathogens using specific lectins or antibodies identified in steps 1 and 2.

The rationale for this approach is that if SARS-CoV expressed antigenic carbohydrate structures, then immunizing animals using the whole virus-based

vaccines would elicit antibodies specific for these structures. In addition, if SARS-CoV displayed a carbohydrate structure that mimicked host cellular glycans, then vaccinated animals may develop antibodies with autoimmune reactivity to their corresponding cellular glycans. By characterizing the SARS-CoV neutralizing antibodies elicited by an inactivated SARS-CoV vaccine, autoantibody reactivity specific for the carbohydrate moieties of an abundant human serum glycoprotein, asialo-orosomucoid (ASOR), was detected [5]. This "chip hit" provides important clues for the selection of specific immunologic probes to further examine whether SARS-CoV expresses antigenic structures that imitate the host glycan. Given that lectin PHA-L is specific for glycoepitopes Tri-II or mII of ASOR (See Fig. 9.1b for an asialo-Tri-II structure of *N*-glycans), this lectin was applied as a structural probe to examine whether SARS-CoV expresses the PHA-L reactive antigenic structure. The results demonstrated that glycoepitopes Tri-II or mII of ASOR are highly expressed by SARS-CoV-infected cells and by the viral particles.

Another study [32] involved the dendritic cell receptor, DC-SIGN, and the endothelial cell receptor, DC-SIGNR, both of which play an important role in pathogen recognition. The ligand-binding properties of these receptors were elucidated using a glycan array fabricated with biotinylated mono- and oligosaccharides immobilized on streptavidin-coated wells. Screening the arrays with extracellular domains of the two cell receptors showed that in addition to the ligands that DC-SIGNR binds, DC-SIGN binds glycans that contain terminal fucose residues. Almost all the carbohydrates screened that bound to the CRD of DC-SIGN had branched terminal structures. Bulky and charged sialic acid residues were found to prevent binding.

Glycan arrays have also been used to investigate the interaction of the gp120 glycoprotein of HIV-1 [29], which interacts with CD4 of human T cells. The binding of gp120 to CD4 initiates events that subsequently allow the gp41 glycoprotein of HIV-1 to insert into the host cell membrane. Understanding how HIV carbohydrates interact with binding partners is expected to aid in developing agents to prevent HIV entry. A microarray of natural and modified glycoproteins, as well as neoglycoproteins were used to reveal the binding profiles of the following four gp120-binding molecules: DC-SIGN, a monoclonal antibody 2G12, cyanovirin-N (CVN), and scytovirin.

The dependence of carbohydrate moieties in their binding to the gp120 was studied by chemically modifying the protein ovalbumin with a high mannose oligosaccharide found on gp120. The four gp120-binding molecules only bind to ovalbumin when the high mannose oligosaccharide is present, indicating that their reactivity with gp120 is mainly dictated by the oligosaccharide residues as opposed to the polypeptide backbone. The binding profiles of the four proteins were investigated in further detail. A microarray composed of the high mannose oligosaccharide and components of the oligosaccharide revealed that both 2G12 and CVN bind to terminal Manα1–2Man linkages whereas scytovirin requires an additional underlying α1–6 trimannoside moiety. DC-SIGN was found to bind to all components investigated.

Aside from gp120 studies, the microarray revealed the novel finding that gp41, which expresses high-mannose oligosaccharides and is known to bind CVN and scytovirin, also binds both DC-SIGN and 2G12. The microarray allows for a rapid screening of fine structural details within a carbohydrate to ascertain the motifs responsible for molecular recognition. Such details will be beneficial in developing prophylaxis agents that prevent or inhibit HIV from infecting a host's cells.

A fourth pathogen-related application [33] involves the influenza A virus that has recently received much public attention due to several recent cases of the avian virus infecting humans. Aside from foreboding commentary of the emergence of a pandemic strain in the near future, virulent episodes from 1918 that claimed more than 50 million lives has left a lasting interest in understanding the immunogenic details of this virus. Influenza A infection is initiated by binding of hemagglutinin (HA), an antigenic protein found on the virus' coat, to carbohydrates on the surface of the host's epithelial cells. HA recognizes sialic acid terminated glycans and its linkage to galactose residues (see Fig. 9.1 for sialic acid terminated glycans in the Tri-II sugar chain configuration). The receptor specificity of various serotypes has been studied using cell-based assays. HA variants adapted to humans recognize an α2–6 linkage whereas strains specific for birds recognize an α2–3 linkage. Recently, researchers have applied carbohydrate microarrays to study the virus in a cell-independent assay [33].

Microarrays are expected to reduce complications involved in the cell studies. Using a combination of genomic sequence analysis and a glycan array displaying 200 carbohydrates, factors determining the specificity of influenza A for birds and mammals were investigated. By screening a variety of HAs and probing their interaction with a glycan array containing sugars with sialic acids attached via α2–3, α2–6, and α2–8 linkages among other glycans, specific mutations were shown to control the specificity of the HA for a given linkage as anticipated by previous studies. In addition, binding specificity was correlated to other fine structural motifs including charge, size, sulfation, fucosylation, and sialylation showing that the microarray can be used to identify different strains of the virus based on a fingerprint of the specificity.

The microarray could distinguish between the binding specificity of two different strains of human HAs, 18NY and 18SC (named after 1918 pandemic strains found in New York and South Carolina). 18SC HA recognizes only α2–6 linkages whereas the 18NY additionally recognizes α2–3 linkages. In the avian 18NY strain, a Glu190Asp mutation confers α2–6 recognition, resulting in virulence towards humans. The strain becomes more suited to α2–6 recognition after a second mutation, Gly225Asp. Thus, only two mutations are required to cross the major species barrier. This study shows that glycan arrays can be used to rapidly screen specificity profiles of pathogens and to predict the emergence of human pathogenic strains.

Another application of carbohydrate microarrays includes the study of carbohydrate-processing enzymes. Nature utilizes enzymes to synthesize carbohydrates. Understanding the specificity of a given enzyme upon carbohydrate

modification, as well as identifying inhibitors of the enzyme aids in treating diseases that rely on carbohydrate interactions is important. For example, NB-DNJ is an inhibitor that prevents a glucosidase enzyme from constructing appropriate carbohydrates on gp120, a glycoprotein of HIV mentioned above, reducing the virus' ability to bind to leucocytes. The specificity of enzymes that modify carbohydrates can be probed by treating a carbohydrate array with a given enzyme. Modified carbohydrates can then be revealed by lectin interactions. In addition, potential inhibitors of the enzyme activity can be screened by mixing selected candidates with the enzyme prior to treating the array. Carbohydrates stabilized on a microtiter plate through hydrophobic interactions have been used to reveal inhibitors of fucosyltransferase, an enzyme responsible for transferring a fucose residue from GDP-fucose to a sialyl-lactosamine to form sialyl-Lewis^x, a tetrasaccharide involved in the inflammatory response in mammalian tissue [34]. By incubating various candidate inhibitors with the enzyme prior to immersing the microarray, four inhibitors with nanomolar K_i values were discovered. The authors indicate that the method is 70% more cost-effective than a previously applied coupled-enzyme assay method.

9.3 Construction of Carbohydrate Microarrays

Developing new methods to fabricate carbohydrate microarrays has been an ongoing topic since 2002 when the first microarrays were reported [28]. Four important requirements involved in creating a functional microarray include (1) the ability to immobilize biological molecules on a flat substrate; (2) the immobilized molecules retain their biorecognition properties; (3) the sensitivity to detect a broad range of specificities; and (4) the ability to incorporate high-throughput equipment in creating the array. Both high- and low-density microarrays have been described. Low-density arrays are created on microtiter plates. High-density arrays are created on glass, metallic, and polymer surfaces. The latter can contain tens of thousands of sugars on one microscope slide for a large-scale characterization of saccharides and their receptors and has a unique advantage in exploring unknown carbohydrate targets and their potential receptors and antibodies. The former is suitable for a more focused biomedical application.

Fig. 9.2 shows the schematics of four approaches for immobilization of saccharides on a chip substrate: (1) noncovalent and nonspecific, (2) noncovalent and specific, (3) covalent and nonspecific, and (4) covalent and specific. Covalent immobilization links sugars on a surface by forming covalent bonds to the substrate. It ensures a stable immobilization of the saccharides regardless of their physicochemical properties. The ability to control the specificity of immobilization provides that a given face of the sugar will not be inactivated through chemical derivatization or burying at the substrate interface. Given that carbohydrates are structurally diverse and that key sugar moieties for biological recognition are frequently unknown to begin with, it remains technically challenging and practically

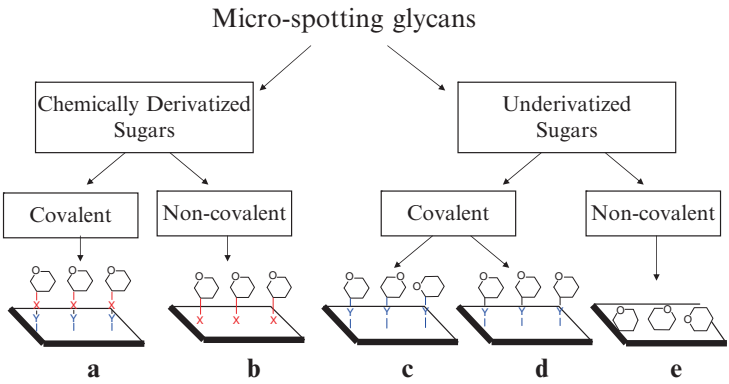

Fig. 9.2 Microspotted sugars can be stabilized on a chip in a variety of ways [35]. Chemically derivatized carbohydrates can be site-specifically immobilized on a substrate derivatized with appropriate functional groups (a). Carbohydrates derivatized with hydrophobic groups such as long hydrocarbon tails adsorb to hydrophobic substrates. Additionally, biotinylated carbohydrates can be stabilized on streptavidin-coated surfaces (b). Photochemical methods can be used to covalently immobilize underivatized carbohydrates to surfaces containing photoactive groups such as aromatic carbonyls or diazo compounds (c). Underivatized carbohydrates can be site-specifically immobilized on hydrazide or amino-oxy derivatized surfaces (d). Underivatized carbohydrates can be physically adsorbed onto polymer surfaces such as nitrocellulose or oxidized polystyrene. The interaction of immobilized carbohydrates with various biological species can be probed using fluorescence as a detection method

difficult to achieve a generally useful method for covalent and site-specific saccharide immobilization for a broad range of applications.

A number of noncovalent methods for presenting carbohydrates on a surface have been developed and are currently in use by many investigators. These include the use of native polysaccharides, glycoproteins, and glycolipids, as well as the use of chemically derivatized carbohydrates [23–25, 28, 36–38]. Hydrophobic effects are utilized by chemically derivatizing the carbohydrates with a hydrophobic group such as a lipid and spotting onto a hydrophobic surface.

Lipid-linked oligosaccharides have been immobilized on commercially available nitrocellulose-coated surfaces [25]. Similarly, sugars derivatized with long tail hydrocarbon chains of 13–15 carbon atoms have been immobilized on microtiter plates [24, 29]. Carbohydrates derivatized with C_8F_{17} fluorocarbon tails can be stabilized on glass slides containing a fluoroalkylsilane coating [40]. In addition, carbohydrates can be conjugated to bovine serum albumin and subsequently immobilized on an appropriate substrate [28, 41, 42]. Another strategy of carbohydrate presentation involves immobilizing biotinylated carbohydrates on streptavidin-coated wells [43] or glass slides [44].

The use of noncovalent immobilization in carbohydrate microarrays is analogous to bioassays that are based on the noncovalent immobilization of a biomolecule on a solid phase. These include the Southern blot for DNA hybridization, Northern blot for mRNA detection, and Western blot and ELISA assays for monitoring

protein and/or carbohydrate-based biomarkers. However, the efficacy of saccharide immobilization by noncovalent bonding is under the influence of the physicochemical properties of the given molecules. The stability of immobilization and exposure of the desired glycoepitopes or antigenic determinants must be examined for each saccharide on the substrate [5, 28, 31, 37, 38].

A number of methods for covalent and site-specific saccharide immobilization have also been developed. For example, maleimide-linked carbohydrates have been attached to thiol-coated surfaces, and vice versa, by formation of a thioether linkage [27, 29, 45]. Other surface linking reactions include Diels–Alder reaction [26], dipolar cycloaddition [46], amine-*N*-hydroxy succinimide (NHS) coupling [47, 48], *p*-aminophenyl-cyanuric chloride coupling [49], dimethylaminopyridine-NHS coupling [50], and the attachment of thiol-derivatized carbohydrates to gold films [51, 52]. In most of these procedures, both a chemically derivatized surface and derivatized carbohydrate are needed.

Methods that allow for arrays to be created from underivatized sugars are important because this avoids altering the native structure of bioactive carbohydrates and is less time-consuming for array construction. A few methods for immobilizing underivatized sugars have been demonstrated. Nitrocellulose-coated glass chips have been used to create polysaccharide microarrays. Although this is the most convenient method presently available, it can only be used for high molecular weight polysaccharides unless lipids are attached (see above), in which case oligosaccharides can be immobilized. Similarly, oxidized black polystyrene substrates immobilize underivatized sugars. The black substrate has the advantage of giving a high signal-to-noise ratio.

Recent methods allow for underivatized sugars to be covalently bound to a surface. Hydrazide-coated glass slides have been shown to react with underivatized mono-, oligo-, and polysaccharides in a site-specific manner [53]. Similarly, oligosaccharide microarrays have been prepared on hydrazide-coated gold slides [54]. In addition, underivatized carbohydrates have been bound to amino [55, 56], aminooxy [53], aminooxyacety [57], and phenylboronic acid-coated [58] slides. Note that in some cases the surface reactions reported were not used to fabricate microarrays. Two other methods for covalently immobilizing underivatized sugars involve derivatizing a surface with photoactive groups that can form covalent bonds to a wide variety of molecules after irradiation with UV light. These methods are discussed below.

9.4 Photons as General Reagents for Covalent Coupling of Carbohydrates on a Chip

When an appropriate functional group within a molecule absorbs a photon, the physical and chemical characteristics of the molecule can be dramatically altered [59]. Fig. 9.3 illustrates the effects of light absorption on the electronic structure

of a molecule when the photon is in the UV/visible region of the electromagnetic spectrum. Electrons in the ground electronic state, S_o, are promoted to a higher energy level, S_1, resulting in an excited singlet state (1). The excited molecule can relax to the ground state by emitting light (fluorescence) (2) or giving off heat (internal conversion) (3). The excited molecule can also undergo a chemical reaction (4) that is not possible in the ground state. In addition, the electron can flip its spin, a process called intersystem crossing (5), resulting in an excited triplet state. From the triplet state the electron can undergo phosphorescence (emission of light from the triplet state) (6), internal conversion (7), or chemical reaction (8).

Exploiting the photoactive nature of various chromophores (chemical units within a molecule that absorb light of a given wavelength) has allowed chemists to form covalent bonds between chemicals that are otherwise nonreactive. This feature has allowed underivatized carbohydrates to be immobilized on surfaces bearing appropriate chromophores. In addition, the use of photons as "traceless" and "weightless" reagents is a convenient, clean, and inexpensive method for fabricating microscale devices.

A variety of photochemical reactions has been performed on carbohydrates [60]. Our emphasis is focused on reactions that involve covalent bond formation between a carbohydrate and another molecule. H-abstraction from a C–H group by a photochemically excited state of carbonyl compounds is a common primary photochemical process. In this reaction, an excited carbonyl compound abstracts a hydrogen atom from a suitable C–H donor to form a pair of radicals. The susceptibility of a hydrogen atom to abstraction will depend on the C–H bond strength and the stability of the resulting radicals. The more substituted a carbon atom, the greater will be the stability of a radical forming at that carbon atom. In addition, inductive and resonance effects will influence the bond strength and stability of the resulting radical.

Studies on model compounds have shown that H-abstraction occurs preferentially at the anomeric center [60], however, when actual carbohydrates were studied, ESR results indicated that H-abstraction occurs preferentially at C–H bonds on C_1–C_4 with varying efficiency [61, 62]. Certain substituents can affect the selectivity

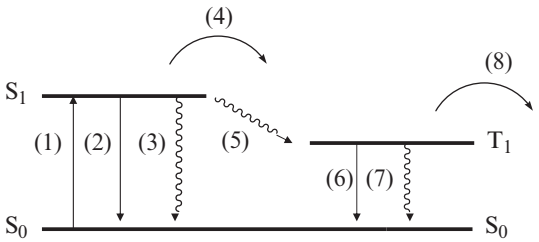

Fig. 9.3 The possible photophysical and photochemical events that can occur upon absorption of a photon by a molecule: (1) singlet–singlet absorption of a photon; (2) fluorescence; (3) internal conversion; (4) reaction from the excited singlet state; (5) intersystem crossing to the triplet state; (6) phosphorescence; (7) internal conversion; and (8) reaction from the triplet state

of the reaction. For example, in D-galacturonic acid hydrogen abstraction occurs predominantly at C_5 due to a combination of steric and stereoelectronic effects from an adjacent carboxyl group [63].

It should be noted that electron-donating substituents such as amines can favor electron transfer as opposed to H-abstraction. The rate for electron transfer is at least an order of magnitude faster than hydrogen abstraction, so sugars such as glycosaminoglycans are expected to favor this pathway. The possibility for electron transfer to occur will depend on the excited state reduction potential of the chromophore and the oxidation potential of the carbohydrate. When proton transfer follows electron transfer, the resulting radicals can recombine to form a covalent bond. Other possible reactions include back transfer, disproportionation, and various rearrangements that are pH-dependent [63].

Photogeneration of carbenes and nitrenes provides another potential method to form covalent bonds to carbohydrates. Irradiation of diazo and azide compounds results in the loss of N_2 and subsequent formation of carbene and nitrene intermediates. These can undergo a variety of reactions that result in covalent bond formation including insertion into sigma and pi bonds, addition of a nucleophile or electrophile and hydrogen abstraction.

Aziridine derivatized polysaccharides have been used to cast films on a surface that react with underivatized sugars after irradiation with UV light [64]. Upon absorption of a photon, the aziridine group loses N_2 to form a highly reactive nitrene. The nitrene presumably reacts with spotted sugars to form a covalent bond. Only polysaccharides were investigated using this surface. Similarly, diazirine derivatized mono- and disaccharides have been synthesized and photochemically immobilized on diamond [65] and poly (styrene) [66] films by photogenerating a carbene that reacts with the film.

A recently reported photochemical method employs a self-assembled monolayer [67] on a glass chip presenting phthalimide chromophores at the air–monolayer interface as shown in Fig. 9.4 [31]. The phthalimide chromophore is known to undergo a variety of photochemical reactions [68, 69]. In the presence of a carbohydrate the most plausible primary process is hydrogen abstraction from a C–H group.

Recombination of the resulting radicals results in a covalent bond. The mechanism is illustrated in Fig. 9.5. After absorption of a photon, the excited phthalimide abstracts a hydrogen atom from a nearby molecule. This creates radical centers on both molecules. The radicals can then recombine to form a covalent bond. Tethering a monolayer of phthalimide-derivatized silanes to a glass surface, SAM **1** (Fig. 9.4), provides a platform for covalently immobilizing sugars on chips. The phthalimide molecule is covalently bound to the glass through a condensation reaction between a trimethoxy silane and Si–OH groups at the glass surface. Bond formation to the substrate as well as van der Waals interactions between the long alkyl chains provide a driving force for the molecules to self-assemble into oriented clusters one molecule thick at the surface. In general the clusters are collectively described as a monolayer of molecules.

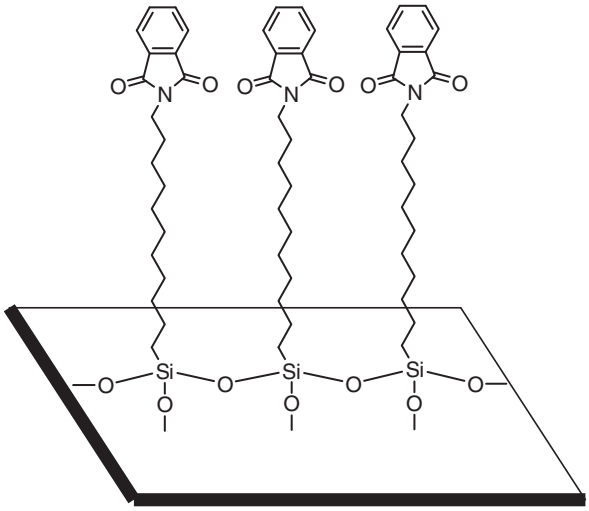

Fig. 9.4 A self-assembled monolayer containing phthalimide endgroups that become reactive when irradiated with UV light

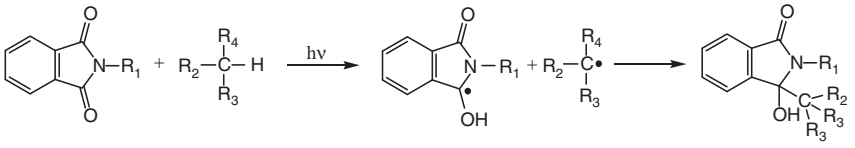

Fig. 9.5 A phthalimide derivative can undergo a photochemical hydrogen abstraction reaction followed by recombination to form a covalent bond

The "two-dimensional" nature of the surface guarantees that the photo-reactive group will present itself at the solid–air interface where the carbohydrates will be adsorbed. This is in contrast to polymeric three-dimensional coatings where van der Waals interactions will dictate which groups migrate to the interface. A key advantage of this photoactive surface is that sugars of all sizes should in principle be stabilized on the substrate because a covalent bond is expected to form. A simple modification of this surface that involves mixing a hydrophilic molecule into the monolayer (discussed below) allows for the use of high-throughput equipment to create microarrays of underivatized poly-, oligo-, and monosaccharides. Microarrays fabricated in this way have proven successful in elucidating fundamental information concerning the immunogenic properties of oligosaccharides found on pathogen glycoproteins.

A first step in pursuing any new methodology of microarray fabrication involves testing the interfacial chemistry between the carbohydrate and surface. In order to

test the photochemical reactivity of SAM **1** towards sugars, films of polysaccharides were spin-coated onto SAM **1**. Ellipsometry, contact angle, and fluorescence measurements were used to demonstrate that polysaccharides can be photochemically immobilized on this surface. In order to show the versatility of the method, a photolithographic patterning experiment was used to show that poly-, di-, and monosaccharides could be immobilized on the surface. Irradiation of homogeneous films of dextran polysaccharides, sucrose, and glucose through a photomask results in stabilized hydrophilic patterns that can be visualized by condensing water onto the surface. This demonstrates that sugar films composed of carbohydrates of any size can be immobilized on a phthalimide monolayer.

In order to be functional at the highest level, immobilized sugars must be accessible to assayed lectins and other such molecules and they must preserve their ability to react with cellular receptors or antibodies of defined specificities. Ideally, the surface must be suitable for patterning by way of conventional robotic spotters used to create microarrays because this allows for an automated production of potentially thousands of different carbohydrate spots on a single chip [38]. In order to perform a reaction at a surface, the thermodynamic properties of the system must favor adsorption of the reacting molecules at the interface. This is pertinent to constructing a microarray in that in order to use a robotic spotter, the surface must favor adsorption of a glycan solution onto the substrate. Otherwise, very little material will leave the pin of the spotter.

It was found that a monolayer consisting of only phthalimide end-groups was inappropriate for spotting. Even when a noticeable amount of material was spotted on SAM **1** or a benzophenone-terminated monolayer (another class of aromatic carbonyls that participates in H-abstraction when irradiated), the surfaces were unable to retain a detectable amount of carbohydrates after irradiation. This is in contrast to the film studies in which a pure monolayer was sufficient to immobilize sugars. The discrepancy is probably a result of the film's sampling a much larger area of the surface in comparison to a spot of approximately 200 μm, making immobilized sugars easier to detect. Also, the mechanical effects of spin-coating may press the sugars into gaps in the monolayer allowing the excited carbonyl more access to the sugar.

In order to make the surface more amenable for spotting, trimethoxyaminopropylsilane was mixed into the surface (PAM) as shown in Fig. 9.6. A ratio of 5:1 amine:phthalimide was found to give a reliable surface for spotting and immobilization. The amine acts as bait to pull the sugars onto the surface through a favorable hydrophilic interaction. The hydrophilic gaps are also expected to put the carbohydrate in a more favorable location for the phthalimide to abstract a hydrogen atom from the sugar. The structure of the surface is probably more complicated than the simple picture shown in Fig. 9.6. If a monolayer is formed, the phthalimide most likely will try to tilt over the amines to reduce the interfacial tension. In addition, it is possible that like molecules cluster or even phase-separate within the mixed monolayer, or those multilayers, oligomers, or polymers form, but these phenomena have not yet been investigated in this system. Regardless of the

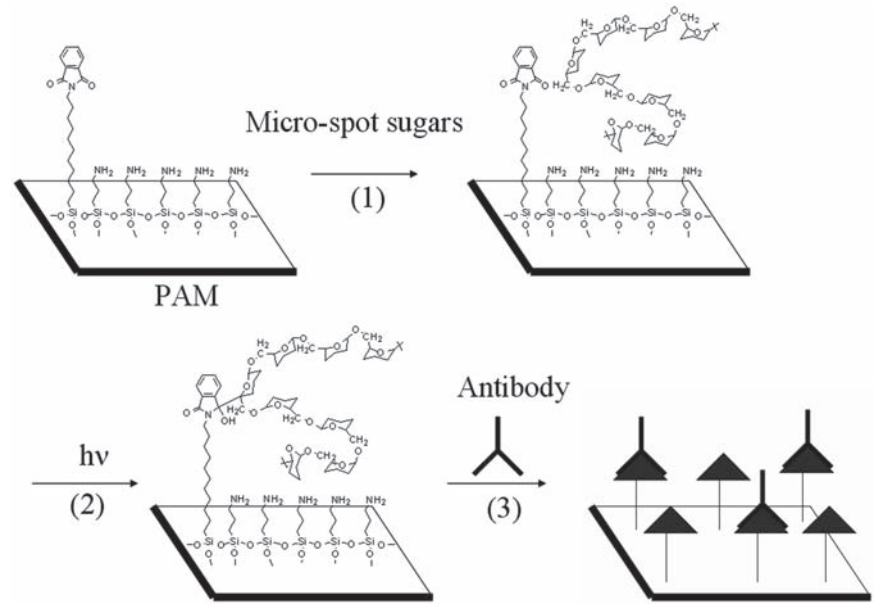

Fig. 9.6 A mixed monolayer containing phthalimide and amine-terminated molecules provides a photoactive surface appropriate for spotting. After sugars are spotted, irradiation with UV light binds the sugars to the surface presumably through covalent bond formation. Antibodies can recognize the corresponding epitopes on the immobilized carbohydrates (triangles)

surface structure, mixing amines into the surface makes the substrate functional in a high-throughput microarray application.

In order to understand the utility of a given method it is critical to screen a variety of sugars with known specificities towards various lectins, antibodies, or other substances that interact with carbohydrates. Steric interactions and the specificity of the immobilization could potentially inhibit recognition. Immobilized polysaccharide dextran antigens were screened against antidextran antibodies. The antibodies were able to recognize the antigenic determinants of photoimmobilized polysaccharides. A more important application of PAM is the ability to assay underivatized oligosaccharides because nitrocellulose-coated surfaces are unable to hold such small molecules.

Photoimmobilized saccharides containing 3–7 glucose and mannose residues were found to recognize the lectin concanavalin A (con A), however, as the size of the oligosaccharide decreased the intensity of the fluorescence signal decreased. Photoimmobilized glucose and mannose monosaccharides were unable to recognize con A. This is most likely due to increased steric hindrance as the size of the sugar decreases. The close proximity of the sugar to the monolayer makes the epitope inaccessible to the protein. In addition, the expected nonspecific nature of the reaction can bury the C_3, C_4, and C_5 hydroxyl groups of the monosaccharide that are required

for binding the lectin. As a sugar decreases in size, the number of biologically active epitopes will decrease along with the probability that the epitope displays itself at the surface. At least some active faces of the sugar are expected to be present at the surface.

Characterizing the immunogenic properties of carbohydrate structures on the surface of pathogens could lead to the development of improved vaccines, drugs, sensors, and diagnostic methods. PAM was recently used to identify immunogenic moieties on the surface of *Bacillus anthracis* spores, rodlike gram-positive bacteria responsible for anthrax infection [70]. Among the various proteins found on the exosporium, the outermost surface of *B. anthracis*, BclA (*Bacillus* collagen-like protein of *anthracis*) is the most prominent. BclA is a glycoprotein containing two types of *O*-linked oligosaccharides: a 324 Da disaccharide and a 715 Da tetrasaccharide. The structure of the tetrasaccharide is shown in Fig. 9.7.

The terminal amide-containing residue was given the name anthrose. The trisaccharide attached to anthrose is made up of rhamnopyranosyl units. The anomeric configuration of the rhamnopyranosyl residue attached to the glycoprotein is unknown. The presence of a given sugar on the surface of the exosporium does not guarantee that the sugar takes part in eliciting an immune response. In order to determine the immunogenic properties of the tetrasaccharide, the α and β conformers of the tetrasaccharide, components of the tetrasaccharide and additional sugars were photoimmobilized on PAM. Incubation of the microarray with

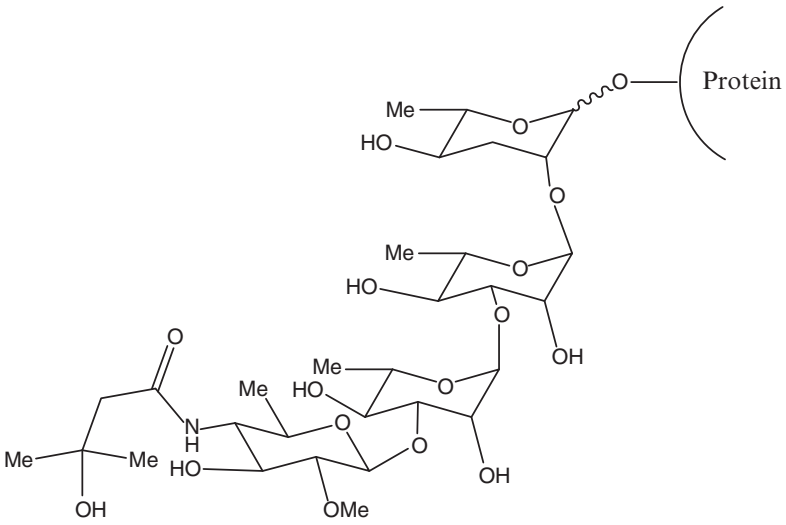

Fig. 9.7 A tetrasaccharide found on the exosporium of *B. anthracis* spores [71]. The terminal monosaccharide residue has been given the name anthrose. Anthrose is attached to a trisaccharide component made up of rhamnopyranosyl residues. The orientation of the anomeric center of the residue attached to the protein is unknown

antibodies elicited by anthrax spore immunization demonstrated that the anthrose-containing tetrasaccharides are specifically recognized by the antibody.

Inhibition assays were also conducted with the microarray. The anthrose monosaccharide was found to inhibit the antibody from binding to the tetrasaccharide. Thus, the terminal anthrose residue, together with the trisaccharide containing rhamnopyranosyl units form a highly specific immunogenic sugar moiety of *B. anthracis* spores. The experimental approach is expected to allow for the high-throughput screening of the saccharide structures found on any pathogen to identify their key antigenic structures.

In summary, we have described a photochemical strategy that allows for carbohydrates to be immobilized on surfaces without chemical modification. This technique offers a clean and simple method to immobilize carbohydrates on a glass chip. A key advantage is that the carbohydrates do not need to be derivatized with a specific functional group for covalent immobilization on a chip surface. However, an intrinsic weakness of this method is that the immobilized saccharides are expected to lack a specific orientation. In a given microspot, the active part of an unknown percentage of the immobilized sugars can get buried at the interface if the photochemical reaction targets a C–H group on the epitope of the sugar. Smaller sugars are expected to be more hindered by this inasmuch as they contain a lesser amount of epitopes.

The photochemical method could be adapted to incorporate reactions mentioned above that result in selective immobilization of carbohydrates. Rather than directly spot carbohydrates onto the photoactive surface, a polymeric scaffold functionalized with a pertinent reactive group (hydrazide groups for underivatized sugars, e.g.) could be photoimmobilized onto the surface before spotting. The polymeric thin film will provide a thicker and more mobile layer of functional groups, potentially increasing the amount of carbohydrates adsorbed and immobilized per spot. In addition, the flexibility of a surface-bound macromolecule in comparison to a small molecule in a monolayer may increase the accessibility of immobilized carbohydrates to lectins, particularly if the assay conditions swell the polymer chain. This carbohydrate microarray platform provides a versatile tool for carbohydrate research. Its potential in biomedical applications is yet to be further explored.

References

1. Apweiler, R., H. Hermjakob, et al. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta, Gen. Subj.* **1473**(1): 4–8.
2. Davis, B.G.F. and J. Antony (2002). *Carbohydrate Chemistry*. Oxford, Oxford University Press.
3. Lindhorst, T.K. (2003). *Essentials of Carbohydrate Chemistry and Biochemistry*. Weinheim, Wiley-VCH.
4. Schwartz, A.L., S.E. Fridovich, et al. (1981). Characterization of the asialoglycoprotein receptor in a continuous hepatoma line. *J. Biol. Chem.* **256**(17): 8878–8881.
5. Wang, D., and J. Lu (2004). Glycan arrays lead to the discovery of autoimmunogenic activity of SARS-CoV. *Physiol. Genomics* **18**(2): 245–248.

6. Rosati, F., A. Capone, et al. (2000). Sperm-egg interaction at fertilization: glycans as recognition signals. *Int. J. Dev. Biol.* **44**(6): 609–618
7. Focarelli, R., G.B. La Sala, et al. (2001). Carbohydrate-mediated sperm-egg interaction and species specificity: A clue from the Unio elongatulus model. *Cells Tissues Organs* **168**(1–2): 76–81.
8. Crocker, P.R. and T. Feizi (1996). Carbohydrate recognition systems: functional triads in cell-cell interactions. *Curr. Opin. Struct. Biol.* **6**(5): 679–691.
9. Ziska, S.E., and E.J. Henderson (1988). Cell surface oligosaccharides participate in cohesion during aggregation of Dictyostelium discoideum. *Proc. Natl. Acad. Sci. USA* **85**(3): 817–821.
10. Geijtenbeek, T.B., D.S. Kwon, et al. (2000). DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* **100**(5): 587–597.
11. Geijtenbeek, T.B., R. Torensma, et al. (2000). Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **100**(5): 575–585.
12. Lindahl, U., L. Thunberg, et al. (1984). Extension and structural variability of the antithrombin-binding sequence in heparin. *J. Biol. Chem.* **259**(20): 12368–12376.
13. Capila, I. and R.J. Linhardt (2002). Heparin-protein interactions. *Angew. Chem. Int. Ed. Engl.* **41**(3): 391–412.
14. Kansas, G.S. (1996). Selectins and their ligands: Current concepts and controversies. *Blood* **88**(9): 3259–3287.
15. Gardinali, M., P. Padalino, et al. (1992). Complement activation and polymorphonuclear neutrophil leukocyte elastase in sepsis. Correlation with severity of disease. *Arch. Surg.* **127**(10): 1219–1224.
16. Karlsson, K.A., J. Angstrom, et al. (1992). Microbial interaction with animal cell surface carbohydrates. *APMIS. Suppl.* **27**: 71–83.
17. Feizi, T., and R.W. Loveless (1996). Carbohydrate recognition by Mycoplasma pneumoniae and pathologic consequences. *Am. J. Respir. Crit. Care. Med.* **154**(4 Pt 2): S133–6.
18. Wang, D. and E.A. Kabat (1996). Carbohydrate antigens (polysaccharides). *Structure of Antigens*. M.H.V.V. Regenmortal (Ed.). Boca Raton, FL, CRC Press. **3**: 247–276.
19. Lee, Y.C., and R.T. Lee (1995). Carbohydrate-protein interactions: Basis of glycobiology. *Acc. Chem. Res.* **28**(8): 321–327.
20. Drickamer, K. and M.E. Taylor (2002). Glycan arrays for functional glycomics. *GenomeBiol.* **3**(12): No pp given.
21. Mammen, M., S.-K. Chio, et al. (1998). Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew. Chem. Int. Ed.* **37**(20): 2755–2794.
22. Wang, D. and E.A. Kabat (1998). Antibodies, specificity. *Encyclopedia of Immunology*. Delves and Roitt (Eds.). London, Academic Press: 148–154.
23. Wang, D. (2004). Carbohydrate antigens. *Encyclopedia of Molecular Cell Biology and Molecular Medicine*. R.A. Meyers (Ed.), Weinheim, Wiley-VCH. **II:** 277–301.
24. Fazio, F., M.C. Bryan, et al. (2002). Synthesis of sugar arrays in microtiter plate. *J. Am. Chem. Soc.* **124**(48): 14397–14402.
25. Fukui, S., T. Feizi, et al. (2002). Oligosaccharide microarrays for high-throughput detection and specificity assignments of carbohydrate-protein interactions. *Nature Biotechnol.* **20**(10): 1011–1017.
26. Houseman, B.T. and M. Mrksich (2002). Carbohydrate arrays for the evaluation of protein binding and enzymatic modification. *Chem. Biol.* **9**(4): 443–454.
27. Park, S., and I. Shin (2002). Fabrication of carbohydrate chips for studying protein-carbohydrate interactions. *Angew. Chem. Int. Ed. Engl.* **41**(17): 3180–3182.
28. Wang, D., S. Liu, et al. (2002). Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells. *Nature Biotechnol.* **20**(3): 275–281.
29. Adams, E.W., D.M. Ratner, et al. (2004). Oligosaccharide and glycoprotein microarrays as tools in HIV glycobiology glycan-dependent gp120/protein interactions. *Chem. Biol.* **11**(6): 875–881.

30. Willats, W.G.T. (2005). Microarrays for the high-throughput analysis of protein-carbohydrate interactions. *Protein Microarrays* 57–69.
31. Carroll, G.T., D. Wang, et al. (2006). Photochemical micropatterning of carbohydrates on a surface. *Langmuir* **22**(6): 2899–2905.
32. Guo, Y., H. Feinberg, et al. (2004). Structural basis for distinct ligand-binding and targeting properties of the receptors DC-SIGN and DC-SIGNR. *Nat. Struct. Mol. Biol.* **11**(7): 591–598.
33. Stevens, J., O. Blixt, et al. (2006). Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. *J. Mol. Biol.* **355**(5): 1143–1155.
34. Bryan, M.C., L.V. Lee, et al. (2004). High-throughput identification of fucosyltransferase inhibitors using carbohydrate microarrays. Bioorg. Med. Chem. Lett. **14**(12): 3185–3188.
35. Shin, I., S. Park, et al. (2005). Carbohydrate microarrays: An advanced technology for functional studies of glycans. *Chem. Euro. J.* **11**(10): 2894–2901.
36. Willats, W.G., S.E. Rasmussen, et al. (2002). Sugar-coated microarrays: A novel slide surface for the high-throughput analysis of glycans. *Proteomics* **2**(12): 1666–1671.
37. Wang, D. (2003). Carbohydrate microarrays. *Proteomics*. **3**(11): 2167–2175.
38. Wang, R., S. Liu, et al. (2005). A practical protocol for carbohydrate microarrays. *Methods in Molecular Biology*. Totowa, NJ, **310**: 241–252.
39. Bryan, M.C., O. Plettenburg, et al. (2002). Saccharide display on microtiter plates. *Chem. Biol*. **9**(6): 713–720.
40. Ko, K.-S., F.A. Jaipuri, et al. (2005). Fluorous-based carbohydrate microarrays. *J. Am. Chem. Soc*. **127**(38): 13162–13163.
41. Manimala, J.C., Z. Li, et al. (2005). Carbohydrate array analysis of anti-Tn antibodies and lectins reveals unexpected specificities: implications for diagnostic and vaccine development. *Chembiochem*. **6**(12): 2229–2241.
42. Manimala, J.C., T.A. Roach, et al. (2006). High-throughput carbohydrate microarray analysis of 24 lectins. *Angew. Chem. Int. Ed. Engl*. **45**(22): 3607–3610.
43. Shao, M.C. (1992). The use of streptavidin-biotinylglycans as a tool for characterization of oligosaccharide-binding specificity of lectin. *Anal. Biochem*. **205**(1): 77–82.
44. Bochner, B.S., R.A. Alvarez, et al. (2005). Glycan array screening reveals a candidate ligand for Siglec-8. *J. Biol. Chem*. **280**(6): 4307–4312.
45. Park, S., M.-R. Lee, et al. (2004). Carbohydrate chips for studying high-throughput carbohydrate-protein interactions. *J. Am. Chem. Soc*. **126**(15): 4812–4819.
46. Bryan, M.C., F. Fazio, et al. (2004). Covalent display of oligosaccharide arrays in microtiter plates. *J. Amer. Chem. Soc*. **126**(28): 8640–8641.
47. Blixt, O., S. Head, et al. (2004). Printed covalent glycan array for ligand profiling of diverse glycan binding proteins. *Proc. Natl. Acad. Sci. USA* **101**(49): 17033–17038.
48. de Paz, J.L., C. Noti, et al. (2006). Microarrays of synthetic heparin oligosaccharides. *J. Am. Chem. Soc*. **128**(9): 2766–2767.
49. Schwarz, M., L. Spector, et al. (2003). A new kind of carbohydrate array, its use for profiling antiglycan antibodies, and the discovery of a novel human cellulose-binding antibody. *Glycobiology* **13**(11): 749–754.
50. Xia, B., Z.S. Kawar, et al. (2005). Versatile fluorescent derivatization of glycans for glycomic analysis. *Nature Meth*. **2**(11): 845–850.
51. Fritz, M.C., G. Hähner, et al. (1996). Self-assembled hexasaccharides: Surface characterization of thiol-terminated sugars adsorbed on a gold surface. *Langmuir* **12**(25): 6074–6082.
52. Revell, D.J., J.R. Knight, et al. (1998). Self-assembled carbohydrate monolayers: Formation and surface selective molecular recognition. *Langmuir* **14**(16): 4517–4524.
53. Lee, M.-R. and I. Shin (2005). Facile preparation of carbohydrate microarrays by site-specific, covalent immobilization of unmodified carbohydrates on hydrazide-coated glass slides. *Organic Lett*. **7**(19): 4269–4272.
54. Zhi, Z.-l., A. K. Powell, et al. (2006). Fabrication of carbohydrate microarrays on gold surfaces: Direct attachment of nonderivatized oligosaccharides to hydrazide-modified self-assembled monolayers. *Anal. Chem*. **78**(14): 4786–4793.

55. Yates, E.A., M.O. Jones, et al. (2003). Microwave enhanced reaction of carbohydrates with amino-derivatized labels and glass surfaces. *J. Mater. Chem*. **13**(9): 2061–2063.
56. Peramo, A., A. Albritton, et al. (2006). Deposition of patterned glycosaminoglycans on silanized glass surfaces. *Langmuir* **22**(7): 3228–3234.
57. Zhou, X. and J. Zhou (2006). Oligosaccharide microarrays fabricated on aminooxyacetyl functionalized glass surface for characterization of carbohydrate-protein interaction. *Biosens. Bioelectron*. **21**(8): 1451–1458.
58. Takahashi, S. and J. Anzai (2005). Phenylboronic acid monolayer-modified electrodes sensitive to sugars. *Langmuir* **21**(11): 5102–5107.
59. Turro, N.J. (1991). *Modern Molecular Photochemistry*. Sausalito, CA, University Science Books.
60. Binkley, E.R. and R.W. Binkley (1998). *Carbohydrate Photochemistry*. In: *ACS Monogr*., 1998; 191.
61. Madden, K.P., and R.W. Fessenden (1982). ESR study of the attack of photolytically produced hydroxyl radicals on a-methyl-D-glucopyranoside in aqueous solution. *J. Am. Chem. Soc*. **104**(9): 2578–2581.
62. Shkrob, I.A., M.C. Depew, et al. (1993). Time-resolved, electron-spin resonance study of radical species derived from naturally occurring carbohydrates. *Chem. Phys. Lett*. **202**(1–2): 133–140.
63. Gilbert, B.C., D.M. King, et al. (1984). Radical reactions of carbohydrates, Part 5. The oxidation of some polysaccharides by the hydroxyl radical: An ESR investigation. *Carbohydrate Res*. **125**(2): 217–235.
64. Angeloni, S., J.L. Ridet, et al. (2005). Glycoprofiling with micro-arrays of glycoconjugates and lectins. *Glycobiology* **15**(1): 31–41.
65. Chevolot, Y., O. Bucher, et al. (1999). Synthesis and characterization of a photoactivatable glycoaryldiazirine for surface glycoengineering. *Bioconj. Chem*. **10**(2): 169–175.
66. Chevolot, Y., J. Martins, et al. (2001). Immobilisation on polystyrene of diazirine derivatives of mono- and disaccharides: Biological activities of modified surfaces. *Bioorg. Med. Chem*. **9**(11): 2943–2953.
67. Ulman, A. (1996). Formation and structure of self-assembled monolayers. *Chem. Rev*. **96**(4): 1533–1554.
68. Kanaoka, Y. (1978). Photoreactions of cyclic imides. Examples of synthetic organic photochemistry. *Acc. Chem. Res*. **11**(11): 407–413.
69. Yoon, U.C., and P.S. Mariano (2001). The synthetic potential of phthalimide SET photochemistry. *Acc. Chem. Res*. **34**(7): 523–533.
70. Wang, D., G.T. Carroll, et al. (2006). Immunogenic sugar moieties of the Bacillus anthracis exosporium (anthrose-based compositions and related methods). United States Provisional Patent Application, Dkt 76323-pro, filed September 15, 2006.
71. Daubenspeck, J.M., H. Zeng, et al. (2004). Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the Bacillus anthracis exosporium. *J. Biol. Chem*. **279**(30): 30945–30953.