Construction of Carbohydrate Microarrays by Using One-Step, Direct Immobilizations of Diverse Unmodified Glycans on Solid Surfaces

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Carbohydrate microarrays have received great attention as high-throughput analytic tools in studies of carbohydrate-mediated biological processes. Most of the methods employed to fabricate glycan microarrays rely on the immobilization of modified glycans on the properly derivatized surfaces. This immobilization strategy requires the availability of modified glycans whose syntheses in many cases are time-consuming and difficult. We have developed a simple and direct immobilization technique that involves a one-step, site-specific attachment of diverse unmodified glycans to the hydrazide-derivatized glass surface. To demonstrate the generality of this direct immobilization method, we examined its use for the construction of carbohydrate microarrays containing a variety of glycans. The results of protein and cell-binding experiments indicate that the glycan microarrays, prepared by using this methodology, are applicable to the rapid evaluation of glycan-mediated biomolecular interactions and the determination of quantitative binding affinities between carbohydrates and proteins.

INTRODUCTION

Carbohydrates are vital components of living systems and a large group of biomolecules that are present mainly in the form of glycoconjugates inside or on the surfaces of cells. Through their interactions with proteins, the glycoconjugates are involved in a wide variety of important biological processes, such as cell differentiation, cell adhesion, signaling, trafficking, and immune response (for some recent reviews, see *refs 1*−*3*). Carbohydrate−protein recognition events also play key roles in many pathological processes, including infection by pathogens, cancer cell metastasis, and inflammation processes (*4, 5*). Consequently, the understanding of glycan−protein interactions at the molecular level provides deep insight into glycan-mediated biological processes and enables the development of more effective therapeutic agents and diagnostic tools in the postgenomics era.

Since 2002, carbohydrate microarrays, which are composed of diverse glycans orderly and densely attached to a single chip substrate, have been extensively developed as reliable and efficient tools for the rapid analysis of carbohydrate-based biomolecular interactions (*6*−*9*). The microarray technology has notable advantages over the use of conventional methods, such as the hemagglutination inhibition assay, enzyme-linked lectin assay, surface plasmon resonance (SPR), and isothermal titration calorimetry (ITC). The most significant advantage is that glycan microarrays enable fast, quantitative, and simultaneous analyses of a number of glycan−protein interactions with small amounts of carbohydrate samples. Another notable feature is that glycans immobilized on microarrays strongly interact with proteins, even in cases where the proteins have low binding affinities to the monovalent carbohydrates in solution. This is due to the fact that immobilized glycans display multivalent binding to proteins caused by a cluster effect. Owing to these advantageous characteristics, carbohydrate microarray-based technologies are applicable to high-throughput analyses of glycan−protein interactions.

A variety of carbohydrate microarray formats that use different surfaces and immobilization methods have emerged over the past few years (for some reviews, see *refs 10*−*16*). The most general method for construction of these microarrays involves site-specific and covalent immobilization of chemically modified carbohydrates to properly derivatized surfaces. For example, we have shown that carbohydrate microarrays can be fabricated by attaching maleimide- or hydrazide-conjugated glycans on respective thiol- or epoxide-coated glass surfaces (*6, 17*−*20*). This immobilization strategy requires the use of properly functionalized sugars, which are typically prepared by multistep synthetic sequences. To circumvent the need for synthetically intensive methods, one-step procedures for the preparation of functionally conjugated sugars have been developed and used for the fabrication of glycan microarrays. For example, amine appended sugars have been prepared by reacting free carbohydrates with 2,6-diaminopyridine in the presence of sodium cyanoborohydride or with *N*-methylaminooxy-containing bifunctional linkers (*21, 22*). The modified sugars, generated in these ways, are then covalently immobilized on the NHS ester-coated surfaces.

However, the requirement for derivatization of glycans by either one-step or multistep sequences remains a significant hurdle for the construction of microarrays containing diverse sugars. In order to avoid the need for functionalized glycans, immobilization strategies that employ unmodified sugars have been developed. One approach involves the attachment of ploysaccharides to the nitrocellulose-coated or the black polystyrene-coated surfaces (*8, 9*). This immobilization strategy is limited by a size dependence and nonspecific adsorption of glycans. An alternative approach that does not require modified sugars involves photoinduced covalent attachment of unmodified sugars to surfaces that are coated with photolabile groups (*23*). A major drawback of this technique is associated with nonspecific attachment of glycans to the surfaces.

An ideal method for the construction of carbohydrate microarrays would rely on site-specific, size-independent covalent attachment of unmodified sugars to proper surfaces. In recent investigations directed at this goal, we have developed a novel, direct, site-specific technique for immobilization of unmodified carbohydrates, including simple carbohydrates, oligosaccharides and polysaccharides, on hydrazide- and aminooxy-derivatized

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surfaces (*24*). This method eliminates the need for laborious and tedious glycan derivatization. To demonstrate the scope and applicability of this approach, carbohydrate microarrays containing a variety of glycans (58 glycans) have been constructed by using one-step, direct attachment of free carbohydrates to a hydrazide-coated surface. These microarrays have been employed for analysis of sugar binding specifities of lectins, antibodies, and bacterial cells. In addition, this microarray format has been applied to the determinination of binding affinities between proteins and glycans. The results of these studies, which have led to the development of facile methods to prepare carbohydrate microarrays and their application to investigations of glycan-mediated biomolecular interactions, are described below.

MATERIALS AND METHODS

General. All chemical and biochemical products were of analytical grade and purchased from commercial suppliers: lectins from Vector Laboratories (Peterborough, UK) and Sigma-Aldrich (St. Louis, MO, USA); Cy3 and Cy5 *N*-hydroxysuccinimide from Amersham Pharmacia biotech (Piscataway, NJ, USA); carbohydrates from Sigma-Aldrich, Calbiochem (Gibbstown, NJ, USA), TCI Corporation (Tokyo, Japan), Acros (Morris Plains, NJ, USA), Senn Chemicals (Dielsdorf, Switzerland), Carbohydrate Synthesis (Oxford, UK), Dextra Laboratories (Reading, UK), and GlycoTech Corporation (Gaithersburg, MD, USA); antidextran antibody from Stemcell Technologies Inc. (Vancouver, BC, Canada) and monoclonal antichondroitin 6-sulfate antibody from MP Biochemicals Inc. (Solon, OH, USA); amine-coated glass slide from Nuricell (Seoul, Korea); SYTO 83 from Molecular Probes (Carlsbad, CA, USA). The solutions of carbohydrates were printed on the surface with a MicroSys 5100 from Cartesian Technologies Inc. (Irvine, CA, USA). Carbohydrate microarrays probed with fluorescent dye labeled-lectins were scanned with an ArrayWoRx Biochip Reader from Applied Precision (Northwest Issaquah, WA, USA).

Preparation of Aminooxy- And Hydrazide-Functionalized Glass Slides Tethered by Oligo(ethylene glycol). Commercially available amine-coated glass slides were kept in a staining jar containing a solution of succinic anhydride (3%) in DMF with gentle shaking for 3 h. The slides were washed with DMF and a solution of diisopropylcarbodiimide (DIC, 3%) and *N*-hydroxysuccinimide (NHS, 3%) in DMF was poured into a staining jar. After gentle shaking for 3 h, the slides were washed with DMF. The slides were immersed into a solution of 4,7,10-trioxa-1,13-tridecanediamine (3%) in DMF with gentle shaking for 3 h. After washing with DMF, the slides were dried by purging with argon gas.

To prepare hydrazide-coated slides, a solution of succinic anhydride (3%) in DMF was poured into a staining jar that contained the slides treated with 4,7,10-trioxa-1,13-tridecanediamine. After gentle shaking for 3 h, the slides were washed with DMF. A solution of DIC (3%) and NHS (3%) in DMF was poured into a staining jar and the slides were gently shaken for 3 h. After washing with DMF, the slides were immersed into a solution of hydrazine monohydrate (3%) in DMF with gentle shaking for 3 h. After washing with DMF, the slides were dried by purging with argon gas. The derivatized slides can be stored at room temperature in a desiccator for several weeks.

To prepare aminooxy-coated slides, a solution of *N*-(2-bromoethoxy)phthalimide (3%) and TEA (3%) in DMF was poured into a staining jar that contained the slides treated with 4,7,10-trioxa-1,13-tridecanediamine. After gentle shaking for 6 h, the slides were washed with DMF. The slides were immersed into a solution of hydrazine monohydrate (3%) in DMF with gentle shaking for 3 h. After washing with DMF, the slides were dried by purging with argon gas. The derivatized slides can be stored at room temperature in a desiccator for several weeks.

Preparation of Hydrazide-Functionalized Glass Slides Tethered by Poly(ethylene glycol). Commercially available amine-coated glass slides were kept in a staining jar containing a solution of poly(ethylene glycol) diglycidyl ether (3%, average $M_n \sim 526$) in 10 mM $NaHCO_3$ with gentle shaking for 1 h. After washing with water, the slides were immersed into a solution of 4,7,10-trioxa-1,13-tridecanediamine (3%) in 10 mM $NaHCO_3$ with gentle shaking for 1 h. They were washed with water and soaked into a solution of succinic anhydride (3%) in DMF with gentle shaking for 3 h. After washing with DMF, the slides were treated with a solution of DIC (3%) and NHS (3%) in DMF with gentle shaking for 3 h. They were washed with DMF and immersed into a solution of hydrazine monohydrate (3%) in DMF with gentle shaking for 3 h. The slides were washed with water and dried by purging with argon gas. The derivatized slides can be stored at room temperature in a desiccator for several weeks.

Printing of Carbohydrates on the Surface. Carbohydrates were dissolved in sodium phosphate buffer (pH 5.0) containing 30% glycerol. A solution of carbohydrates (1 nL, 30 mM) from a 384-well plate was printed in predetermined places on a hydrazide or aminooxy-coated glass slide with a distance of 250 μm between the centers of adjacent spots. After completion of printing, the slide was placed into a humidity chamber at 50 °C for 12 h, and then, a compartmentalized plastic film that was coated by adhesive on one side (thickness: 0.1−0.2 mm) was attached to the glass slide (*17*). The slide was washed with PBS (pH 7.4) containing 0.1% Tween 20 and a solution of 0.1% Tween 20 possessing 1% BSA was dropped in the blocks. After incubation for 0.5 h, the slide was washed with the same buffer without BSA.

Detection of Carbohydrate−Protein Interactions. The printed slides were probed with fluorescent dye-labeled lectins (1−10 μg/mL) or unlabeled antibodies in PBS (pH 7.4) containing 0.1% Tween 20 for 1 h at room temperature. For ConA binding, $MnCl_2$ and $CaCl_2$ were added at final concentrations of 1 mM. The unbound lectins or antibodies were then removed by washing with the same buffer and rinsed with water. After drying by purging with argon gas, the slide treated with fluorescent dye-labeled lectins was scanned by using an ArrayWoRx scanner. The slide probed with unlabeled antibodies was further treated with FITC-secondary IgG antibody and then scanned by using a microarray scanner. Fluorescence intensity was analyzed using *ImaGene 6.1* software from BioDiscovery.

Detection of Glycan−Pathogen Interactions. *E. coli* ORN 178 and 208 strains were grown in LB media at 37 °C for 12 h and then harvested by centrifugation. The collected cells were suspended with PBS buffer containing $CaCl_2$ (1 mM) and $MnCl_2$ (1 mM). SYTO 83 (50 μM) was added to the suspension of bacterial cells, and then, the mixture was poured onto the carbohydrate microarray. After 30 min, unbound cells were removed by gentle washing with PBS buffer and water. The slide was scanned by using a microarray scanner. Fluorescence intensity was analyzed using *ImaGene 6.1* software from BioDiscovery.

Determination of Dissociation Constants for Protein−Glycan Interactions Using Carbohydrate Microarrays. The carbohydrate microarray containing fucose, Fucα1,2Gal, Fucα1,3GlcNAc, Fucα1,4GlcNAc, and GalNAcα1,3(Fucα1,2)Gal was treated with a solution of 0.1% Tween 20 possessing 1% BSA at room temperature for 30 min. The microarray was washed with PBS (pH 7.4) containing 0.1% Tween 20 and then probed with various concentrations (0.1 nM−4 μM)

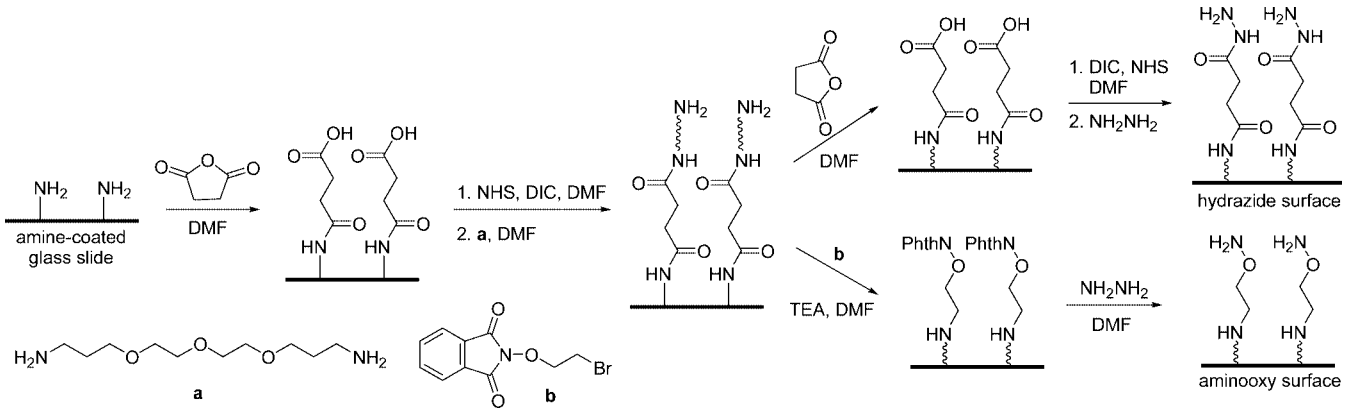


Figure 1. Preparation of hydrazide- and aminooxy-derivatized glass slides from amine-coated glass slides.

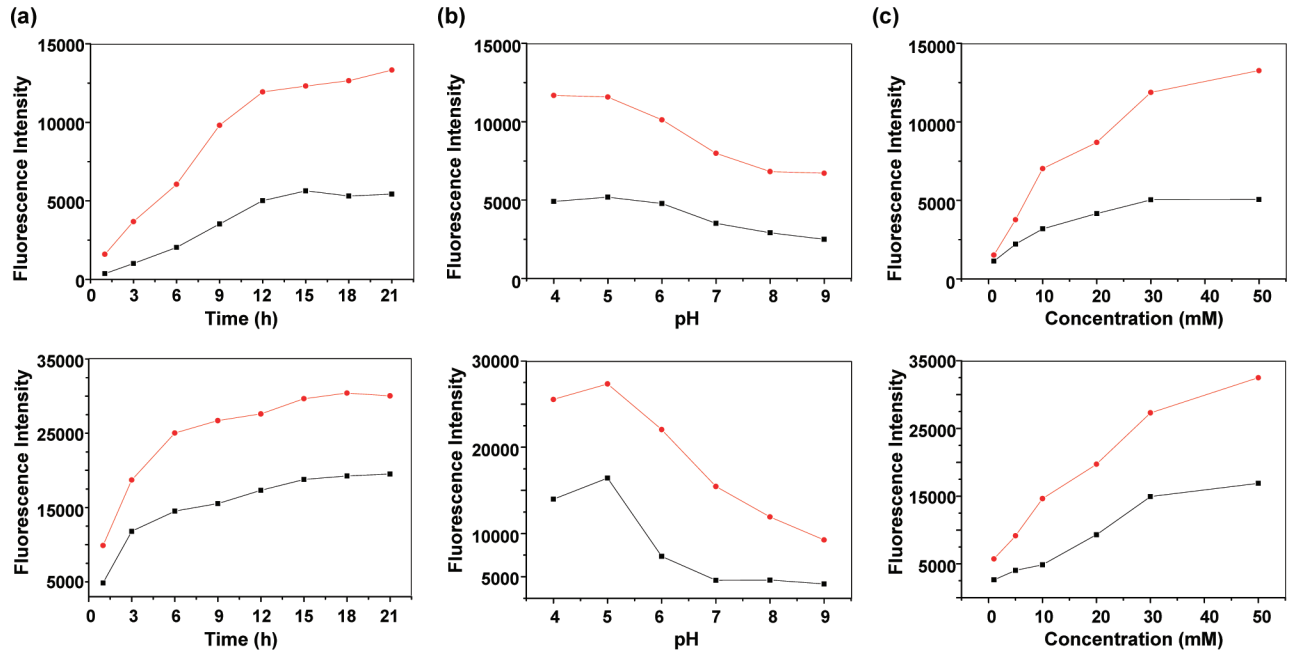


Figure 2. Immobilization of fucose (top) and *N,N′*-diacetylchitobiose (bottom) on the hydrazide (red line) and aminooxy (black line) functionalized glass surfaces as a function of (a) incubation time, (b) pH, and (c) concentration. Fluorescence intensities were measured after probing immobilized sugars with Cy5-AA and Cy3-WGA, respectively.

of Cy5-AA in PBS buffer containing 0.1% Tween 20 for 1 h at room temperature. The unbound proteins were removed by washing with PBS containing 0.1% Tween 20 by gentle shaking. After drying by purging with argon gas, the slide was scanned by using a microarray scanner. The fluorescence intensity of spots was measured and used for data processing. The means for replicate spots for each glycan was calculated, and then the mean and standard deviations for the three replicate experiments were determined and used for subsequent K_d measurement.

Apparent dissociation constants were obtained by the commercial nonlinear regression program *Origin 8.0*. Fluorescence intensity of replicate spots was plotted as a function of AA concentration. Nonlinear line-fitting to the equation was carried out,

$$Fl = Fl_{max}[P]_o/(K_d+[P]_o)$$

where Fl is the fluorescence intensity of spots for AA binding at a given concentration, Fl_{max} is the maximum fluorescence intensity, $[P]_o$ is the initial concentrations of the lectin, and K_d is the equilibrium dissociation constant for AA-surface-linked glycan interactions (*20*).

RESULTS AND DISCUSSION

The identification of suitable functional groups that efficiently react with free glycans is a prerequisite for the development of direct immobilization processes for the construction of carbohydrate microarrays. Reactions of aminooxy or hydrazide groups with unmodified carbohydrates, known to take place chemoselectively, have been widely employed for the synthesis of various glycoconjugates (*25, 26*). We have used this technique, involving covalent immobilization of unmodified glycans on the aminooxy- or hydrazide-derivatized surfaces, for the facile fabrication of carbohydrate microarrays.

Glass slides, functionalized by aminooxy and hydrazide groups, are prepared from the commercially available amine-modified glass slides by the route shown in Figure 1. The surface amino groups are reacted with succinic anhydride in order to produce carboxylic acid groups, which are then reacted with *N*-hydroxysuccinimide (NHS) and diisopropylcarbodiimide (DIC) to generate activated NHS ester. Reaction with 4,7,10-trioxa-1,13-tridecanediamine (**a**) results in insertion of oligo-

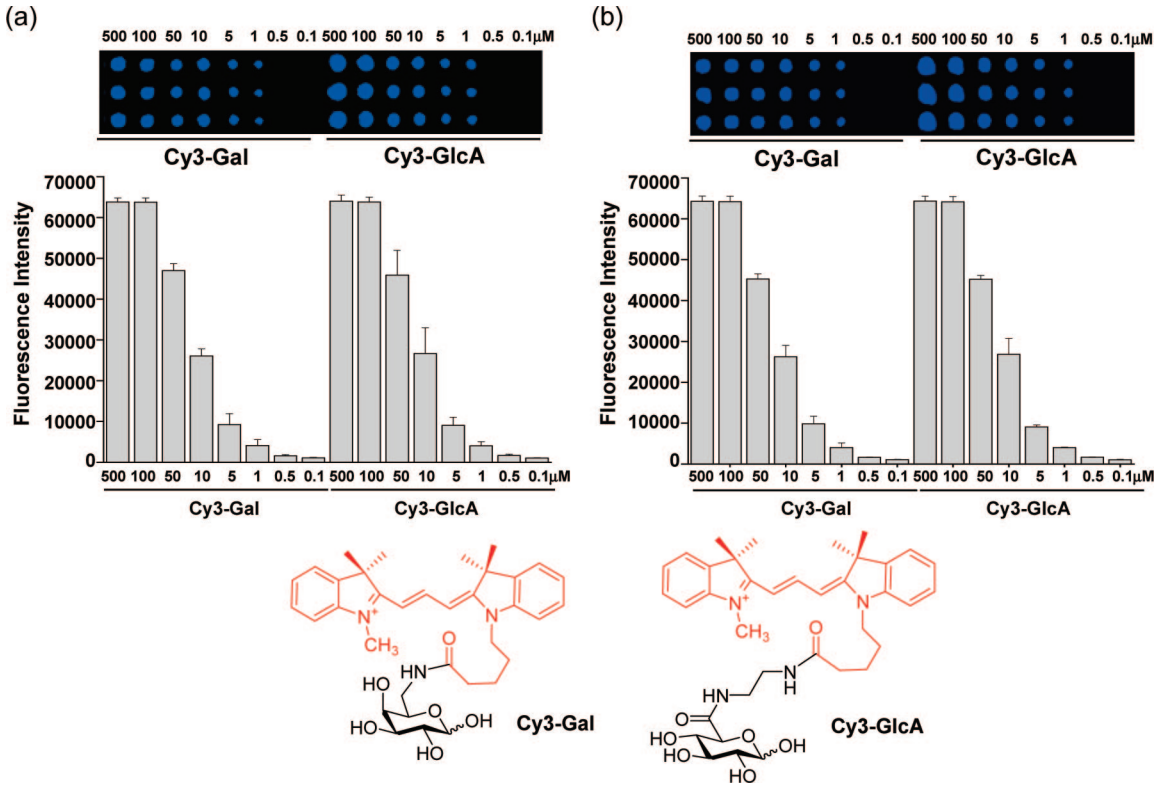


Figure 3. Immobilization of various concentrations (0.1−500 μM) of Cy3-Gal and Cy3-GlcA on (a) hydrazide and (b) aminooxy-coated glass slides.

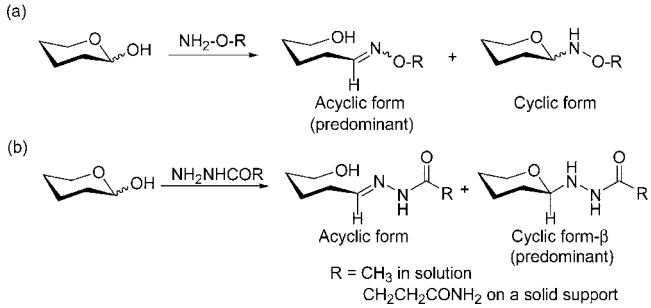


Figure 4. Products obtained from reactions of glycans with (a) aminooxy and (b) hydrazide groups.

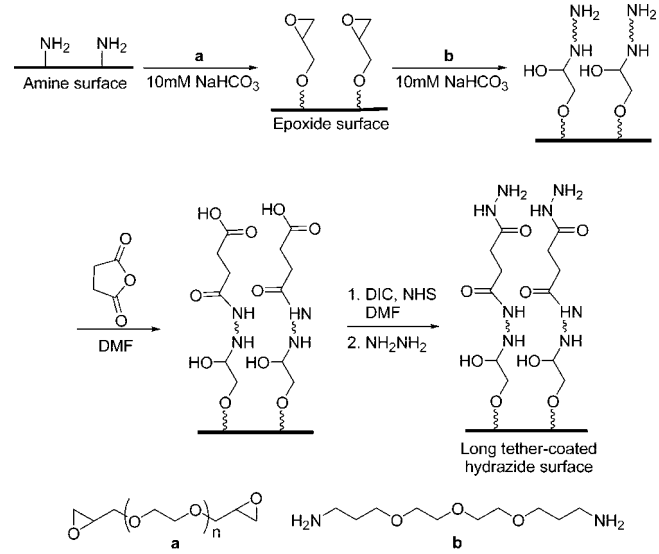
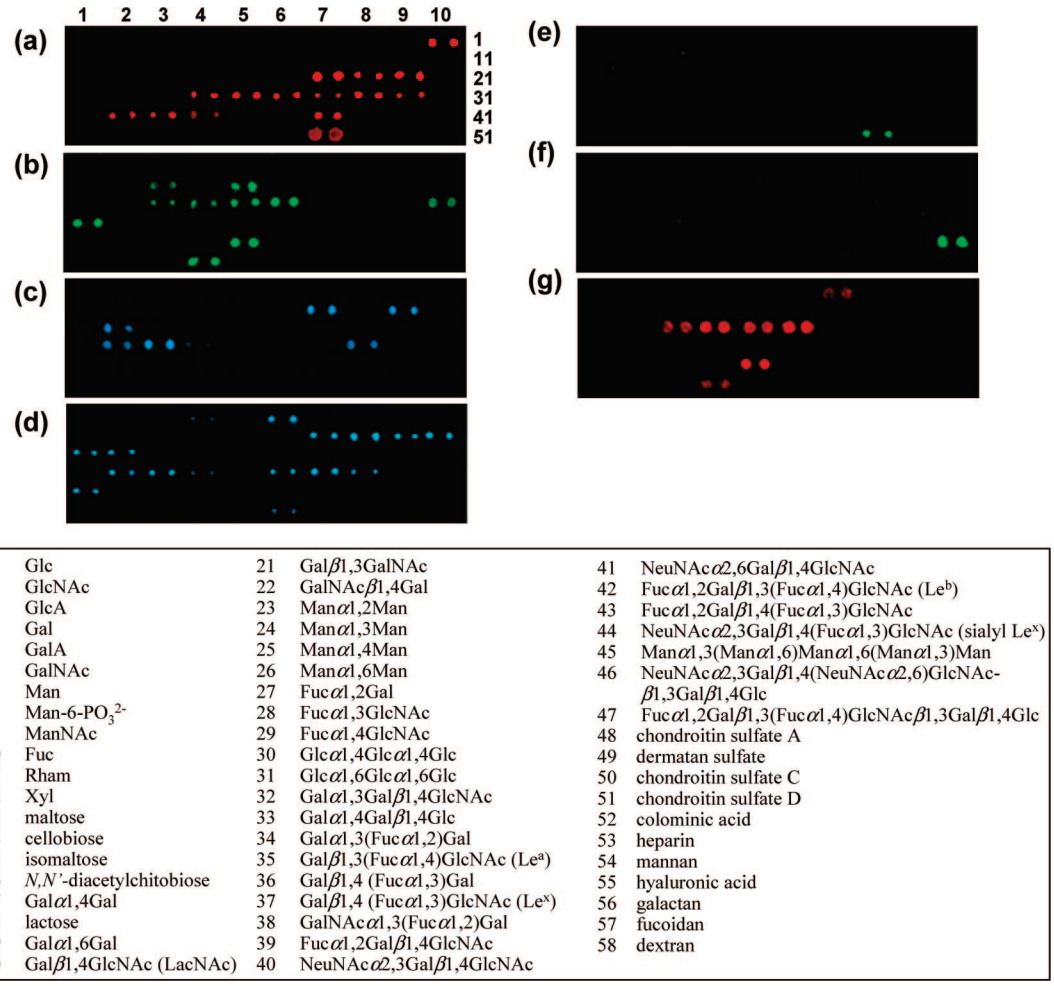


Figure 5. Preparation of hydrazide-coated glass slides containing a PEG linker.

(ethylene glycol) tether, which will serve to increase the accessibility of proteins to glycan ligands and to reduce nonspecific adsorption of proteins to the substrate during protein binding. The resulting amine-derivatized surfaces are sequentially treated with succinic anhydride, DIC-NHS, and hydrazine to prepare the target hydrazide-coated glass slide. The aminooxy-coated slide is obtained by treatment of the amine-derivatized slide with *N*-(2-bromoethoxy)phthalimide (**b**) followed by hydrazine-induced removal of phthaloyl group. Although the surface modification processes described above require synthetic procedures, they simply and reproducibly generate derivatized glass slides that can be stored in a desiccator for months.

Optimized conditions (temperature, time, pH, and concentration) have been probed for glycan immobilization on the hydrazide- and aminooxy-derivatized glass slides. To determine the optimized immobilization temperature and time, fucose and *N,N′*-diacetylchitobiose (30 mM in sodium phosphate buffer containing 30% glycerol, pH 5.0) were printed on both derivatized substrates by using a robotic pin-type microarrayer. Since ligation reactions are generally performed at relatively high temperature (*25*), the resulting slides were kept in a humid chamber for 1−21 h at temperatures of 22, 37, or 50 °C. To measure the extent of glycan immobilization, the slides were incubated with Cy5-*Aleuria aurantia* (Cy5-AA) and Cy3-wheat germ agglutinin (Cy3-WGA) for 0.5−1 h. The carbohydrate microarrays prepared at 50 °C show the highest fluorescence intensities after probing with proteins (data not shown). It has been reported previously that unmodified glycans are immobilized on a hydrazide-coated gold surface and an aminooxy-acetyl-coated glass surface at 18 °C and room temperature, respectively (*27, 28*). However, our results show that the glycans are not efficiently attached to hydrazide- and aminooxy-coated glass surfaces at low temperature. Finally, microarrays obtained using incubation time greater than 12 h incubation at 50 °C display similarly high fluorescence intensities (Figure 2a). The results of these experiments indicate that unmodified glycans



1	Glc	21	Galβ1,3GalNAc	41	NeuNAcα2,6Galβ1,4GlcNAc
2	GlcNAc	22	GalNAcβ1,4Gal	42	Fucα1,2Galβ1,3(Fucα1,4)GlcNAc (Le^b)
3	GlcA	23	Manα1,2Man	43	Fucα1,2Galβ1,4(Fucα1,3)GlcNAc
4	Gal	24	Manα1,3Man	44	NeuNAcα2,3Galβ1,4(Fucα1,3)GlcNAc (sialyl Le^x)
5	GalA	25	Manα1,4Man	45	Manα1,3(Manα1,6)Manα1,6(Manα1,3)Man
6	GalNAc	26	Manα1,6Man	46	NeuNAcα2,3Galβ1,4(NeuNAcα2,6)GlcNAc-β1,3Galβ1,4Glc
7	Man	27	Fucα1,2Gal		
8	Man-6-PO_3^{2-}	28	Fucα1,3GlcNAc	47	Fucα1,2Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glc
9	ManNAc	29	Fucα1,4GlcNAc	48	chondroitin sulfate A
10	Fuc	30	Glcα1,4Glcα1,4Glc	49	dermatan sulfate
11	Rham	31	Glcα1,6Glcα1,6Glc	50	chondroitin sulfate C
12	Xyl	32	Galα1,3Galβ1,4GlcNAc	51	chondroitin sulfate D
13	maltose	33	Galα1,4Galβ1,4Glc	52	colominic acid
14	cellobiose	34	Galα1,3(Fucα1,2)Gal	53	heparin
15	isomaltose	35	Galβ1,3(Fucα1,4)GlcNAc (Le^a)	54	mannan
16	*N,N′*-diacetylchitobiose	36	Galβ1,4 (Fucα1,3)Gal	55	hyaluronic acid
17	Galα1,4Gal	37	Galβ1,4 (Fucα1,3)GlcNAc (Le^x)	56	galactan
18	lactose	38	GalNAcα1,3(Fucα1,2)Gal	57	fucoidan
19	Galα1,6Gal	39	Fucα1,2Galβ1,4GlcNAc	58	dextran
20	Galβ1,4GlcNAc (LacNAc)	40	NeuNAcα2,3Galβ1,4GlcNAc		

Figure 6. Fluorescence images of glycan microarrays containing 58 glycans probed with (a) Cy5-AA, (b) FITC-ConA, (c) Cy3-BS-I, (d) Cy3-RCA_{120}, (e) antidextran antibody followed by incubation with FITC-labeled antiantibody, (f) antichondroitin 6-sulfate antibody followed by incubation with FITC-labeled antiantibody, and (g) *E. coli* ORN178 strain preincubated with SYTO 83 (see Supporting Information for quantitative fluorescence intensity data).

are efficiently immobilized after 12 h incubation at 50 °C. It is important to note that extensive washing of the slides with PBS buffer after immobilization of the carbohydrates does not affect lectin binding, demonstrating that the covalent linkages between carbohydrates and aminooxy or hydrazide groups on the surface are stable.

To investigate the effects of pH and concentration on immobilization efficiencies, fucose and *N,N′*-diacetylchitobiose were immobilized on both substrates at pHs between 4 and 9 and concentrations in the range 1−50 mM for a the fixed incubation time (12 h) and temperature (50 °C). Probing of the slides with proteins reveals that 20−30 mM concentration of carbohydrates and pH 4−5 are ideal for efficient immobilization (Figure 2b,c). Although glycans immobilized at lower concentrations (1−5 mM) can interact with proteins, higher concentrations (20−30 mM) of glycans are desired in order to obtain reproducible and reliable binding results, especially in the case of glycans that are only weakly bound to proteins. It is worthwhile mentioning that the immobilization concentrations used in this procedure are relatively high in comparison with those used in other methods. However, this immobilization technique leads to efficient construction of microarrays, since a little of underivatized glycans (spotting volume: 1 nL) are required.

An interesting observation made in these experiments is that carbohydrates immobilized on hydrazide-coated glass slides exhibit more intense fluorescence signals after lectin binding than those immobilized on aminooxy-coated slides. There are two possible explanations that account for this result. The first is that hydrazide groups on the surface react with free glycans more efficiently than do aminooxy groups. To examine this possibility, galactose (Gal) and glucuronic acid (GlcA), linked to a fluorescence probe (Cy3) at their C-6 positions, were immobilized on both types of substrate under the same conditions (0.1−500 μM concentrations for 12 h at 50 °C). As shown in Figure 3, the two glycans were attached to both substrates with similar efficiencies, indicating that two functional groups on the surface have a similar reactivity with glycans.

A second possible explanation focuses on differences in the nature of anomeric linkage involved in sugar attachment to the surfaces. It is known that reactions of unmodified glycans with aminooxy group-possessing substances produce acyclic adducts preferentially (Figure 4a) (*26, 29*). However, it has been reported that cyclic products with β-configuration at the anomeric centers are produced predominantly in reactions of carbohydrates with substances containing hydrazide groups (Figure 4b) (*25, 30, 31*). Consequently, the nature of the linkage present in products obtained from reactions of carbohydrates with hydrazide groups was investigated. For this study, four carbohydrates (glucose, mannose, fucose, and maltose) were reacted with hydrazide derivatives in solution and on a solid support (pH 5, 50 °C, 12 h). NMR analyses of mixtures obtained from these reactions show that β-anomeric adducts are produced predominantly (more than 90%) in both cases (*24*). Therefore, it is likely that the stronger binding of proteins to carbohydrates attached to hydrazide-modified surface rather than aminooxy-coated sur-

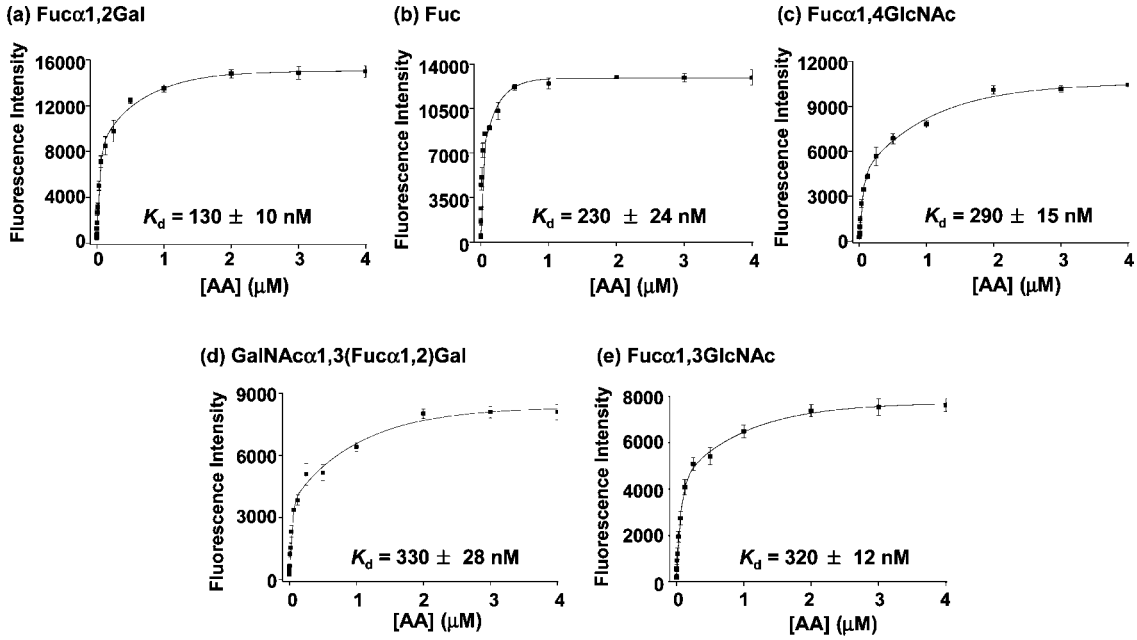


Figure 7. Determination of dissociation constants (K_d) for (a) AA-Fucα1,2Gal, (b) AA-fucose, (c) AA-Fucα1,4GlcNAc, (d) AA-GalNAcα1,3(Fucα1,2)Gal, and (e) AA-Fucα1,3GlcNAc interactions using carbohydrate microarrays.

faces results from a higher ratio of cyclic to acyclic linkages present in the former system.

The effect of insertion of a tether between hydrazide groups and the surface on protein binding was examined. For this purpose, hydrazide-coated glass slides containing poly(ethylene glycol) (PEG) were prepared by the procedure shown in Figure 5. A commercially available amine-coated glass slide is first treated with poly(ethylene glycol) diglycidyl ether (**a**) to place epoxide groups on the surface. The resulting substrate is then reacted with diamine **b** to introduce amino groups. The PEG-containing hydrazide-functionalized substrates are obtained by sequential reactions with succinic anhydride, DIC-NHS, and hydrazide. The results of protein binding experiments show that hydrazide substrates containing a long spacer arm give higher S/N ratios (around 1.5 times) than those arising from hydrazide substrates containing a shorter linkage. As a consequence, hydrazide-coated glass slides tethered by the long spacer arm were used throughout the remainder of this study. To our knowledge, these hydrazide-modified surfaces containing PEG were prepared for the first time for the construction of microarrays.

Prior to applications of carbohydrate microarrays, we measured the number of carbohydrates immobilized on the surface. To this end, various concentrations (500 μM−0.5 μM) of Cy3-Gal were printed on the hydrazide-coated slide, and the fluorescence intensity of the printed spots was measured before and after immobilization. The density of maximum carbohydrate immobilized on the surface was measured to be $10^{14}-10^{15}$ molecules/cm^2, a similar value to carbohydrates attached to the glass surface by other methods (see Supporting Information) (*32*).

To demonstrate the utility of this immobilization method and the application of the arrayed glycans to the rapid analysis of glycan−protein recognition events, 58 glycans (1 nL, 30 mM) shown in Figure 6 were printed in duplicate on a hydrazide-derivatized glass slide in a microarray format. The glycans comprise simple carbohydrates, oligosaccharides, and polysaccharides. The microspotted slide was kept at 50 °C for 12 h, washed with water, and dried. Since lectins serve as important models for studying glycan−protein interactions at the molecular level, the glycan microarrays were probed with the four fluorescent dye-labeled lectins, Cy5-AA, FITC-ConA, Cy3-*Bandeiraea simplicifolia* I (Cy3-BS-I), and Cy3-*Ricinus communis* agglutinin 120 (Cy3-RCA$_{120}$). Fluorescence data of carbohydrate microarrays obtained after probing with lectins are shown in Figure 6. The data demonstrate clearly that Cy5-AA interacts selectively with its cognate fucose-containing ligands and that it does not bind to the other sugars on the microarrays (Figure 6a). The carbohydrate microarrays treated with FITC-ConA exhibits binding to α-mannose and α-glucose containing ligands, a result that is consistent with the established specificity of this lectin (Figure 6b). However, this lectin does not recognize monomeric mannose and glucose that are attached to the surface with β-configurations.

Binding specificities of two galactose/*N*-acetylgalactosamine binding proteins, Cy3-labeled BS-I and RCA$_{120}$, were determined by using these microarrays. The results of microarray experiments indicate that BS-I recognizes terminal α-galactose and α-*N*-acetylgalactosamine residues preferentially. In contrast, BS-I rarely interacts with monomeric galactose and *N*-acetylgalactosamine owing to the fact that these glycans are immobilized to the surface with β-configuration (Figure 6c). However, RCA$_{120}$ was shown to bind to a broader range of galactose/*N*-acetylgalactosamine containing glycans than does BS-1 (Figure 6d). RCA$_{120}$ recognizes terminal galactose and *N*-acetylgalactosamine residues as well as galactan, but it rarely binds to Le^a (*33*). Subsequently, binding specificities of antibodies were analyzed by using carbohydrate microarrays. The results of experiments, in which carbohydrate microarrays were probed with antidextran and antichondroitin 6-sulfate antibodies followed by treatment with FITC-secondary IgG antibody, show that each antibody interacts with the corresponding antigen selectively (i.e., antidextran antibody binds to dextran and antichondroitin 6-sulfate antibody binds to chondroitin sulfate C) (Figure 6e,f). The observations made in this effort demonstrate that carbohydrate microarrays prepared by a direct immobilization method can be effectively employed to assess carbohydrate-binding properties of proteins.

Many bacteria, including pathogens, express specific lectins on pili. Pathogenic properties are caused by the initial binding of pathogens to host cells through specific glycan−protein interactions. In order to demonstrate that the fabricated glycan microarrays can be used to detect pathogens, microarrays containing 58 glycans were incubated with *E. coli* ORN178

strain, which was pretreated with SYTO 83. In accord with the fact that the ORN178 strain expresses a mannose-binding protein, a gene product of *fimH*, on pili, the microspots containing mannose epitopes were found to display fluorescence (Figure 6g) (*34, 35*). In contrast, glycan microarrays treated with the ORN208 strain which lacks the *fimH* gene do not show fluorescence signals in the regions containing these glycans (data not shown).

Recently, we developed a procedure in which carbohydrate microarrays are used to determine binding affinities of glycans for protein (*20*). The microarrays, prepared through the one-step, direct immobilization method described above, were applied to measuring dissociation constants (K_d) associated with lectin surface-linked glycan interactions. For this study, the five fucose-containing glycans, fucose, Fucα1,2Gal, Fucα1,3GlcNAc, Fucα1,4GlcNAc, and GalNAcα1,3(Fucα1,2)Gal, were printed in replicates of five on a hydrazide-derivatized surface. The resulting microarrays were incubated with various concentrations (0.1 nM-4 μM) of Cy5-AA. After 1 h incubation, the microarrays were thoroughly washed with PBS buffer to remove unbound lectin. Fluorescence intensities of microspots were quantitated by using a microarray scanner. The K_d values of Fucα1,2Gal, fucose, Fucα1,4GlcNAc, GalNAcα1,3(Fucα1,2)Gal, and Fucα1,3GlcNAc are determined to be 1.3×10^{-7} M, 2.3×10^{-7} M, 2.9×10^{-7} M, 3.3×10^{-7} M, and 3.2×10^{-7} M, respectively (Figure 7). These studies demonstrate clearly that this immobilization technique is suitable for measuring quantitative binding affinities of glycans for protein.

CONCLUSIONS

The investigation described above has led to the development of a simple and efficient method for the construction of carbohydrate microarrays that relies on site-specific and covalent immobilization of unmodified carbohydrates on a hydrazide-coated surface. The insertion of a hydrophilic poly(ethylene glycol) linker between hydrazide groups and the surface leads to the desired orientational flexibility and increased accessibility of binding partners to the glycan ligands. Protein and cell-binding experiments show that, irrespective of their size, all types of carbohydrates undergo efficient covalent bonding to the hydrazide-coated surface. The glycan microarrays, generated in this way, are applicable to the rapid analysis of carbohydrate−protein recognition events, the detection of pathogens, and measurements of quantitative binding affinities of glycans for protein. This immobilization method can be readily modified to enable the identification and quantification of binding patterns of biologically relevant materials. As such, the technique should open new avenues for elucidating glycan−protein interactions. Of great significance is the fact that this free glycan based immobilization procedure can be used by scientists who lack organic synthesis experience.

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Supporting Information Available: Synthesis of Cy3-labeled Gal and GlcA, measurement of the density of an immobilized carbohydrate, and quantitative analysis of fluorescence intensity of a carbohydrate microarray probed with proteins and cells. This material is available free of charge via Internet at http://pubs.acs.org.

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