ORGANIC LETTERS

2005
Vol. 7, No. 19
4269−4272

Facile Preparation of Carbohydrate Microarrays by Site-Specific, Covalent Immobilization of Unmodified Carbohydrates on Hydrazide-Coated Glass Slides

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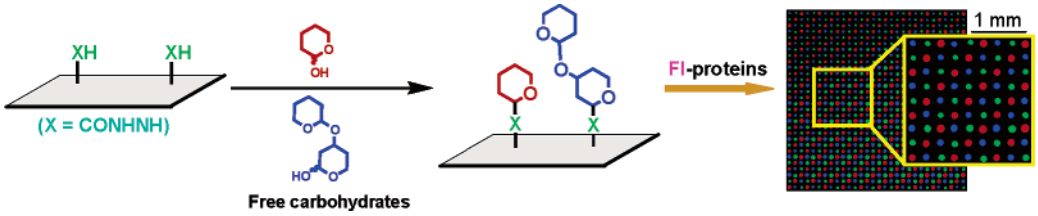
Received July 24, 2005

ABSTRACT



A new, simple and efficient immobilization method to attach mono-, di-, oligo-, and polysaccharides to hydrazide-coated glass slides was developed. Protein and cell-binding experiments show that the carbohydrate microarrays prepared by this method are applicable for the rapid analysis of protein–carbohydrate interactions and fast detection of pathogens.

Carbohydrate microarrays have received considerable attention in the post-genomic era as part of advanced technologies used in functional glycomics.[1] These microarrays can be used for (1) high-throughput analysis of glycan−protein interactions, (2) characterization of carbohydrate-processing enzymes, (3) quantitative determination of binding affinities, (4) analysis of glycomes, and (5) detection of pathogens.[1] The most general method for the preparation of carbohydrate microarrays is site-specific and covalent attachment of chemically modified carbohydrates to appropriately derivatized surfaces (Figure 1a).[2] For example, we have fabricated carbohydrate microarrays by immobilizing maleimide- or hydrazide-linked carbohydrates on thiol- or epoxide-derivatized glass slides, respectively.[2a,b,j] Carbohydrate microarrays also have been prepared by site-specifically but noncovalently attaching neoglycolipids to the underivatized surface (Figure 1b).[3] Although these two approaches can be used to

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10.1021/ol051753z CCC: $30.25
Published on Web 08/24/2005

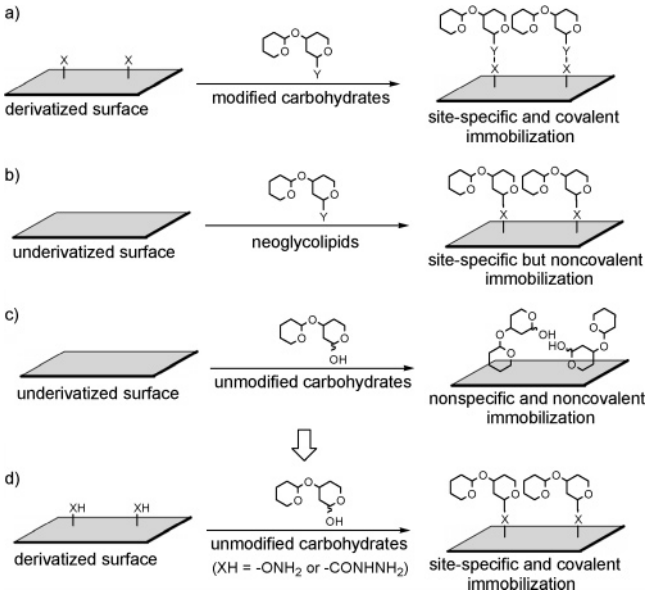

Figure 1. Immobilization strategies for the preparation of carbohydrate microarrays.

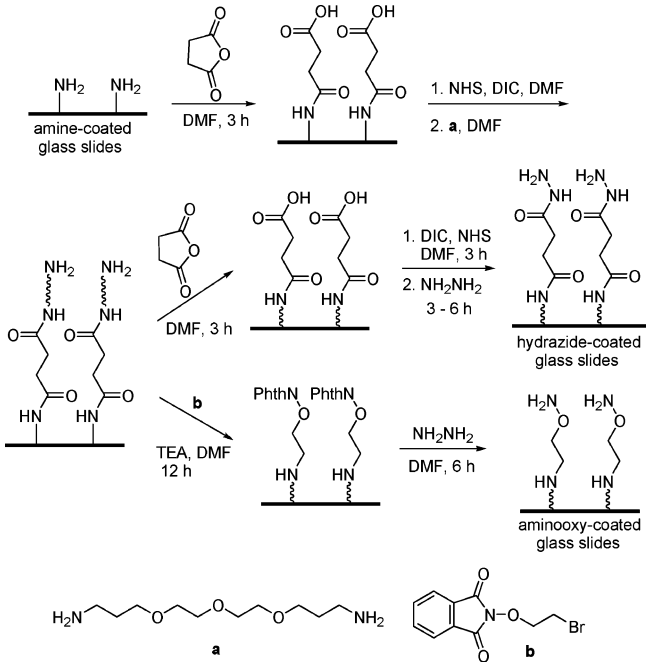

Figure 2. Preparation of aminooxy- and hydrazide-coated glass slides.

efficiently immobilize modified carbohydrates on the surface, the required synthetic manipulations are sometimes difficult and time consuming.

Alternatively, glycan microarrays have been obtained from the nonspecific and noncovalent adsorption of unmodified carbohydrates on underivatized surfaces (Figure 1c).[4] Although this technique is useful for the fabrication of the polysaccharide arrays, it has the disadvantage of being size dependent and nonspecific in the immobilization of carbohydrate probes. A potentially superior approach to construct the carbohydrate microarrays would involve covalent, site-specific, and size-independent immobilization of unmodified carbohydrates (Figure 1d). Herein we describe the results of recent studies, in which a facile method to prepare carbohydrate microarrays on derivatized glass slides has been developed. In addition, this new technique has been applied to the high-throughput analysis of protein binding and the detection of pathogens.

Initial consideration was given to identify functional groups that efficiently and selectively react with unmodified carbohydrates. Reactions of aminooxy or hydrazide groups with free carbohydrates are known to be chemoselective and have been widely used for the synthesis of various glycoconjugates.[5,6] Consequently, these processes were used for the site-specific and covalent immobilization of free carbohydrates on glass slides. The required aminooxy- and hydrazide-coated glass slides were prepared according to the sequence shown in Figure 2. The long tether of 4,7,10-trioxa-1,13-tridecanediamine (**a**) was employed in the linking processes to reduce steric hindrance and nonspecific interactions during protein binding to carbohydrates on the surface.

Conditions (temperature, time, pH, and concentration) for the immobilization reactions were then optimized. To find the proper temperature and time for immobilization, fucose and *N*,*N*′-diacetylchitobiose (30 mM in sodium phosphate buffer containing 30% glycerol, pH 5.0) were printed on an aminooxy- or hydrazide-derivatized surface and the resulting slides were incubated in a humid chamber at 22, 37, and 50 °C. After 1−21 h incubation, the slide was probed with Cy5-*A. aurantia* (AA) and Cy3-*T. vulgaris* (TV or wheat germ agglutinin) for 0.5−1 h.[7] Carbohydrate chips prepared at 50 °C showed the highest fluorescent intensity among the tested temperature (data not shown). In addition, fucose and *N*,*N*′-diacetylchitobiose-immobilized arrays obtained from greater than 12 h incubation at 50 °C exhibited similarly high fluorescent intensities (Figure 3).

Next, we examined the optimal immobilization pH and concentration at a fixed time (12 h) and temperature (50 °C). It was found that about 30 mM concentration of carbohydrates and pH 4−5 were ideal for efficient immobilization.[8]

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(8) See the Supporting Information.

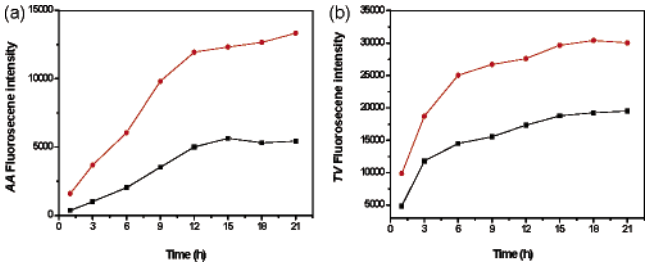

Figure 3. Time-dependent immobilization of (a) fucose and (b) *N*,*N*′-diacetylchitobiose on the aminooxy (black line) and hydrazide (red line) coated glass slides.

It is worth noting that extensive washing of the slides with PBS buffer after immobilizing the carbohydrates does not affect lectin binding. This observation demonstrates that the covalent linkages between carbohydrates and aminooxy or hydrazide groups on the surface are stable. Interestingly, higher fluorescence signals after lectin probing were observed for carbohydrates immobilized on hydrazide-coated glass slides than those immobilized on aminooxy-coated slides. It is known that reactions between carbohydrates and substances containing aminooxy groups generate acyclic products preferentially (Figure 4a).[6,9] In contrast, it has been

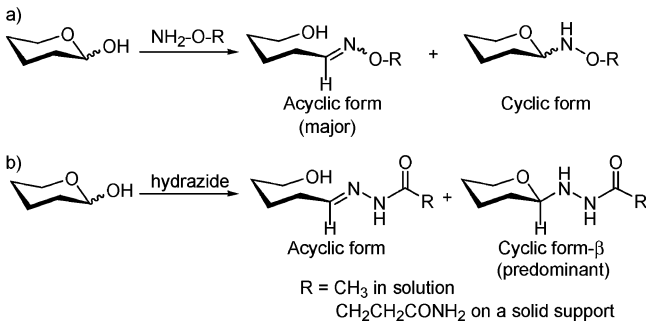

Figure 4. Adducts obtained from reactions of carbohydrates with (a) aminooxy and (b) hydrazide groups.

reported that cyclic adducts with β-configuration are produced predominantly in reactions of carbohydrates with hydrazide-containing compounds (Figure 4b).[5,10] Since the hydrazide-coated glass slides were used for the construction of carbohydrate microarrays in our further studies (vide infra), the anomeric structures of adducts obtained from

(9) Reactions between carbohydrates and N-alkylated aminooxy groups generated cyclic adducts predominantly. However, the cyclic adducts were formed in low yield in the case of some carbohydrates. See: (a) Carrasco, M. R.; Brown, R. T.; Serafimova, I. M.; Silva, O. *J. Org. Chem.* **2003**, *68*, 195. (b) Carrasco, M. R.; Nguyen, M. J.; Burnell, D. R.; MacLaren, M. D.; Hengel, S. M. *Tetrahedron Lett.* **2002**, *43*, 5727. (c) Peri, F.; Dumy, P.; Mutter, M. *Tetrahedron* **1998**, *54*, 12269.

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Table 1. Carbohydrates Used for the Fabrication of Carbohydrate Microarrays

monosaccharides	disaccharides	oligosaccharides
1. Glc	11. maltose	17. Fucα1,3(Galβ1,4)Glc (FucLac)
2. GlcNAc	12. cellobiose	18. NeuNAcα2,3Galβ1,4-GlcNAc (NeuNAcLacNAc)
3. GlcA	13. *N*,*N*′-diacetyl-chitobiose	19. sialyl Le^x
4. Gal	14. lactose	20. Fucα1,2Galβ1,3-(Fucα1,4)GlcNAc-β1,3Galβ1,4Glc
5. GalNAc	15. Galβ1,4GlcNAc (LacNAc)	polysaccharides
6. Man	16. Manα1,6Man (mannobiose)	21. mannan
7. ManNAc		
8. Fuc		
9. Rham		
10. Xyl		

reactions of carbohydrates with hydrazide groups were determined. NMR analysis of mixtures obtained by reactions of four carbohydrates (Glc, Man, Fuc, and maltose) with hydrazide groups in solution and on a solid support (pH 5, 50 °C, 12 h) showed that β-anomeric products were generated predominantly.[8] On the basis of these observations, we concluded that stronger binding of proteins to carbohydrates immobilized on hydrazide-coated slides than their aminooxy-coated counterparts could be a consequence of the higher ratio of cyclic to acyclic linkages obtained in the former system.

To profile protein binding with the glycan microarrays, 21 mono-, di-, oligo-, and polysaccharides (30 mM in 30% glycerol in sodium phosphate buffer at pH 5.0 except mannan (1 mg/mL)) shown in Table 1 were printed on hydrazide-coated surfaces by using a pin-type microarrayer. After incubation for 12 h at 50 °C in a humid chamber, the glycan microarrays were probed with Cy3-TV, Cy5-AA, FITC-ConA, and anti-sialyl Le^x antibody (Figure 5a−d). Probing with Cy3-TV showed that this lectin bound strongly to *N*,*N*′-diacetylchitobiose (**13**), less tightly to GlcNAc (**2**) and sialyl Le^x (**19**), and weakly to GalNAc (**5**), LacNAc (**15**), and NeuNAcLac NAc (**18**). The glycan microarrays treated with

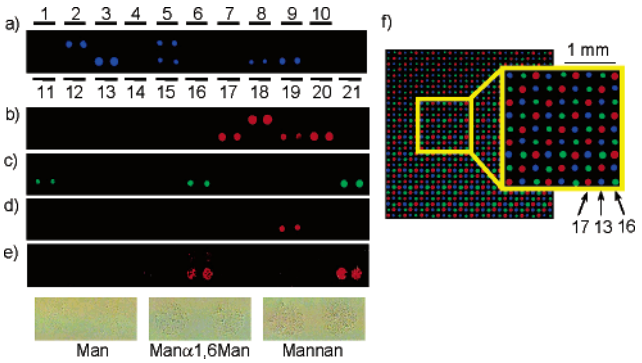

Figure 5. Fluorescence images of carbohydrate microarrays containing 21 carbohydrates probed with (a) Cy3-TV, (b) Cy5-AA, (c) FITC-ConA, (d) anti-sialyl Le^x antibody followed by incubation with Cy5-anti-antibody, (e) *E. coli* ORN178 preincubated with PI (bottom; phase contrast micrographs in the region of Man, Manα1,6Man, and mannan), and (f) a fluorescence image of 900 microspots (30 × 30) consisting of *N*,*N*′-diacetylchitobiose (**13**), Manα1,6Man (**16**), and FucLac (**17**).

Cy5-AA exhibited strong lectin binding to Fuc (**8**), FucLac (**17**), and hexasaccharide **20** but very weak binding to sialyl Le^x (**19**). Microarrays probed with FITC-ConA showed strong lectin binding to mannan (**21**), and less tight binding to Manα1,6Man (**16**) and very weak binding to maltose (**11**).[11] The relative binding affinities of the lectins with the carbohydrate microarrays are consistent with those observed for solution-based assays.[7] The carbohydrate microarrays were also probed with the anti-sialyl Le^x antibody. As expected, the antibody only recognized sialyl Le^x (**19**).

Many bacteria, including pathogens, express specific carbohydrate-binding proteins on pili. The initial attachment of pathogens to host cells through specific carbohydrate−protein interactions confers pathogenic properties.[12] To demonstrate an ability to detect pathogens, the glycan microarrays were treated with *E. coli* ORN178 and ORN208, preincubated with propidium iodide (PI). Since the ORN178 strain expresses a mannose-binding protein, a gene product of *fimH*, on the cell surface, the microspots containing Manα1,6Man and mannan exhibit fluorescence signals with stronger intensity in the region of mannan than Manα1,6Man (Figure 5e).[2e,13,14] In addition, the ORN178 strain also recognized the microspot containing β-anomeric mannose with a very weak binding affinity.[14] In contrast, glycan microarrays treated with the ORN208 strain that lacks the *fimH* gene did not show a fluorescence signal.

To demonstrate the applicability of this methodology to the preparation of carbohydrate microarrays containing a number of microspots, we constructed a slide containing 900 (30×30) microspots. This array was then probed with Cy3-TV, Cy5-AA, and FITC-ConA. As shown in Figure 5f, the three carbohydrates on this slide were selectively recognized by the corresponding lectins.

In conclusion, we have developed a new method for the facile fabrication of carbohydrate microarrays by using the site-specific and covalent immobilization of free carbohydrates on modified glass surfaces. Protein and cell-binding experiments show that, irrespective of their size, all types of carbohydrates undergo efficient covalent bonding to the hydrazide-derivatized surface to generate carbohydrate microarrays that are applicable for the rapid analysis of specific carbohydrate−protein interactions and fast detection of pathogens.[15]

Acknowledgment. This work was supported by grants from the NRL program (KOSEF) and the Protein Chip Technology Program (MOST).

Supporting Information Available: Fabrication of carbohydrate microarrays, optimization of immobilization, and NMR spectra of reaction mixtures of carbohydrates and hydrazide in solution and on a solid support. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) However, ConA did not recognize mannose, glucose, and *N*-acetylglucosamine owing to an improper β-configuration at the anomeric position.

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(15) The possibility that mannan is immobilized on the surface by nonspecific adsorption is not ruled out. However, lectin binding studies demonstrate that the modified glass slides are suitable for immobilization of this polysaccharide.