J|A|C|S
ARTICLES
Published on Web 03/26/2004

Carbohydrate Chips for Studying High-Throughput Carbohydrate−Protein Interactions

Sungjin Park, Myung-ryul Lee, Soon-Jin Pyo, and Injae Shin*

Contribution from the Department of Chemistry, Yonsei University, Seoul 120-749, Korea

Received October 22, 2003; E-mail: injae@yonsei.ac.kr

Abstract: Carbohydrate−protein interactions play important biological roles in living organisms. For the most part, biophysical and biochemical methods have been used for studying these biomolecular interactions. Less attention has been given to the development of high-throughput methods to elucidate recognition events between carbohydrates and proteins. In the current effort to develop a novel high-throughput tool for monitoring carbohydrate−protein interactions, we prepared carbohydrate microarrays by immobilizing maleimide-linked carbohydrates on thiol-derivatized glass slides and carried out lectin binding experiments by using these microarrays. The results showed that carbohydrates with different structural features selectively bound to the corresponding lectins with relative binding affinities that correlated with those obtained from solution-based assays. In addition, binding affinities of lectins to carbohydrates were also quantitatively analyzed by determining IC_{50} values of soluble carbohydrates with the carbohydrate microarrays. To fabricate carbohydrate chips that contained more diverse carbohydrate probes, solution-phase parallel and enzymatic glycosylations were performed. Three model disaccharides were in parallel synthesized in solution-phase and used as carbohydrate probes for the fabrication of carbohydrate chips. Three enzymatic glycosylations on glass slides were consecutively performed to generate carbohydrate microarrays that contained the complex oligosaccharide, sialyl Le^x. Overall, these works demonstrated that carbohydrate chips could be efficiently prepared by covalent immobilization of maleimide-linked carbohydrates on the thiol-coated glass slides and applied for the high-throughput analyses of carbohydrate−protein interactions.

Introduction

Specific interactions between carbohydrates and proteins occur in a wide variety of important biological processes, including cell−cell communication, cell adhesion, fertilization, differentiation, development, inflammation, and tumor cell metastasis.[1] These interactions also initiate infection of host cells by bacteria and viruses.[1] Therefore, understanding of the molecular basis for carbohydrate−protein interactions not only provides valuable information on biological processes in living organisms but it also aids the development of potent biomedical agents.[2]

To date, biophysical and biochemical methods have been mainly used to probe the details of carbohydrate−protein interactions. For example, X-ray crystallographic and NMR spectroscopic techniques have been employed to determine binding modes between carbohydrates and proteins.[3] The results of studies using surface plasmon resonance (SPR) spectroscopy and isothermal titration calorimetry (ITC) have provided information on the binding affinities of carbohydrates to proteins.[4] In addition, specifically modified synthetic carbohydrates have been used to elucidate the molecular basis of carbohydrate−protein interactions.[5]

In contrast, much less attention has been given to the development of high-throughput methods to probe these interactions. During the past decade, DNA chips and protein chips have been fabricated and utilized in genomic, transcriptomic, and proteomic investigations. DNA chips have been extensively used for studying how patterns of gene expression change in diseases and for simultaneously tracking the activities of many genes.[6] Protein chips, which are more difficult to fabricate than DNA chips owing to easy denaturation of protein probes, have been exploited in high-throughput studies of molecular interactions[7,8] and in profiling protein expression in normal and

(1) For recent reviews, see (a) Roth, J. *Chem. Rev.* **2002**, *102*, 285. (b) Ritchie, G. E.; Moffatt, B. E.; Sim, R. B.; Morgan, B. P.; Dwek, R. A.; Rudd, P. M. *Chem. Rev.* **2002**, *102*, 305. (c) Zachara, N. E.; Hart, G. W. *Chem. Rev.* **2002**, *102*, 431. (d) Bertozzi, C. R.; Kiessling, L. L. *Science* **2001**, *291*, 2357.

(2) (a) Danishefsky, S. J.; Allen, J. R. A. *Angew. Chem., Int. Ed.* **2000**, *39*, 836. (b) Walker, F.; Vicens, Q.; Westhof, E. *Curr. Opin. Chem. Biol.* **1999**, *3*, 694. (c) Sears, P.; Wong, C.-H. *Angew. Chem., Int. Ed.* **1999**, *38*, 2300.

(3) (a) Mechref, Y.; Novotny, M. V. *Chem. Rev.* **2002**, *102*, 321. (b) Wormald, M. P.; Petrescu, A. J.; Pao, Y.-L.; Glithero, A.; Elliott, T.; Dwek, R. A. *Chem. Rev.* **2002**, *102*, 371. (c) Vyas, N. K. *Curr. Opin. Struct. Biol.* **1991**, *1*, 732. (c) Wyss, D. F.; Choi, J. S.; Li, Jing.; Knoppers, M. H.; Willis, K. J.; Arulanandam, A. R. N.; Smolyar, A.; Reinherz, E. L.; Wagner, G. *Science* **1995**, *269*, 1273.

(4) (a) Dam, T. K.; Brewer, C. F. *Chem. Rev.* **2002**, *102*, 387. (b) Green, R. J.; Frazier, R. A.; Shakesheff, K. M.; Davies, M. C.; Roberts, C. J.; Tendler, S. J. B. *Biomaterials* **2000**, *21*, 1823. (c) Mann, D. A.; Kanai, M.; Maly, D. J.; Kiessling, L. L. *J. Am. Chem. Soc.* **1998**, *120*, 10575.

(5) Lee, Y. C.; Lee, R. T.; Rice, K.; Ichikawa, Y.; Wong, T.-C. *Pure Appl. Chem.* **1991**, *63*, 499.

(6) (a) Perou, C. M. *Nature* **2000**, *406*, 747. (b) Ramsey, G. *Nat. Biotechnol.* **1998**, *16*, 40. (c) Marshall, A.; Hodgson, J. *Nat. Biotechnol.* **1998**, *16*, 27. (d) DeRisi, J. L.; Lyer, V. R.; Brown, P. O. *Science* **1997**, *278*, 680.

(7) (a) Templin, M. F.; Stoll, D.; Schrenk, M.; Traub, P. C.; Vöhringer, C. F.; Joos, T. O. *Trends Biotechnol.* **2002**, *20*, 160. (b) Weinberger, S. R.; Dalmasso, E. A.; Fung, E. T. *Curr. Opin. Chem. Biol.* **2001**, *6*, 86.

10.1021/ja0391661 CCC: $27.50

diseased states.[9] Much in the same way that DNA chips and protein chips are used as high-throughput analytic tools, carbohydrate chips have the potential of serving as useful high-throughput analytical tools for elucidating recognition events between carbohydrates and proteins in glycomic researches.

Recent efforts have led to the development of carbohydrate chips by either covalent or noncovalent immobilization strategy.[10] Wang et al. and Feizi et al. successfully fabricated carbohydrate microarrays by immobilizing various oligosaccharides on nitrocellulose or nitrocellulose-coated glass slides without the use of chemical linking techniques.[10a,b] The immobilization efficiency of carbohydrates was size-dependent; the smaller carbohydrates were less retained on the nitrocellulose surface than the larger ones after extensive washing. In addition, Mrksick et al. fabricated carbohydrate arrays on gold surfaces via Diels−Alder reactions between cyclopentadiene-possessing carbohydrates and benzoquinone-coated gold surface.[10c] The results indicated that this immobilization method was highly efficient and oligo(ethylene glycol)-linked carbohydrate monolayers displayed minimal nonspecific adsorption with proteins.

We also developed a new method for constructing carbohydrate microarrays that relied on covalent immobilization of carbohydrates on glass slides via chemoselective ligation between maleimide-linked sugars and thiol-derivatized glass surface.[10d] We demonstrated that linker lengths and immobilization concentrations of carbohydrates were important factors in governing protein binding to these microarrays. Importantly, the results showed that the specificity of protein−carbohydrate interactions on the solid surfaces resembled that observed in solution.

In our more recent work in this area, carbohydrate chips containing more diverse carbohydrate probes have been fabricated to analyze the binding affinities of carbohydrates to several lectins. Furthermore, three disaccharides were synthesized by solution-phase parallel glycosylations, and their binding affinities to lectins on the solid surface were measured and compared with those of monosaccharides. Finally, a carbohydrate chip containing the complex oligosaccharide, sialyl Le^x, was also prepared by three consecutive glycosyl transferase-catalyzed reactions. The results of these studies, which have demonstrated that carbohydrate microarrays can be efficiently prepared and used in high-throughput analyses of carbohydrate−protein interactions, are described below.

Results and Discussion

Synthesis of Maleimide-Linked Carbohydrates. We previously demonstrated that maleimide-linked carbohydrates with suitable length of tethers on glass slides strongly bound to lectins

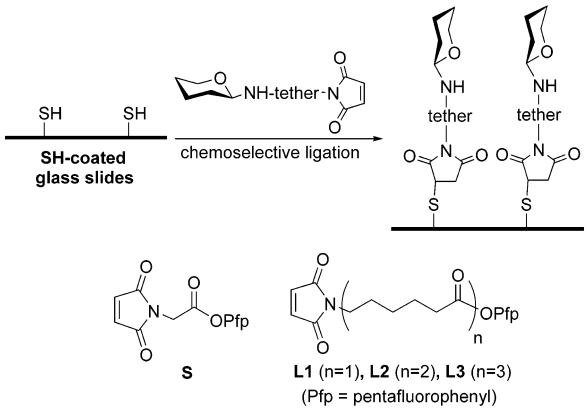
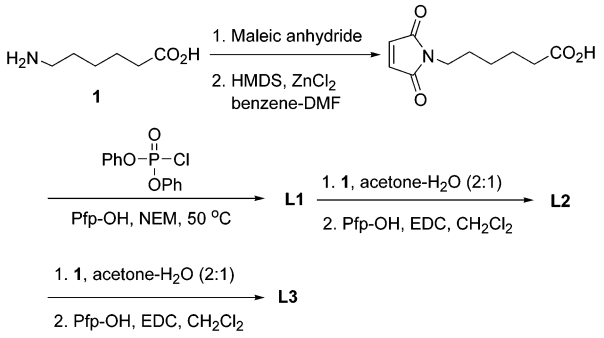


Figure 1. Fabrication of carbohydrate microarrays by immobilizing maleimide-linked carbohydrates on thiol-coated glass slides via chemoselective ligation.

Scheme 1. Synthesis of Linkers **L1**, **L2**, and **L3**



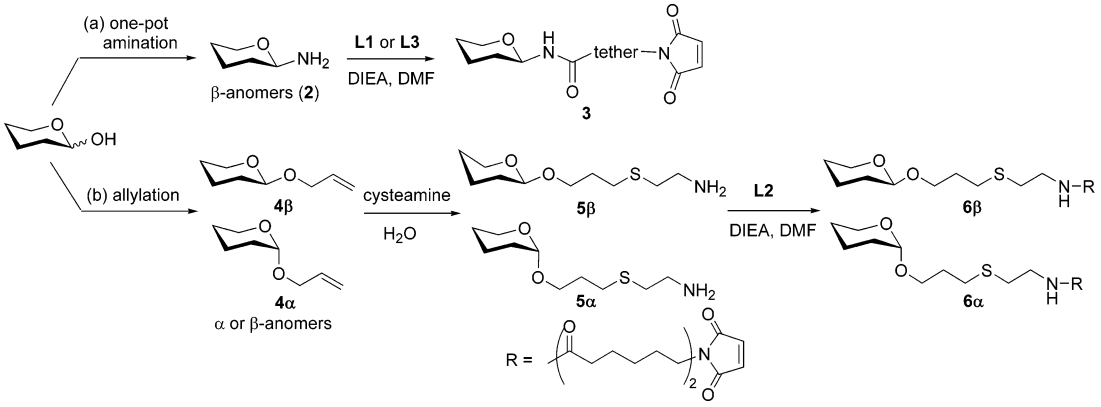
(Figure 1).[10d] For example, carbohydrates linked by **L1**, **L2**, and **L3** bound to lectins above 0.5 mM immobilization concentrations. However, carbohydrates coupled to the shortest linker **S** exhibited very weak interactions with lectins below 5 mM immobilization concentrations. Thus, **L1**, **L2**, or **L3** was used as a linker for the current studies to fabricate carbohydrate microarrays containing more diverse carbohydrate probes.

The linkers **L1**, **L2**, and **L3** were prepared from 6-aminohexanoic acid (**1**) by the procedure shown in Scheme 1. The amino moiety in **1** was first converted to a maleimide group by reaction with maleic anhydride followed by treatment with hexamethyldisilazide (HMDS) and $ZnCl_2$.[11,12] The resulting acid was treated with pentafluorophenol (Pfp-OH), diphenyl chlorophosphate, and *N*-ethylmorpholine (NEM) to give **L1**.[13] Linker **L2** was prepared by coupling **L1** to **1** and a subsequent esterification with Pfp-OH and *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide·HCl (EDC). Repetition of this procedure with **L2** provided **L3**.

The maleimide-linked carbohydrates were prepared by either a one-pot amination or an allylation approach (Scheme 2). The one-pot amination of carbohydrates gave β-glycosylamines **2**,[14] which were coupled to the bifunctional cross-linker **L1** or **L3** to produce *N*-linked carbohydrates **3a**−**g** (Figure 2a). Although this route successfully produced various carbohydrate probes, this had a limitation to give only β-anomeric carbohydrate probes.

(8) (a) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760. (b) Zhu, H.; Klemic, J. F.; Chang, S.; Bertone, P.; Casamayor, A.; Klemic, K. G.; Smith, D.; Gerstein, M.; Reed, M. A.; Snyder, M. *Nat. Genet.* **2000**, *26*, 283.
(9) (a) Merchant, M.; Weinberger, S. R. *Electrophoresis* **2000**, *21*, 1164. (b) Fung, E. T.; Thulasiraman, V.; Weinberger, S. R.; Dalmasso, E. A. *Curr. Opin. Biotech.* **2001**, *12*, 65.
(10) (a) Wang, D.; Liu, S.; Trummer, B. J.; Deng, C.; Wang, A. *Nat. Biotech.* **2002**, *20*, 275. (b) Fukui, S.; Feizi, T.; Galustian, C.; Lawson, A. M.; Chai, W. *Nat. Biotech.* **2002**, *20*, 1011. (c) Houseman, B. T.; Mrksich, M. *Chem. Biol.* **2002**, *9*, 443. (d) Park, S.; Shin, I. *Angew. Chem., Int. Ed.* **2002**, *41*, 3180. (e) Fazio, F.; Bryan, M. C.; Blixt, O.; Paulson, J. C.; Wong, C.-H. *J. Am. Chem. Soc*. **2002**, *124*, 14397. (f) Willats, W. G. T.; Rasmussen, S. E.; Kristensen, T.; Mikkelsen, J. D.; Knox, J. P. *Proteomics* **2002**, *2*, 1666. (g) Köhn, M.; Wacker, R. Carsten, P.; Schröder, H.; Soulère, L.; Breinbauer, R.; Niemeyer, C. M.; Waldmann, H. *Angew. Chem., Int. Ed.* **2003**, *42*, 5830. (h) Mellet, C. O.; Fernández, J. M. G. *ChemBioChem.* **2002**, *3*, 819. (i) Love, K. R.; Seeberger, P. H. *Angew. Chem., Int. Ed.* **2002**, *41*, 3583.
(11) Reddy, P. Y.; Kondo, S.; Fujita, S.; Toru, T. *Synthesis* **1998**, 999.
(12) (a) Shin, I.; Jung, H-j.; Lee, M-r. *Tetrahedron Lett.* **2001**, *42*, 1325. (b) Shin, I.; Jung, H.-j.; Cho, J. *Bull. Korean Chem. Soc.* **2000**, *21*, 845.
(13) Pöchlauer, P.; Hendel, W. *Tetrahedron* **1998**, *54*, 3489.
(14) (a) Vetter, D.; Gallop, M. A. *Bioconjugate Chem.* **1995**, *6*, 316. (b) Vetter, D.; Tate, E. M.; Gallop, M. A. *Bioconjugate Chem.* **1995**, *6*, 319.

Scheme 2. Two Synthetic Routes for the Synthesis of Maleimide-Linked Carbohydrate Probes



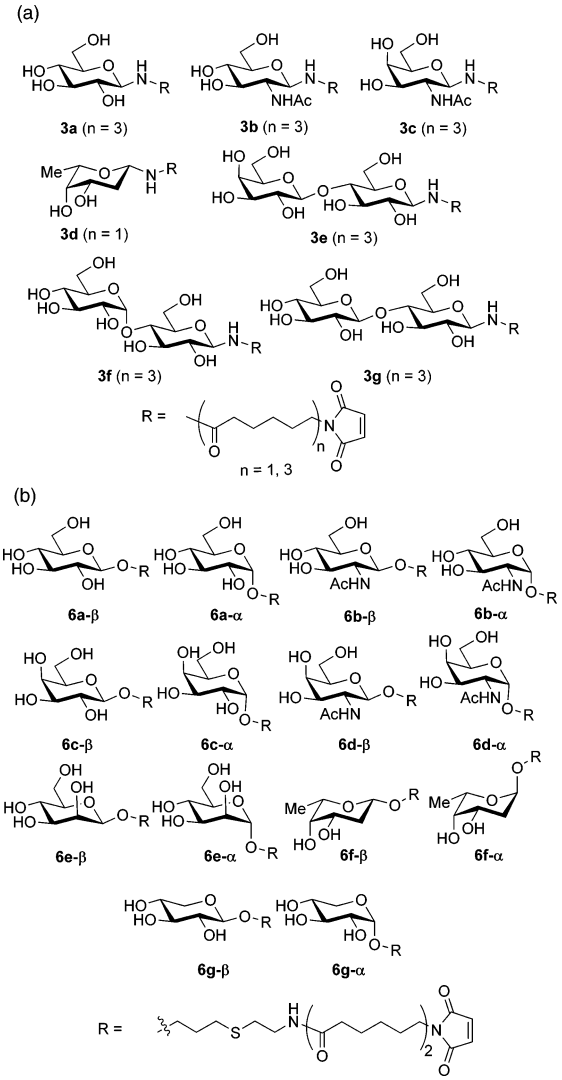
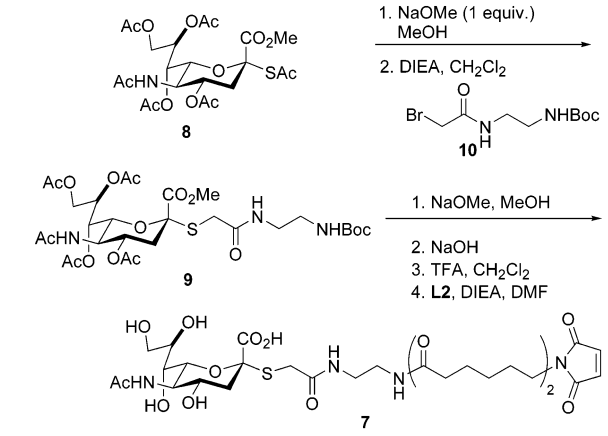


Figure 2. Structures of (a) N-linked and (b) O-linked carbohydrate probes for the fabrication of carbohydrate microarrays.

Alternatively, the allylation of carbohydrates was performed to prepare both α-and *β*-anomeric maleimide-linked carbohydrates as well as to examine the difference of binding affinities between N-linked and O-linked carbohydrates to lectins (Scheme 2b). The α and *β*-allyl glycosides (**4**) were reacted with cysteamine according to the known procedures,[15] and the resulting amines (**5**) were coupled to **L2** to yield O-linked α- and *β*-anomeric maleimide-linked carbohydrates **6a**−**g** (Figure 2b).

Scheme 3. Synthesis of a Maleimide-Linked Sialic Acid Probe



Furthermore, the sialic acid probe **7** was prepared from the procedure shown in Scheme 3. The known thiosialoside **8** was transformed to **9** by selective deacetylation of the thioacetate group with 1 equiv NaOMe and a subsequent coupling of the resulting thiol to bromoacetylated compound **10**.[16] Removal of all the protecting groups in **9** followed by coupling to **L2** provided **7**.

Fabrication of Carbohydrate Microarrays. Carbohydrate microarrays (spot size, ca. 100-μm diameter) composed of 22 carbohydrate probes were fabricated by in duplicate printing solutions of maleimide-connected carbohydrates **3**, **6**, and **7** (1 nL, 1.0 mM) on a thiol-derivatized glass slide with a high-precision pin-type microarrayer. Each solution of the carbohydrates used in this process was prepared by dissolving the carbohydrates in phosphate-buffered saline (PBS) containing 40−50% glycerol to suppress undesired evaporation of the nanodroplets during spotting and immobilization. After 5- or 10-h immobilization in a humid chamber (55% humidity), the glass slides were treated with 1% *N*-ethylmaleimide (NEM) in water to remove unreacted thiol groups. This process prevents oxidative disulfide-bond formation between surface thiols and cysteine residues of proteins used in the next incubation step. A blocked plastic film (0.1−0.2-mm thickness), which was coated by adhesive at one side, was then carefully attached to the microspotted slide (Figure 3). Use of the blocked plastic film facilitates compartmentalization that is required for (1) simultaneous incubation with several lectins, (2) enzymatic glycosylation of carbohydrate probes, and (3) determination of

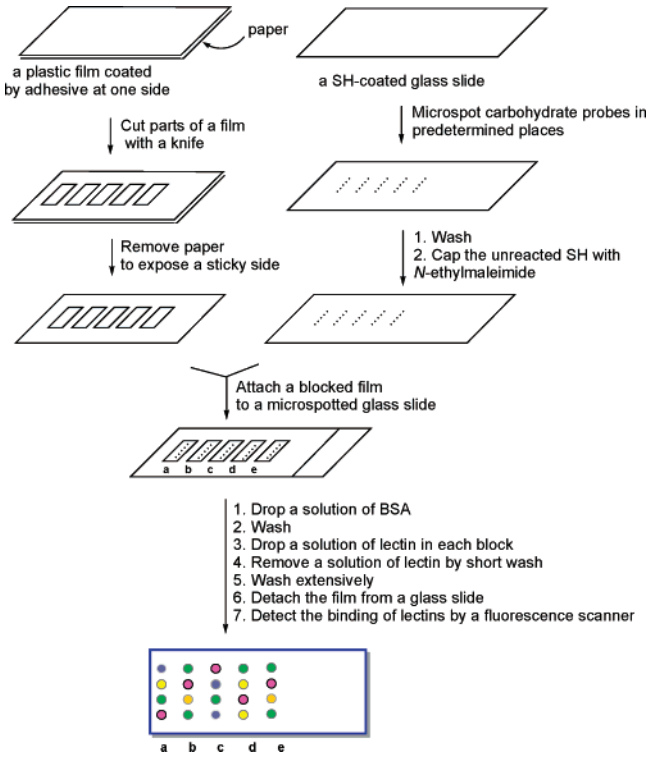


Figure 3. A flow chart to fabricate carbohydrate microarrays blocked by a plastic film.

IC_{50} values (vide infra). The fabricated carbohydrate microarrays can be stored in a desiccator for several months without degradation.

Investigations of Protein−Carbohydrate Interactions with Carbohydrate Microarrays. To profile protein binding with the carbohydrate microarrays, each block containing 22 carbohydrate probes was treated with 3% bovine serum albumin (BSA) in PBS containing 0.2% Tween 20 to minimize nonspecific binding of proteins on the surface. Next, the BSA-treated blocks were probed with solutions of the FITC-labeled lectins (1−10 μg/mL) such as FITC-*Concanavalin A* (FITC-ConA), FITC-*E. cristagalli* (FITC-EC), FITC-*T. vulgaris* (FITC-TV), FITC-*N. pseudonarcissus* (FITC-NPA) and FITC-*A. aurantia* (FITC-AA) in PBS containing 0.1% Tween 20 for 0.5−1 h.[17,18] After extensive washing of the lectin-treated slide with the same buffer, lectin binding was visualized and quantitated by using a fluorescence scanner.

Extensive washing (three times with PBS containing 0.1% Tween 20 for 5−10 min) of the blocks after incubation with the FITC-labeled lectins was important for diminishing background fluorescent signals. The retention of measurable fluorescent signals on carbohydrate probes, even after extensive washing, suggested that their binding to lectins on the solid surface was strong. It was well known that carbohydrate-binding proteins interacted only weakly with monovalent carbohydrates but they bound to multivalent carbohydrates strongly owing to cluster effects.[19] Thus, carbohydrates immobilized on the glass slide appear to display multivalency.

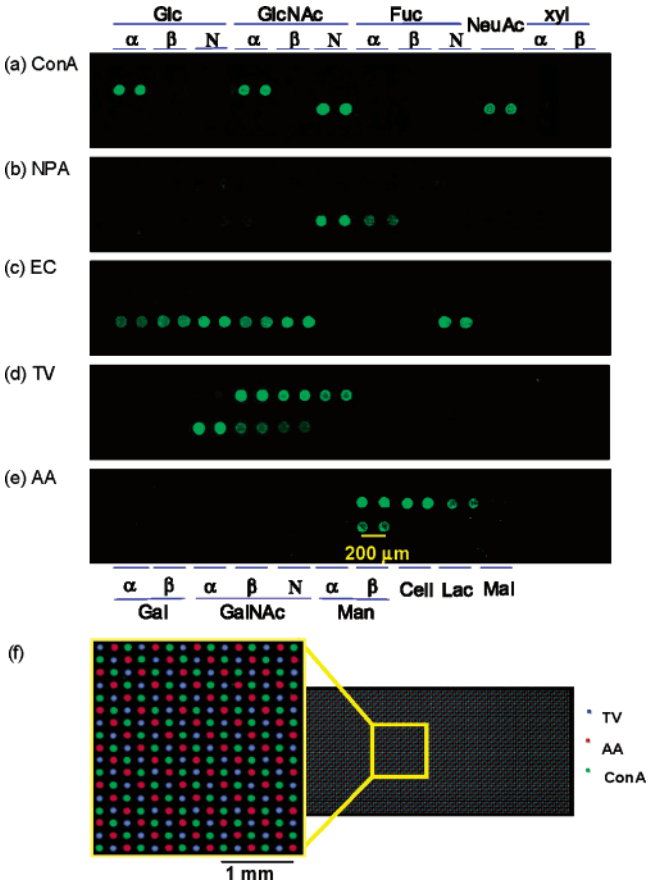


Figure 4. Fluorescence images of carbohydrate microarrays containing 22 carbohydrates probed with (a) FITC-ConA, (b) FITC-NPA, (c) FITC-EC, (d) FITC-TV, (e) FITC-AA, and (f) fluorescence image of 12000 microspots (60 × 200) consisting of β-GlcNAc, α-Man, and α-Fuc probed with a mixture of Cy3-TV, Cy5-AA, and FITC-ConA. The α and β symbols denote O-linked α and β-anomeric carbohydrates (**6**) and N denotes N-linked carbohydrates (**3**).

Figure 4 shows the fluorescence images of carbohydrate microarrays probed with five FITC-labeled lectins. The carbohydrate microarray incubated with ConA (an α-Man/α-Glc binding lectin) exhibited strong binding of α-Man (**6e**-α), lower binding of α-Glc (**6a**-α), and weak binding of maltose (**3f**) and α-GlcNAc (**6b**-α) (Figure 4a).[18] These results are consistent with binding tendencies determined by isothermal titration calorimetry (ITC).[20] In contrast, β-Man (**6e**-β), β-Glc (**3a**, **6a**-β), β-GlcNAc (**3b**, **6b**-β), and cellobiose (**3g**) with β-glucose at nonreducing terminus were not recognized by ConA. This demonstrated that the anomeric configuration of the glucose, mannose, and GlcNAc on the solid surface governed lectin binding. On the other hand, when the carbohydrate microarray was treated with NPA, a Man binding lectin which does not bind to Glc, fluorescent signals were observed only for Man

(15) (a) Lee, R. T.; Lee, Y. C. *Carbohydr. Res.* **1974**, *37*, 193. (b) Wong, T. C.; Townsend, T. R.; Lee, Y. C. *Carbohydr. Res.* **1987**, *170*, 27. (c) Utille, J.-P.; Priem, B. *Carbohydr. Res.* **2000**, *329*, 431. (d) Garegg, P. J.; Norberg, T. *Carbohydr. Res.* **1976**, *52*, 235. (e) Rosenberg, H. J.; Riley, A. M.; Marwood, R. D.; Correa, V.; Taylor, C. W.; Potter, B. V. L. *Carbohydr. Res.* **2001**, *332*, 53.

(16) Fitz, W.; Rosenthal, P. B.; Wong, C.-H. *Bioorg. Med. Chem.* **1996**, *4*, 1349.

(17) The lectin of *T. vulgaris* (TV) is also known as wheat germ agglutinin (WGA).

(18) (a) Liener, I. E.; Sharon, N.; Goldstein, I. J. *The Lectins: Properties, Functions, and Applications in Biology and Medicine*; Academic Press: Orlando, FL, 1986; pp 35−244. (b) Van Damme, E. J. M.; Psumans, W. J.; Pusztai, A.; Bardocz, S. *Handbook of Plant Lectins: Properties and Biomedical Applications*; John Wiley & Sons: Chichester, U.K., 1998.

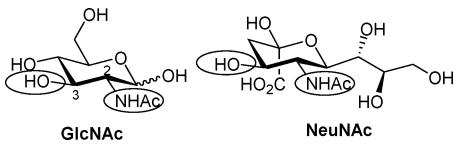
(19) For reviews, see (a) Mannen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754. (b) Lee, Y. C.; Lee, R. T. *Acc. Chem. Res.* **1995**, *28*, 321.

(20) Dissociation constants (K_d) of methyl α-mannoside, methyl α-glucoside, maltose, methyl α-GlcNAc, and Manα1,6Man-α-OMe for ConA determined by ITC were 0.12, 0.51, 0.76, 0.93, and 0.12 mM, respectively. Mandal, D. K.; Kishore, N.; Brewer, C. F. *Biochemistry* **1994**, *33*, 1149.

(**6e**-α and β) and the intensity of microspots of α-Man (**6e**-α) was greater than that of β-Man (**6e**-β) (Figure 4b).[21] The relative fluorescence intensity in the region of α-Man (**6e**-α) for ConA and NPA showed that ConA bound to α-Man more strongly than NPA. The dissociation constants (K_d) of Manα1,3Man for ConA and NPA determined by ITC were reported to be 0.07 and 2.00 mM, respectively.[20,22] Thus, binding experiments using both the carbohydrate chips and the ITC method indicated that ConA interacted more strongly with Man than NPA.

The carbohydrate microarray incubated with EC (a GalNAc/Gal binding lectin) showed fluorescent signals in the regions of Gal, GalNAc, and lactose (Figure 4c).[18] The α-GalNAc (**6d**-α), *N*-linked β-GalNAc (**3c**), and lactose (**3e**) bound to EC with similar binding affinities. However, O-linked Gal (**6c**-α and β) and β-GalNAc (**6d**-β) interacted with the lectin with the reduced binding affinities. Interestingly, N-linked β-GalNAc (**3c**) exhibited stronger fluorescence intensity than O-linked β-GalNAc (**6d**-β). This suggested that the nature of the anomeric linkage in maleimide-linked monosaccharides affected their binding affinities to the lectin. The relative binding affinities of EC with the carbohydrate microarray were similar to those observed for solution-based assays.[23]

When the carbohydrate microarray was probed with TV (a GlcNAc binding lectin), the lectin recognized α-GlcNAc (**6b**-α) strongly and O-linked β-GlcNAc (**6b**-β) with the reduced binding affinity (Figure 4d).[18] Microspots of N-linked β-GlcNAc (**3b**) exhibited weaker fluorescent signals than those of O-linked β-GlcNAc (**6b**-β). It was also revealed that TV bound to O-linked α-GalNAc (**6d**-α) and O-linked β-GlcNAc (**6b**-β) with similar binding affinities but to β-GalNAc (**3c**, **6d**-β) with reduced binding affinity.[18,24] To analyze the difference of binding affinity between α-GlcNAc (**6b**-α) and α-GalNAc (**6d**-α) for TV, we determined concentrations (IC_{50}) of methyl *N*-acetyl-α-glucosaminide (α-GlcNAc-OMe) to inhibit 50% of TV binding to α-GlcNAc (**6b**-α) and α-GalNAc (**6d**-α) on the microarray. Microspots of α-GlcNAc and α-GalNAc were incubated with a series of mixtures of Cy3-TV (1 μg/mL) and α-GlcNAc-OMe (~10 μM to 0.4 M) for 1 h. After extensive washing of the carbohydrate chip, the amount of bound lectin was quantitated by measuring fluorescence intensity. As shown in Figure 5a and b, α-GlcNAc (IC_{50} = 8.9 mM for α-GlcNAc−OMe) competed more efficiently with α-GlcNAc−OMe for TV than α-GalNAc (IC_{50} = 3.5 mM for α-GlcNAc−OMe).[25] Because of configurational similarity of GlcNAc and *N*-acetylneuraminic acid (NeuNAc) at positions C-2 (NHAc) and C-3 (OH), TV was known to recognize terminal NeuNAc.[24b] However, it was revealed that monomeric thiosialoside **7** on the glass slide did not bind to the lectin.



The carbohydrate microarray probed with FITC-AA (a Fuc binding lectin) showed strong binding of O-linked Fuc (**6f**-α and β) but weak binding of N-linked β-Fuc (**3d**) (Figure 4e).[18] Microspots of N-linked β-Fuc (**3d**) displayed weaker fluorescent signals than those of O-linked β-Fuc (**6f**-β). It was intriguing that AA also bound to O-linked β-Man (**6e**-β) with a similar affinity as it did to N-linked β-Fuc (**3d**). Since this was an unexpected result, the binding affinity of O-linked β-Man (**6e**-β) to AA was assessed by determining concentrations (IC_{50}) of methyl α-fucoside (α-Fuc-OMe) and allyl α-fucoside (α-Fuc-OAllyl) that inhibit 50% of AA binding to the O-linked β-Man (**6e**-β).[26] For this purpose, microspots of O-linked β-Man (**6e**-β) were incubated with a series of mixtures of Cy5-AA (1 μg/mL) and α-Fuc-OMe or α-Fuc-OAllyl (1 nM−0.01 M) for 1 h, and then the amount of bound lectin was quantitated by measuring fluorescence intensity. In this manner, the IC_{50} values of α-Fuc-OAllyl and α-Fuc-OMe were determined to be 6.2 and 3.5 μM, respectively (Figure 5c and d). The results suggested that O-linked β-Man (**6e**-β) was a poor ligand for AA and α-Fuc-OMe was a little better ligand for the lectin than α-Fuc-OAllyl.

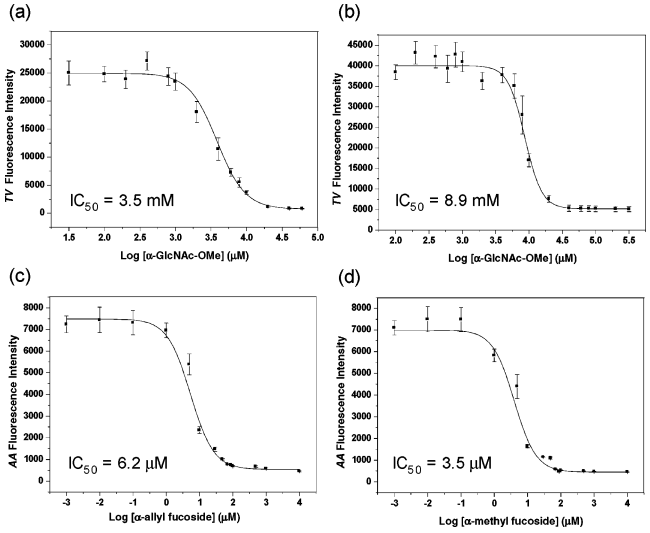
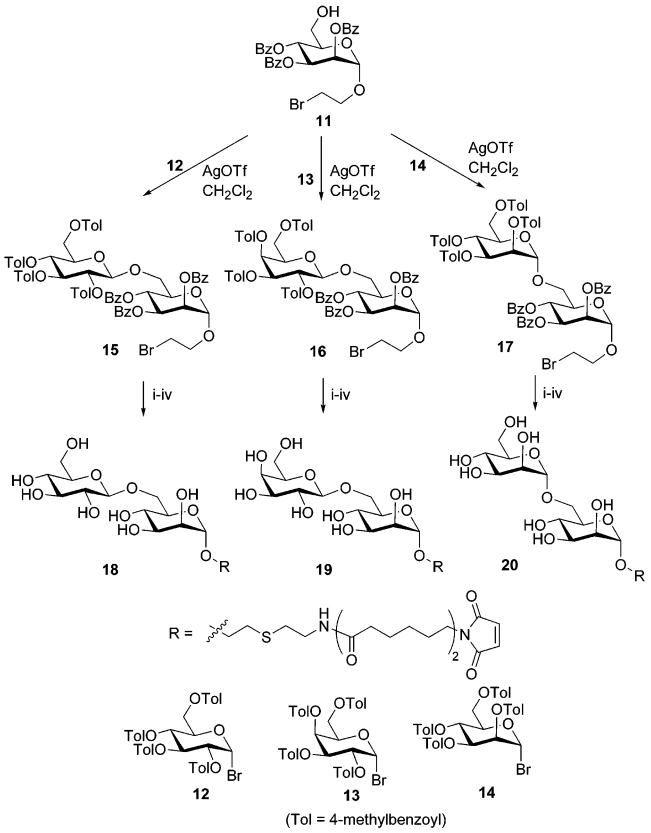


Figure 5. Determination of concentrations (IC_{50}) of soluble methyl *N*-acetyl-α-glucosaminide (α-GlcNAc-OMe) to inhibit 50% of TV binding to α-GlcNAc (a) and α-GalNAc (b) on the carbohydrate microarray, and determination of concentrations (IC_{50}) of soluble allyl α-fucoside (c) and methyl α-fucoside (d) to inhibit 50% of AA binding to β-Man on the carbohydrate microarray.

To demonstrate that carbohydrates on the chips containing several microspots selectively bind to proteins, we printed 12000 microspots (60 × 200) composed of α-Fuc (**6f**-α), α-Man (**6e**-α), and O-linked β-GlcNAc (**6b**-β) on a thiol-coated slide in an alternate fashion. The microarray was then probed with a mixture of Cy3-TV, Cy5-AA, and FITC-ConA. As shown in Figure 4f, α-Fuc, α-Man, and β-GlcNAc selectively bound to the corresponding lectins. The lectin-binding experiments described above clearly demonstrated that carbohydrate chips were ideally suited for high-throughput analysis of specific carbohydrate−protein interactions.

(21) Kaku, H.; Van Damme, E. J. M.; Peumans, W. J.; Goldstein, I. J. *Arch. Biochem. Biophys.* **1990**, *279*, 298.
(22) Sauerborn, M. K.; Wright, L. M.; Reynolds, C. D.; Grossmann, J. G.; Rizkallah, P. J. *J. Mol. Biol.* **1999**, *290*, 185.
(23) Kaladas, P. M.; Kabat, E. A.; Iglesias, J. L.; Lis, H.; Sharon, N. *Arch. Biochem. Biophys.* **1982**, *217*, 624.
(24) (a) Monsigny, M.; Roche, A.-C.; Sene, C.; Maget-Dana, R.; Delmotte, F. *Eur. J. Biochem.* **1980**, *104*, 147. (b) Wright, C. S. *J. Mol. Biol.* **1980**, *141*, 267. (c) Bhavanandan, V. P.; Kaltlic, A. W. *J. Biol. Chem.* **1979**, *254*, 4000.
(25) Goldstein, I. J.; Hammarström, S.; Sundblad, G. *Biochim. Biophys. Acta* **1975**, *405*, 63.
(26) It was reported that AA did not bind to Man up to 50 mM concentration. However, it seems that AA weakly binds to Man on the solid surface maybe because of cluster effect. (a) Kochibe, N.; Furukawa, K. *Biochemistry* **1980**, *19*, 2841. (b) Sudakevitz, D.; Imberty, A.; Gilboa-Garber, N. *J. Biochem.* **2002**, *132*, 353.

Scheme 4. Solution-Phase Parallel Synthesis of Three Disaccharides. Conditions: (i) $HSCH_2CH_2NHBoc$, TEA, DMF, 50 °C; (ii) NaOMe, MeOH; (iii) TFA; (iv) **L2**, DIEA, DMF



Preparation of Maleimide-Linked Carbohydrate Probes by Solution-Phase Parallel Glycosylation. Carbohydrate microarrays composed of various carbohydrate probes are potentially more useful for understanding recognition events between carbohydrates and proteins. Recently, combinatorial synthesis of carbohydrates have been developed to prepare diverse carbohydrate libraries.[27] In the current effort, we synthesized three model maleimide-linked disaccharides (Manα1,6Man, Glcβ1,6Man, and Galβ1,6Man) by solution-phase parallel glycosylation (Scheme 4). The 2-bromoethyl α-mannoside **11** was glycosylated with three glycosyl bromides **12**−**14** in the presence of silver triflate (AgOTf) as an activator to give the 1,6-linked disaccharides **15**−**17**.[27c] The bromoethyl group was used as an anomeric substituent because it could be readily transformed into other functional groups.[27c,28] The three disaccharides (**15**−**17**) were converted to maleimide-linked carbohydrates **18**−**20** by reaction with Boc-protected cysteamine, removal of all the protecting groups, and a subsequent coupling of the resulting amines to **L2**.

The three disaccharides were printed in duplicate on a thiol-coated slide along with four monosaccharides (**6b**-α, **6d**-β, **6e**-α, and **6f**-β) as controls and then were probed with five FITC-labeled lectins (ConA, NPA, EC, TV, and AA). As shown in Figure 6, ConA bound to Manα1,6Man (**20**) and O-linked α-Man (**6e**-α) with similar binding affinities.[20] However, NPA interacted with Manα1,6Man (**20**) more strongly than it did with O-linked α-Man (**6e**-α). Microspots treated with EC exhibited stronger fluorescent signals in the region of O-linked β-GalNAc (**6d**-β) than Galβ1,6Man (**19**). As anticipated, TV and AA only bound to the monosaccharide controls.

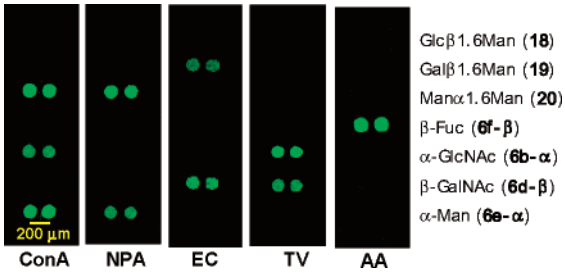


Figure 6. Fluorescence images of microspots containing mono- and disaccharides probed with FITC-labeled lectins.

Enzymatic Transformations of Carbohydrates on the Carbohydrate Microarrays. Enzymatic glycosylation processes serve as alternatives to chemical glycosylations for the synthesis of complex carbohydrates.[27f,29] To examine the usefulness of enzymatic transformations of carbohydrates on carbohydrate microarrays, we prepared sialyl Le^x from GlcNAc by three consecutive glycosyl transferase-catalyzed reactions.

The *N*-linked β-GlcNAc (**3b**) was in quadruplicate microspotted on the thiol-coated glass slide and then compartmentalized by using a plastic film to create separate blocks. Efficient immobilization of β-GlcNAc was confirmed by probing one block with Cy3-TV (Figure 7a). The remaining blocks were treated with β-1,4-galactosyl transferase (GalT) in the presence of UDP-Gal for 15 h at 37 °C in a humid chamber.[10c] To prove successful enzymatic galactosylation, one block was incubated with Cy5-EC. High fluorescent signals were observed in the region of microspots containing LacNAc. The GalT-incubated block was further treated with α-2,3-sialyl transferase (SialT) and CMP-NeuAc under the above-mentioned conditions.[29] The resulting microarray was then probed with Cy5-EC. No fluorescence was observed, indicating that LacNAc was converted to NeuNAcα2,3LacNAc.

Finally, a solution of α-1,3-fucosyl transferase (FucT) and GDP-Fuc was added to a block containing NeuNAcα2,-3LacNAc.[10e] After 15 h at 37 °C in a humid chamber, the glass slide was rinsed with PBS three times, and then the enzymatic fucosylation was repeated under the same conditions to bring about more fucosylation of NeuNAcα2,3LacNAc.[30] The block was then treated sequentially with mouse anti-sialyl Le^x and goat Cy5-anti-antibody. As shown in Figure 7a, microspots of the synthetic sialyl Le^x displayed visible fluorescence. A single fucosylation leads to poor glycosylation on the basis of low fluorescence intensity.

The selective glycosylations of carbohydrates by glycosyl transferases on carbohydrate microarrays was also examined. For this purpose, GlcNAc (**3b**) and α-Fuc (**6f**-α) were alternately printed on a glass slide to produce 100 microspots (5 × 20). The slide was then incubated with GalT and UDP-Gal under

(27) (a) Marcaurelle, L. A.; Seeberger, P. H. *Curr. Opin. Chem. Biol.* **2002**, *6*, 289. (b) Seeberger, P. H.; Haase, W.-C. *Chem. Rev.* **2000**, *100*, 4349. (c) Takahashi, T.; Adachi, M.; Matsuda, A.; Doi, T. *Tetrahedron Lett.* **2000**, *41*, 2599. (d) Sofia, M. J. *Mol. Divers.* **1998**, *3*, 75. (e) Liang, R.; Yan, L.; Loebach, J.; Ge, M.; Uozumi, Y.; Sekanina, K.; Horan, N.; Gildersleeve, J.; Thompson, C.; Smith, A.; Biswas, K.; Still, W. C.; Kahne, D. *Science* **1996**, *274*, 1520. (f) Koeller, K. M.; Wong, C.-H. *Chem. Rev.* **2000**, *100*, 4465.
(28) Kieburg, C.; Sadalapure, K.; Lindhorst, T. K. *Eur. J. Org. Chem.* **2000**, 2035.
(29) Gijsen, H. J. M.; Qiao, L.; Fitz, W.; Wong, C.-H. *Chem. Rev.* **1996**, *96*, 443.
(30) Seitz, O.; Wong, C.-H. *J. Am. Chem. Soc.* **1997**, *119*, 8766.

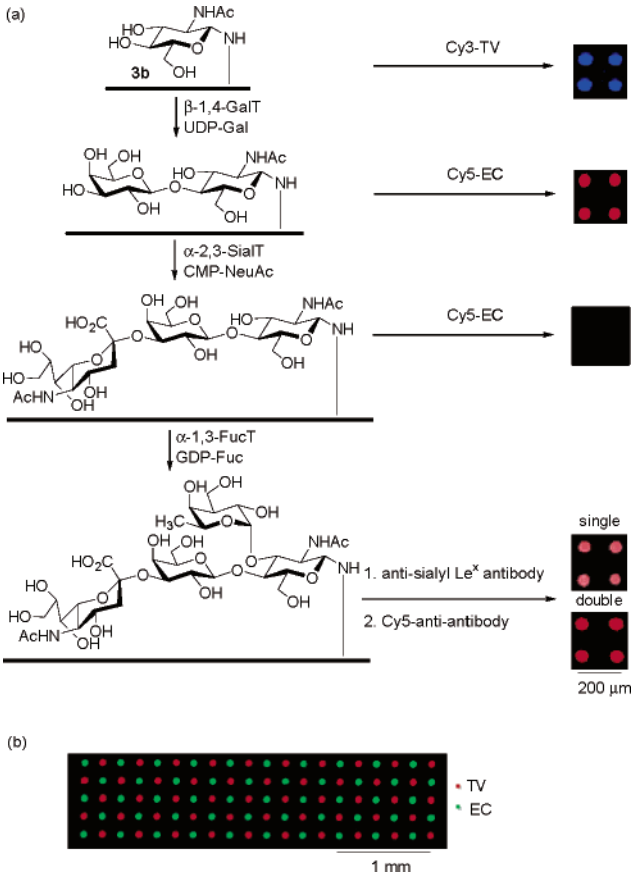


Figure 7. (a) Enzymatic synthesis of sialyl Le^x from GlcNAc by three glycosyl transferases. (b) Selective glycosylation of GlcNAc by galactosyl transferase.

the glycosylation conditions given above. The results of probing the carbohydrate chip with a mixture of Cy5-AA and FITC-EC show that only GlcNAc was converted to LacNAc by GalT (Figure 7b). These observations showed that carbohydrate probes were efficiently and selectively transformed by glycosyl transferases to produce carbohydrate microarrays that contained more complex and perhaps more diverse carbohydrates.

Conclusion

In the studies described above, we demonstrated that carbohydrate microarrays were suitable for sensitive, high-throughput detection of binding between carbohydrate ligands and proteins. We found that the nature of the anomeric linkage in maleimide-linked monomeric carbohydrates affected their protein binding affinities. In addition, we showed that the carbohydrate microarrays could be used to quantitatively analyze binding affinities between carbohydrates and lectins. We also showed that solution-phase parallel glycosylations could be employed to prepare carbohydrate microarrays that contained diverse probes. Finally, we demonstrated that enzymatic glycosylations on the glass slides could be applied for the synthesis of complex oligosaccharides such as sialyl Le^x.

In addition to their uses in high-throughput profiling of protein binding, carbohydrate microarrays might be applicable to high-throughput screening of potential inhibitors for carbohydrate-binding proteins required for the development of novel therapeutic agents. Moreover, they might be used to identify or characterize novel carbohydrate-binding proteins or carbohydrate-processing enzymes, such as glycosyl transferases, glycosidases, and sulfotransferases. Finally, carbohydrate microarrays might play a potential role in the diagnoses of diseases which correlate with the presence or absence of carbohydrate-binding molecules.

Experimental Section

General. All materials were purchased from commercial suppliers: solvents and reagents from Aldrich, Acros, and Senn Chemicals; thiol-derivatized glass slides from Biometrix Technology; lectins from Vector labs and Sigma; glycosyl transferases, substrates, and antibodies from Calbiochem; Cy3 and Cy5 *N*-hydroxysuccinimide ester from Amersham Pharmacia biotech. The solutions of carbohydrates were microspotted with a MicroSys 5100 from Cartesian Technologies. Carbohydrate microarrays probed with fluorescent dye-labeled lectins were scanned with a Scanarray 5000 from Packardbiochip.

Microspotting of Carbohydrates. The carbohydrate probes were dissolved in PBS (pH 6.8) containing 40−50% glycerol. The solution of carbohydrates (1 nL, 1.0 mM) from a 384-well plate was microspotted in predetermined places on a thiol-coated glass slide with a distance of 200 μm between the centers of adjacent spots (spot size, ca. 100 μm in diameter). After completion of printing, the slide was placed in a humid chamber (55% humidity) at room temperature for 5 or 10 h and then immersed into water containing 1% *N*-ethylmaleimide (NEM) for 15 min with gentle shaking. The slide was washed with PBS (pH 6.8) containing 0.1% Tween 20 for 1 h and then rinsed with water. After drying by purging with Ar gas, a compartmentalized plastic film which was coated by adhesive at one side (thickness: 0.1−0.2 mm) was attached to the glass slide. The solution of 0.2% Tween 20 possessing 3% BSA was dropped in the blocks and then incubated for 30 min. The slide was washed with the same buffer without BSA (3 × 15 min).

Detection of Lectin−Carbohydrate Interactions. Blocks containing microspots were probed with fluorescent dye-labeled lectins (1−10 μg/mL) in PBS (pH 6.8) 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 were then removed by gentle shaking in the same buffer (3 × 5−10 min). After removal of a plastic film, the slide was scanned by using a Scanarray 5000.

Quantitative Analysis of Lectin Binding to Microspots. The carbohydrate probes were microspotted in predetermined places on the glass slide. After attaching a compartmentalized plastic film to the microspotted slide, each block was incubated with a series of mixtures of fluorescent dye-labeled lectin and an inhibitor in PBS (pH 6.8) containing 0.1% Tween 20 for 1 h. Fluorescence intensity was measured by using a Scanarray 5000.

Enzymatic Synthesis of Sialyl Le^x on the Carbohydrate Microarrays. The slide microspotted by GlcNAc (**3b**, 1 mM) in predetermined places was compartmentalized with a plastic film. A solution (15 μL) of GalT (23 mU), $MnCl_2$ (10 mM), and UDP-Gal (0.1 mM) in HEPES buffer (50 mM, pH 7.5) was dropped into each block and the slide was placed into a humid chamber for 15 h at 37 °C. The slide was washed with PBS (pH 6.8) containing 0.1% Tween 20 for 3 × 10 min and then dried by purging with Ar gas. For sialylation of LacNAc, a solution (15 μL) of SialT (1 mU), $MnCl_2$ (5 mM), alkaline phosphatase (20 μU), and CMP-NeuNAc (0.1 mM) in HEPES buffer (100 mM, pH 7.0) was dropped in each block and then placed in the humid chamber for 15 h at 37 °C. The slide was washed with the above-mentioned buffer. For fucosylation of NeuNAcα2,6LacNAc, a solution (15 μL) of FucT (1 mU), $MnCl_2$ (15 mM), alkaline phosphatase (20 μU), and GDP-Fuc (0.1 mM) in MES buffer (50 mM, pH 6.0) was dropped in each block and then placed in the humid chamber for 15 h at 37 °C. The slide was washed with the same buffer.

To examine successful sialylation, one block after incubation with SialT was treated with 0.2% Tween 20 containing 3% BSA for 30 min and then probed with Cy5-EC (10 μg/mL). To test efficient

fucosylation, after incubation with FucT one block was incubated with anti-sialyl Le^x antibody (5 μg/mL) in PBS (pH 7.2) containing NaCl (500 mM) and 0.02% Tween 20 for 1 h at room temperature and then was probed with Cy5-anti-antibody (10 μg/mL) in the same buffer for 1.5 h at room temperature.

Selective Enzymatic Glycosylation on the Glass Slide. The solutions of GlcNAc (**3b**, 1 mM) and Fuc (**6f**-α, 1 mM) were alternately microspotted on the thiol-derivatized glass slide. The carbohydrate microarrays were incubated with a solution (15 μL) of GalT (23 mU), $MnCl_2$ (10 mM), and UDP-Gal (0.1 mM) in HEPES buffer (50 mM, pH 7.5) in the humid chamber for 15 h at 37 °C. The slide was probed with a solution (50 μL) of FITC-EC and Cy5-AA in PBS (pH 6.8) containing 0.1% Tween 20 for 1 h at room temperature.

Acknowledgment. This work was supported by Ministry of Science & Technology (M10213050001-02B1505-00210).

Supporting Information Available: Detail synthetic procedures and NMR spectra for carbohydrate probes (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0391661