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Fabrication of Carbohydrate Chips for Studying Protein–Carbohydrate Interactions**

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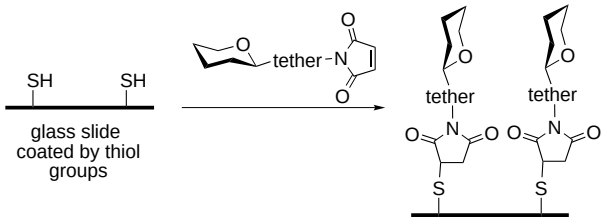
Carbohydrates play a central role in living organisms as recognition markers to enable cell adhesion, fertilization, differentiation, development, and tumor-cell metastasis through carbohydrate–protein interactions.[1] Interestingly, it is through these biomolecular interactions that bacteria and viruses adhere to the host cells and confer pathogenic properties.[1] Therefore, it is important to determine the molecular basis for specific protein–carbohydrate recognition events. Details of carbohydrate–protein interactions have been investigated mainly by biophysical and biochemical approaches, including X-ray crystallography and NMR spectroscopic studies of carbohydrate–protein complexes, mutagenesis, and synthesis of nonnatural sugar analogues.[2] Despite the established approaches to investigate carbohydrate–protein interactions, high-throughput methods have not been developed.

In the last decade, biological microchips (biochips) have been fabricated for a variety of applications. For instance, DNA chips (gene chips) are extensively used for tracking the activity of many genes at once and for studying changes in gene expression in diseased states.[3] Additionally, protein chips have been exploited for the high-throughput study of molecular interactions and biochemical activities,[4,5] and profiling protein expression in normal and diseased states.[6] Ziauddin and Sabatini have developed cell microarrays for the functional analysis of gene products in parallel.[7] Analogously, carbohydrate-based arrays may provide a new tool for the high-throughput study of carbohydrate–protein interactions.[8]

In many cases, carbohydrate-binding proteins recognize terminal and/or penultimate saccharides. Furthermore, they exhibit a strong affinity for multivalent carbohydrates with suitable spacing and orientation (cluster effect) but bind to monovalent oligosaccharides weakly.[9] Thus, we reasoned that carbohydrate chips that contain immobilized mono- and disaccharides with suitable distances between the carbohydrate probes on the glass surface could provide a useful tool for elucidating recognition events between carbohydrates and proteins at a molecular level.

An efficient immobilization technique of carbohydrates on the surface is essential for successful fabrication of carbohydrate chips. Recently, we prepared glycopeptides/glycoproteins by chemoselective ligation of maleimide-linked sugars to cysteine-possessing peptides/proteins.[10,11] Herein we report how this method was applied to immobilize carbohydrates on glass microscope slides (7.5 cm × 2.5 cm). As shown in Scheme 1, maleimide-linked sugars are attached through stable thioether linkages to the slide coated with thiol groups.



Scheme 1. Immobilization of maleimide-linked carbohydrates on thiol-derivatized slides.

Carbohydrates were appended to linkers L*n* of various lengths (L1, L2, L3, and L4) by coupling glycosylamines obtained from one-pot amination of carbohydrates to bifunctional cross-linkers such as **1** (Schemes 2 and 3).[10] Furthermore, we also prepared L2-NH_2 and L4-NH_2, which lack the carbohydrate moiety, to examine the nonspecific binding of lectins to linkers (Scheme 2).

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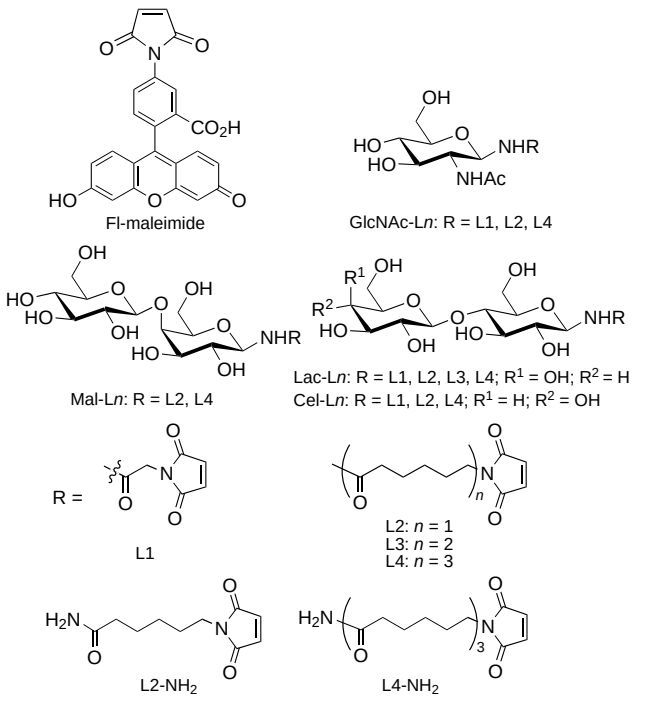
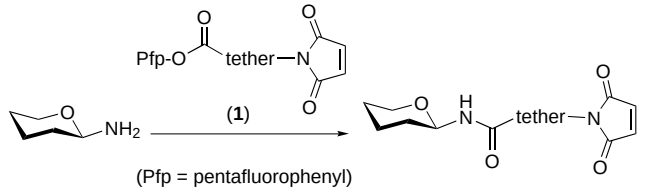


Scheme 2. Structure of carbohydrate probes and linkers.



Scheme 3. Glycosylamines were coupled to bifunctional cross-linkers such as **1** to produce carbohydrate probes, which were immobilized on the thiol-derivatized glass slides.

We immobilized maleimide-conjugated carbohydrate probes (GlcNAc, lactose (Lac), cellobiose (Cel), or maltose (Mal)) on glass slides derivatized with thiol groups. A high-precision pin-type microarrayer was used to microspot carbohydrate probes (100–120 μm in diameter). Carbohydrate probes were dissolved in phosphate-buffered saline (PBS)/glycerol (1:1) to avoid undesired evaporation of the nanodroplets during spotting and ligation reactions. To examine the effect of the concentration of immobilized carbohydrates on the subsequent protein binding, we printed carbohydrates at various concentrations (5.0, 1.0, 0.5, and 0.1 mM) on the slide. After the coupling reaction (5 or 10 h), the unreacted thiols on the slide were capped with *N*-ethylmaleimide (NEM; 1%) to prevent disulfide-bond formation of the surface thiol groups with cysteine residues of proteins used in the subsequent step through air oxidation. Next, we treated the slide with a solution of bovine serum albumin (BSA; 3%) in PBS buffer that contained Tween 20 (0.2%) to reduce background fluorescence caused by non-specific binding of fluorophore-labeled proteins. Subsequently, the microspotted slides were probed with fluorescein-labeled lectins (FITC-lectins) such as Concanavalin A (ConA), *Erythrina cristagalli* (EC), and *Triticum vulgaris* (TV) in PBS buffer that contained Tween 20 (0.1%) for 1 h.[12] After extensive washing of the lectin-treated slides with the same buffer, the fluorescence intensity of microspots on the slides was determined by using a laser scanner.

Interestingly, carbohydrate chips obtained after a coupling reaction of either 5 h or 10 h exhibited similar lectin-binding patterns. The results showed that 5 h was adequate for efficient immobilization of carbohydrate probes onto the glass slides. In contrast, washing the slides thoroughly after incubation with FITC-lectins was critical to reduce background fluorescence. For example, short washes (3 × 1 min) of the slide gave rise to a high background, whereas three washes for 5–10 min consistently resulted in a low background. The microspots that contained L2-NH_2 and L4-NH_2 were used as negative controls and did not show any fluorescent signal after incubation with FITC-lectins, thus demonstrating that lectins do not bind to the linkers alone (data not shown).

As shown in Figure 1 A, the microspots that contained fluorescein as a positive control exhibited similar fluorescence intensity at carbohydrate concentrations greater than 2 μM,

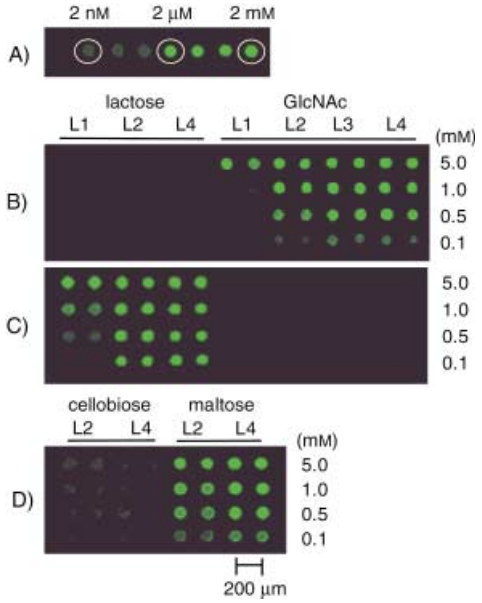


Figure 1. Lectin binding to carbohydrate microarrays. A) Fluorescence image of fluorescein (concentration of immobilized fluorescein decreases 10-fold from right to left); B) fluorescence images of lactose and GlcNAc-containing microspots probed with FITC-TV; C) lactose and GlcNAc-containing microspots probed with FITC-EC; D) cellobiose and maltose-containing microspots probed with FITC-ConA.

but were weakly visible at carbohydrate concentrations lower than 0.2 μM. To investigate the lectin-binding patterns with carbohydrate chips, the slides containing immobilized lactose (Galβ1,4Glc) and GlcNAc with linkers of various lengths were probed with FITC-TV (a GlcNAc-binding lectin) and FITC-EC (a GalNAc/Ga-binding lectin).[12] As expected, only the microspots that contained GlcNAc retained fluorescent signals after treatment with FITC-TV, whereas the lactose-bearing microspots specifically bound to FITC-EC (Figures 1 B and C). In addition, we found that the binding affinity of TV and EC for carbohydrates on the slide depends on both

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the concentration of the immobilized carbohydrate probes and on the length of linkers. The carbohydrates connected by the shortest linker (L1) bound to lectins weakly, unless their concentrations were high (5 mM). However, carbohydrates tethered to longer linkers (L2, L3, or L4) bound to the lectins with similar binding affinity at concentrations above 0.5 mM.

We then examined the difference in binding affinity and selectivity for recognition epitopes that differed in the anomeric configuration. As shown in Figure 1D, the slide with maltose (Glcα1,4Glc) and cellobiose (Glcβ1,4Glc) was probed with FITC-ConA (an αMan/αGlc-binding lectin).[12] As anticipated, maltose with α-glucose at the nonreducing terminus was bound tightly to the ConA; however, cellobiose with β-glucose showed a weak affinity for the lectin. The binding pattern of ConA by the immobilization concentration and the length of linkers (L2 and L4) is similar to that of TV. Overall, we demonstrated that immobilized carbohydrate probes (0.5–5 mM) connected by long linkers (L2–L4) bind to lectins with high specificity and strong affinity. Based on these observations, immobilized GlcNAc-L4 and lactose-L4 (5 mM) were printed on a slide to produce 12000 microspots (60 × 200) and subsequently probed with FITC-TV. As shown in Figure 2, only the microspots that contained GlcNAc were visible.

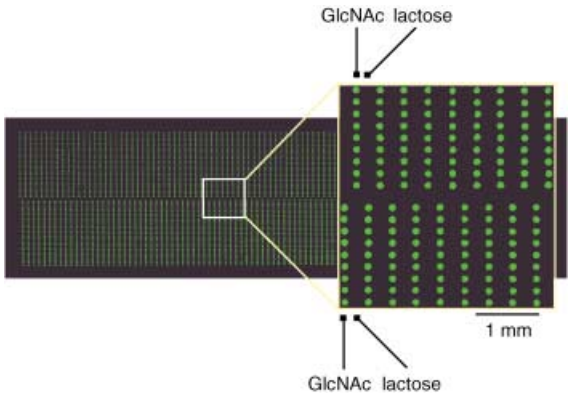

Figure 2. Carbohydrate microarrays consisting of 12000 microspots (60 × 200).

In summary, we have developed carbohydrate chips with mono- and disaccharides that are covalently immobilized on glass slides and can be recycled without detaching carbohydrate probes from the slide. Linkers of suitable lengths and the concentration of the immobilized carbohydrate probes are important for strong binding to proteins. The specificity of protein–carbohydrate interactions on carbohydrate chips resembles that in solution. The results described herein demonstrate that carbohydrate chips are practical for evaluating high-throughput protein–carbohydrate interactions. We believe that they could be useful for high-throughput screening of inhibitors of carbohydrate-binding proteins to develop novel therapeutic agents.

Experimental Section

Microchip production: Thiol-derivatized glass slides and FITC-lectins were purchased from Biometrix Technology (Korea) and Sigma, respectively. Samples were spotted with a MicroSys 5100 from Cartesian Technologies and the slides probed with FITC-lectins were scanned with a Scanarray 5000 from Packardbiochip.

Microspotting of carbohydrates: The carbohydrate probes (1 nL with concentrations of 5.0, 1.0, 0.5, 0.1 mM) dissolved in PBS/glycerol (1:1) from a 384-well plate were printed on a thiol-coated glass slide with a distance of 200 μm between the centers of adjacent spots by using a MicroSys 5100 (spot size: 100–120 μm in diameter). Subsequently, the slides were left at room temperature for 5 h and then the unreacted thiols were capped by immersing them into a solution of PBS buffer (pH 6.8) that contained *N*-ethylmaleimide (1%) for 15 min with gentle shaking. The slides were then washed with PBS buffer (pH 6.8) that contained Tween 20 (0.1%) for 1 h followed by doubly distilled H_2O (three times) and then dried by purging with Ar gas. Subsequently, the slides were treated with buffer that contained Tween 20 (0.2%) and BSA (3%) for 1 h and then rinsed with the same buffer without BSA.

Detection of protein–carbohydrate interactions: The slides were probed with FITC-lectin (1 $\mu g\,mL^{-1}$) in PBS buffer that contained Tween 20 (0.1%) for 1 h at room temperature. For FITC–ConA binding, $MnCl_2$ and $CaCl_2$ were added at with final concentrations of 1 mM. After incubation, unbound lectins were removed by gentle shaking in the same buffer (3 × 5–10 min). The slides were scanned by using a Scanarray 5000.

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