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Carbohydrate Microarrays for Assaying Galactosyltransferase Activity

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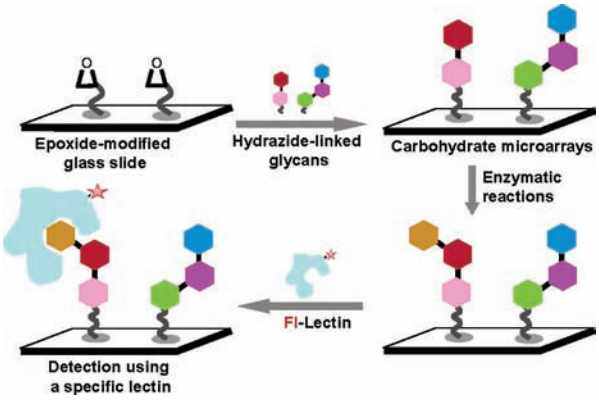
ABSTRACT



Carbohydrate microarrays have been used recently for the rapid analysis of glycan–protein or glycan–cell interactions and for the detection of pathogens. As a demonstration of its significance and versatility, the microarray technology has been applied in this effort to assay glycosyltransferase activities. In addition, carbohydrate microarray based methods have been employed to quantitatively determine binding affinities between lectins and carbohydrates.

Glycans in living organisms play a critical role in both normal physiological and detrimental disease processes through their interactions with proteins.[1] Owing to their significance in biological research and biomedical applications, the development of rapid and sensitive methods to elucidate the nature and consequence of glycan−protein interactions has become the target of a number of recent investigations. In 2002, we and others reported a novel, carbohydrate microarray based technology for this purpose.[2] An important advantage over other conventional methods is that a large number of glycan−protein interactions can be simultaneously analyzed by using a small amount of carbohydrate samples. In addition, glycans attached to surfaces resemble those on cell surfaces, and consequently, they may interact with proteins in a similar way to when they are located on cell surfaces. Furthermore, glycans immobilized on solid surfaces in a properly spaced and oriented manner display a cluster effect promoted multivalency which leads to enhancement in their interactions with proteins. These unique properties allow carbohydrate microarrays to be ideally applied in glycomics research for the rapid assessment of glycan−protein interactions.[3,4]

A major driving force for the expanded use of glycan microarrays is the development of new experimental proto-

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cols in which they can be applied. A potentially important application of these microarrays is to assay glycosyltransferase activities. To date, only a few examples of enzymatic reactions taking place on the carbohydrate microarrays have been reported.[2c,4a] However, this microarray technology has been rarely used in assaying glycosyltransferase activities.[5] Herein, we describe the results of our recent studies which show that carbohydrate microarrays can play an important role in methods for assaying galactosyltransferase activity. The approach requires only a tiny amount of immobilized acceptor substrates (picomoles), and enzymatic catalytic activities are readily assessed by measuring the amount of a product formed in a time-dependent manner. The level of time-dependent product conversion is determined by using fluorescence detection of lectin recognition of carbohydrate products.

In recent studies, we developed a novel, efficient, chemoselective immobilization method for the fabrication of chemical microarrays which relies on the reaction of hydrazide-conjugated compounds with epoxides derivatized on solid surfaces.[4b] In the current effort, we employed this immobilization strategy to prepare carbohydrate microarrays. Twenty hydrazide-conjugated carbohydrate probes were prepared by first reacting 2-aminoethyl glycosides with carboxylic acids on resins in the presence of coupling reagents and subsequently releasing the products with 1% triethylsilane (TES) in TFA (Figure 1). Carbohydrate microarrays (spot size, ca. 120 μm diameter), composed of 20 carbohydrate probes, were prepared by duplicate printing of solutions of hydrazide-linked glycans **1**−**20** (1 nL, 1 mM) on epoxide-coated glass slides by using a pin-type microarrayer. After 3 h immobilization in a humid chamber with 60% humidity, the glass slides were treated with 2-aminoethanol to remove the unreacted epoxide groups.

Before using the microarrays to assay galactosyltransferase activities, their binding preferences for galactose-binding proteins, such as *Pseudomonas aeruginosa* agglutinin-I lectin (PA-IL), *Bandeiraea simplicifolia* I (BS-I), and *Ricinus communis* agglutinin 120 (RCA_{120}), were examined to select an appropriate lectin for the detection of galactose moieties transferred by β-1,4-galactosyltransferase (β-1,4-GalT). Quite

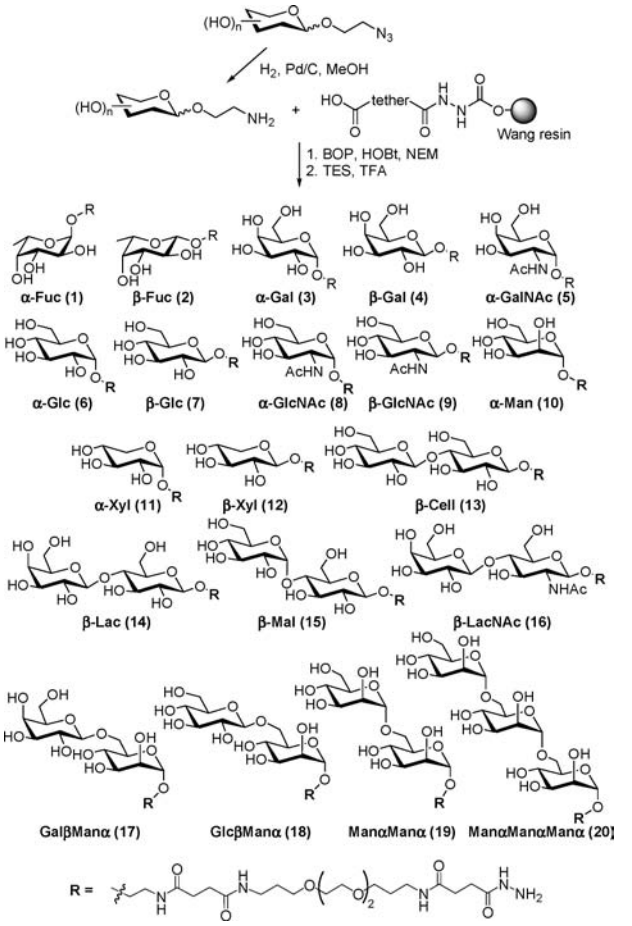

Figure 1. Preparation and structures of carbohydrate probes used for fabrication of the carbohydrate microarrays (HOBt = 1-hydroxybenzotriazole, BOP = 1-benzotriazolyloxytris(dimethylamino)-phosphonium hexafluorophosphate, NEM = *N*-ethylmorpholine).

different galactose-binding patterns were observed when the carbohydrate microarrays were probed with three galactose-binding lectins. The results show that Cy5-PA-IL has a binding preference for α-Gal (**3**), β-Gal (**4**), and Gal1,6βMan (**17**), and it rarely binds to β-Lac (**14**) and β-LacNAc (**16**) (Figure 2a). The binding tendency of PA-IL is consistent with findings obtained by using the microtiter plate lectin-binding assay and inhibition of carbohydrate−lectin interactions with sugar ligands.[6] Additional studies with these microarrays show that Cy3-BS-I recognizes α-Gal (**3**) and α-GalNAc (**5**), an observation that is in agreement with hemagglutination assay results (Figure 2b).[7] RCA_{120} was found to bind to a more diverse set of galactose-containing glycans (Figure 2c). Because this lectin recognizes α-Gal (**3**), β-Gal (**4**), and Gal1,6βMan (**17**) as well as β-Lac (**14**) and β-LacNAc (**16**),[8] it was selected for studies probing the detection of galactose moieties transferred by β-1,4-GalT.

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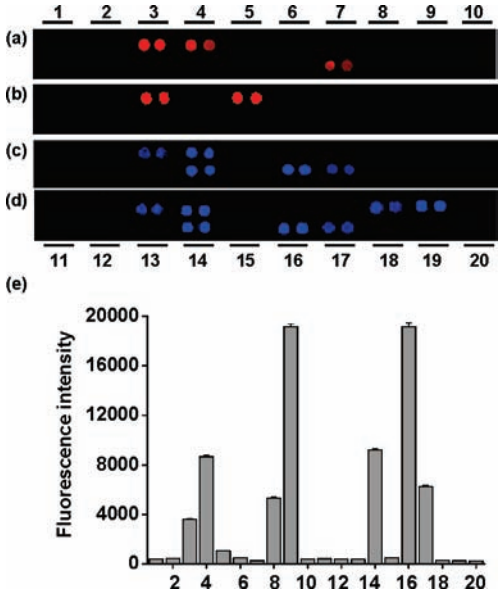

Figure 2. Carbohydrate microarrays probed with (a) Cy5-PA-1L, (b) Cy5-BS-I, (c) Cy3-RCA_{120}, and (d) Cy3-RCA_{120} after enzymatic reaction with β-1,4-GalT. (e) Quantitative analysis of fluorescence intensity of a carbohydrate microarray probed with Cy3-RCA_{120} after enzymatic reaction with β-1,4-GalT.

To profile β-1,4-GalT activity, glycan microarrays containing 20 carbohydrates were incubated with 1 mU β-1,4-GalT (a final concentration of 0.5 μM) in the presence of 10 mM Mn^{2+} and 1 mM UDP-Gal at 37 °C. After 3 h incubation, the slides were washed with a hot SDS solution (35 mM in PBS buffer) under sonication to reduce background fluorescence signals after probing with Cy3-RCA_{120}.[5c] Notably, omission of this step results in a high background fluorescence. Probing the enzyme-treated microarrays with Cy3-RCA_{120} leads to fluorescence patterns that demonstrate that both α- (**8**) and β-GlcNAc (**9**) are converted to α- and β-LacNAc by this enzyme (Figure 2d).[9] The fluorescence intensity at the region of β-GlcNAc is larger than that for α-GlcNAc (Figure 2d and 2e). In addition, the fluorescence intensity of β-LacNAc converted from β-GlcNAc (**9**) by β-1,4-GalT is almost the same as that of β-LacNAc (**16**), suggesting that a 3 h reaction time is sufficient to bring about complete conversion of β-GlcNAc to β-LacNAc.

The relative binding affinities of α- and β-LacNAc for RCA_{120} were determined. One approach to the synthesis of α-LacNAc, required for this purpose, involves chemical glycosylation of protected α-bromoethyl GlcNAc with several protected galactosyl donors (e.g., galactosyl bromide, galactosyl trichloroacetimidate, and thioglycosides) in the presence of a promoter (BF_3·OEt_2, AgOTf, or NIS-TfOH). Unlike the related process used to prepare β-LacNAc, the chemical glycosylations are not successful. Next, we explored an enzymatic glycosylation route using β-galactosidase from *Bacillus circulans*, an enzyme that is known to catalyze glycosylation reactions of GlcNAc derivatives.[10] Incubation of unprotected α-2-azidoethyl GlcNAc and *p*-nitrophenyl galactoside with *B. circulans* β-galactosidase for 3 h at 55 °C led to the formation of 2-azidoethyl α-LacNAc in 78% yield. Hydrazide-conjugated α- and β-LacNAc were printed on the epoxide-modified glass slides, and then the resulting microarray was incubated with Cy3-RCA_{120}. The observation that microspots containing α- and β-LacNAc show similar fluorescence intensities indicates that RCA_{120} has a similar binding affinity for both of these compounds.

To determine quantitative binding affinities of α- and β-LacNAc for RCA_{120}, dissociation constants (K_d) for lectin-surface-linked LacNAc interactions were measured by using carbohydrate microarrays. Although carbohydrate microarrays have been applied to determine IC_{50} values of soluble inhibitors,[2c,4a] dissociation constants associated with protein−glycan interactions have never yet been measured by using this technology. To determine dissociation constants, α- and β-LacNAc (1 mM immobilization concentration) were printed on the epoxide-derivatized surfaces and the resulting microarrays were probed with various concentrations (0.12 nM−1 μM) of Cy3-RCA_{120}.[11] 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. As shown in Figure 3a, the K_d values of α- and β-LacNAc were determined to be 34 nM and 33 nM, respectively (see Supporting Information for the method employed to determine K_d values). These K_d values are similar to those (26.3 nM for α-LacNAc and 25.3 nM for β-LacNAc) obtained by using a conventional SPR technology (Figure 3b and 3c).[12] Therefore, the higher fluorescence intensity observed at the region of β-GlcNAc after enzymatic reaction as compared to α-GlcNAc indicates that β-GlcNAc is a better substrate for β-1,4-GalT.

We then examined time-dependent glycosylation of α- and β-GlcNAc immobilized on the surface by β-1,4-GalT to determine the relative enzymatic glycosylation rates (Figure 4). In these studies, we measured the initial velocity of glycosylation of two substrates by an enzyme. Carbohydrate microarrays containing α- and β-GlcNAc (1 mM), along with α- and β-LacNAc (1 mM) as controls, were fabricated and then treated with 1 mU β-1,4-GalT in the presence of 10 mM Mn^{2+} and 1 mM UDP-Gal at 37 °C for various time periods (0−60 min). After washing with a hot SDS solution under sonication, the enzyme-treated microarrays were probed with Cy3-RCA_{120}. On the other hand, the fluorescence intensities of various concentrations (10^{-3}−1 mM) of α- and β-LacNAc immobilized on the surface were measured after probing with Cy3-RCA_{120}. Because fluorescence intensities

(9) The β-1,4-GalT activity was also examined by using carbohydrate arrays containing 10 carbohydrates (ref 2c). This work showed that although β-GlcNAc was converted to the corresponding product, α-GlcNAc is not a substrate for this enzyme unlike our results. HPLC analysis of enzymatic products (see Supporting Information) and the present work demonstrate that both α- and β-GlcNAc are substrates for this enzyme.

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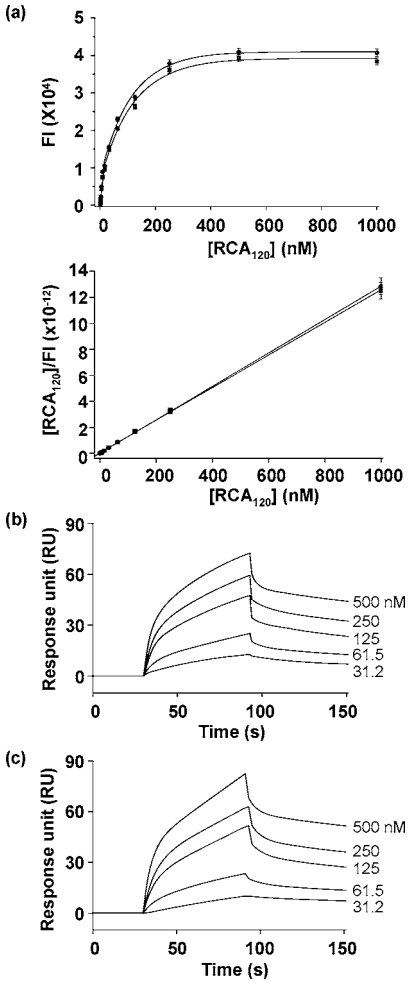

Figure 3. (a) Determination of dissociation constants (K_d) for RCA_{120}-surface-linked LacNAc interactions using carbohydrate microarrays (●, α-LacNAc; ■, β-LacNAc; FI, fluorescence intensity) (K_d values were determined by using the equation $[P]_o/FI = K_d/Fl_{max} + [P]_o/Fl_{max}$, see Supporting Information). Determination of dissociation constants for RCA_{120}-surface-linked (b) α- and (c) β-LacNAc interactions using SPR technology (RCA_{120} concentrations: 500, 250, 125, 61.5, and 31.2 nM).

of α- and β-LacNAc on the surface and concentrations of these carbohydrates show a linear relationship, the fluorescence intensities of products converted from α- and β-GlcNAc by an enzyme were used to determine the relative enzymatic glycosylation rates. The results obtained by using this procedure show that the relative rates of glycosylation of β- to α-GlcNAc by β-1,4-GalT are 11:1. For comparison purposes, enzymatic reactions in solution were also performed. The

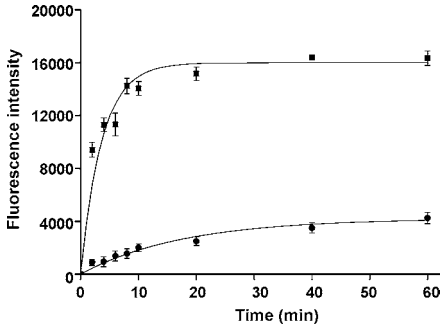

Figure 4. Time-dependent glycosylation of α- (●) and β-GlcNAc (■) immobilized on the surface by β-1,4-GalT.

ratio of β- to α-GlcNAc glycosylation rates is determined to be 10:1 in solution (1 mM α- or β-azidoethyl GlcNAc, 10 mM Mn^{2+}, 1 mM UDP-Gal, and 1 mU β-1,4-GalT at 37 °C).[13] Therefore, the ratio of enzymatic glycosylation rates determined by using the carbohydrate microarray method is similar to that for the corresponding solution reactions.

In conclusion, we have demonstrated the usefulness of carbohydrate microarrays in assaying glycosyltransferase activities. Importantly, this technology requires only minute amounts of glycosyl acceptors. In addition, applications of these microarrays to determine quantitative binding affinities between carbohydrates and proteins have been demonstrated. The findings should open new applications of carbohydrate microarrays in the field of functional glycomics.

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Supporting Information Available: Experimental procedure, enzymatic reactions on carbohydrate microarrays, and determination of dissociation constants using carbohydrate microarrays. This material is available free of charge via the Internet at http://pubs.acs.org.

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