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A functional carbohydrate chip platform for analysis of carbohydrate–protein interaction

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Abstract
A carbohydrate chip based on glass or other transparent surfaces has been suggested as a potential tool for high-throughput analysis of carbohydrate–protein interactions. Here we proposed a facile, efficient, and cost-effective method whereby diverse carbohydrate types are modified in a single step and directly immobilized onto a glass surface, with retention of functional orientation. We modified various types of carbohydrates by reductive amination, in which reducing sugar groups were coupled with 4-(2-aminoethyl)aniline, which has di-amine groups at both ends. The modified carbohydrates were covalently attached to an amino-reactive NHS-activated glass surface by formation of stable amide bonds. This proposed method was applied for efficient construction of a carbohydrate microarray to analyze carbohydrate–protein interactions. The carbohydrate chip prepared using our method can be successfully used in diverse biomimetic studies of carbohydrates, including carbohydrate–biomolecule interactions, and carbohydrate sensor chip or microarray development for diagnosis and screening.

Online supplementary data available from stacks.iop.org/Nano/21/215101/mmedia
(Some figures in this article are in colour only in the electronic version)

1. Introduction
Over the past several decades, gene cloning and protein engineering techniques have advanced markedly, and it is now feasible to identify functions of the entire human genome and all encoded proteins. Whereas research on genomics and proteomics has been extremely active, glycomics has only recently become prominent in efforts to clarify important roles of carbohydrates. Inside the cell, monosaccharides are utilized as energy sources, and complex carbohydrates (glycans) have critical roles in determination of biological functions (protein folding, stability, and pharmacokinetics) and affect diverse physical processes including specific molecular recognition and communication when the glycans are conjugated with lipids and proteins [1–4]. To understand the biological functions of carbohydrates, efficient carbohydrate immobilization methods onto appropriate surfaces are essential, and carbohydrate libraries and effective analytical screening methods are then required [5–11].

In recent years, diverse techniques for carbohydrate immobilization have been reported; some involve noncovalent immobilization onto specific surfaces or membranes, and others immobilize carbohydrates covalently onto gold or glass surfaces [12–21]. As carbohydrates and glyco-conjugates are recognized to be the most complex biomaterials in nature, and as the amounts of naturally occurring glycans of interest are extremely low and thus difficult to obtain in sufficient quantities [22], in vitro carbohydrate study is challenging. Therefore, for carbohydrate immobilization, it is most important to develop facile and efficient methods, including simple modes of carbohydrate modification and direct immobilization techniques to reduce handling amount and loss of carbohydrates. In addition to the requirement

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for facile and efficient immobilization, functional sites on carbohydrates must be exposed (to allow proper functional orientation) for the study of specific biomolecular interactions. Thus, immobilized carbohydrates with functional orientation can mimic natural structures shown on the cell surface. However, the complexity of and non-functional groups in carbohydrates make it difficult to immobilize such materials in an oriented manner on surfaces, with retention of inherent structure and functionality.

Previously, we developed a facile method for carbohydrate modification and direct immobilization onto a gold surface using a reductive amination reaction [23]. However, gold-based systems have limited capability for high-throughput analysis with fluorescence detection [24, 25]. A carbohydrate chip based on glass or other transparent surfaces has been suggested as a potential tool for high-throughput analysis with a chip based on glass or other transparent surfaces has been suggested as a potential tool for high-throughput analysis with fluorescence detection [24, 25]. A carbohydrate chip based on glass or other transparent surfaces has been suggested as a potential tool for high-throughput analysis with a chip based on glass or other transparent surfaces has been suggested as a potential tool for high-throughput analysis with fluorescence detection [24, 25]. A carbohydrate chip based on glass or other transparent surfaces has been suggested as a potential tool for high-throughput analysis with a chip based on glass or other transparent surfaces has been suggested as a potential tool for high-throughput analysis with fluorescence detection [24, 25].

2. Materials and methods

2.1. Carbohydrate modification

One hundred millimolar solutions of six disaccharides (lactose (Junsei, Tokyo, Japan), galactose (Sigma, St Louis, MO, USA), maltose (Junsei), mannobiose (V-Labs, Los Angeles, CA, USA), diacetylgalactosamine mannose (V-Labs), and acetylglucosamine mannose (V-Labs)), GM1 pentasaccharide (Alexis Biochemicals, San Diego, CA, USA) and two GM1 analogs (asialo GM1 and GM3) (Alexis Biochemicals) were dissolved in water, and 4-(2-aminoethyl)aniline (Sigma) was dissolved in glacial acetic acid to 100 mM. The carbohydrate and 4-(2-aminoethyl)aniline solutions were mixed well and incubated in sealed tubes for 1 h at 37 °C. Next, freshly prepared reducing reagent, 100 mM dimethylamine borane (Fluka, Milwaukee, WI, USA), was added to each reaction solution and the sealed tubes incubated for 1 h at room temperature. Samples were then transferred into glass vials and heated for 1 h at 50 °C under nitrogen gas streaming [23]. All modified carbohydrates were dissolved in print buffer (150 mM phosphate; pH 8.5, containing 5% (v/v) glycerol, 0.1 mg ml⁻¹ BSA, and 0.04% (v/v) Tween 20) and held at 4 °C. Without further treatment steps, modified carbohydrates were immobilized onto glass surfaces after confirmation of modifications by high performance liquid chromatography (HPLC), matrix-assisted laser desorption/immobilization/time-of-flight mass spectrometry (MALDI-TOF MS), and nuclear magnetic resonance (NMR) analyses.

2.2. HPLC analysis

Modified carbohydrate samples were separated by normal-phase HPLC (Shimadzu, Tokyo, Japan) on a C18 column (3.6 mm × 100 mm; Alltech, Deerfield, IL, USA). Samples were eluted using a linear gradient of acetonitrile (10–50%, v/v) in 0.1% (v/v) trifluoroacetic acid (TFA), and monitored by a photodiode array (PDA) detector at 254 nm.

2.3. MALDI-TOF MS analysis

To improve the ionization efficiency of MALDI-TOF MS, modified carbohydrate samples were desalted using Zip-tip C18 (Millipore, Billerica, MA, USA) and next eluted onto MALDI target plates using matrix solution (10 mg 2,5-dihydroxybenzoic acid (DHB; Sigma) dissolved in 20% (v/v) acetonitrile and 0.1% (v/v) TFA). A 1 μl sample was mixed with the same volume of DHB matrix solution and loaded onto the MALDI target; sample spots were allowed to vacuum dry at room temperature. All mass spectra were acquired in the reflection mode using a Voyager DE-STR (PerSeptive Biosystems, Flamingham, MA, USA) in the Korean Basic Science Institute. Spectra were obtained in the mass range 100–2000 Da using ~200 laser shots. Internal calibration was performed employing the 4700 Cal Mix (Applied Biosystems, Forster City, CA, USA) [26].

2.4. NMR analysis

¹H NMR spectra of modified carbohydrates were acquired at 20 °C (solvent D₂O) using an ABANCE 300 FT-NMR spectroscope (300 MHz; Bruker, Germany). Data were reported as follows: chemical shifts (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), and coupling constants (Hz).

2.5. Preparation of carbohydrate chip

NH₂-modified carbohydrates in print buffer were spotted onto NHS-activated glass slides (GmbH, Jena, Germany) [27] using a Microsys 5100 microarrayer (Cartesian Technologies, Ann Arbor, MI, USA) with the Chip Maker 2 pin (Telecom International, Sunnyvale, CA, USA) at 75% humidity in a class 10000 clean room and incubated overnight under the same humidity conditions to achieve tight immobilization [28].

2.6. Carbohydrate–lectin interaction assay

After immobilization, each carbohydrate microarray slide was submerged in blocking solution (50 mM ethanolamine in 50 mM sodium borate buffer, pH 8.0) for 1 h to deactivate remaining functional groups; this was achieved in a clean 50 ml conical tube using gentle agitation. Next, each slide was removed from the blocking solution and rinsed with wash buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄; pH 7.5, with 0.5% (v/v) Tween 20) and wash buffer II (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, and 1.4 mM KH₂PO₄; pH 7.5). For interaction analysis, 40 μl rhodamine-conjugated lectin (concanavalin A (Con A; Vector Laboratories, Burlingame, CA, USA), Ricinus communis agglutinin I (RCA₁₂₀; Vector Laboratories), and wheat germ agglutinin (WGA; Vector Laboratories)) or fluorescein isothiocyanate (FITC)-conjugated Vibrio cholerae
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Figure 1. Strategy for carbohydrate modification and direct immobilization onto a glass surface. R: free saccharide (acyclic reducing sugar, see table 1), R²: R–CH₂–NH–Ph.

Table 1. Carbohydrates and their lectins in the present work.

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Sequence</th>
<th>Lectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltose</td>
<td>Glc[β1-4]Glc</td>
<td>ConA³</td>
</tr>
<tr>
<td>Mannobiose</td>
<td>Man[α1-3]Man</td>
<td>ConA</td>
</tr>
<tr>
<td>Lactose</td>
<td>Gal[β1-4]Glc</td>
<td>RCA₁₂₀</td>
</tr>
<tr>
<td>Galactobiose</td>
<td>Gal[β1-6]Gal</td>
<td>RCA₁₂₀</td>
</tr>
<tr>
<td>N,N′-diacetylchitobiose</td>
<td>GlcNAc[β1-4]GlcNAc</td>
<td>WGA²</td>
</tr>
<tr>
<td>β1,2-N-acetylglucosamin mannose</td>
<td>GlcNAc[β1-2]Man</td>
<td>WGA</td>
</tr>
</tbody>
</table>

a) Rhodamine-conjugated concanavalin A; ConA.
b) Rhodamine-conjugated Ricinus communis agglutinin I; RCA₁₂₀.
c) Rhodamine-conjugated wheat germ agglutinin; WGA.
d) FITC-conjugated Vibrio cholera toxin B subunit; CtxB. (Underline indicates terminal sugar.)

Toxin B subunit (FITC–CtxB; Sigma) were incubated under a supported coverslip for 1 h in a 75% humidity chamber (wash buffer was used for lectin dilution) and the slide washed with wash buffer/wash buffer II. A commercial confocal laser scanner (GSI Lumonics, Wilmington, MA, USA) was used for image acquisition.

3. Results and discussion

3.1. Modification of carbohydrates

We modified various types of carbohydrates (table 1) by reductive amination, in which reducing sugar groups were coupled with 4-(2-aminoethyl)aniline (NH₂–Ph–CH₂–CH₂–NH₂), which has di-amine groups at both ends (figure 1). The reductive amination is generally used in pyridylamination (PA) or 2-aminobenzamide (2-AB) reaction for glycan analysis [29–32]. The modified carbohydrates were covalently attached to an amino-reactive NHS-activated glass surface by formation of stable amide bonds.

By formation of a stable coupling between carbohydrate and a linker with a phenyl group (Ph), we could easily purify and analyze modified carbohydrates using HPLC. All carbohydrate modifications were conducted at 37°C for 1 h, and solvent was directly evaporated under N₂ streaming. With disaccharides (lactose and maltose), HPLC analysis showed 88.8% and 92.9% purity, respectively (figure S1 in supplementary data available at stacks.iop.org/Nano/21/215101/mmedia). This method significantly simplified the modification process, greatly reduced carbohydrate loss during modification, and showed high derivatization efficiency. Compared to other modification methods with multiple steps, introduction of highly reactive and simple reductive amination contributed to reduced carbohydrate loss during modification with the selected linker. Because reductive amination reaction occurs at a relatively low temperature in a short reaction time and does not need other additive reagents, a high yield of modified carbohydrate was possible. After purification by HPLC and subsequent analysis by MALDI-TOF MS, we found that the molecular weights of all modified carbohydrates, including lactose and maltose, were identical to the calculated values (figure 2). In addition to determining which of the amine groups in the selected linker was used in coupling, we performed NMR analysis and found that the spectrum contained features consistent with a –CH₂–NH–Ph– moiety (for lactose (figure 3(a)), ¹H NMR (300 MHz, D₂O, δ): 7.40–7.33 (m, 4H; Ph), 4.09–4.04 (m, 1H; 2-H), 2.63 (s, 2H; 5-H₂); for maltose (figure 3(b)), ¹H NMR (300 MHz, D₂O, δ) 7.37 (d, J = 8.55 Hz, 2H; 5-H), 7.28 (d, J = 8.59 Hz, 2H; 4-H), 3.97–3.93 (m, 1H; 3-H), 2.63 (s, 2H; 8-H₂); other
Figure 2. MALDI-TOF MS spectra of modified (a) lactose (Lac–NH₂) and (b) maltose (Mal–NH₂). The MS profiles indicated successful synthesis of Lac–NH₂ and Mal–NH₂ (calculated molecular weights: 463) through reductive amination between the 4-(2-aminoethyl)aniline linker (using the primary amine close to the phenyl group) and carbohydrate (employing an aldehydic endgroup). In addition, two disaccharide-containing products (disaccharide-linker-disaccharide; calculated molecular weight: 789) were observed, because the linker had two amine groups.

NMR data including the free linker are shown in figures S2–S4 in supplementary data available at stacks.iop.org/Nano/21/215101/mmedia). Consequently, the amine close to the phenyl group was mainly coupled with carbohydrate. Although we suspected that another amine might react easily because the group can be a strong electron donor, contrary results were seen with both lactose and maltose. Therefore, we surmise that the amine close to the phenyl group (aniline terminus) is preferred for coupling because either an intermediate or the final product might be more stable during or after reaction. When sole amine group exists, reductive amination occurs mainly by nucleophilic attack. However, for the system of two amines, reactivity might be determined by other factors like conjugation stability of intermediate or product. The single product peak shown by NMR analysis might support this hypothesis (figure 3). Overall, the MS and NMR analyses confirmed that all carbohydrates, including six disaccharides, GM1 pentasaccharide, and two GM1 analogs, were successfully modified.

3.2. Construction of carbohydrate chip platform

We directly immobilized the NH₂-modified carbohydrates onto an NHS-coated glass surface without additional treatment.
A microarray with six disaccharides was chosen as a suitable format (6 × 6, 12 repeats, figure S5 in supplementary data available at stacks.iop.org/Nano/21/215101/mmedia) and used for assays of lectins binding to carbohydrates (figure 4). Three forms of rhodamine-conjugated lectins were used to detect the terminal sugars of six disaccharides. Mannobiose (Man–Man) showed greater affinity than did maltose (Glc–Glc) in the ConA assay (figure 4(a)) and this result was in agreement with the fact that ConA exhibits a higher affinity for D-mannose [33, 34]. In the case of the RCA lectin assay (figure 4(b)), lactose (Gal–Glc) showed a higher affinity than did galactobiose (Gal–Gal) because RCA displays a greater affinity for structures with terminal β-1,4-linked galactose residues [35]. Also, in the WGA assay (figure 4(c)), N,N′-diacetylchitobiose (GlcNAc–GlcNAc) showed a much greater affinity than did β-1-2N-acetylgalcosamine mannose (GlcNAc–Man), owing to the presence of two β-1,4-linked N-acetylgalcosamines [36, 37]. The lectin binding results showed that the modified carbohydrates retained their terminal sugars after reductive amination and were immobilized on the glass surface with retention of functional orientation. We thus found that the carbohydrate chip platform constructed by our efficient and direct immobilization method could be successfully used to identify specific structural features of carbohydrate–lectin interactions.

We investigated the effects of carbohydrate concentration on immobilization and detection (figure 4(d)). Fluorescence imaging was adequate for detection at carbohydrate levels of at least 1 mM, but at levels less than 1 mM fluorescence intensities were markedly decreased and detection was rather...
difficult. Thus, subsequent microarrays were constructed using 20 mM levels of modified carbohydrates. Next, we checked the lectin detection limit using WGA as a model (figure 4(e)). At concentrations greater than 100 μg ml\(^{-1}\), fluorescence intensities were similar (data not shown), but intensities decreased with reduction in lectin concentration. The lectin WGA was detectable to 50 ng ml\(^{-1}\) (1.39 nM) (figure 4(e)). Although the limit of detection of lectin concentration might be dependent both on the concentration of immobilized carbohydrate and lectin properties, we concluded that the carbohydrate chip prepared in this study was effective in the sensing of carbohydrate–protein interactions, thus mimicking the association between lectin and carbohydrate on the cell surface. Based on these results, we considered that a critical factor in carbohydrate–protein interaction analysis might be the immobilized carbohydrate concentration rather than lectin level. We surmise that a biological system would have evolved to express relatively high levels of carbohydrates on the cell surface for purposes of detection of and/or communication with low concentrations of environmental materials.

Therefore, the results collectively indicate that our proposed method using commercially available 4-(2-aminoethyl) aniline as both coupling reagent and linker is highly efficient in immobilization of carbohydrates onto a glass surface for functional construction of a carbohydrate chip. Chemical synthesis of a linker with a polyethylene glycol rather than an alkyl chain, and with other functional groups for surface binding, might improve the current carbohydrate microarray platform with respect to efficiency of lectin binding. In addition, linkers of appropriate length for optimal accessibility and conjugation of analytical groups such as fluorescent tags will assist in advancing carbohydrate-related research.

### 3.3. Application of carbohydrate chip tool for interaction analysis between GM1 pentasaccharide and *V. cholerae* toxin protein

To demonstrate a practical application of our chip platform for carbohydrate–protein interaction analysis, we modified the GM1 pentasaccharide and analogs thereof (asialo GM1 and GM3; table 1) and immobilized these materials onto a glass surface (figure S5 in supplementary data available at stacks.iop.org/Nano/21/215101/mmedia). The interaction between GM1 and *V. cholerae* toxin proteins has been widely regarded as a good model of carbohydrate–protein interaction [38–41]. FITC–CtxB was used as partner lectin because the GM1 pentasaccharide can interact with the sole CtxB subunit. The GM1 pentasaccharide–CtxB interaction showed strong affinity and specificity, displaying a high fluorescence intensity (figure 5). However, analog analyses using asialo GM1 and GM3, which lack terminal sialic acid (Neu5Ac) and galactose-\(N\)-acetylgalactosamine (Gal–GalNAc), respectively, yielded low fluorescence intensities. Thus, it was clear that deletion of the terminal sugar significantly affected interaction (figures 5(b) and (c)). Interestingly, we found that lactose (which has the same structure after deletion of three sugars from GM1) did not show any fluorescence; that is, the residual Gal–Glc structure could not interact with CtxB. Indeed, it has been reported that three monosaccharides (terminal Gal, Neu5Ac, and GalNAc) are responsible for ~99% of the GM1 pentasaccharide–CtxB interaction [42]. Therefore, using the carbohydrate chip constructed by our method, we successfully analyzed the specific structural interaction between GM1 pentasaccharide and *V. cholerae* toxin.
4. Conclusions

We herein developed a simple and cost-effective method, in which the di-amine group of commercially available 4-(2-aminoethyl)aniline as both coupling reagent and linker was successfully coupled with the aldehyde group of the terminal reducing sugars of diverse carbohydrates, and the modified carbohydrates could be directly immobilized onto a glass surface for efficient construction of a carbohydrate chip. The functional carbohydrate chip prepared using our facile and efficient modification and immobilization method can be successfully used in diverse biomimetic studies of carbohydrates, including carbohydrate–biomolecule interactions, and carbohydrate sensor chip or microarray development for diagnosis and screening.

Acknowledgments

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