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Trityl-derivatized carbohydrates immobilized on a polystyrene microplate

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ABSTRACT

Carbohydrate biosensors, including carbohydrate arrays, are attracting increased attention for the comprehensive and high-throughput investigation of protein–carbohydrate interactions. Here, we describe an effective approach to fabricating a robust microplate-based carbohydrate array capable of probing protein binding and screening for inhibitors in a high-throughout manner. This approach involves the derivatization of carbohydrates with a trityl group through an alkyl linker and the immobilization of the trityl-derivatized carbohydrates (mannose and maltose) onto microplates noncovalently to construct carbohydrate arrays. The trityl carbohydrate derivative has very good immobilization efficiency for polystyrene microplates and strong resistance to aqueous washing. The carbohydrate arrays can probe the interactions with the lectin *Concanavalin A* and screen this protein for the well-known inhibitors methyl α-D-mannopyranoside and methyl α-D-glucopyranoside in a high-throughput manner. The method described in this paper represents a convenient way of fabricating robust noncovalent carbohydrate arrays on microplates and offers a convenient platform for high-throughput drug screening.

1. Introduction

Protein–carbohydrate interactions are biologically important processes that govern many critical cellular functions in living organisms, such as cell recognition, cell communication and cell adhesion.[1–3] These interactions play critical roles in various human disorders such as HIV infections and cancers, and therefore extensive efforts have been directed to the development of therapeutic reagents (e.g., antibodies and small ligands) capable of inhibiting such interactions. The wide variety of natural or synthetic compounds,[4,5] however, imposes a big hurdle on the discovery of compounds that have potential for treating disease. Therefore, it is highly desirable to develop a high-throughout tool for drug candidate screening. In this regard, carbohydrate arrays (glycoarrays), prepared by attachment of carbohydrates to a solid surface in a spatially discrete pattern, have received considerable attention because of their ability to screen a large pool of samples in small quantities in a high-throughput manner[6–8] (e.g., using a 96-well microplate).

In recent years, a number of carbohydrate arrays have been developed by attaching carbohydrates to solid supports such as glass slides, gold surface and polystyrene microplates through either covalent or noncovalent immobilization strategies.[7–17] Examples include nitrocellulose- and gold-coated glass arrays as well as microplate-based arrays.[18–24] Among these methods, the noncovalent type is attractive in the sense that this approach avoids the covalent modification of chemically inert solid supports (e.g., polystyrene microplates), which is often challenging and complicated.[15,17,25] Noncovalent carbohydrate immobilization can be achieved by means of hydrophobic interactions. Carbohydrate arrays of this type are particularly promising because polystyrene microplates are the most commonly used samplers and compatible with most microplate readers provided by commercial companies. Moreover, the microtiter plate approach can be coupled with ELISA assays to probe the carbohydrate- binding interactions.[15] In this category, microplate-based carbohydrate arrays fabricated from lipid-bearing carbohydrates for screening for carbohydrate–protein interactions were first reported by the Wong group.[26] In this work, they developed a number of aliphatic alkyl chains as noncovalent linkers to attach carbohydrates to microtiter plates and further improved this strategy to synthesize and immobilize sugars to microtiter plates in situ so as to avoid the difficult handling of glycolipids.[19] Despite this encouraging result, the alkyl-derivatized carbohydrates are usually easily detached from polystyrene microplates in the presence of aqueous solutions, presumably due to their weaker hydrophobic forces relative to solvation forces. Besides, new microarrays have been fabricated by immobilizing fluorous-tagged sugars on fluorous-derivatized glass slides. The noncovalent interactions in the fluorous-based arrays are strong enough to resist detergents used in binding assays.[21] The fluorous derivatives are, however, rather costly, thus limiting their routine

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use in carbohydrate studies. As such, it is highly desirable to develop a strong and cost-effective noncovalent immobilizing agent for construction of microplate-based carbohydrate arrays.

It is generally believed that π–π interactions can occur between phenyl rings.[27,28] This interesting phenomenon inspired us to devise a noncovalent aromatic immobilizing agent for constructing carbohydrate arrays on phenyl-rich polystyrene microplates. Trityl linkers represent an excellent class of noncovalent immobilizing agents for this purpose, because the trityl group contains three phenyl rings that allow π–π interactions. Thus, trityl-derivatized carbohydrates are expected to bind strongly to polystyrene microplates. This carbohydrate array can serve as a robust and high-throughput drug screening tool for searching for promising drug candidates.

Here, we report the fabrication of a new and robust microplate-based carbohydrate array from trityl-derivatized carbohydrates. In this approach, the carbohydrate (e.g., mannose) is conjugated with a trityl group, which is responsible for attaching to polystyrene surface through an alkyl chain as a spacer. The alkyl linker can enhance carbohydrate accessibility for protein binding. The trityl-derivatized mannose performs good immobilization efficiency against polystyrene microplates and strong resistance against aqueous washing. The mannose-immobilized carbohydrate array can probe the interactions with the lectin Con A (*Concanavalin A*)[29] and can also be used to screen Con A against inhibitors in a high-throughput manner. In addition, the compatibility of the trityl immobilizing agent with the disaccharide maltose was also studied.

2. Results

2.1. Synthesis of trityl-derivatized carbohydrates

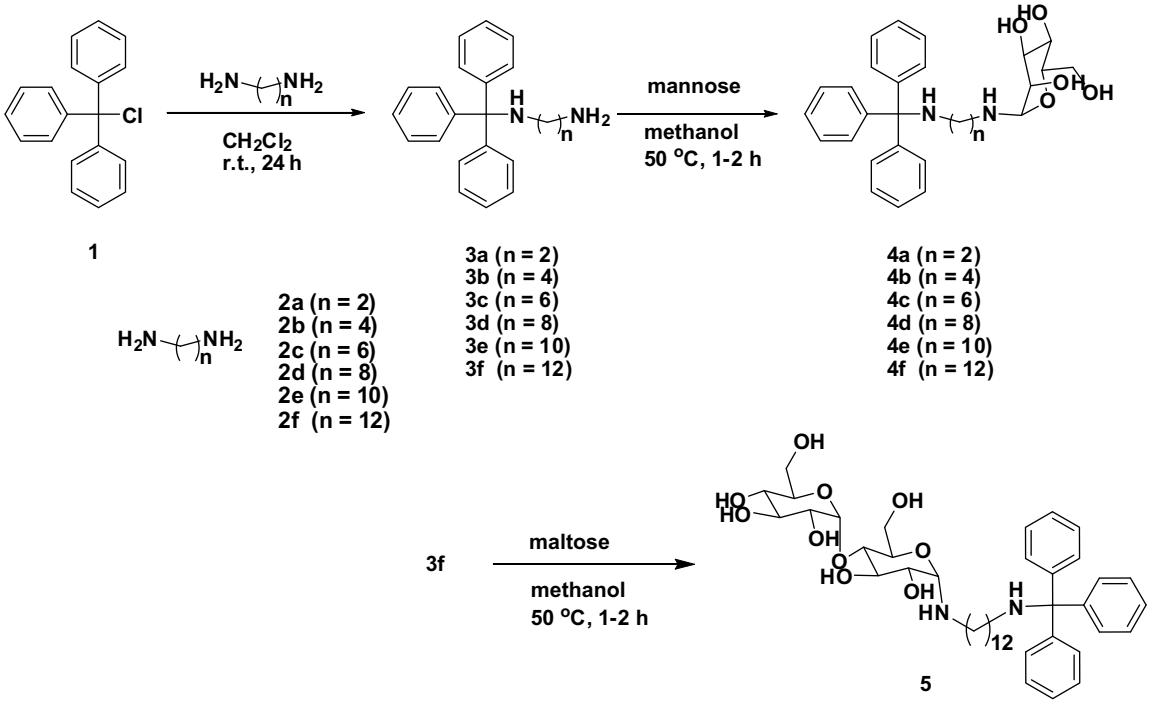
A series of trityl-derivatized mannoses (**4a–f**) were prepared[30,31] (Scheme 1). The preparation of the mannose derivatives requires only a simple two-step synthetic approach. The trityl group was first conjugated with a diaminoalkyl chain, which was subsequently allowed to couple with mannose. The desired targets were purified by column chromatography on silica gel in synthetically useful yields. The anomeric configurations of the glycosylamines were determined by measuring the coupling constants and chemical shifts of the respective products. Analysis of these data and those reported in the literature[30] indicated that the glycosylamines were of the α-configuration and in the pyranose form.

2.2. Fabrication of trityl-derivatized carbohydrate arrays

The mannose derivatives were then noncovalently immobilized on polystyrene microplates to fabricate trityl-derivatized carbohydrate arrays (Fig. 1). To investigate whether **4a–f** can bind to polystyrene microplates, phenol–sulfuric acid assay was performed. This colorimetric assay can determine carbohydrate concentrations by reacting phenol with carbohydrates to give chromogenic products, which absorb strongly at ~490 nm.[32] The absorbance at 490 nm (A_{490}) of **4a–f** immobilized on the microplate before and after washing with deionized water was determined. All the mannose derivatives exhibit strong visible absorbance at 490 nm (A_{490} >0.8) compared to the phenol–sulfuric acid solution (A_{490} = ~0.1) before washing with deionized water. The A_{490} of each mannose derivative remains virtually similar (Table 1) after washing with deionized water, indicating that the mannose derivatives are able to bind to the microplate.

2.3. Lectin-binding assay

The abilities of the trityl-derivatized carbohydrate arrays with **4a–f** to bind to concanavalin A (Con A) were studied by fluorescence spectroscopy. Briefly, the wells immobilized with trityl-derivatized carbohydrates were first washed with deionized water and then treated with phosphate buffer containing bovine serum albumin (to reduce nonspecific binding).[33] The wells were then incubated with fluorescein–Con A, followed by extensive washing with phosphate buffer. In all cases, the mannose derivatives gave a stronger fluorescence signal compared to the control (the trityl



Scheme 1. Synthetic procedures for trityl-derivatized carbohydrates (**4a–f** and **5**).

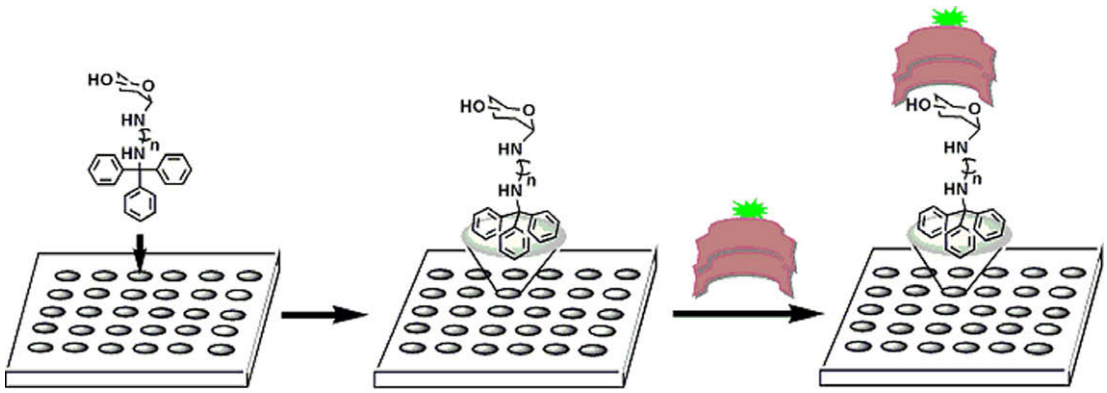
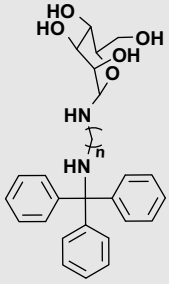


Figure 1. Fabrication of a trityl-derivatized carbohydrate array. A 40-μL aliquot of the stock solution of each sample (100 mM) was pipetted into a 96-well polystyrene microplate, which was subsequently dried by evaporation.

Table 1
Retention of various trityl-derivatized carbohydrates (**4a**–**f**) subjected to three washing cycles with deionized water

Trityl-derivatized carbohydrates			Retention[a] (%)
	4a (n = 2) 4b (n = 4) 4c (n = 6) 4d (n = 8) 4e (n = 10) 4f (n = 12)	**4a** **4b** **4c** **4d** **4e** **4f**	70 ± 11 90 ± 11 88 ± 18 99 ± 12 94 ± 19 91 ± 16

Trityl-derivatized carbohydrates

[a] The retention of (**4a**–**f**) was calculated by dividing the A_{490} of washed trityl-derivatized carbohydrates by the A_{490} of unwashed trityl-derivatized carbohydrates. Values are given as mean ± SD.

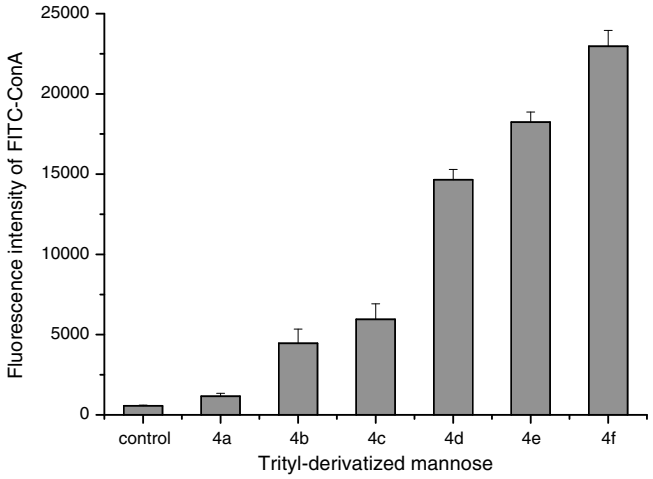


Figure 2. Fluorescence signals of fluorescein–Con A with carbohydrate arrays immobilized with various trityl-derivatized mannoses. Compounds **4a**–**f** and the trityl group (control) were immobilized onto microplates in similar quantity (4 μmol). The carbohydrate array was first washed with deionized water and then treated with BSA in phosphate buffer for 1 h. The microplate was then incubated with fluorescein–Con A for 1 h, followed by extensive washing with phosphate buffer and fluorescence measurements with a microplate reader. The error bars were obtained by five fluorescence measurements ($n = 5$).

group) after binding to fluorescein–Con A, indicating that these trityl-derivatized carbohydrates (**4a**–**f**) are able to bind to Con A (Fig. 2). Moreover, the fluorescence intensity of the complexed fluorescein–Con A increased with the length of the alkyl linker (Fig. 2). Because **4f** gave the strongest fluorescence signal, this mannose derivative was used for further studies.

In order to optimize the sensing function of the **4f**-glycoarray, different concentrations of **4f** (0–200 mM) were immobilized on a microplate, and their response was examined by the fluorescein–Con A assay. As shown in Figure 3, the fluorescence signal of complexed fluorescein–Con A increases as a function of **4f** concentration in the concentration range of 0–100 mM. Further increasing the concentration of **4f**, however, resulted in a fluorescence plateau, and even a slight decrease of fluorescence signal appeared at a concentration of 160 mM. Because the carbohydrate array constructed from 100 mM **4f** gave a maximum response, this concentration was chosen to fabricate trityl-derivatized carbohydrate arrays.

We then studied the immobilization efficiency of **4f** on polystyrene microplates. This mannose derivative was immobilized on a polystyrene microplate for different time intervals (2–28 h), and the relative amount of immobilized **4f** was examined by the fluorescein–Con A assay as described above. In all cases, the fluorescence signals of complexed fluorescein–Con A were virtually similar (Fig. S15, see Supplementary data), indicating that **4f** can be completely immobilized on a microplate within 2 h.

As many protein–carbohydrate interactions take place in an aqueous medium, the robustness of the **4f**-glycoarray in this environment was investigated. The resistance of **4f** against aqueous washing was studied by the fluorescein–Con A assay. The fluorescence signal of the **4f**-glycoarray complexed with fluorescein–Con A showed no significant decrease compared to the unwashed sample with that after six washing cycles (Fig. S16, see Supplementary data). However, in the case of trityl-free mannose subjected to similar washing steps, no fluorescein–Con A was found to bind to the microplate, implying that this sugar is unable to bind to polystyrene microplates without the trityl agent (data not shown). No fluorescence signal was detected when the **4f**-glycoarray was washed with ethanol, indicating that **4f** can be easily removed from microplates by this organic solvent (data not shown). These observations indicate that trityl-derivatized mannose can bind strongly to polystyrene microplates and is very resistant against aqueous washing rather than organic solvent.

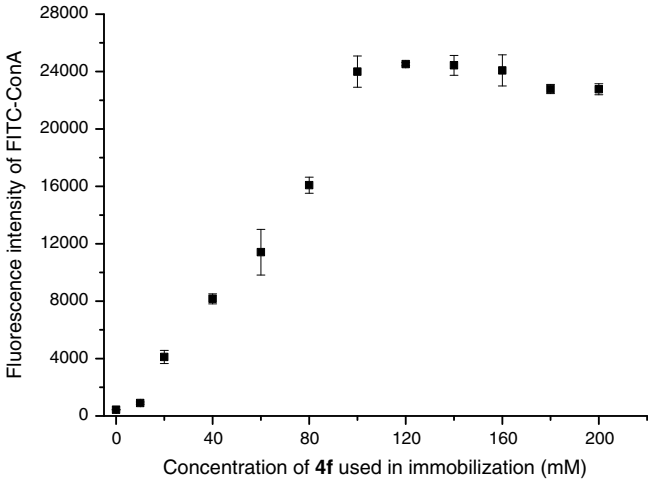


Figure 3. Plot of the fluorescence signals of fluorescein–Con A with the **4f**-glycoarray against different concentrations of **4f**. Different concentrations of **4f** (0–200 mM) were immobilized on a microplate, and the binding to fluorescein–Con A was examined by fluorescence measurements. The error bars were obtained by five fluorescence measurements ($n = 5$).

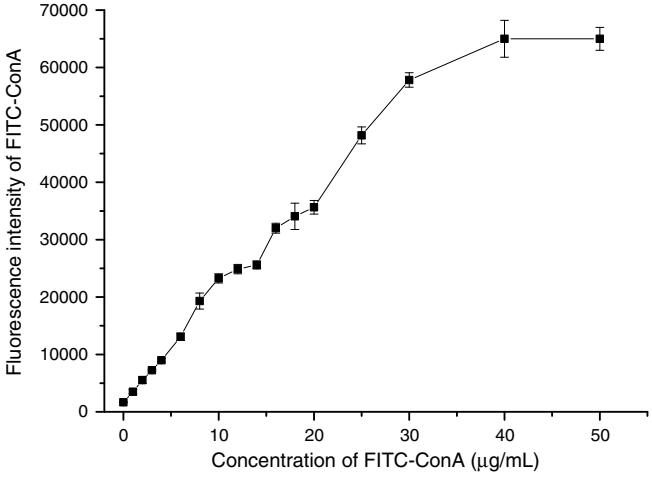


Figure 4. Plot of the fluorescence signals of fluorescein–Con A with the **4f**-glycoarray against different concentrations of fluorescein–Con A. The glycoarray was washed with deionized water (3 cycles) and then treated with BSA in phosphate buffer. The microplate was incubated with different concentrations of fluorescein–Con A (0–50 μg/mL), and the fluorescence signal was measured. The error bars were obtained by five fluorescence measurements ($n = 5$).

The working range for the **4f**-glycoarray was then studied by the fluorescein–Con A assay. Figure 4 shows the fluorescence response of the carbohydrate array complexed with different concentrations of fluorescein–Con A. The fluorescence response of the carbohydrate array shows good linearity up to 30 μg/mL fluorescein–Con A and reaches a plateau in the concentration range of 40–50 μg/mL of fluorescein–Con A, presumably due to the saturated binding of Con A to the carbohydrate array. The detection limit of the **4f**-glycoarray for Con A was determined to be 0.12 μg/mL.

2.4. Inhibitor screening

Methyl α-D-mannopyranoside and methyl α-D-glucopyranoside were used in the competitive binding studies of mannose with fluorescein–Con A. The BSA-treated **4f**-glycoarray was incubated with fluorescein–Con A in the presence of different concentrations of the inhibitors. In both cases, the fluorescence signal of fluorescein–Con A decreases with the increasing concentration of inhibitors (Fig. 5). The IC_{50} values for methyl α-D-mannopyranoside and methyl α-D-glucopyranoside were determined to be 2.7 and 5.7 mM, respectively.

2.5. Carbohydrate compatibility

In order to investigate the compatibility of the trityl immobilizing agent with oligosaccharides, we constructed a carbohydrate array with maltose (consisting of two glucose units) using the same approach and conditions and studied its sensing function by the fluorescein–Con A assay. The phenol–sulfuric acid array showed that the trityl-derivatized maltose (**5**) can bind to polystyrene microplates, as revealed by the strong A_{490} of **5** (washed extensively with deionized water) compared to that of the phenol–sulfuric acid solution (Fig. S17, see Supplementary data).

Fluorescence measurements indicated that the **5**-glycoarray can also sense the binding to Con A (Fig. S18, see Supplementary data); the fluorescence response of the carbohydrate array increases linearly in the concentration range of 0–12 μg/mL of fluorescein–Con A. Beyond this concentration range, the fluorescence response of the carbohydrate array levels off to give a plateau, presumably arising from the saturated binding of fluorescein–Con A to **5**. The lower saturation point of **5** (12 μg/mL) compared to that of **4f** (40 μg/mL) is likely to be due to the strong binding of maltose to Con A.[34]

The effects of methyl α-D-mannopyranoside and methyl α-D-glucopyranoside on the Con A/**5** binding were investigated by the inhibitor screening assay described above. No significant

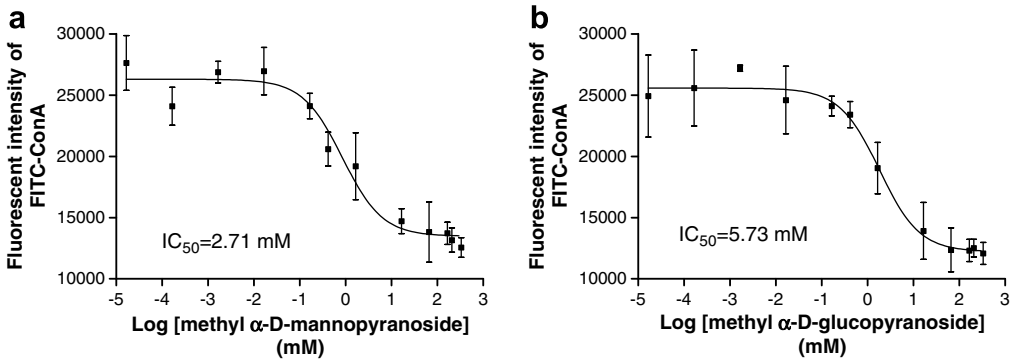


Figure 5. Determination of the IC_{50} values of (a) methyl α-D-mannopyranoside and (b) methyl α-D-glucopyranoside against the Con A/mannose binding using the **4f**-glycoarray. Fluorescein-Con A (10 μg/mL, in phosphate buffer) was first incubated with different concentrations of the inhibitors methyl α-D-glucopyranoside and methyl α-D-mannopyranoside (10 nM–400 mM) for 1 h. A 40-μL portion of each solution was then pipetted into the BSA-treated carbohydrate arrays, followed by incubation for another 1 h. The microplate was then washed with the same buffer for three cycles (5 min each), and the fluorescence signal of each well was measured.

inhibition was observed when the concentration of the inhibitors was increased to 400 mM (i.e., IC_{50} >400 mM). This observation is presumedly to be due to the strong interactions of Con A with maltose.

3. Discussion

Carbohydrate-mediated biological events such as protein–carbohydrate interactions are important in many living organisms. Many human disorders such as cancers and viral infections involve these biological processes, and therefore the development of new therapeutic agents for inhibiting protein–carbohydrate interactions has attracted much attention in recent years. In response to this growing research field, carbohydrate arrays are also under active development because of their potential application in high-throughput drug screening. In this regard, noncovalent carbohydrate arrays are an attractive choice because they can be easily constructed compared to the covalent ones, which usually require complicated chemical modifications on the solid supports. In the former, robustness is particularly important, and therefore it is necessary to use a strong immobilizing agent to firmly anchor the carbohydrate of interest.

In this study, we developed a new trityl immobilizing agent for the construction of microplate-based carbohydrate arrays. The trityl immobilizing agent has a very high propensity to bind to polystyrene microplates, as revealed by the phenol–sulfuric acid assay; the A_{490} values of **4a–f** recorded before and after washing are similar, with ~70% and 90% retention for **4a** and **4b–f**, respectively (Table 1). The lower retention for **4a**, which has a shorter alkyl spacer, is presumably due to their lower resistance to aqueous solution.

Previous studies have shown that the linker attached to the solid support confers access to carbohydrate–binding proteins/cells on the carbohydrate, and the length of the linker affects the binding of proteins to carbohydrate arrays.[8,17,35,36] In our case, we found that the fluorescence signal of the control is much lower than those of **4a–f** (Fig. 2). Moreover, extending the length of the alkyl linker can enhance the binding to Con A, as revealed by the increasing fluorescence signal of fluorescein–Con A with **4a–f** (Fig. 2). This observation can be attributed to the fact that increasing the length of the alkyl linker is likely to lead to the greater accessibility of the trityl-derivatized mannose to fluorescein–Con A, which facilitates the binding between these two species. This observation was also obtained in a previous study.[36] We attempted to further study the effects of alkyl linkers with more than 12 carbons. However, these alkyl linkers are not commercially available, and therefore their effects were not studied.

It has been reported that increasing the density of carbohydrates immobilized on the surface can facilitate protein–carbohydrate binding.[22] Excessive carbohydrate density, however, may lead to a weakening rather than enhancing effect on the binding to carbohydrate-binding proteins.[16] In our case, the fluorescence response reached the maximum level with 100 mM **4f**, implying that the well of the microplate can be fully coated at this carbohydrate concentration (Fig. 3). With **4f** concentration larger than 160 mM, a slight decrease in fluorescence intensity appears, indicating that the mannose–Con A binding becomes less favourable under this condition.

The fluorescein–Con A assays showed that the trityl-derivatized carbohydrate array is very robust. For example, the **4f**-glycoarray was found to bind to fluorescein–Con A to a similar extent before and after six cycles of aqueous washing. This observation implies that the trityl-derivatized mannose can bind strongly to polystyrene microplates and is highly resistant to aqueous washing. In contrast, most alkyl-derivatized carbohydrates were significantly lost from the polystyrene microplates after five cycles of aqueous washing (~50–100% loss).[26] These observations reveal that the trityl immobilizing agent binds more strongly to microplates than the alkyl type. This interesting phenomenon is likely to arise from the strong π–π interactions between the phenyl rings of the trityl group and polystyrene. The strong π–π interactions appear to allow the trityl-derivatized mannose (**4f**) to be efficiently immobilized on the microplate within 2 h.

The trityl-derivatized carbohydrate array devised in this study can serve as a high-throughput drug screening tool. The **4f**-glycoarray can screen Con A against methyl α-D-mannopyranoside and methyl α-D-glucopyranoside and distinguish the inhibitory activities of these two inhibitors. The IC_{50} values determined by the carbohydrate array indicate that methyl α-D-mannopyranoside is more effective in inhibiting the mannose–Con A binding than methyl α-D-glucopyranoside (Fig. 5). This observation is in good agreement with previous findings that methyl α-D-mannopyranoside is a stronger inhibitor of Con A binding than methyl α-D-glucopyranoside.[37]

The trityl immobilizing agent is also compatible with oligosaccharides. As similar to the case of the mannose derivatives, the disaccharide maltose can also be immobilized on polystyrene microplates after modified with the trityl immobilizing agent (Fig. S17) and used to probe the binding to Con A (Fig. S18). These observations highlight the high applicability of the trityl immobilizing agent in the construction of carbohydrate arrays with different carbohydrates.

In summary, we have developed an effective approach to fabricating a robust microplate-based carbohydrate array. The key element in this noncovalent carbohydrate array is the trityl immobilizing agent, which anchors firmly the carbohydrate of interest on polystyrene microplates. The trityl-derivatized carbohydrate array is very robust, apparently due to the strong π–π interactions between the phenyl rings of the trityl group and polystyrene. This carbohydrate array can be used to probe protein–carbohydrate interactions and to screen for inhibitors in a high-throughput manner. The trityl immobilizing agent is compatible with oligosaccharides and therefore has high applicability in the fabrication of various carbohydrate arrays. Moreover, the trityl immobilizing agent is more resistant to aqueous washing than the lipid-type immobilizing agent[26] and less expensive than the fluorous linker.[21] With these advantageous properties, the trityl-derivatized carbohydrate array should find its applications in a variety of areas, ranging from glycomic research to clinical diagnosis.

4. Experiment

4.1. Materials and instrumentation

NMR spectra were recorded with a Bruker DPX 400 MHz spectrometer. The ESIMSs were measured with a VG-PLATFORM spectrometer. Trityl chloride, 1,2-ethylenediamine, 1,4-diaminobutane, 1,6-diaminohexane, 1,8-diaminooctane, 1,10-diaminodecane and 1,12-diaminododecane were purchased from Sigma–Aldrich. D-Mannose and maltose were obtained from Sigma–Aldrich.

Bovine serum albumin (BSA), methyl α-D-glucopyranoside (99%), methyl α-D-mannopyranoside (99%) and polyethylene glycol sorbitan monolaurate (Tween 20) were obtained from Sigma–Aldrich. Fluorescein-labelled Concanavalin A (FITC-Con A, 104 kDa) was purchased from Vector Laboratories, Inc. Microtiter plates with a hydrophobic surface (no. 3631, 96 wells, flat clear bottom black polystyrene, nontreated) were obtained from Corning. All luminescence measurements were conducted on a BMG Labtech POLARstar microplate reader equipped with a microcomputer.

The wavelengths of the band pass filters for excitation and emission were 490 and 520 nm, respectively.

4.2. Synthesis of *N*-(*N*′-tritylaminoalkyl)-α-D-glycosylamines (4a–f and 5)

A solution of trityl chloride (1.39 g, 5 mmol, dissolved in 50 mL of CH_2Cl_2) was added dropwise to alkyldiamine (**2a**–**f**, 20 mmol) dissolved in 15 mL of CH_2Cl_2 at room temperature, followed by stirring at room temperature for 24 h.[31] The mixture was then diluted with 50 mL of $CHCl_3$ and washed with 5% aq Na_2CO_3 and satd brine. The organic phase was dried with $MgSO_4$, and the solvent was evaporated. The concentrated fraction was purified by column chromatography (silica gel) using 8:2:0.1 CH_2Cl_2–MeOH–NH_4OH as the eluent to give *N*-tritylalkyldiamine (**3a**–**f**, yield: 60–70%).

N-Tritylalkyldiamine (**3a**–**f**, 10 mmol) was dissolved in 10 mL of anhyd MeOH and then mixed with D-mannose (1.80 g, 10 mmol) at 50 °C with stirring for 1–2 h.[30] The solvent was then removed by evaporation, and the concentrated fraction was purified by column chromatography (silica gel) using 9:1 CH_2Cl_2–MeOH as the eluent to give *N*-(*N*′-tritylaminoalkyl)-α-D-manno-pyranosylamines (**4a**–**f**, yield: 50–60%).

4.2.1. *N*-(*N*′-Tritylaminoethyl)-α-D-mannopyranosylamine (4a)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.37 (d, $J \sim 7.6$ Hz, 6H, $CH_{Ar's}$), 7.26 (t, $J \sim 7.6$ Hz, 6H, $CH_{Ar's}$), 7.15 (t, $J \sim 7.2$ Hz, 3H, $CH_{Ar's}$), 3.66 (s, 1H, H1), 3.57–3.60 (m, 2H, H6), 3.49 (m, 2H, H2, H3), 3.13–3.24 (m, 2H, H4, H5), 2.84–2.97 (m, 2H, $NHCH_2$), 1.91 (m, 2H, $NHCH_2$). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 146.09, 145.97, 128.71, 128.63, 127.93, 127.80, 126.28 ($CH_{Ar's}$), 87.42 (C1), 76.87 (C3), 74.79 (C2), 72.01 (C5), 70.90 (C4), 70.83 ($C(Ph)_3$), 60.87 (C6), 45.96, 43.21 ($NHCH_2CH_2$). (+) ESIMS: *m/z* 465.4, $[M+H]^+$; HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{27}H_{33}N_2O_5$, 465.2389; found, 465.2399.

4.2.2. *N*-(*N*′-Tritylaminobutyl)-α-D-mannopyranosylamine (4b)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.36 (d, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.25 (t, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.14 (t, $J \sim 7.2$ Hz, 3H, $CH_{Ar's}$), 3.85 (s, 1H, H1), 3.59–3.63 (m, 2H, H6), 3.46–3.52 (m, 2H, H2, H3), 3.21–3.26 (m, 2H, H4, H5), 2.82–2.93 (m, 2H, $NHCH_2$), 1.92 (m, 2H, $NHCH_2$), 1.34–1.44 (m, 4H, CH_2CH_2). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 146.23, 128.66, 128.36, 128.30, 127.79, 126.23 ($CH_{Ar's}$), 87.85 (C1), 76.77 (C3), 74.95 (C2), 72.07 (C5), 70.96 (C4), 70.88 ($C(Ph)_3$), 66,24 (C6), 45.87, 43.53 ($NHCH_2$), 28.60, 27.89 (CH_2CH_2). (+) ESIMS: *m/z* 493.6, $[M+H]^+$; HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{29}H_{37}N_2O_5$, 493.2702; found, 493.2699.

4.2.3. *N*-(*N*′-Tritylaminohexyl)-α-D-mannopyranosylamine (4c)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.36 (d, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.25 (t, $J \sim 7.6$ Hz, 6H, $CH_{Ar's}$), 7.14 (t, $J \sim 7.2$ Hz, 3H, $CH_{Ar's}$), 3.86 (s, 1H, H1), 3.59–3.65 (m, 2H, H6), 3.42–3.49 (m, 2H, H2, H3), 3.21–3.26 (m, 2H, H4, H5), 2.83–2.91 (m, 2H, $NHCH_2$), 1.91 (m, 2H, $NHCH_2$), 1.19–1.43 (m, 8H, 4 × CH2). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 146.31, 146.25, 128.65, 128.33, 127.75, 127.51, 126.17 ($CH_{Ar's}$), 87.77 (C1), 74.94 (C3), 72.00 (C2), 70.87 (C5), 66.26 (C4), 61.15 ($C(Ph)_3$), 61.07 (C6), 45.86, 43.61 (2 × NHCH2), 30.98, 30.07, 27.48, 27.35 (4 × CH2). (+) ESIMS: *m/z* 521.4, $[M+H]^+$; HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{31}H_{41}N_2O_5$, 521.3015; found, 521.3022.

4.2.4. *N*-(*N*′-Tritylaminooctyl)-α-D-mannopyranosylamine (4d)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.36 (d, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.25 (t, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.14 (t, $J \sim 7.2$ Hz, 3H, $CH_{Ar's}$), 3.87 (s, 1H, H1), 3.60–3.64 (m, 2H, H6), 3.49 (m, 2H, H2, H3), 3.22–3.24 (m, 2H, H4, H5), 2.81–2.93 (m, 2H, $NHCH_2$), 1.91 (m, 2H, $NHCH_2$); 1.19–1.41 (m, 12H, 6 × CH2). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 146.73, 128.94, 128.82, 128.08, 126.41 ($CH_{Ar's}$), 87.77 (C1), 78.33 (C3), 75.20 (C2), 71.94 (C5), 70.84 (C4), 68.06 ($C(Ph)_3$), 62.15 (C6), 45.32, 43.77 (2 × NHCH2), 30.48, 29.55, 29.48, 27.36 (CH_2). (+) ESIMS: *m/z* 549.4, $[M+H]^+$; HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{33}H_{45}N_2O_5$, 549.3328; found, 549.3324.

4.2.5. *N*-(*N*′-Tritylaminodecyl)-α-D-mannopyranosylamine (4e)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.36 (d, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.25 (t, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.14 (t, $J \sim 7.2$ Hz, 3H, $CH_{Ar's}$), 3.87 (s, 1H, H1), 3.60–3.64 (m, 2H, H6), 3.49 (m, 2H, H2, H3), 3.22–3.32 (m, 2H, H4, H5), 2.79–2.93 (m, 2H, $NHCH_2$), 1.91 (m, 2H, $NHCH_2$), 1.19–1.41 (m, 16H, 8 × CH2). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 146.73, 128.82, 128.07, 126.41 ($CH_{Ar's}$), 87.76 (C1), 78.33 (C3), 75.18 (C2), 71.93 (C5), 70.84 ($C(Ph)_3$), 68.05 (C4), 62.14 (C6), 44.20, 43.76 (2 × NHCH2), 30.48, 29.52, 29.45, 27.35 (CH_2). (+) ESIMS: *m/z* 577.5, $[M+H]^+$; HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{35}H_{49}N_2O_5$, 577.3641; found, 577.3632.

4.2.6. *N*-(*N*′-Tritylaminododecyl)-α-D-mannopyranosylamine (4f)

^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.36 (d, $J \sim 8$ Hz, 6H, $CH_{Ar's}$), 7.25 (t, $J \sim 7.6$ Hz, 6H, $CH_{Ar's}$), 7.14 (t, $J \sim 7.2$ Hz, 3H, $CH_{Ar's}$), 3.87 (s, 1H, H1), 3.59–3.64 (m, 2H, H6), 3.49 (m, 2H, H2, H3), 3.22–3.24 (m, 2H, H4, H5), 2.80–2.93 (m, 2H, $NHCH_2$), 1.92 (t, $J \sim 4.8$ Hz, 2H, $NHCH_2$), 1.19–1.41 (m, 20H, 10 × CH2). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 146.71, 128.80, 128.05, 126.40 ($CH_{Ar's}$), 87.78 (C1), 78.33 (C3), 75.21 (C2), 73.61 (C5), 71.09 (C4), 70.83 ($C(Ph)_3$), 62.00 (C6), 43.74 ($NHCH_2$), 30.48, 29.54, 29.49, 29.06, 27.51, 27.35 (CH_2). (+) ESIMS: *m/z* 605.5, $[M+H]^+$; HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{37}H_{53}N_2O_5$, 605.3954; found, 605.3961.

4.2.7. *N*-(*N*′-Tritylaminododecyl)-α-D-maltosylamine (5)

N-Trityldodecyldiamine (**3f**, 10 mmol) was dissolved in 10 mL of anhyd MeOH and then mixed with maltose (3.60 g, 10 mmol) at 50 °C with stirring for 1–2 h.[30] The solvent was then evaporated, and the concentrated fraction was purified by column chromatography (silica gel) using 9:1 CH_2Cl_2–MeOH as the eluent to give *N*-(*N*′-tritylaminododecyl)-α-D-maltosylamine **5** (yield: 70%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.36 (d, $J \sim 7.6$ Hz, 6H, $CH_{Ar's}$), 7.22 (t, $J \sim 7.2$ Hz, 6H, $CH_{Ar's}$), 7.12 (t, $J \sim 7.2$ Hz, 3H, $CH_{Ar's}$), 5.0 (d, $J \sim 3.6$ Hz, 1H, CH), 4.31 (d, $J \sim 3.2$ Hz, 1H, CH), 3.37–3.69 (m, 10H, CH), 3.24–3.29 (m, 2H, CH), 2.93–3.08 (m, 2H, $NHCH_2$), 1.92–1.94 (m, 2H, $NHCH_2$), 1.35–1.39 (m, 4H, 2 × CH2), 1.16–1.18 (m, 16H, 8 × CH2). ^{13}C NMR (DMSO-d_6, 400 MHz): δ 146.69, 128.79, 128.04, 126.41 ($CH_{Ar's}$), 101.41, 91.28, 80.73, 77.63, 76.44, 73.88, 73.51, 70.84, 70.41, 61.33 (CH), 49.09, 46.05, 43.72 ($NHCH_2$), 30.49, 29.58, 29.47, 29.06, 27.34, 26.90 (CH_2). (+) ESIMS: *m/z* 767.2, $[M+H]^+$; HRESIMS: *m/z* $[M+H]^+$ calcd for $C_{43}H_{63}N_2O_{10}$, 767.4483; found, 767.4490.

4.3. Fabrication of trityl-derivatized carbohydrate arrays

A series of 100 mM stock solutions of **4a**–**f** and **5** (in MeOH) were prepared. A 40-μL portion of each sample was pipetted into a 96-well polystyrene microplate, which was subsequently dried by evaporation. Each well was then washed three times with 250 μL of deionized water.

4.4. Phenol–sulfuric acid assay

A 40-μL portion of trityl-derivatized carbohydrates (100 mM **4a**–**f** and **5** in MeOH) was pipetted into a 96-well microplate, and the plate was incubated at room temperature to allow the solvent to evaporate. The wells were then washed with deionized water (3 cycles). The phenol–sulfuric acid assay was performed according to a literature method.[32] Briefly, a 5% phenol solution (14 μL) was

added to each well, followed by the addition of concentrated H_2SO_4 (70 μL). The mixture was incubated for 30 min at room temperature, and the absorbance at 490 nm (A_{490}) measured to determine the amount of mannose immobilized on the microtiter plate. For comparison, similar visible absorption measurements were also performed with the phenol–sulfuric acid solution (without mannose) and the trityl-derivatized carbohydrate controls (**4a**–**f** and **5**), which were prepared as described above except that no washing was carried out. The amount of immobilized **4a**–**f** and **5** was estimated from the ratio of the A_{490} of immobilized **4a**–**f** and **5** (subjected to 3 washing cycles with deionized water) to the A_{490} of the corresponding control (unwashed).

4.5. Con A binding studies

Trityl-derivatized carbohydrates (**4a**–**f** and **5**) were immobilized on 96-well microplates as described above and subsequently incubated with 3% BSA in PBST buffer (phosphate-buffered saline containing 0.2% Tween 20, 0.1 M, pH 6.8) for 1 h. BSA is widely used as a blocking agent to inhibit nonspecific binding by hydrophobic interactions in many ELISA, immunoblotting and immunohistochemical studies.[38,39] The BSA-treated wells were then incubated with FITC-Con A in PBST buffer for 1 h, and subsequently washed with the same buffer three times (5 min each). The binding of the Con A/mannose complex was monitored by fluorescence measurements using a microplate reader. For comparison, similar immobilization and fluorescence assays were performed with the trityl group (control).

4.6. Inhibitor screening

Compounds **4f** and **5** (40 μL, 100 mM) were immobilized on 96-well microplates as described above, followed by incubation with 3% BSA in PBST buffer. FITC-Con A (10 μg/mL, in PBST buffer) was first mixed with different concentrations of the inhibitors methyl α-D-glucopyranoside and methyl α-D-mannopyranoside (10 nM–400 mM), and each mixture was incubated for 1 h. A 40-μL portion of each solution was then pipetted into the BSA-treated carbohydrate arrays, followed by incubation for another 1 h. The wells were then washed with PBST buffer for three cycles (5 min each), and the fluorescence signal of each well was measured by a microplate reader.

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Supplementary data

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