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PAPER

Glyco-macroligand microarray with controlled orientation and glycan density†

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We report a new type of glycan microarray, namely, oriented and density-controlled glyco-macroligand microarray based on end-point immobilization of glycopolymer that was accompanied with boronic acid (BA) ligands in different sizes as detachable "temporary molecular spacers". Briefly, an *O*-cyanate chain-end functionalized lactose-containing glycopolymer was pre-complexed with polyacrylamide–BA, lysozyme–BA, and bovine serum albumin (BSA)–BA conjugates as macromolecular spacers first and then immobilized onto an amine-functionalized glass slide *via* isourea bond formation both at pH 10.3, respectively. Subsequently, the macromolecular spacers were detached from the immobilized glycopolymers at pH 7.4 so as to afford the oriented and density controlled glycopolymer microarrays. The spaced glycopolymer microarray showed enhanced lectin (*Arachis hypogaea*) binding compared to a non-spaced one. Among them, the polyacrylamide–BA spaced glycopolymer showed the highest level of lectin binding compared to lysozyme–BA- and BSA–BA-spaced glycopolymers. Furthermore, SPR results confirmed the same trend of density-dependent lectin binding as the glycoarray. This glyco-macroligand microarray platform permits variations of glycan density in the polymer, glycopolymer density and its orientation on the microarray surface and thus will provide a versatile tool for profiling glycan recognition for both basic biological research and practical applications.

Introduction

The glycan microarray has become a powerful high-throughput tool for examining binding interactions of carbohydrates with lectins, antibodies, cells, and viruses.[1] Recently, it has been applied to clinical antibody detection and profiling,[2] vaccine development,[3] biomarker discovery,[4] and drug screening[5] applications. Nevertheless, they are still not perfect for clinical applications and novel microarray designs are much needed to improve the performance of this technology. Two critical limitations prevent wide and potential applications of the glycan microarray technology. First, the detection is limited by restricted epitopes available for microarray fabrication from both synthesis and isolation from nature sources. Second, the features of glycan presentation on the microarray surface such as density and orientation of glycans have a substantial effect on protein recognition related to both affinity and specificity. Conventional glycan microarrays were made by directly immobilizing glycans onto microarray surfaces by either physical absorption or covalent conjugation, which directly mimics the multivalent display of carbohydrate epitopes on the cell surface. However, this two dimensional (2D) surface immobilization chemistry usually results in low signal intensity and substantial non-specific binding of target proteins due to an insufficient number of accessible glycans and the presence of surface–protein interactions. To overcome this limitation, recently, glycan density on the array surface has been recognized as a very important feature of carbohydrate recognition and thus has been investigated extensively.[6–12]

A general strategy for modulating glycan density is to vary the concentration of the monovalent glycans in the printing solution that is being spotted onto a microarray slide. However, this approach does not generate variations in density in the molecular level and often yields inconsistent results. Particularly, often variations in density gradient over the spot are seen when the surface is not saturated with diluted printing solution. Alternatively, new strategies have been developed, in which multivalent glycoconjugates with varying density were first synthesized, and then printed onto a solid support to generate a microarray of multivalent glycans in different densities. For example, Pieters and coworkers attached mannose-functionalized dendrimers to a microarray surface to produce a microarray of glycodendrimers of valencies ranging from monovalent to octavalent.[13] This microarray chip enabled rapid real time evaluation of multivalency effects on binding of lectins *Concanavalin* A (Con A) and *Galanthus nivalis* (GNA) to mannose. On the other hand, Gildersleeve and coworkers fabricated an array of multivalent

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glycoconjugates by attaching linker-functionalized monosaccharides and oligosaccharides to BSA as neoglycoprotein.[2] In this approach, the densities were modulated by varying the number of sugars attached to BSA. The authors evaluated density-dependent binding of lectins, monoclonal antibodies, and polyclonal human serum antibodies and found that subpopulations of antibodies in humans could recognize different densities of the same glycan antigen.

Another key feature of the glycan presentation in glycan microarray is the glycan orientation. Cell surface glycans are spatially displayed such as glycoprotein scaffolds with three-dimensional (3D) geometries. However, conventional glycan arrays present glycans on a 2D substrate and thus have limited activity. Early study confirmed that surface-bound multivalent glycan ligands show higher avidity to protein receptors compared to immobilized monomeric glycans.[14] Recently, Bertozzi's group[15] and our group[16] reported oriented glycopolymer microarrays based on end-point immobilization of glycopolymers to mimic 3D natural cell surface glycan display. In these approaches, the densities and orientations of the glycan ligands are determined by the polymer structure rather than by features of the underlying microarray surface. These parameters should therefore be more controllable than in the case with conventional 2D glycan microarrays. Nevertheless, the density of the immobilized glycopolymer on the array surface is still uncontrollable, and thus, the possibility to access the multivalent glycans in parallel might be limited and thus does not facilitate maximum protein binding affinity and specificity. In this study, we report an oriented and density controlled glycopolymer microarray formation based on end-point immobilization of glycopolymer combined with a molecular spacing technique. Briefly, an *O*-cyanate chain-end functionalized glycopolymer was pre-complexed with boronic acid ligands in different sizes and then immobilized onto an amine-functionalized glass slide *via* isourea bond formation at high pH value (pH 10.3 buffer). Once the immobilization is complete, the spacer boronic acid ligands were released from the immobilized glycopolymers at a reduced pH (pH 7.4 buffer) so as to afford the oriented and density controlled glycopolymer microarray (Fig. 1). This glyco-macroligand microarray platform will facilitate both affinity and specificity of protein binding and thus will provide a versatile tool for profiling glycan recognition for both basic biological research and practical applications.

Experimental

Syntheses of *O*-cyanate chain-end functionalized glycopolymers

Glycopolymers were synthesized according to our previously reported method.[16] Briefly, in a three-necked flask, 4-chloroaniline (21.6 mg, 1.69×10^{-4} mol) and sodium nitrite (14.1 mg, 2.04×10^{-6} mol) in a mixture of 2 mL water and THF (1 : 1, v/v) were dissolved. To the above mixture, HBF_4 (66 mg, 7.51×10^{-4} mol) was added and allowed to react for 30 min at 0 °C under Ar atmosphere. Following this, a degassed mixture of 2-*N*-acryoyl-aminoethoxyl 4-*O*-(β-D-galatopyranosyl)-β-D-glucopyranoside (186 mg, 2.65×10^{-5} mol), acryl amide (210 mg, 2.65×10^{-4} mol) and NaOCN (55.2 mg, 8.49×10^{-4} mol) dissolved in 1 mL of water was added into the flask containing diazonium salt. The reaction solution was thus heated at 65 °C for 16 h, and then was filtered to remove any precipitates. The resultant mixture is separated from any inorganic salts and impurities by dialysis against deionized water for 2 days at room temperature to afford the glycopolymer (248 mg). The conversion yield was about 60%, which was determined by weight for the resultant glycopolymer. Four kinds of glycopolymers with different ratios of pendant glycan and molecular weights (**1a**, **1b**, **1c** and **1d**) were obtained by using different ratios of glycomonomer (GM) to acryl amide (AA) and were characterized by ^{1}H NMR spectroscopy (see ESI†, Table S1 and Fig. S1–S4).

Syntheses of boronic acid (BA) ligands

Synthesis of lysozyme–BA. To 5 mL ice cold 0.05 M MES (pH 6), add 100 mg of lysozyme (6.9 μmol), 20 mg of aminophenyl boronic acid (APBA) (130 μmol) and 10 mg of EDC with constant stirring. The reaction was allowed to take place for 2 h at room temperature, then adjust the pH to 7 and let the reaction continue overnight at room temperature. Separate the salts and the unreacted APBA by centrifugation in a 10 kDa cut-off filter tube for 30 min. The resultant lysozyme–BA conjugate was

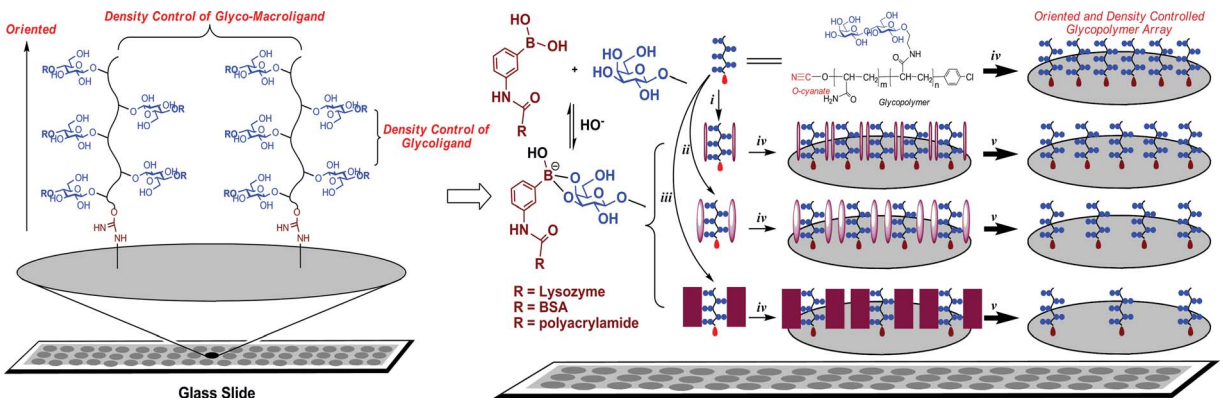

Fig. 1 Schematic illustration of orientation and density controlled glycopolymer microarray formations based on end-point immobilization of glycopolymers that were accompanied with boronic acid (BA) ligands in different sizes as temporary molecular spacers followed by releasing the BA ligands from the immobilized glycopolymers. *Reaction conditions*: (i) R_1–BA, $NaHCO_3$ buffer (pH 10.3), (ii) R_2–BA, $NaHCO_3$ buffer (pH 10.3), (iii) R_3–BA, $NaHCO_3$ buffer (pH 10.3), (iv) $NaHCO_3$ buffer (pH 10.3), (v) 1 mM glucose, PBS buffer (pH 7.4).

characterized by SDS-PAGE and Alizarin Red S (ARS) assay (see ESI†, Fig. S5A).

Synthesis of BSA–BA. To 5 mL ice cold 0.05 M MES (pH 6), add 100 mg of BSA (1.5 μmol), 20 mg of APBA (130 μmol) and 10 mg of EDC with constant stirring. After 2 h, adjust the pH to 7 and let the reaction sit overnight at room temperature. Separate the salts and the unreacted APBA by centrifugation in a 10 kDa cut-off filter tube for 30 min. The resultant BSA–BA (BSA–BA) conjugate was characterized by SDS-PAGE and ARS assay (see ESI†, Fig. S5B).

Glycopolymer microarray formation and lectin binding assay

A MicroCaster microarray tool (Whatman, spot size 500 μm diameter) was inked with a solution of glycopolymer (**1a**, 4.8×10^{-5} mM) in $NaHCO_3$ buffer (pH 10.3), then was pressed onto an amine functionalized glass slide (Xenopore, Co) at room temperature for 10 min. The glass slide was then incubated in a humidifier chamber at room temperature for 4 h and then washed with $NaHCO_3$ buffer (pH 10.3) for 30 min (3 times) to remove un-reacted glycopolymer, followed by washing with 0.2% PBST for 30 min. The glass slides were then incubated with lectin-FITC (*Arachis hypogaea*, FITC-labeled, Sigma, 0.2 mg, 1.61×10^{-9} mol) PBS containing 0.2% Tween 20 (PBST) solution at room temperature for 3 h followed by extensive washing with PBST buffer for 30 min. Finally, the glass slide was subjected to fluorescence imaging and the fluorescence intensity was recorded by using a Typhoon 9410 Variable Model Imager (Amersham Biosciences, USA). Under the same condition, glycopolymer microarrays with glycopolymer **1b**, **1c**, and **1d** were conducted, respectively.

Density controlled glycopolymer microarray formation and lectin binding assays

Glycopolymer (**1b**, 3 mg, 7.7×10^{-5} mmol) was mixed with a polyacrylamide–BA ligand (3 mg, 1.5×10^{-4} mmol) in 3 mL of 10.3 pH $NaHCO_3$ buffer and was stirred at room temperature for 4 h. The MicroCaster microarray tool (Whatman, spot size 500 μm diameter) was inked with the glycopolymer–boronic acid complex solution (1 mg mL^{-1}, 1 : 2 mol) and then was printed onto an amine functionalized glass slide in $NaHCO_3$ buffer (pH 10.3). The glass slide was then incubated in a humidifier chamber for 4 h and then washed with $NaHCO_3$ buffer (pH 10.3) for 30 min (3 times) to remove un-reacted glycopolymer and boronic acid ligands. Detachment of boronic acid ligand from the immobilized glycopolymer was performed by incubating the glass slide with 7.4 pH PBS buffer for 30 min and followed by 7.4 pH PBS buffer with 1 mM glucose for 10 min. Boronic acid ligands detached from the glycopolymer were confirmed by the ARS assay (see ESI†, Fig. S8). The glass slide was washed with PBST buffer for 30 min then incubated with lectin-FITC (*Arachis hypogaea*, FITC-labeled, Sigma, 0.2 mg, 1.61×10^{-9} mol) in 0.2% PBST solution at room temperature for 3 h, followed by extensive washing with PBST buffer for 30 min and subjected to fluorescent imaging and the fluorescence intensity was recorded as above. Under the same condition, density controlled glycopolymer microarray formation of glycopolymer **1b** spaced with polyacrylamide–BA in different ratios (as shown in Fig. 4) was prepared and their lectin binding was tested, respectively.

A similar protocol was followed for glycopolymer **1b** microarray formation spaced with other two boronic acid ligand conjugates (BSA–BA and Lyz–BA) in different ratios (Fig. 5) and their lectin (*Arachis hypogaea*) binding assays, respectively.

A similar protocol was followed for spaced glycopolymer **1b** microarray formation spaced with these three boronic acid ligands in different ratios and their lectin *Ricinus communis* (RCAI, FITC-labeled, EY Laboratories, Inc) binding assays, respectively (Fig. 6).

Control experiments for spaced glycopolymer microarray formation

Glycopolymer (**1b**, 3 mg) was mixed with unmodified lysozyme (3 mg) in 3 mL of 10.3 pH $NaHCO_3$ buffer and was stirred at room temperature for 4 h. The MicroCaster array tool (Whatman, spot size 500 μm diameter) was inked with the glycopolymer/BSA solution (1 mg mL^{-1}) and then was printed onto an amine functionalized glass slide in $NaHCO_3$ buffer (pH 10.3). The glass slide was then incubated in a humidifier chamber for 4 h and then washed with $NaHCO_3$ buffer (pH 10.3) for 30 min (3 times) The glass slide was then incubated with lectin-FITC (*Arachis hypogaea*, FITC-labeled, Sigma, 0.2 mg, 1.61×10^{-9} mol) in 0.2% PBST solution at room temperature for 3 h followed by extensive washing with PBST buffer for 30 min and subjected to fluorescent imaging and the fluorescence intensity was recorded as above. The same protocols were followed for glycopolymer treated with unmodified BSA, respectively (Fig. 5).

The same protocols were followed for printing a glycopolymer with a hydroxyl chain end group and its complex with polyacrylamide–BA, respectively.

Surface Plasmon Resonance (SPR) assay for immobilized glycopolymers spaced with boronic acid ligands

Glycopolymer immobilization onto SPR chip. A CM5 chip (GE health science) surface was activated with EDC/NHS 1 : 1 for 6 min at 10 μL min^{-1}, treated with 70 μL of 0.1 M ethylenediamine in 8.5 pH 0.1 M borate buffer at 10 μL min^{-1}, followed by 70 μL of 1 M ethanolamine 8.5 pH 0.1 M borate buffer for 7 min at 10 μL min^{-1}. Once the amine modified surface was generated, glycopolymer (**1b**) was immobilized by flowing glycopolymer solution in 10.3 pH $NaHCO_3$ buffer (0.5 mg mL^{-1}) for 7 min at 10 μL min^{-1} followed by washing with 10.3 pH $NaHCO_3$ buffer for 5 min (flow cell 2). Immobilization of the glycopolymer was confirmed by observing the increase in response from the base line.

Boronic acid ligands spaced glycopolymer immobilization onto SPR chip. A CM5 chip (GE health science) was modified as mentioned earlier to create amine functionalization. Glycopolymer pre-modified with polyacrylamide–BA (0.5 mg mL^{-1}, 1 : 2 mol) in pH 10.3 $NaHCO_3$ buffer was flowed for 7 min at 10 μL min^{-1}. Immobilization of the polyacrylamide–BA modified glycopolymer was confirmed by the increase in response from the base line, followed by washing with 10.3 pH $NaHCO_3$ buffer for

5 min. Polyacrylamide–BA was released from the immobilized glycopolymer by flowing with 7.4 pH PBS buffer for 7 min. The same procedure was followed to immobilize the lysozyme–BA pre-modified glycopolymer (**1b**).

Lectin binding onto immobilized glycopolymer. The binding of lectin to the immobilized glycopolymer was studied by flowing lectin (*Arachis hypogaea*, Sigma). Different concentrations of lectin ranging from 0.125 nM to 2 nM in 7.4 pH PBST buffer were flowed through the immobilized glycopolymer for 2 min at 10 μL min^{-1}; and the amplification of response with respect to increase in lectin concentration was illustrated.

Results and discussion

In our previous study, we have demonstrated that an *O*-cyanate chain-end functionalized glycopolymer could be immobilized onto amine-functionalized silica gel and glass slide surfaces *via* isourea bond formation.[16] To fully exploit the potential of the oriented immobilized glycopolymer, herein, we examined molecular control of both the density of glycan in the glycopolymer and the glycopolymer itself on the microarray surface. First, we examined the glycan density effect for lectin binding in the glycopolymer by synthesizing a series of glycopolymers with different ratios of pendant glycan and molecular weights. Four glycopolymers were synthesized by our previously reported method (see ESI†).[16] Then, glycopolymer microarrays (spot size about 500 μm diameter) were fabricated by microcontact stamping (MicroCaster) of glycopolymers onto amine functionalized glass slides (Xenopore Co.) in $NaHCO_3$ buffer (pH 10.3). The glass slides were incubated in a humidifier chamber for 4 h and then washed for 30 min (3 times) with respective buffers followed by washing with PBST to minimize the nonspecific binding of proteins onto the surface. Then, the glass slides were incubated with lectin-FITC (*Arachis hypogaea*, FITC-labeled, Sigma) solution in PBST buffer for 3 h, followed by extensive washing with PBST buffer for 30 min, and are finally subject to fluorescence imaging. As shown in Fig. 2, glycopolymer (**1b**) with a 1 to 30 ratio of lactose and acryl amide (LT/AA) showed the highest level of lectin binding (Fig. 2B) compared to the glycopolymers with 1 to 18 (**1a**) (Fig. 2A), 1 to 54 (**1c**) (Fig. 2C) and 1 to 51 (**1d**) (Fig. 2D) ratios of lactose and acryl amide. These results indicated that the glycan density in the polymer has an impact on the lectin binding. We chose glycopolymer **1b** for our continued density controlled glycopolymer microarray study below.

The molecular imprinting technique has been recognized as a powerful tool for the preparation of synthetic polymers with molecular sized cavities that are capable of molecular recognition.[17,18] This technique has been used for chromatographic separations,[19] solid phase extraction,[20] binding assay[21] and sensors,[22,23] and drug delivery[24] applications. Boronic acid–carbohydrate complex formation is a pH-dependent reversible process and has been used for the preparation of molecularly imprinted polymers targeting sugars.[25–28] In the present study, we envisioned that the reversible interaction between boronic acid and carbohydrate can be used to modulate the density of immobilized glycopolymer on the microarray surface by using boronic acid ligands of different sizes as "temporary spacing molecules". Briefly, the glycopolymer was pre-complexed with boronic acid ligands. These assemblies were then printed onto the microarray surface, in which the boronic acid ligands temporarily occupy the space between the immobilized glycopolymers. Once the immobilization was complete, the spacer boronic acid ligands are removed leaving empty space behind and thus afforded density controlled glycopolymer microarray. The key factor for this innovative fabrication of density controlled glycopolymer microarray is the compatible conditions: first, the basic condition for both boronic acid and carbohydrate complex formation and *O*-cyanate-based isourea bond formation immobilization and second, the neutral condition for removal of spacer boronic acid ligands from the microarray surface (Fig. 1).

The *O*-cyanate chain end functionalized lactose-containing glycopolymer **1b**, which showed the highest lectin binding above, was used as a model glyco-macroligand for oriented and density controlled glycopolymer microarray formation. Three macroboronic acid ligand (**2**), lysozyme–BA (**2a**, M_w: about 15 kDa), BSA–BA (**2b**, M_w: about 70 kDa) and polyacrylamide–BA (**2c**, M_w: about 10 kDa) conjugates were designed and synthesized as spacing molecules to vary the immobilized glycopolymer density since they have different molecular sizes and BA conjugation

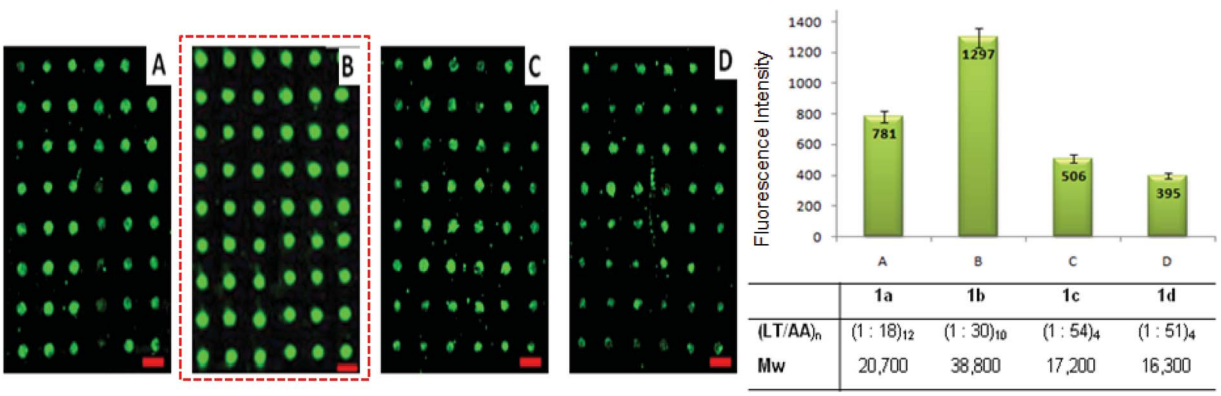

Fig. 2 Fluorescence images of glycopolymer microarrays with different glycan densities after incubation with lectin (*Arachis hypogaea*, FITC-labeled, Sigma): (A) glycopolymer **1a** (LT/AA, 1 : 18), (B) glycopolymer **1b** (LT/AA, 1 : 30), (C) glycopolymer **1c** (LT/AA, 1 : 54), and (D) glycopolymer **1d** (LT/AA, 1 : 51). LT: lactose and AA: acrylamide. Bar size: 500 μm.

density on it can be variable. Lysozyme–BA **2a** and BSA–BA **2b** conjugates were synthesized by amidation of carboxylic acid groups in lysozyme and BSA with aminophenyl boronic acid in the presence of EDC, respectively (see ESI†, Fig. S5 and S6 and Table S2). Polyacrylamide–BA conjugate **2c** was prepared as per our previously reported method.[29] Lysozyme–BA and BSA–BA conjugates were characterized by SDS-PAGE. As shown in Fig. 3, the coomassie staining clearly showed large molecular weight shifts for the protein–BA conjugates compared to unmodified BSA and lysozyme (Fig. 3A). Interestingly, the gel stained with Alizarin Red S (ARS PBS buffer (pH 7.4) showed similar molecular weight shifts for the conjugates but the color of the bands is brown compared to the yellow band of the unmodified lysozyme and BSA (Fig. 3B) (for detailed characterization see ESI†, Fig. S5). The ability of the boronic acid ligands to specifically bind with carbohydrate was characterized by the ARS-binding assay.[30] ARS displays a dramatic color change in response to binding of boronic acid and has been used as a general reporter for studying carbohydrate–boronic acid binding interactions. In the present study, galactose (Gal) was used as a model compound to determine the binding between carbohydrate and boronic acid in the three ligands. As a result, the galactose–boronic acid complex formed immediately upon adding galactose into the reaction solution of boronic acid ligands with ARS, a corresponding change in UV absorption was recorded (ESI†, Fig. S5).

The general process for oriented and density controlled glycopolymer microarray formation includes three steps as shown in Fig. 1. First, prior to immobilization, the complexes formation between glycopolymer (**1b**) and spacing boronic acid ligands (**2**) was conducted by mixing **1b** and **2** in $NaHCO_3$ buffer (pH, 10.3) for 4 h at room temperature. Next, the MicroCaster array tool (Whatman, spot size 500 μm diameter) was inked with the solution of the glycopolymer–boronic acid ligand complex in $NaHCO_3$ buffer (pH, 10.3), then was pressed onto an amine functionalized glass slide (Xenopore, Co) for 10 min at room temperature. The glass slide was then incubated in a humidifier chamber for 4 h at room temperature. Finally, the spacing boronic acid ligands were detached from the immobilized glycopolymer by incubating the glass slides in PBS buffer (pH 7.4) solution in the presence of high concentration glucose (1 mM) as

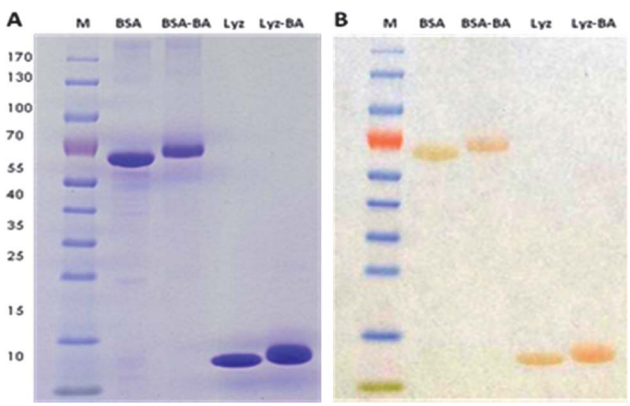

Fig. 3 SDS-PAGE characterization of BSA–BA and lysozyme–BA conjugates: (A) stained with coomassie blue and (B) stained with ARS in PBS buffer (pH 7.4) for 30 min.

well, and followed by washing with PBS buffer (pH 7.4) for 30 min (3 times) so as to afford the expected oriented and density controlled glycopolymer microarrays. The ARS assay was also used to confirm the boronic acid ligands detached from the immobilized glycopolymer, in which PBS buffer solution containing ARS turned to brown color from red color upon falling of detached boronic acid ligands from the immobilized glycopolymers on the microarray surface (see ESI†, Fig. S7).

For protein binding, the resultant glycoarray glass slides were washed with PBS containing 0.2% Tween 20 (PBST) first to minimize nonspecific protein binding, then incubated with β-galactose specific lectin (*Arachis hypogaea* (PNA) FITC-labeled, Sigma) solution in PBST buffer (pH 7.4, 0.2 mg mL^{-1}) for 3 h at room temperature followed by extensive washing with PBST buffer for 1 h. The slides then were subjected to fluorescence imaging. As a result, all spaced glycopolymer microarrays showed enhanced lectin binding compared to a non-spaced one (Fig. 4). Among them, the polyacrylamide–BA spaced glycopolymer showed the highest enhanced lectin binding (Fig. 4A), while the BSA–BA spaced glycopolymer and lysozyme–BA spaced glycopolymer showed moderately enhanced lectin binding (Fig. 4B and C). Significantly, glycopolymer microarrays spaced with boronic acid ligands in different sizes showed different levels of lectin bindings (Fig. 4A–C). Specifically, the polyacrylamide–BA spaced glycopolymer showed its highest level of lectin binding with the glycopolymer/polyacrylamide–BA ratio of 1 to 6 (mol) (Fig. 4A3), while the lysozyme–BA spaced glycopolymer showed its highest level of lectin binding with the glycopolymer/lysozyme–BA ratio of 1 to 2 (mol) (Fig. 4B2), and the BSA–BA spaced glycopolymer showed its highest lectin binding with the glycopolymer/BSA–BA ratio of 1 to 0.6 (mol) (Fig. 4C3), both of which are lower than that of the polyacrylamide–BA spaced glycopolymer microarray (Fig. 4A3). These results indicated that glycopolymer microarrays with different densities can be made with different boronic ligands in different ratios and hence revealed relevant protein bindings.

It should be noted that both the glycopolymer and the boronic acid ligands have multi-binding sites and thus could afford a mixture of conjugates or form aggregates due to the multivalent interactions. In the current study, low concentrations (mg/mL) of both polymers were used and no aggregates were observed. The spaced glycopolymer microarray formations were reproduced with the highest lectin binding (Fig. 5A–C). In addition, control experiments for glycopolymer microarray formation with unmodified lysozyme and BSA showed no enhanced lectin binding compared to glycopolymer alone microarray (Fig. 5C1, C2, D). On the other hand, no glycopolymer microarray formed when the chain end *O*-cyanate group was converted to a hydroxyl group in the glycopolymer[16] (Fig. 5C3 and C4). Overall, these results indicated the successful oriented and density controlled glycopolymer microarray formation and that the immobilized glycopolymer density had a substantial effect on its lectin recognition.

Next, the oriented and density controlled glycopolymer microarrays described above were also tested for lectin *Ricinus communis* (RCAI, FITC-labeled, EY Laboratories, Inc) binding, which recognizes β-galactose too. Interestingly, different from lectin *Arachis hypogaea* bindings above, RCAI showed stronger bindings to the polyacrylamide–BA spaced glycopolymer with

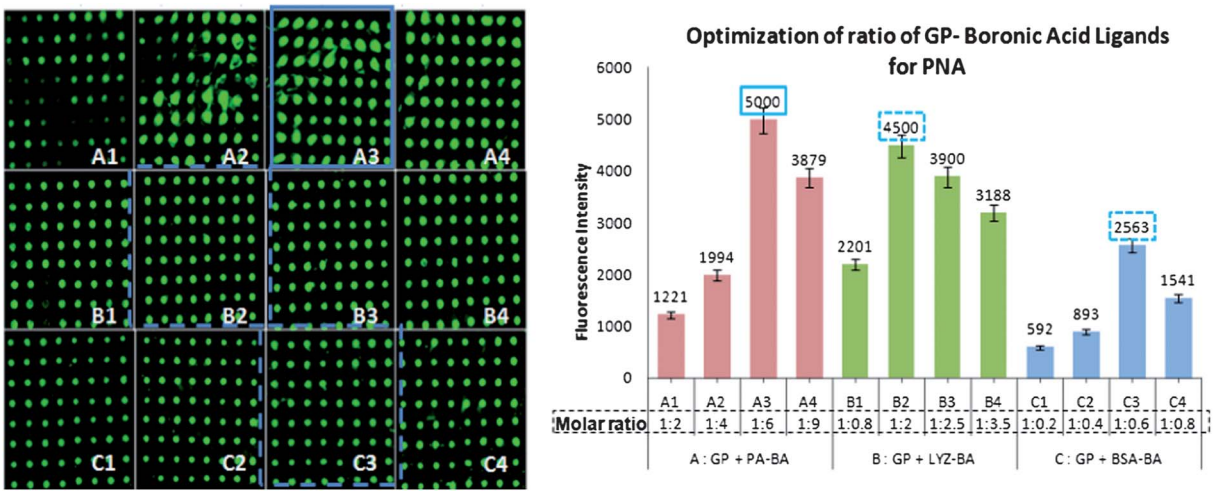

Fig. 4 Fluorescence images of density controlled glycopolymer microarrays spaced with boronic acid ligands in different molar ratios after incubation with lectin (*Arachis hypogaea* (PNA), FITC-labeled, Sigma) and their fluorescence intensities: (A1–A4) glycopolymer spaced with polyacrylamide–BA in different ratios, (B1–B4) glycopolymer spaced with lysozyme–BA in different ratios, and (C1–C4) glycopolymer spaced with BSA–BA in different ratios. Bar size: 500 μm. GP: glycopolymer, PA–BA: polyacrylamide–BA, and LYZ–BA: lysozyme–BA.

the glycopolymer/polyacrylamide–BA ratio of 1 to 4 (mol) (Fig. 6A2) and lysozyme–BA spaced glycopolymer with the glycopolymer/lysozyme–BA ratio of 1 to 2 (mol) (Fig. 6B2), and BSA–BA spaced glycopolymer showed stronger lectin binding with the glycopolymer/BSA–BA ratio of both 1 to 0.4 and 1 to 0.6 (mol) (Fig. 6C2 and C3). Both lectin *Ricinus communis* and lectin *Arachis hypogaea* have four binding sites and about 120 kDa molecular weight. However, they prefer different spacer distance and glycan density. This different binding preference might be due to different location geometry of their binding sites in the protein or the protein conformation. Overall, these observations indicated that arrayed glycopolymers with controlled ligand density facilitate optimized protein binding, which will be important for studying glycan–protein interaction, such as assessing multivalent glycans in parallel in a microchip format.

The interaction of lectin with oriented multivalent glycans in different densities was also investigated with surface plasmon resonance (SPR). The major advantages of this assay are that it is a label free assay and monitors binding in real time. Three different density controlled immobilization glycopolymers were made onto the CM5 chip (GE health science), in which the active ester NHS was first converted to the amine surface by reacting with ethylene diamine and followed by *O*-cyanate-based glycopolymer immobilization similarly as described for glycopolymer microarray formation above. Briefly, the CM5 chip was treated with EDC/NHS first, then with ethylene diamine in borate buffer (pH 8.5), followed by 1 M ethanolamine in borate buffer (pH 8.5)

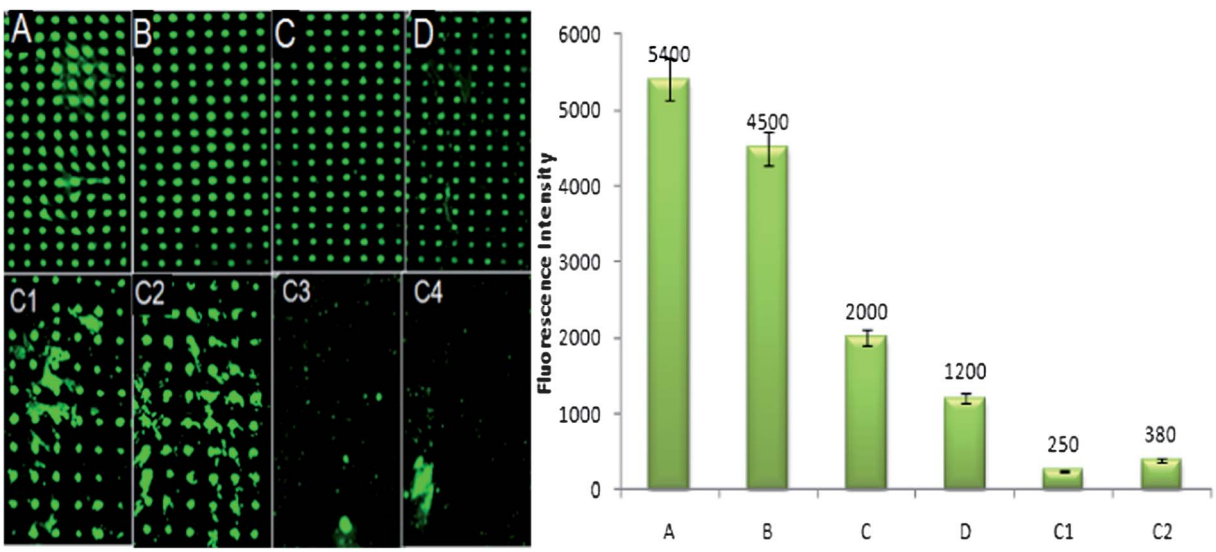

Fig. 5 Fluorescence images of density controlled glycopolymer microarrays after incubation with lectin (*Arachis hypogaea*, FITC-labeled, Sigma): (A) Gglycopolymer spaced with polyacrylamide–BA (1 : 6 mol), (B) glycopolymer spaced with lysozyme–BA (1 : 2 mol), (C) glycopolymer spaced with BSA–BA (1 : 0.6 mol), (C1) glycopolymer treated with lysozyme (1 : 2 mol), (C2) glycopolymer treated with BSA (1 : 0.6 mol), (C3) glycopolymer-OH, (C4) glycopolymer-OH treated with polyacrylamide–BA (1 : 6 mol), and (D) glycopolymer alone. Bar size: 500 μm.

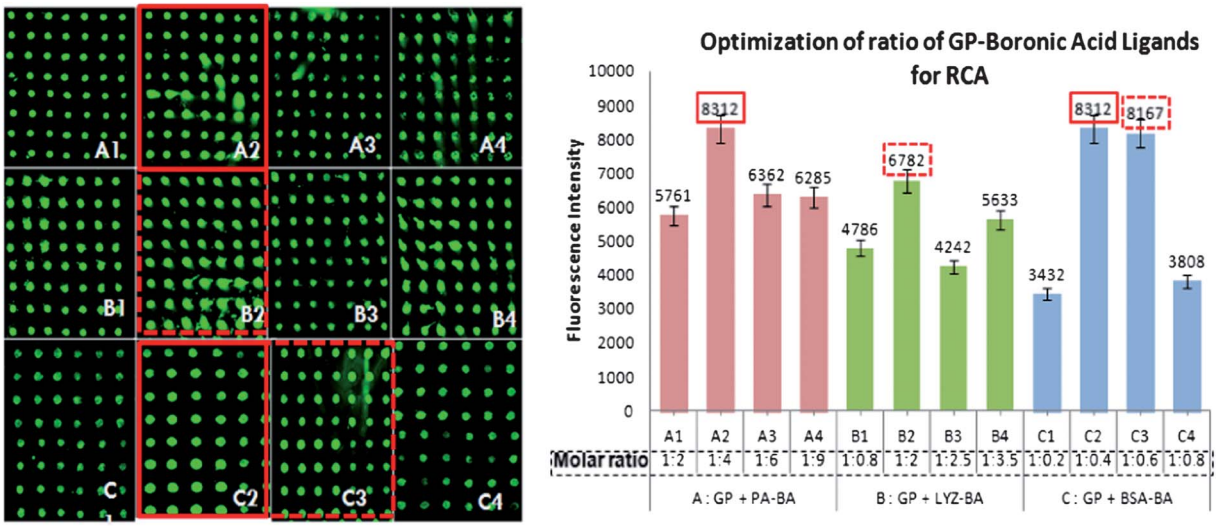

Fig. 6 Fluorescence images of density controlled glycopolymer microarrays spaced with boronic acid ligands in different molar ratios after incubation with lectin *Ricinus communis* (RCAI, FITC-labeled, EY Laboratories, Inc) and their fluorescence intensities: (A1–A4) glycopolymer spaced with polyacrylamide–BA in different ratios, (B1–B4) glycopolymer spaced with lysozyme–BA in different ratios, and (C1–C4) glycopolymer spaced with BSA–BA in different ratios. Bar size: 500 μm. GP: glycopolymer, PA–BA: polyacrylamide–BA, and LYZ–BA: lysozyme–BA.

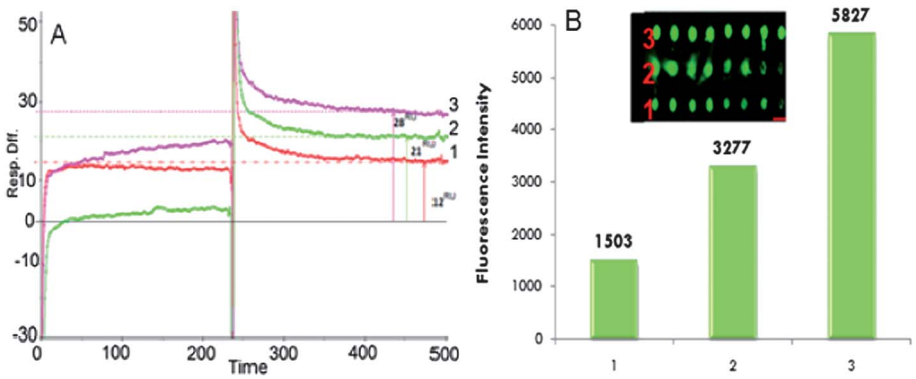

Fig. 7 Specific binding of lectin (*Arachis hypogaea*, FITC-labeled, Sigma) onto oriented and density controlled glycopolymers on the SPR surface (A) and microarray (B): (1) glycopolymer, (2) glycopolymer spaced with lysozyme–BA (1 : 2 mol), (3) glycopolymer spaced with polyacrylamide–BA (1 : 6 mol). Bar size: 500 μm.

to quench all NHS on the chip surface. Once the amine modified surface is formed, glycopolymer, glycopolymer/lysozyme–BA, and glycopolymer/polyacrylamide–BA complexes (5 mg mL^{-1}, $NaHCO_3$ buffer (pH 10.3) were flowed over the chip for 7 min at 10 μL min^{-1} and followed by washing with $NaHCO_3$ buffer (pH 10.3) for 5 min, respectively. Finally, flowing PBS (pH 7.4) buffer for 7 min at 10 μL min^{-1} to remove the boronic acid ligands afforded the oriented and density controlled glycopolymer on the SPR chip surface. Binding of lectin to the immobilized glycopolymers was assessed by flowing lectin (*Arachis hypogaea*, Sigma) over the chip at various concentrations (0.125 nM, 0.25 nM, 0.5 nM, 1 nM, 2 nM) and recording the SPR sensorgrams (10 μL min^{-1}, 25 °C). As shown in Fig. 7, glycopolymer spaced with different boronic acid ligands showed different levels of lectin bindings. Among them, the polyacrylamide–BA spaced glycopolymer showed the highest lectin binding (Fig. 7A, track 3), while glycopolymer alone showed the least lectin binding (Fig. 7A, track 1). These results are consistent with glycoarray results above (Fig. 7B). These results further demonstrated that the density of multivalent glycans plays a critical role for enhancing their interactions with proteins.

Conclusions

We have demonstrated an oriented and density controlled glycopolymer microarray formation based on end-point immobilization of glycopolymer that was spaced with boronic acid ligands of different sizes. Our studies confirmed that end-point immobilized glycopolymers with density control of both glycan in the polymer and glycopolymer on the microarray surface can effectively engage glycan-binding proteins and function as novel glycan microarray format. Lectins that bind the same glycan are affected by the glycopolymer density in unique ways. The reported well-defined glycopolymer microarray in both orientation and glycan density can be utilized for various biological analyses such as profiling the glycan–protein interactions and clinical antibody detection and profiling, vaccine development, biomarker discovery, and drug screening applications.

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