

Fabrication of an Oriented Fc-Fused Lectin Microarray through Boronate Formation**

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In the development of protein microarrays,^[1] the accessibility of surface-protein active sites and the stability of surface proteins may be influenced significantly by the orientation of the proteins on the solid surface. Therefore, various site-specific immobilization strategies have been developed.^[2] Most of the noncovalent methods for site-specific protein immobilization are based on the use of affinity tags, for example, on binding between nickel nitrilotriacetic acid (Ni-NTA) and histidine-tagged proteins,^[3] or rely on the highly specific avidin–biotin interaction.^[4] The fabrication of protein microarrays with site specifically oriented proteins attached through highly robust and extremely stable covalent linkages has been demonstrated by the use of express protein ligation,^[5] Staudinger ligation,^[6–8] and copper(I)-catalyzed 1,2,3-triazole formation.^[9,10] Alternatively, antibody-binding proteins, such as protein G, have been used. Protein G specifically recognizes and captures the Fc region of an antibody to allow optimal exposure of the antigen-binding domain (Fab) on the surface.^[11] Furthermore, the immobilization of glycoproteins, such as antibodies, through oxidation and Schiff base formation at the carbohydrate moiety has been shown to provide better accessibility to the antigen-binding site.^[12] However, under oxidation conditions the binding activity of the protein may be lost. Thus, new strategies for glycoprotein immobilization that minimize protein destruction and enable site-specific covalent bond formation are urgently needed.^[13]

Boronic acids (BAs) are known to form a stable but reversible cyclic ester (boronate) with the *cis* diol of a saccharide in aqueous media at room temperature.^[14] Accordingly, the BA–saccharide interaction has been exploited for the development of aqueous sugar sensors,^[15] and BAs have also been employed to immobilize a glycosylated enzyme on a gold electrode without significant loss of enzyme activity.^[16] Recently, Hindsgaul and co-workers developed a fluorescently tagged BA derivative for glycoprotein sensing by the naked eye.^[17] Traditionally, lectins, carbohydrate-binding proteins, have been immobilized randomly on solid supports and used as probes for glycan detection in various cellular carbohydrate-binding experiments.^[18] However, it remains a challenge to fabricate an oriented lectin microarray on a BA-based surface that maintains the high carbohydrate-binding activity of glycosylated lectins but minimizes the background interaction between the surface BAs and the polysaccharides to be detected.

Herein, we describe the development of a BA-based surface for the oriented and covalent fabrication of Fc-fused lectin microarrays through the formation of stable boronates. In this conjugation approach, Fc-fused lectins were immobilized covalently on BA-modified glass slides. The binding activity of the immobilized lectins was compared to those of the products of noncovalent oriented immobilization by protein G and random covalent attachment by Schiff base formation (Figure 1). The extracellular domain of human decin-1 (A isoform), a well-characterized receptor for (1→3)- β -D-glucan,^[19] fused with the Fc domain of human IgG1 (immunoglobulin G1) at its N terminus (Fc-dec1)^[20] was chosen as a model lectin to prove our concept. Biotin-labeled

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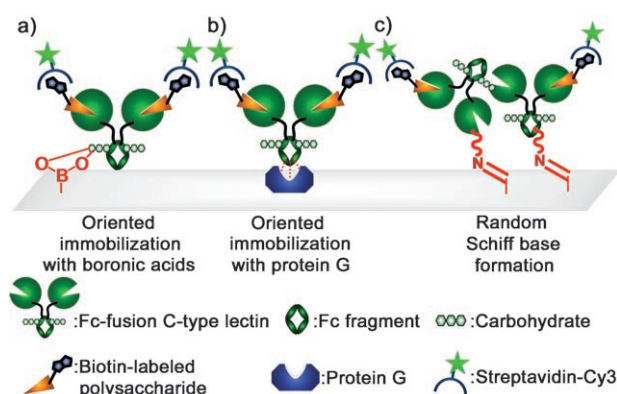


Figure 1. Illustration of the strategies used to generate microarrays of Fc-fused C-type lectin for detecting the biotin-labeled polysaccharide of *G. lucidum*: a) oriented covalent immobilization through boronate formation; b) oriented noncovalent immobilization through protein G/Fc recognition; c) random Schiff base formation.

F3 (F3-biotin; F3 is the bioactive fraction of highly antitumor active immunomodulating *Ganoderma lucidum* polysaccharide extracts)^[21] was used as a probe to investigate the influence of the immobilization strategy on the binding activity of the protein.

To create a boronate-based conjugation approach for oriented and covalent immobilization of the Fc-fused protein, we functionalized aldehyde slides with *m*-aminophenylboronic acid (APBA) and amino-terminated tri(ethylene glycol)-linked phenylboronic acid (APBA-TEG). The tri(ethylene glycol) (TEG) linker could prevent nonspecific adsorption because of its hydrophilic nature, and could also minimize any detrimental interaction between the attached protein and the solid surface.^[22] Since there is only one putative glycosylation site in the stalk region of human dectin-1,^[23] and this site is distant from the carbohydrate-recognition domain, the BA-based slides could be used to specifically capture the carbohydrate moiety on the Fc domain or stalk region (Figure 2). Fc-dectin-1 was spotted on the slides and incubated at 4°C for 12 h. After blocking with BSA (bovine serum albumin) and following incubation with F3-biotin, the binding activity of immobilized lectin was visualized by staining with streptavidin-Cy3.

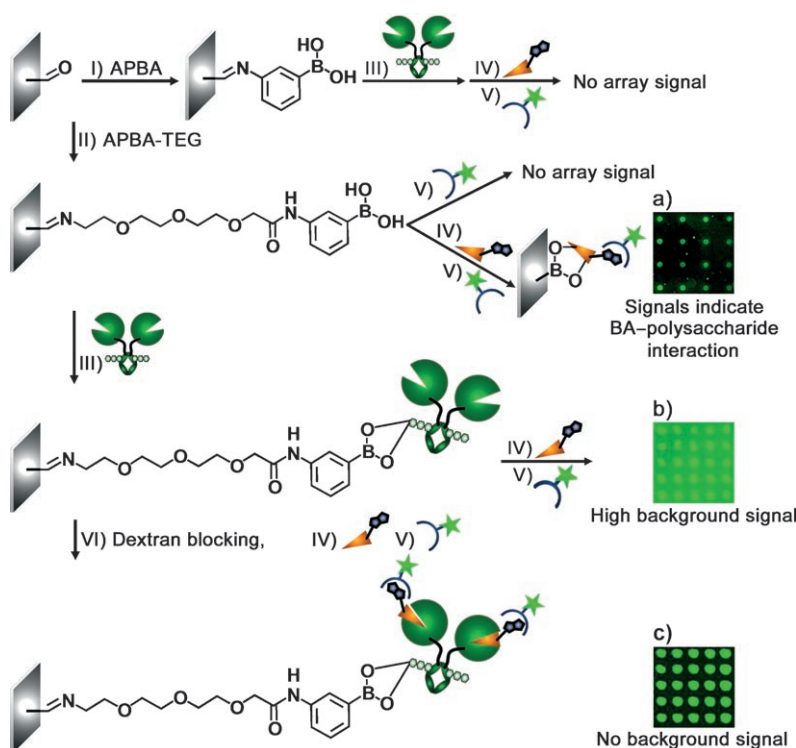


Figure 2. Schematic presentation of the fabrication of a covalent oriented lectin microarray through boronate formation. The aldehyde slides were incubated with I) APBA or II) APBA-TEG (100 mM) at 4°C for 12 h; III) Fc-dectin-1 (2 mg mL⁻¹) was printed, and the slides were incubated at 4°C for 12 h and then treated with 5% BSA; IV) F3-biotin (0.1 mg mL⁻¹), 25°C, 1 h; V) streptavidin-Cy3 (10 µg mL⁻¹), 25°C, 1 h; VI) dextran (1 µM), 4°C, 4 h. a) The APBA-TEG microarray was incubated with 5% BSA and then with F3-biotin, and was then stained with streptavidin-Cy3. Signals indicate the interaction of BAs with the polysaccharide. b) Binding results for the Fc-fused dectin-1 microarray fabricated by boronate formation with BSA as the surface-blocking reagent. c) Binding results for the Fc-fused dectin-1 microarray fabricated by boronate formation with dextran as the surface-BA-blocking reagent (see Figure 3).

The results showed an extremely low binding activity of the APBA surface, possibly as a result of a low amount of immobilized lectin. Furthermore, without the use of TEG, the immobilized protein may be denatured during the conjugation step.^[24] Since the Fc carbohydrate moiety is located in the interchain region of the Fc domain, the surface BAs were unable to interact with these carbohydrates. Although the longer linker in APBA-TEG significantly alleviates surface steric hindrance and therefore facilitates boronate formation, there was serious background noise on the microarray surface (Figure 2a,b).

After substantial experimentation, we found that the high background signal was mainly due to an undesired interaction between the BAs and F3-biotin (Figure 2a). Surface BAs can potentially react with any polysaccharide; therefore, the undesired BA–target carbohydrate interaction should be avoided in the development of a BA-based protein-microarray platform. In an attempt to suppress the undesired boronate formation, we conducted a chemical competition assay (Figure 3a) with the following blocking reagents: ethylene glycol (R¹), glycidol (R²), glycerol (R³), tris(hydroxymethyl)aminomethane (R⁴), dextran (R⁵), glycolic acid (R⁶), nitrilotriacetic acid (R⁷), and ethylenedinitrilotetraacetic acid

(R⁸). The intensity of the fluorescence signal decreased as the number of hydroxy groups in the blocking reagent increased. In particular, the dextran-blocked surface showed the lowest signal in the assay (Figure 3b). This observation may be attributed to the multiple diol interactions of dextran with surface BAs to form stable boronates. Thus, dextran was chosen as the optimal blocking reagent for the BA-based microarray (Figure 2c).

The influence of the immobilization strategy on the binding activity of surface lectins was also investigated. To fabricate lectin microarrays, Fc-dectin-1 (2 mg mL⁻¹) was immobilized on glass slides by the use of the three immobilization strategies delineated in Figure 1. The aldehyde- and BA-modified slides were used for covalent random Schiff base formation and oriented immobilization by boronate formation, respectively, whereas the protein-G-modified slide was used for noncovalent oriented immobilization by specific recognition between the Fc domain and protein G (20 µM).

Quantitative analysis of the fluorescence intensities (Figure 4) showed that the binding activities of the Fc-dectin-1 microarrays created by oriented boronate formation and protein G/Fc recognition were 16- and 5-fold higher, respectively, than that of the microarray formed by random covalent immobilization. Notably, as a result of increased hydrophilicity, the spots on the BA-modified slide were bigger than those on the aldehyde-modified slide. To gain insight into whether oriented noncovalent immobilization results in higher F3-biotin binding as a result of increased availability of lectin

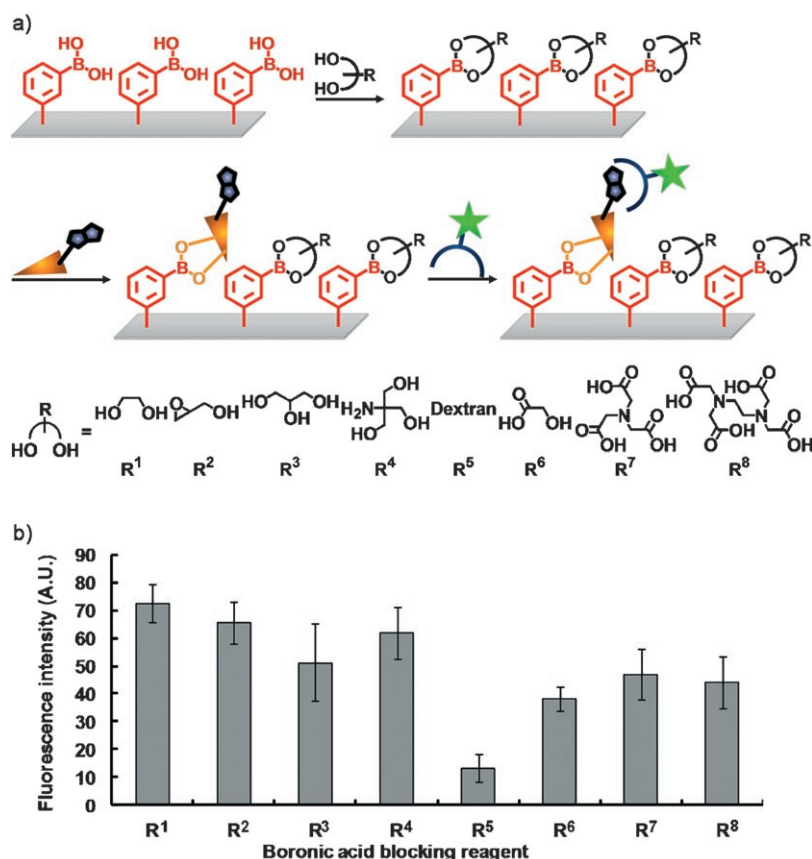


Figure 3. a) Scheme of the chemical competition assay for optimizing the blocking reagent. After blocking of the BA-modified slides with different blocking reagents, F3-biotin (1 mg mL^{-1} , 25°C , 16 h) was printed on the slides, which were then stained with streptavidin-Cy3 ($10 \text{ }\mu\text{g mL}^{-1}$, 25°C , 1 h). b) Fluorescence intensities of the products of the reaction sequence in (a).

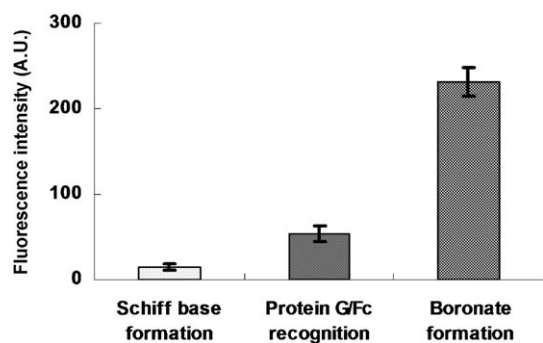


Figure 4. Comparison of the fluorescence images for F3-biotin binding to Fc-fused lectin-1 microarrays fabricated by random Schiff base formation (left), noncovalent oriented immobilization (middle), and covalent oriented immobilization (right). (For color microarray images, see Figure S2 in the Supporting Information).

active sites or an increase in the amount of lectin bound to the surface, Fc-fused lectin-1 was spotted at five different concentrations (0.25 , 0.5 , 1 , 2 , and 4 mg mL^{-1}) on the protein G and aldehyde slides, respectively, and incubated at 4°C for 24 h to ensure complete conjugation.

The results (Figure 5) showed that the surface was not saturated by the lectin at the concentration used. The protein-

G-based slide (oriented) consistently displayed higher F3 binding than that of aldehyde slide (random). We could therefore conclude that lectin-1 binding activity is affected significantly by its orientation on the surface. When F3-biotin diluted 100-fold ($1 \text{ }\mu\text{g mL}^{-1}$) was tested, the BA-based slide still provided a detectable signal, whereas protein-G- and aldehyde-based slides showed very weak or no binding activity (see Figure S1 in the Supporting Information). These results demonstrate the advantage of boronate formation for the oriented presentation of proteins.

Taken together, these results clearly demonstrate the superiority of BA surfaces for the covalent and oriented immobilization of Fc-fused lectin-1. The greater F3-biotin-binding efficiency of BA-based Fc-fused lectin microarrays may be due to the extremely small size of the BA relative to protein G. The distribution of BAs at a higher density on the slide surface results in an increase in the effective concentration of Fc-fused lectin molecules on the microarray. Furthermore, the noncovalent nature of protein G/Fc recognition may result in the leakage of Fc-lectin-1 during washing steps. Also, during the covalent immobilization of protein G in a random manner on the solid surface, the generation of some inactive conformations could occur^[25] and thus result in a lower signal.

Finally, we demonstrated the generality and quantitative assessment of this BA-based method with TREM-like transcript 2 (TLT-2), which has been shown to recognize specifically a number of Gram-negative and Gram-positive bacteria and some yeasts.^[26] We selected TLT-2 as the next target because it can also bind to F3-biotin.^[20] TLT-2 was fused to Fc, and the resulting fusion protein (Fc-TLT-2) was immobilized on

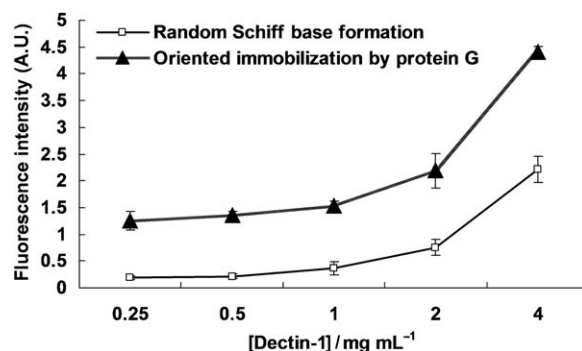


Figure 5. Comparison of F3-biotin binding by Fc-fused lectin-1 microarrays on protein G and aldehyde slides. Fluorescence signals were generated by staining with streptavidin-Cy3, and the signal intensities were quantified. The bars show the average of the mean fluorescence signal intensities of five spots for the indicated microarray. (For color microarray images, see Figure S3 in the Supporting Information).

aldehyde-, protein-G-, and BA-modified surfaces as described previously. On the basis of the fluorescence intensity of the arrays after treatment with F3-biotin and then streptavidin-Cy3 (Figure 6), both forms of oriented immobilization of Fc-TLT-2 led to higher F3-biotin binding than that observed following random immobilization, and the TLT-2 binding activity was highest for the BA-based microarray. Thus, the developed method appears to be of broad utility for the fabrication of other Fc-fused lectins.

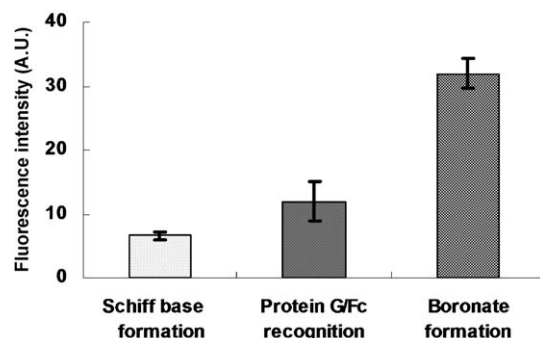


Figure 6. Comparison of the fluorescence images for F3-biotin binding to Fc-fused TLT-2 microarrays fabricated by random Schiff base formation (left), noncovalent oriented immobilization (middle), and covalent oriented immobilization (right). (For color microarray images, see Figure S4 in the Supporting Information).

The X-ray crystal structure of the mouse C-type lectin-like domain (CTLD) of dectin-1^[27] (very similar to human dectin-1; see Figure S5a in the Supporting Information) revealed that four out of ten lysine and arginine residues on the protein surface are located near the carbohydrate-binding site. The formation of Schiff bases at amine groups on these residues during covalent random immobilization may result in partial or complete blockage of the carbohydrate-binding site. In contrast, the glycosylation site(s) in the stalk region or Fc domain of Fc-dectin-1 (for a putative structure, see Figure S5b in the Supporting Information) is far away from the carbohydrate-binding site; therefore, boronate formation would have minimal impact on the binding activity of the lectins towards target carbohydrates.

In conclusion, we have developed a novel method to produce a stable, covalent, and highly active protein microarray. The binding activities of the immobilized Fc-fused lectins varied according to their orientation and density on the microarray surfaces. Because of improved surface exposure of the carbohydrate-binding site, the Fc-fused lectin microarray produced by boronate formation provides higher target sensitivity. Although the formation of the boronate is a reversible reaction in aqueous solution, the protein microarray fabricated from the BA-modified slide is very stable as a result of multiple reaction sites between the oligosaccharide of the Fc domain and BAs on the surface. The method reported herein should also be applicable to the fabrication of microarrays of other glycoproteins, such as antibodies.

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