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Fabrication of Antibody Microarrays by Light-Induced Covalent and Oriented Immobilization

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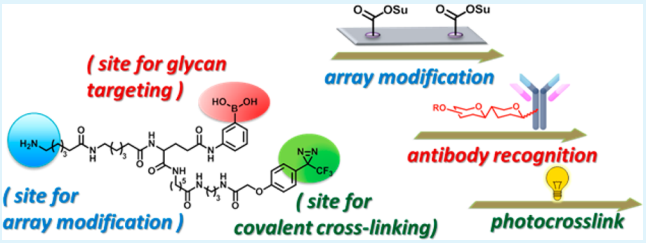
Supporting Information

ABSTRACT: Antibody microarrays have important applications for the sensitive detection of biologically important target molecules and as biosensors for clinical applications. Microarrays produced by oriented immobilization of antibodies generally have higher antigen-binding capacities than those in which antibodies are immobilized with random orientations. Here, we present a UV photo-cross-linking approach that utilizes boronic acid to achieve oriented immobilization of an antibody on a surface while retaining the antigen-binding activity of the immobilized antibody. A photoactive boronic acid probe was designed and synthesized in which boronic acid provided good affinity and specificity for the recognition of glycan chains on the Fc region of the antibody, enabling covalent tethering to the antibody upon exposure to UV light. Once irradiated with optimal UV exposure (16 mW/cm^2), significant antibody immobilization on a boronic acid-presenting surface with maximal antigen detection sensitivity in a single step was achieved, thus obviating the necessity of prior antibody modifications. The developed approach is highly modular, as demonstrated by its implementation in sensitive sandwich immunoassays for the protein analytes *Ricinus communis* agglutinin 120, human prostate-specific antigen, and interleukin-6 with limits of detection of 7.4, 29, and 16 pM, respectively. Furthermore, the present system enabled the detection of multiple analytes in samples without any noticeable cross-reactivities. Antibody coupling via the use of boronic acid and UV light represents a practical, oriented immobilization method with significant implications for the construction of a large array of immunosensors for diagnostic applications.



KEYWORDS: *protein immobilization, protein microarray, boronic acid, immunosensor, antigen detection, carbohydrate*

INTRODUCTION

The stability and accessibility of a surface-bound protein's active site may be influenced significantly by the orientation of the protein on the solid support.[1,2] For the development of immunobiosensors, highly efficient surface immobilization of antibodies (Abs) is essential for the successful implementation of microarray screening, which relies upon the specific recognition and binding of Abs to an antigen. The Abs are typically immobilized on a fixed solid surface and are used to detect a wide range of analytes, including microbial pathogens, bacterial toxins, drugs, and metabolic biomarkers.[3,4] The most common strategies for Ab immobilization include noncovalent immobilization or random covalent immobilization.[5] However, these methods suffer from reduced immunoaffinity of the immobilized Abs because of steric blockage of the essential binding sites. Therefore, various site-specific, i.e., oriented, Ab conjugation strategies have been developed.[6] A single carbohydrate moiety is present in the constant region (Fc) of Abs; this moiety can be oxidized to generate reactive aldehyde groups that selectively bind to surfaces via Schiff base formation.[7,8] Although this technique usually provides a high coupling yield, the resulting aldehyde groups may potentially react with other amine groups in the Ab, and the oxidative chemicals used can result in a loss of biological activity.[9] To overcome these limitations, Ab-binding proteins such as protein A, G, or L have been used to site-specifically immobilize Abs in protein-based microarrays.[10] These Ab arrays usually exhibit significantly improved antigen-binding ability and sensitivity compared to those employing random covalent coupling or physical adsorption methods.[11] However, generating a uniform, oriented Ab on the surface with preserved antigen-binding activity still presents a major challenge and is the subject of ongoing investigations.[12,13] To preserve the biological recognition activity of the captured Ab, an ideal Ab immobilization method should enable immobilization solely at a single attachment point with a proper orientation propitious for antigen binding under mild conditions in aqueous buffer solutions while avoiding the use of other chemical reagents.

Boronic acids (BAs) rapidly and reversibly form cyclic boronate esters with *cis*-diols in both nonaqueous and aqueous milieus.[14,15] In recent years, BAs have received much attention as saccharide binders and potential sensors[16,17] and in protein and drug delivery[18,19] and affinity chromatography.[20] The interaction between BAs and carbohydrates has also been

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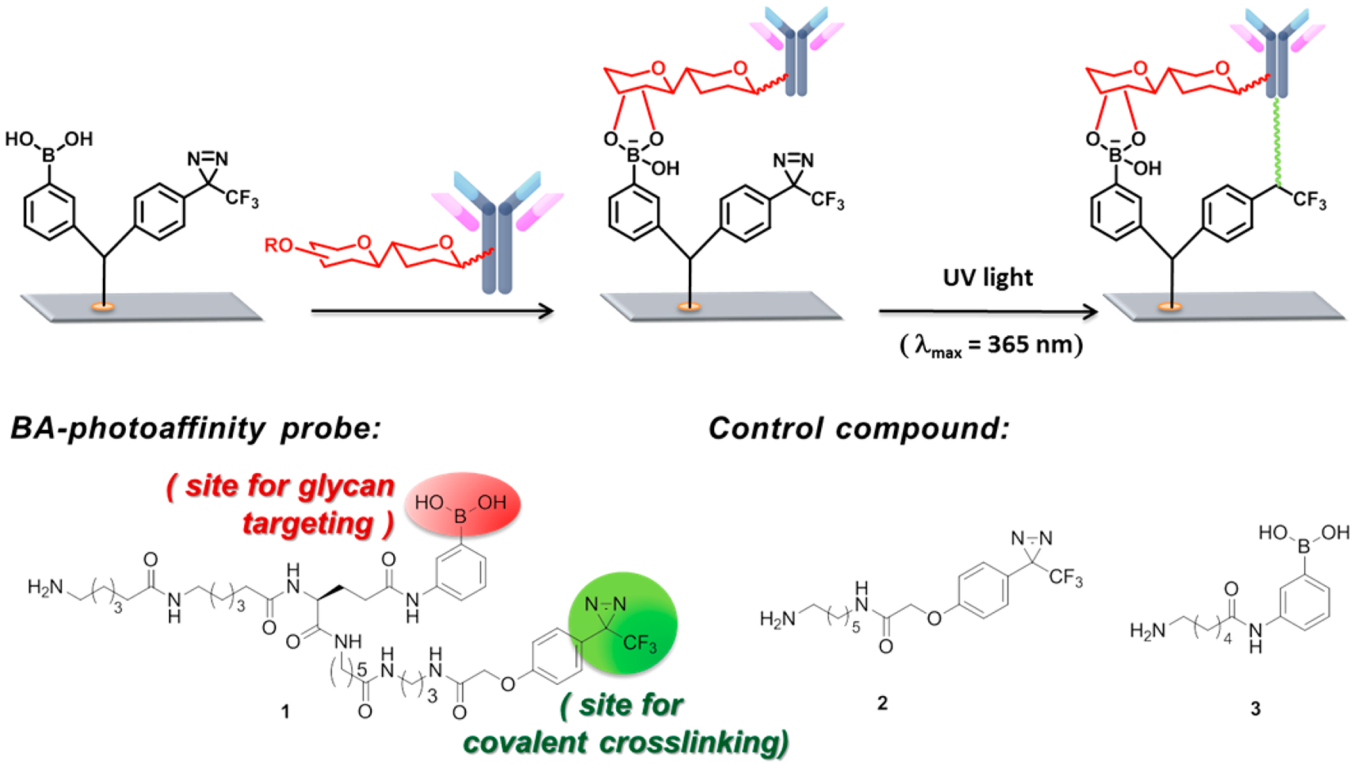

Figure 1. Light-induced covalent and oriented immobilization of Abs on BA-presenting surfaces. Upon UV irradiation (λ_{max} = 365 nm), the (trifluoromethyl)phenyldiazirine functional groups in **1** generate highly reactive carbenes that spontaneously undergo insertion reactions to permanently tether the Ab on the surface. The preservation of the biological activity after Ab photoattachment was characterized by immunoassays. Structures of the BA-based photoaffinity probe (**1**) and control compounds (**2** and **3**) used in this study.

exploited for the oriented, reversible immobilization of glycoproteins onto cellulose beads.[21] These interactions have been assessed to determine the presence of a glycoprotein using a fluorescently tagged BA derivative to enable direct visualization.[22] Recently, BA groups have been implemented in chemoselective glycoprotein immobilization on solid supports such as glass with complete conservation of glycans.[23] We previously used BA-coated glass slides to fabricate carbohydrate and protein microarrays using unprotected carbohydrates and the glycan chains of Fc-fusion proteins, respectively, to generate oriented protein microarrays through boronate formation.[24,25] When BA-based coupling strategies were used to produce protein microarrays, the binding capacities of the microarrays were typically orders of magnitude higher than those of the cognate analytes. Because the carbohydrate region is located in the Fc domain, which is not involved in antigen-binding activity,[7] interactions between BA and the carbohydrate chains are not likely to affect the biological activity of the immobilized Abs. This approach was further extended to other BA-presenting substrates, such as magnetic nanoparticles and graphite-modified electrodes, to immobilize Abs through the Fc region, leaving the variable light-chain regions (Fab) available for enhanced binding to antigen epitopes.[26,27] BA self-assembled monolayers (SAMs) have also been used to fabricate horseradish peroxidase (HRP)-conjugated Ab for the development of an amperometric immunosensor.[28] However, the reversibility of the bond between BAs and carbohydrates permits protein release, and thus the array sensors do not remain stable during subsequent washing steps.[29] Therefore, in addition to BAs, the use of a mixed monolayer of reactive epoxy groups has been suggested for the covalent attachment of glycoprotein−HRP to heterobifunctional boronate−epoxy SAMs on gold to prevent protein release.[29] Generating a universal tool for the oriented, covalent surface immobilization of Abs with minimal Ab modification remains a major challenge.[6]

In this work, we evaluated the applications of a versatile microarray platform on glass slides that had been derivatized with multiple copies of **1** suitable for oriented and irreversible attachment of an Ab to a surface (Figure 1). To permit interaction with carbohydrate moieties at the Fc region of the Abs and visualization of the covalently attached targets, **1** contains a BA and a (trifluoromethyl)phenyldiazirine photoreactive cross-linker, respectively. We demonstrated that the probe can be photoactivated to attach Abs covalently to surfaces in one step. The use of BAs in combination with a photoactivatable reagent enables the immobilization of Abs in a uniform manner while retaining the antigen-binding activity to enhance the antigen detection efficiency of the functionalized surface. As a proof-of-concept, the model protein analytes *Ricinus communis* agglutinin (RCA_{120}), a useful lectin surrogate for the bioterrorism agent ricin toxin; human prostate-specific antigen (PSA), a biomarker for prostate cancer; and interleukin (IL)-6, a pleiotropic cytokine, were tested. Finally, we demonstrated the feasibility of the use of this functional Ab microarray platform for the simultaneous detection of proteins in samples containing mixtures of analytes.

EXPERIMENTAL SECTION

Materials. All starting materials and reagents were obtained from commercial sources and used as received unless otherwise noted. 6-Aminohexanoic acid and Fmoc-L-glutamic acid 5-*tert*-butyl ester [Fmoc-Glu(OtBu)-OH] were purchased from Acros and AnaSpec,

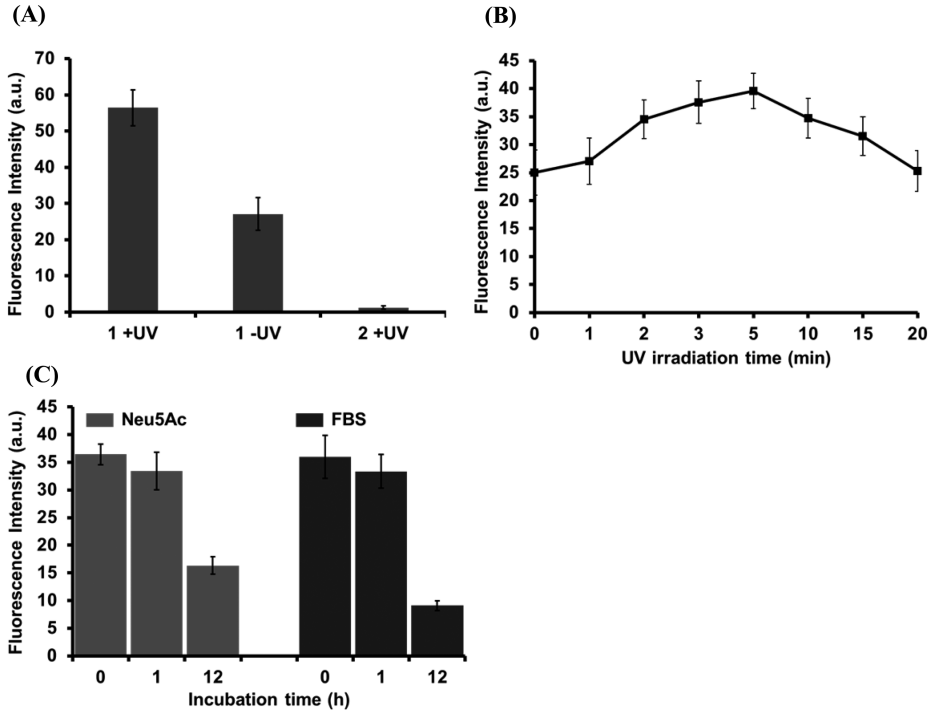

Figure 2. BA-Pi of an Ab on a **1**-coated surface through boronate formation. (A) Effect of UV light and boronate formation on the surface immobilization of the Ab. (B) Effect of UV irradiation time on the surface photoimmobilization of the Ab. (C) Relative changes in the fluorescence intensities of **1**-coated surfaces without photoirradiation after exposure to different competitive reagents, Neu5Ac (100 mM in PBS) (left) and FBS (right), for 0−12 h at rt prior to antigen incubation. The antigen-binding efficiency of the immobilized Ab was measured using fluorescein-RCAI (25 μg/mL).

respectively. *N*-Hydroxysuccinimide (NHS)-activated glass slides (Nexterion H) were purchased from Schott, North America. Bovine serum albumin (BSA), *R. communis* agglutinin 120 (RCA_{120}), dextran (D-1662), concanavalin A (biotin conjugate), and streptavidin−Cy3 conjugate were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Mouse monoclonal antiprostate-specific antigen Ab, mouse monoclonal anti-interleukin (IL)-6 Ab, human IL-6 full-length protein, biotinylated rabbit anti-IL6 Ab, and biotinylated antiprostate-specific antigen polyclonal Ab were purchased from Abcam. Fluorescein-labeled *R. communis* agglutinin I, goat monoclonal anti-ConA Ab, and biotinylated anti-RCAI&II Ab were obtained from Vector Laboratories. Prostate-specific antigen (PSA, human seminal fluid) and unconjugated monoclonal rabbit anti-RAC (ricin alpha chain) Abs were purchased from Merck and EY Laboratories, respectively. Deionized water with a resistivity of >18 MΩ·cm was obtained from an ultrafiltration system (Milli-Q, Millipore) and passed through a 0.22 μm filter to remove particulate matter.

Synthesis of BA-Based Photoaffinity Probe (1). The synthesis of **1** is outlined in Scheme S1 and described in detail in the Supporting Information (SI). An orthogonally protected Fmoc-Glu derivative, which was obtained from an amide coupling reaction between Fmoc-Glu(OBu-t)-OH and 6-aminocaproic acid methyl ester, was employed (Scheme S1 in the SI). The synthetic procedures of control compounds **2** and **3** are also mentioned in the SI.

Preparation of 1-Coated Glass Slides. Nexterion H glass slides were treated with a 25 mM solution of **1** in dimethyl sulfoxide (DMSO)/water (1:3, v/v, adjusted to pH 9.0 with 2 M NaOH) at room temperature (rt) for 6 h to react with the NHS ester groups. The slides were washed with deionized water and then dried by centrifugation. Unreacted NHS esters were deactivated by immersing the slides in a solution containing ethanolamine (25 mM) in borate buffer (100 mM, pH 9.2) at rt for 1 h. The slides were sequentially washed with phosphate-buffered saline (PBS, pH 7.4) and PBS containing 0.05% Tween-20 (PBST), followed by rinsing with deionized water. The rinsed slides were dried by centrifugation, and the coated slides could be stored safely at 4 °C for several weeks in the dark until use.

General Ab Microarray Printing. Monoclonal capture Abs (6.6 μM) were prepared in a print buffer (PBS containing 0.005% Tween 20, pH 7.4) and dispensed using a robotic contact arrayer (AD1500 Arrayer, BioDot) fitted with Stealth Pins SMP3 (ArrayIt Corp.) onto **1**-coated glass slides. The printing process was performed at a relative humidity of 94%, and the temperature was maintained below 26 °C. The arrayer was programmed to deliver approximately 6 nL of an Ab solution per spot in a 5 × 5 array with an average spot diameter of ~340 μm. The spacing between spots was 0.4 mm. The needle dwell time in the wells was 5 s, and the pins underwent three wash cycles between visits. The printed slides were maintained at 4 °C for 12 h to mediate boronate formation with the surface-conjugated BAs. A total of 14 blocks were printed on each slide, and all slides were divided into a 2 × 7 format using a permanent marker to prevent cross-contamination when different samples were applied in neighboring blocks. After incubation, unbound Abs were washed away with PBS (pH 7.4) and deionized water prior to exposure to UV light (see below).

Amidation of NHS Esters Using a BA Derivative (3) and a Photoaffinity Group (2). A Nexterion H glass slide was immersed in a solution containing **3** and **2** (1:1, 25 mM final concentration) in DMSO/water (1:3, v/v, adjusted to pH 9.0 with 2 M NaOH) overnight. The remaining activated esters on the surface were deactivated using ethanolamine, and the slide was washed and dried prior to microarray printing as described above.

BA-Based Photoimmobilization (BA-Pi) of Abs. The above Ab-coated slides were exposed to 365 nm UV light at a distance of 7.5 cm using a Blak-Ray high-intensity UV lamp (B-100AP, Sunway Scientific Instrument, Taiwan) at 4 °C for a period of 5 min to covalently immobilize the Ab onto the slides. After the photoaffinity reaction, the microarray slides were washed with PBST and deionized water to remove unbound Abs. The remaining BAs on the surface were then blocked by treatment with dextran (100 μM) containing 1% BSA in water at rt for 2 h to prevent nonspecific adhesion/interactions.

Following a wash, the Ab microarray slides were treated with a diluted solution of analyte (20 μL) in assay buffer (PBST containing 0.1% BSA) and probed with a biotinylated secondary Ab when streptavidin−Cy3 was used to detect the presence of the secondary Ab. After a final wash, the slide was dried, and the signal intensity was detected using a microarray fluorescent scanner (see below).

Microarray Image Acquisition and Data Analysis. All images were acquired using the microarray slide scanner Nova Ray detection platform (Alpha Innotech, USA). Fluorescent signals were detected using FITC and Cy3 filters. The fluorescence intensity of the spots was quantified using the *ArrayVision* software package (version 8.0) with correction for local background. All proteins were analyzed in triplicate, and the data represent an average of 18−20 spots for a given antigen concentration. The mean intensity of each spot was taken as a single data point for analysis.

RESULTS AND DISCUSSION

In this work, we investigated the immobilization of Abs using BAs as an affinity headgroup and covalently cross-linked Abs to surfaces using the photoactivatable reagent via UV-light irradiation. As outlined in Figure 1, when Abs are introduced, BAs direct the formation of a cyclic boronate diester with the diol groups of glycans in the nonantigenic Fc region,[26,27,29] thus preventing the Abs from binding to the solid surface via their antigen-binding Fab domains. (Trifluoromethyl)-phenyldiazirine was chosen as the cross-linker because of its relatively small size and its excellent ability to be cross-linked at a wavelength (~365 nm) that imparts minimal photochemical damage to biomolecules.[30] We and others have previously demonstrated that diazirine cross-linkers are able to capture carbohydrate−glycoprotein interactions.[31,32] Upon UV irradiation, the diazirine functional groups generate highly reactive carbene species, which subsequently trigger a rapid insertion into proximal amino acid residues, resulting in irreversible tethering of the Ab to the surface (Figure 1). This method essentially facilitates the direct, covalent attachment of an Ab to a surface with the bond distal from the antigen-capturing site, thus improving accessibility to the analyte. The immobilization technique is site-specific and enables precise Ab orientation, which may facilitate the preservation of the activity of the immobilized Ab.

Significance of BAs and UV Light in BA-Pi of Abs. To immobilize Abs using the BA-Pi strategy, highly amine-reactive NHS-activated Nexterion H glass slides were incubated with **1** (Scheme S1 in the SI) to yield BA-functionalized slides (Figure 1). Then, a rabbit anti-RAC (A-chain) mAb (RCA Ab) was spotted on the slides and incubated at 4 °C to enable association with the surface-conjugated BAs overnight. After washing, the slides were exposed to brief UV irradiation (λ_{max} = 365 nm; 16 mW/cm^2) for 5 min to covalently immobilize the Ab onto the surface. To prevent undesired interactions between the glycoproteins (analytes) and surface-coated BAs, dextran was used to block the surface BAs.[23,25] The dextran-blocked surface is hydrophilic and therefore can suppress nonspecific binding. The activity of immobilized Abs could be assessed by a direct-detection format using fluorescently labeled lectins or in a sandwich assay in which bound antigens were detected using a biotinylated secondary Ab followed by a streptavidin−fluorophore conjugate. The resulting slides were then incubated with fluorescein-conjugated *R. communis* agglutinin I (fluorescein-RCAI, 25 μg/mL) to evaluate the activity of the immobilized Abs. As shown in Figure 2A, the photoirradiated slide provided better binding activity than the slide without UV irradiation, demonstrating that the covalent linkage prevented the escape of Abs from the surface during the washing steps. Notably, the slide that only contained the diazirine compound (**2**) without the BA functionality produced no apparent signal after photoirradiation and incubation with fluorescein-RCAI. These results confirm that boronate formation results in Ab immobilization and that the surfaces resist nonspecific protein adsorption.

Effect of the UV Irradiation Time and Determination of the Concentration of 1 on the Surface for the Effective Immobilization of Ab. The influence of the UV irradiation time on the efficiency of photo-cross-linking between **1** and Ab was next evaluated. The procedures are the same as those described for BA-Pi of the Ab except that the UV irradiation time was changed. As shown in Figure 2B, quantitative analyses of emission intensities revealed that the fluorescence intensity depends on the UV irradiation time. The maximum intensity was reached using a UV exposure (365 nm; 16 mW/cm^2) of 5 min. However, prolonged irradiation (>5 min) resulted in a decrease in the fluorescence signal, possible due to damage to the Ab with longer UV irradiation times. UV-irradiated Abs have been shown to degrade on solid surfaces, and a decrease in the antigen-binding ability was observed when benzophenone was used as a photoactivatable group.[33] In addition, we also determined that a 12 h association for the formation of the boronate diester between the surface BAs and the Ab is sufficient to produce a fluorescence signal after UV exposure, and a longer incubation time (18 h) had no effect on fluorescein-RCAI binding (Figure S1 in the SI).

Neutral pyranose saccharides, such as glucose (Glc),[15,34] and acidic monosaccharides, such as *N*-acetylneuraminic acid (Neu5Ac),[18] bind BAs with high affinity. Therefore, these monosaccharides should compete with immobilized Abs for boron complexation. As expected, when Neu5Ac (100 mM in PBS) was applied to a microarray of anti-RCA Ab on a **1**-coated surface without UV irradiation, the retention of boronated Abs after fluorescein-RCAI binding was diminished (Figure 2C, left). However, no apparent decrease in the fluorescence intensity was observed when Glc was employed under similar conditions (Figure S2 in the SI), largely because of the greater affinity of BAs for Neu5Ac than for Glc.[19] Strong suppression of the fluorescence signal was also observed when the slide was treated with a more complex biofluid, fetal bovine serum (FBS), in which boronate is prone to degrade, resulting in Ab leakage (Figure 2C, right).[25] Diluted FBS (diluted 1:10 in PBS, pH 7.4) similarly suppressed the fluorescent signal (data not shown). To verify that the Abs were permanently tethered onto the **1**-coated surface, after UV exposure, the slides were treated with FBS and diluted FBS at rt for 1−12 h prior to antigen binding. As is evident from Figure S3A in the SI, no significant change in the fluorescence intensity was observed, confirming that the Abs remained on the surface and maintained their antigen-binding activity and highlighting the necessity of a photo-cross-linker. In addition, incubation with Glc (100 mM) at pH 7.4 (PBS buffer) or with other BA-interacting polyols such as glycerol, which has been used to release native glycans from a boronated glycoprotein complex,[23] as a competitor had no significant effect on fluorescein-RCAI binding (Figure S3B in the SI). These results support the conclusion that Abs immobilized using the BA-Pi strategy described above are most likely irreversibly attached to the **1**-coated surface upon UV irradiation.

The relative antigen-binding activity of the anti-RCA Ab was measured to define the optimum concentration of **1** needed to

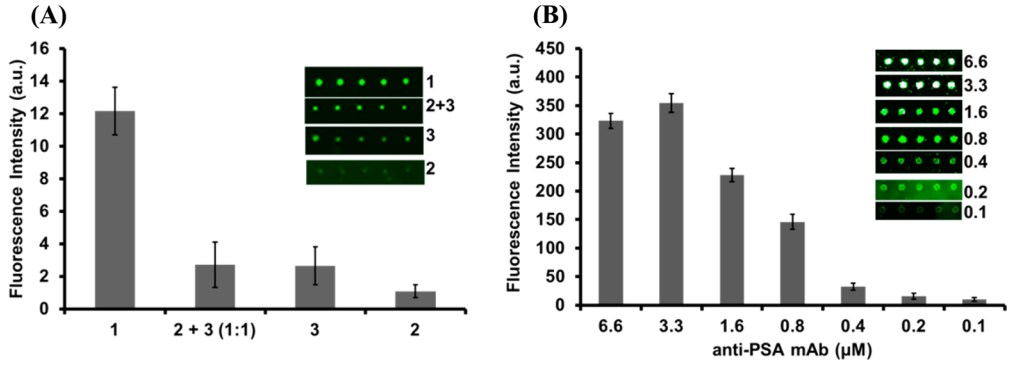

Figure 3. (A) Direct comparison of the photo-cross-linking of Ab to different substrates derived from **1** and an equimolar mixture of **2** and **3**. Representative fluorescence images obtained from different substrates are shown in the inset. (B) Antigen-binding efficiency as a function of the surface-immobilized Ab concentration. Anti-PSA mAb (0.1−6.6 μM) was immobilized on a **1**-coated surface (see the Experimental Section), incubated with PSA (1 μg/mL), and visualized with biotinylated goat anti-PSA pAb (10 ng/mL) and streptavidin−Cy3. Representative fluorescence images at the indicated Ab concentrations (μM) are shown in the inset.

generate an oriented, immobilized Ab microarray surface. We used RCA_{120} as an antigen and a second biotinylated Ab directed toward a different epitope of RCA_{120} in a sandwich immunoassay. The anti-RAC Ab (6.6 μM) was spotted onto slides fabricated using various concentrations of **1** (1−75 mM). The slides were then reacted with RCA_{120} (1 μg/mL), and the binding abilities were evaluated using biotinylated goat anti-RCAI&II pAb (10 ng/mL) and streptavidin−Cy3. Signal saturation was observed when the concentration of compound **1** was higher than 10 mM (Figure S4 in the SI). Therefore, the optimum BA-Pi conditions for Ab microarray fabrication to obtain the maximum signal were defined as 25 mM compound **1** and UV exposure for 5 min (365 nm; 16 mW/cm^2).

Roles of BA, the Photoreactive Group, and the Ab Concentration in the BA-Pi Strategy. To determine the roles of BA and the photoactive group in **1**, a comparative analysis of the antigen-binding ability of Ab using **3** and **2** was investigated. As an analyte, we employed IL-6, an ∼20 kDa glycoprotein that is a key molecule in the regulation of the immune response.[35] A mixture of **2** and **3** (molar ratio 1:1) was reacted with NHS-activated glass slides (see the Experimental Section for details), followed by BA-Pi to immobilize the anti-IL-6 Ab (IL-6 Ab). IL-6 was then used to evaluate the activity of the immobilized Ab on the microarray using a sandwich immunoassay similar to that used for RCAI. The presence of IL-6 on the microarray was quantified by the emission intensity of the fluorescent signal detected using biotinylated anti-IL6 pAb (1 ng/mL) and streptavidin−Cy3. As shown in Figure 3A (and Figure S5 in the SI), a **1**-coated surface produced a ∼5.5-fold enhancement of the emission intensity compared to the surface coated with a mixture of **2** and **3**, while a very weak signal was detected from the surfaces coated with **2**. However, the control substrate separately treated with **3** produced a signal comparable to that of the mixed surface, which was attributed to the reversible but covalent attachment of Abs to BAs that were not completely removed by the stringent buffer wash. These results clearly demonstrate that Ab immobilization is greatly influenced by both the surface capturing group (i.e., BA) and the photoreactive group present on the same molecule.

The enhanced cross-linking of **1** is critical for oriented Ab immobilization for superior antigen capture and to provide stable, covalent immobilization. To further confirm our results, PSA was chosen as the analyte, and the BA-Pi and sandwich immunoassay was repeated. PSA is an ∼34 kDa serine protease that is produced predominantly by epithelial cells of the human prostate gland and is secreted into the prostatic fluid. PSA has been recognized as a useful tumor marker for prostate cancer in men.[36] The fluorescent signal observed for the anti-PSA Ab arrays generated on **1** was 4.3-fold higher than that observed for the mixed **3**- and **2**-coated surface (Figure S6 in the SI). We attribute the high antigen-binding activities of the Abs immobilized on the **1**-coated surface to two factors. First, BAs preferentially interact with the carbohydrate moiety of Fc and permit exposure of the Fab domain, enabling intimate contact between the heavy chain and the surface to facilitate cross-linking. Second, the short lifetime of the carbene generated from **1** ensures cross-linking with Abs that bind to BAs in close proximity, thereby permanently tethering Abs to the surface. Notably, the generated carbenes may be rapidly quenched by intramolecular rearrangements or by solvent when Abs are far away and not bound to BAs. This feature of Ab photo-cross-linking on a **1**-coated surface enables a one-step, direct immobilization approach without the requirement for oxidative modification of the Fc region of the Ab. We expect that the developed BA-Pi method will reduce the loss of Ab activity by eliminating unfavorable orientations or multisite attachment, both of which are inevitable when common Ab immobilization techniques are employed.

To evaluate the effect of the Ab concentration used to fabricate the Ab microarray, we conducted experiments using various concentrations of anti-PSA Ab (0.1−6.6 μM) to detect PSA. Figure 3B shows the PSA (1 μg/mL) binding ability of the immobilized Abs on a **1**-coated surface. Binding analysis of the captured Abs revealed low surface coverage at anti-PSA Ab concentrations of less than 3.3 μM. In addition, there was a clear trend of increased antigen binding with increasing Ab cross-linking at an anti-PSA Ab concentration of 3.3 μM, above which the signal tended to plateau, indicating that antigen access to the surface-immobilized Abs was slightly hindered. A similar trend was also observed when anti-ConA, anti-IL-6, and anti-RAC (A-chain) Abs were cross-linked on a **1**-coated glass surface (Figure S7 in the SI), indicating the suitability of the boronate-directed oriented cross-linking approach for immunoassays and for applications such as the development of multiplex screening platforms.

Detection of Individual Antigens. To demonstrate the utility of the developed method, the use of the fabricated Ab microarrays to detect three antigens was investigated. We

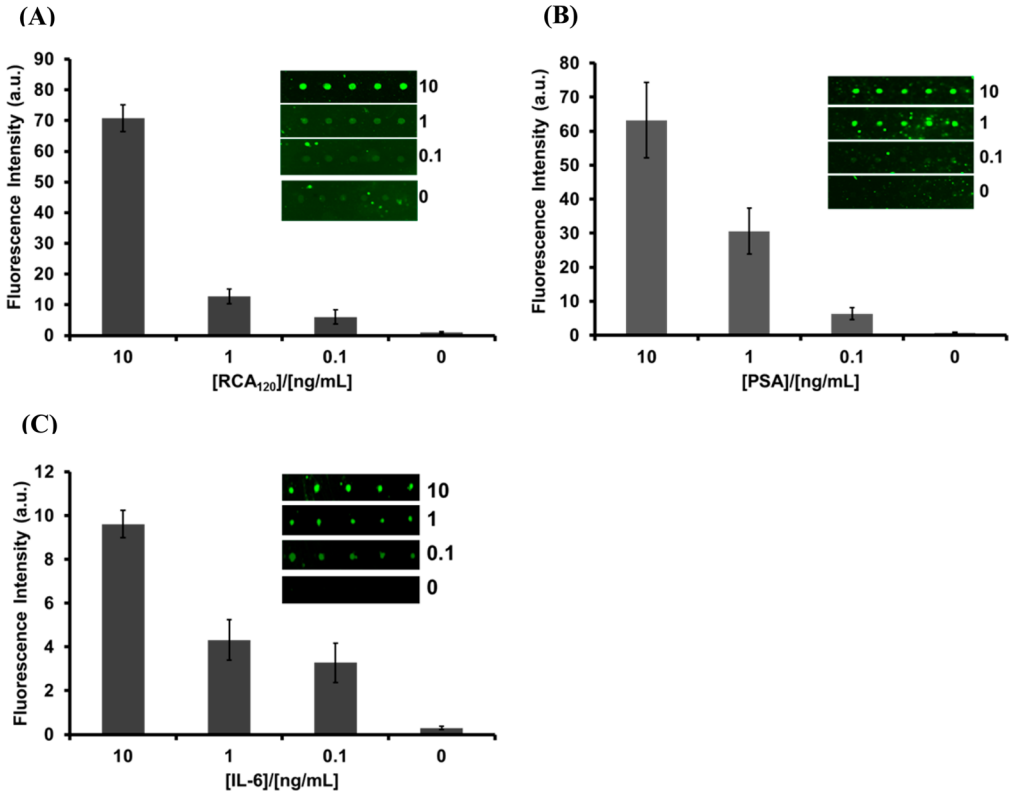

Figure 4. Functional Ab microarrays for the detection of a single antigen. Ab microarrays were fabricated on a **1**-coated slide, as described in the Experimental Section for each analyte: (A) detection of RCA_{120}; (B) detection of PSA; (C) detection of IL-6. The LOD was determined as follows: LOD = signal of the blank + 3 SDs of the blank. The concentrations of each biotinylated secondary Ab are specified in the text. Corresponding fluorescence images at the indicated antigen concentration [ng/mL] are shown in the inset. Fluorescent signals were obtained by staining with streptavidin−Cy3. Mean signals and the SD are shown.

selected RCA_{120}, a low cytotoxic surrogate for the bioterrorism agent ricin toxin; IL-6, an interleukin that is known to have proinflammatory properties;[35] and PSA, which is used as a biomarker for prostate cancer in clinical diagnosis.[36] Anti-RAC Ab microarrays were exposed to RCA_{120} concentrations of 0, 0.01, 0.1, 1, 10, 100, 1000, and 10000 ng/mL for 1 h. The detection of bound antigen was subsequently achieved by treatment with biotinylated goat anti-RCAI&II pAb (10 ng/mL) and subsequent staining with streptavidin−Cy3. Increasing the RCA_{120} concentration resulted in an increased fluorescence emission intensity from the arrayed spots, and a detectable signal was produced at concentrations as low as 1 ng/mL (Figures 4A and S8 in the SI). The limit of detection [LOD, the concentration corresponding to 3 standard deviations (SDs) over the background signal] of the microarray for RCA_{120} was 0.89 ng/mL (7.4 pM). Figure 4B shows the corresponding plot of the fluorescence intensity as a function of the PSA concentration. The Ab microarrays could reproducibly detect PSA concentrations as low as 0.98 ng/mL (29.0 pM), which is sufficient for measuring clinically relevant diagnostic PSA concentrations (range 4−10 ng/mL).[37] The sensitivity of the BA-mediated Ab cross-linking readout is more than 2-fold higher than that of other methods that rely on HRP-anti-PSA-based detection.[28] In Figure 4C, the plot of the fluorescence intensity as a function of the IL-6 concentration is shown. Bound IL-6 was detected using biotinylated rabbit anti-IL6 pAb (10 ng/mL). In this study, the lowest detected concentration of IL-6 on the **1**-coated surface was 0.32 ng/mL (16 pM), a sensitivity that is comparable to that of the radioimmunoassay method.[38]

Simultaneous Detection of Multiple Antigens. To demonstrate the potential of the fabricated Ab microarrays for multiplex detection, the three different Abs were immobilized by the BA-Pi method, and the resulting microarray was used to detect multiple antigens in the same sample. Anti-IL-6, anti-PSA, and anti-RAC Abs were spotted (12 replicate spots per Ab) in a 2 × 6 array format onto a **1**-coated glass slide, as described previously, to detect the presence of the three target antigens, IL-6 (10 μg/mL), PSA (1 μg/mL), and RCA_{120} (1 μg/mL). Parts A−D of Figure 5 show representative fluorescence patterns obtained when these three antigens were assayed either individually (Figure 5A−C) or as a mixture of antigens (Figure 5D). Detection was achieved by exposure of each microarray to biotinylated Abs aimed at each of the target antigens: rabbit anti-IL6 pAb (100 ng/mL), goat anti-PSA pAb (10 ng/mL), and goat anti-RCAI&II pAb (10 ng/mL). Notably, to obtain a fluorescent signal similar to that obtained for PSA and RCA_{120}, a 10-fold higher concentration of the biotinylated anti-IL6 Ab was required, consistent with the concentration used in other immunoassays based on ELISA.[39] The fluorescent signal in the microarray was visualized by staining with streptavidin−Cy3 (10 μg/mL).

As shown in Figure 5A−C, each antigen produced distinct fluorescent signals at appropriate regions where the cognate Ab had been cross-linked. In addition, parts D and H of Figure 5 clearly indicate the fluorescence pattern resulting from a solution containing all three antigens. Furthermore, the detection was highly specific, with no noticeable cross-reactivity detected between the two antigens and Abs, confirming the selectivity of the functional Ab arrays (Figure 5E−G).

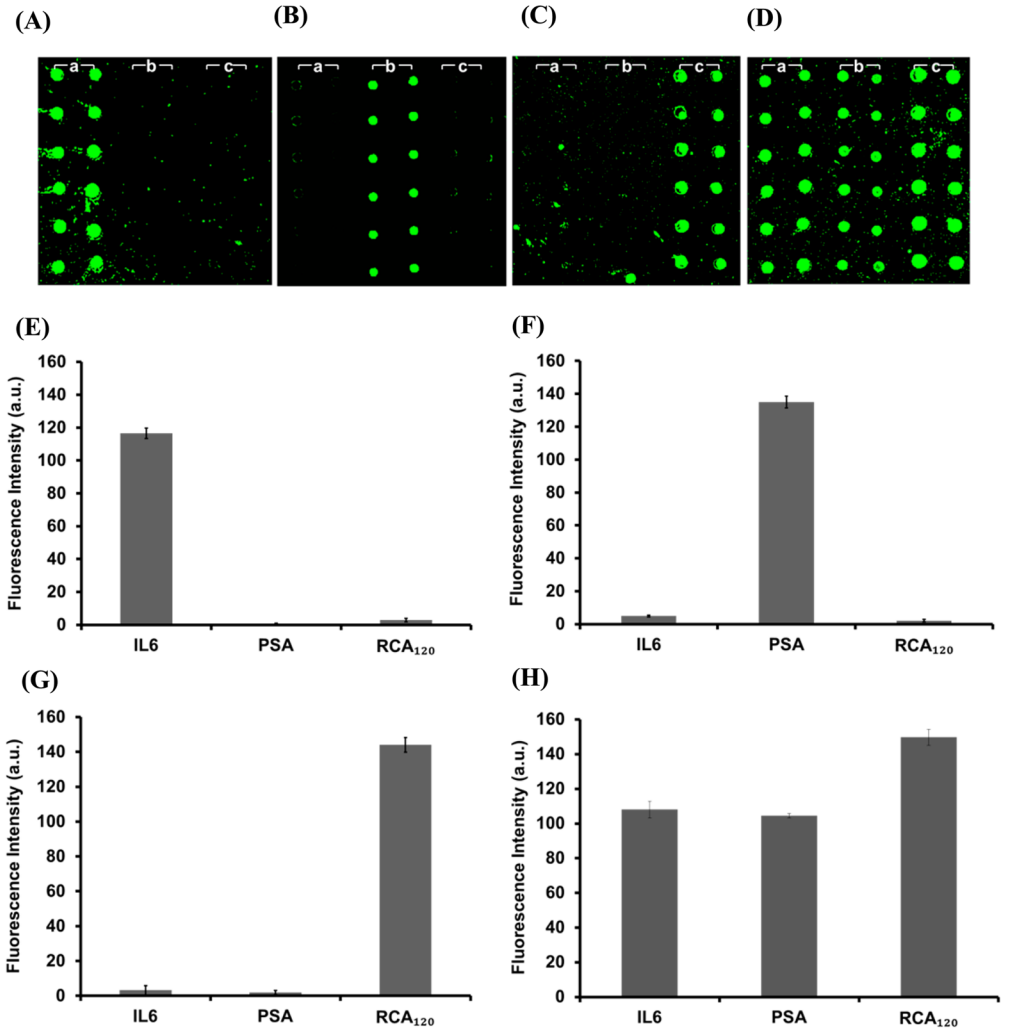

Figure 5. Functional Ab microarrays for the simultaneous detection of multiple antigens. Ab microarrays were fabricated on a **1**-coated slide for each antigen, as described in the Experimental Section. A microarray was patterned onto two columns with 12 replicate capture Ab spots corresponding to three different antigens as follows: (a) Ms anti-IL-6 mAb; (b) Ms anti-PSA mAb; (c) rabbit anti-RAC (A-chain) mAb. Three samples containing a single antigen (A-C) and one sample containing a mixture of the three antigens (D) were incubated with the microarrays for 1 h. The detection of bound antigen was visualized with the corresponding biotinylated Abs and streptavidin−Cy3. Representative fluorescence image of the microarray: (A) IL-6; (B) PSA; (C) RCA_{120}; (D) all three antigens. (E−H) Comparison of the fluorescent signals produced in parts A−D, corresponding to either single-antigen detection (E−G) or multiple-antigen detection (H). Each data point represents the mean ± SD of the 12 spots printed for the captured Ab.

However, both IL-6 and PSA produced relatively low fluorescent signals when detected as part of a multiantigen immunosensing solution (Figure 5H). A possible explanation for this observation is that both PSA and IL-6 may bind to the peptidoglycan of RCA_{120} as a result of its capacity to function as a lectin, thus reducing the effective concentrations of PSA and IL-6 available for binding to their corresponding Abs.

In summary, we have developed a straightforward method for oriented Ab immobilization via boronate formation. We also demonstrated that the immobilized Abs could be tethered irreversibly to the BA surface by UV irradiation for enhanced antigen detection sensitivity. Using this method, a functional Ab microarray was constructed that enabled the detection of analytes including RCA_{120}, PSA, and IL-6 at low picomolar concentrations. Moreover, we demonstrated that the microarray assay could be used to detect the presence of multiple antigens in multiple samples without any detectable cross-reactivities. We anticipate that the method described here will be a simple and universal platform for fabricating Ab microarrays for the analysis of low-concentration analytes in real-time diagnostic and biosensing applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General procedures, synthesis, and characterization details of BA-photoaffinity probe **1** and compounds **2** and **3**, effect of time on boronate diester formation between immobilized Ab and surface-conjugated BAs (Figure S1), effect of Glc on Ab-boronate immunocomplex on **1**-coated surfaces without photoirradiation (Figure S2), relative changes in the fluorescence intensities of a photoirradiated surface after exposure to different reagents prior to antigen incubation (Figure S3), effect of the BA-photoaffinity probe (**1**) concentration on surface immobilization of Abs (Figure S4), fluorescence images of photo-cross-linking of anti-IL-6 mAb to different substrates (Figure S5), direct comparison of photo-cross-linking of anti-PSA mAb between different substrates (Figure S6), surface immobilization of different Abs (Figure

S7), and LOD of RCA_{120} (Figure S8). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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