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Fluorogenic Affinity Gels Constructed from Clickable Boronic Acids

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ABSTRACT: Boronate affinity gel was prepared by immobilizing azide-functionalized boronic acid on alkyne-modified Sepharose. Different azide-functionalized boronic acids were synthesized from 2-, 3-, and 4-aminophenylboronic acids. One of the azide-functionalized boronic acids, 3BII, displayed weak and unexpected fluorescence emission. Using Cu(I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition (CuAAC) reaction (click chemistry), the azide-functionalized boronic acid could be immobilized on alkyne-modified Sepharose 4B under very mild reaction condition. Immobilization of boronic acid 3BII also led to a new fluorogenic affinity gel, which displayed dose-dependent fluorescence intensity change upon binding fructose and glucose at physiological pH. The clickable boronic acids may be used as simple and modular building blocks to construct molecular recognition materials and surfaces for different applications including bioseparation, sensing and controlled drug delivery. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: gels; functionalization of polymers; biopolymers and renewable polymers; molecular recognition; separation techniques

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INTRODUCTION

Phenylboronic acids have been investigated extensively in the past to design effective affinity materials for separation and detection of bioactive *cis*-diol compounds.[1–3] For affinity separation purposes, phenylboronic acid derivatives are often immobilized on solid supports through different chemical coupling reactions, or introduced into polymer matrices via free radical polymerization.[4–12] Immobilization on solid support also allows boronic acid to be reused after regeneration using simple washing and filtration steps. To afford straightforward immobilization, new boronic acid derivatives carrying clickable tags are becoming very attractive,[13,14] because these new affinity ligands can be conjugated to solid supports through high efficiency and versatile Cu-catalyzed azide-alkyne cycloaddition (CuAAC),[15,16] which is one of the most widely used Click reactions.[17]

In a previous work, we reported an alkyne-functionalized clickable boronic acid, and used it to prepare affinity gels for effective separation of glycoproteins.[18] The clickable boronic acid was constructed by introducing terminal alkyne into commercially available 3-aminophenyl boronic acid (APBA). To further extend the scope of clickable boronic acids, in this work we intended to: (1) introduce terminal azide into different phenylboronic acids, (2) develop new methods to modify Sepharose (crosslinked agarose) with terminal alkyne groups, and (3) prepare hydrophilic boronate affinity gels using CuAAC under mild reaction conditions. This paper reports the details of the synthesis of the boronic acids, the procedures used for Sepharose modification, the Cu(I)-catalyzed ligand immobilization, and the characterization of the soft materials. Among the three azide-functionalized boronic acids, boronic acid 3BII displayed unexpected fluorescence property. For the affinity gel derived from boronic acid 3BII, its fluorescence response to fructose and glucose was further investigated. This is the first time that simple click-conjugation of boronic acid with agarose resulted in stable affinity gel with unprecedented fluorogenic property under physiological conditions. The fluorogenic boronate affinity gel has potential applications for separation and sensing of saccharides and glycosylated biomolecules. The new synthetic pathways developed in this work may also be extended to other systems to construct chemical sensors and drug delivery materials.

MATERIALS AND METHODS

Materials

Phenylboronic acids (2-, 3-, and 4-aminophenylboronic acid), bromoacetyl bromide, propargyl chloroformate, $CuSO_4$, sodium ascorbate, sodium azide, epichlorohydrin, ammonium hydroxide solution (25%), Alizarin Red S (ARS), KCN, and 2,3-naphthalene-dicarboxaldehyde (NDA) were purchased from Sigma-Aldrich. Sepharose 4B was obtained from GE Healthcare. All solvents purchased from commercial resources were of analytical or HPLC grade.

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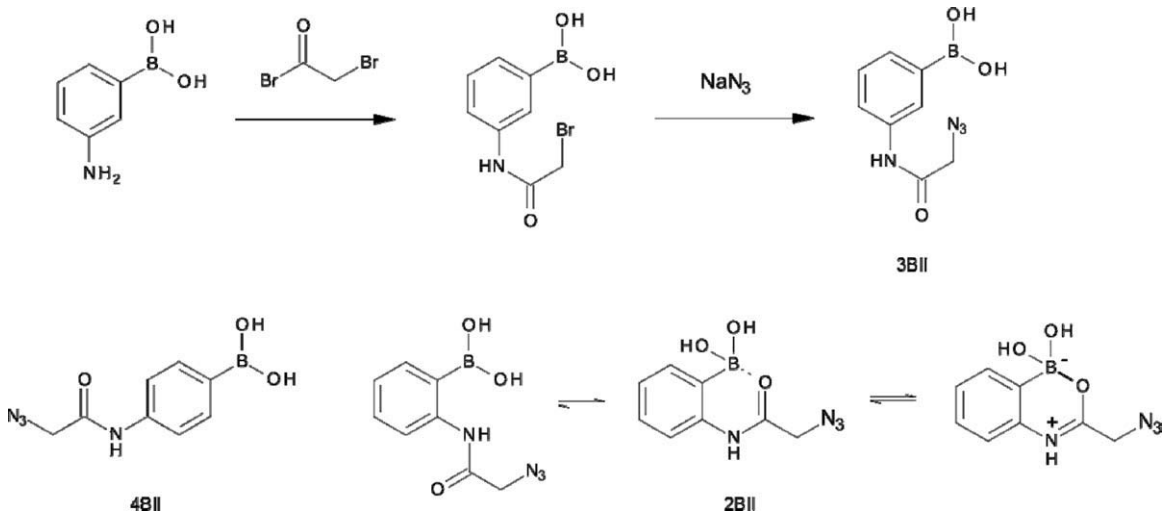

Figure 1. Synthesis of clickable boronic acids.

Synthesis of Clickable Boronic Acids

The clickable boronic acids were synthesized by introducing terminal azide into commercially available aminophenyl boronic acid, as exemplified by the synthesis of 3BII (Figure 1).

Boronic Acid 3BII. Briefly, 3-aminophenylboronic acid hemisulfate (1.488 g, 8 mmol) and $NaHCO_3$ (2.016 g, 24 mmol) were dissolved in 80 mL of water. The solution was cooled to 0°C on a magnetic stirrer before bromoacetyl bromide (3.680 g, 18 mmol) was added drop wise. The mixture was first stirred at 0°C for 2 h, and then at room temperature for 16 h. After reaction, the precipitate was filtered off, and then redissolved in 20 mL of 0.5*M* NaOH. The pH of the solution was adjusted to 2.0 by addition of concentrate HCl, which led to precipitation of the intermediate 3BI. The intermediate was filtered off and freeze-dried to give a white powder. Yield: 92%. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ/ppm = 4.04 [2H, s], 7.30 [1H, t, J = 7.98 Hz], 7.52 [1H, d, J = 7.10 Hz], 7.70 [1H, d, J = 7.98 Hz], 7.84 [1H, s], 10.33 [1H, s]. MS (ESI+) (M+Na^+): Calc'd, 279.9755; found, 280.1422, 282.1577.

Compound 3BI (1.37 g, 5.31 mmol) was dissolved in 21 mL of dimethylformamide (DMF). After addition of sodium azide (0.36 g, 5.54 mmol), the mixture was stirred at room temperature overnight. After the reaction, 35 mL of water was added to bring the reaction mixture into a clear solution with pH = ~ 7. The pH was adjusted to 2 by adding concentrate HCl. This solution was extracted with ethyl acetate. The organic phase was separated, dried over anhydrous sodium sulfate, followed by removal of solvent using a rotavap. The product was dried in vacuum for three days to give greenish oil, which was re-dissolved in 5 mL of ethyl acetate. Removal of the solvent finally gave a greenish powder. Yield: 58%. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ/ppm = 4.03 [2H, CH_2, s], 7.30 [1H, aromatic, t, J = 8.03 Hz], 7.52 [1H, aromatic, d, J = 7.10 Hz], 7.70 [1H, aromatic, d, J = 7.98 Hz], 7.86 [1H, aromatic, s], 10.09 [1H, NH, s]. MS (ESI+) (M+Na^+): Calc'd, 243.0665; found, 242.3520.

Boronic Acid 4BII. Boronic acid 4BII was obtained as a white powder. Yield: 40%. ^{1}H-NMR (DMSO-d_6, 400 MHz): δ/ppm = 4.05 [2H, CH_2, s], 7.55 [2H, aromatic, d, J = 8.40 Hz], 7.75 [2H, aromatic, d, J = 8.40 Hz], 10.17 [1H, NH, s]. MS (ESI+) (M+DMF+H^+): Calc'd, 294.1375; found, 294.4395.

Boronic Acid 2BII. Boronic acid 2BII was obtained as a brownish powder. Yield: 45%. ^{1}H-NMR (DMSO-d_6/D_2O = 6/1, 400 MHz): δ/ppm = 4.10 [2H, CH_2, s], 7.09 [1H, aromatic, t, J = 8.00 Hz], 7.38 [1H, aromatic, t, J = 7.20 Hz], 7.70 [1H, aromatic, d, J = 7.20 Hz], 8.10 [1H, aromatic, d, J = 8.00 Hz]. MS (ESI+) (M+DMF+H^+): Calc'd, 294.1375; found, 294.5024.

Modification of Sepharose 4B with Alkyne Groups

Modification of Sepharose 4B with terminal alkyne groups was achieved in three steps. Sepharose 4B (10 mL) was washed with water (20 mL) for five times before it was suspended in a mixture of 2.5 mL water and 2.5 mL of 2*M* NaOH. Epichlorohydrin (3 mL) was added to the suspended Sepharose. The pH of this suspension was ~13. The mixture was rotated in a DNA hybridization oven at 40°C overnight. The Sepharose was then washed with water on a glass filter.

The epoxy-activated Sepharose (10 mL) was reacted with 5 mL of NH_4OH (25%) at 40°C overnight in a DNA hybridization oven. The Sepharose was washed with water (30 mL) for six times on a glass filter. After the washing, the amine-functionalized Sepharose was collected and kept in water at 4°C until further use.

The amino-functionalized Sepharose 4B (10 mL) was suspended in a mixture of 2.5 mL of water and 2.5 mL of 2*M* NaOH. Propargyl chloroformate (300 μL) was added to the suspended Sepharose at room temperature. The pH of this suspension was ~13. The mixture was stirred in a DNA hybridization oven at 40°C overnight. The Sepharose gel was then washed with water (30 mL) for five times on a glass filter before it was suspended in water and stored at 4°C.

Immobilization of Boronic Acids Using CuAAC

Sepharose-3BII. Boronic acid 3BII (220 mg, 1 mmol) was dissolved in 10 mL of methanol: water (1: 1). The alkyne-modified Sepharose (5 mL) was added to the 3BII solution, followed by addition of 10 μL of 100 m*M* $CuSO_4$ and 50 μL of 100 m*M* sodium ascorbate. The suspension was purged with a gentle flow of nitrogen gas for 5 min, and then stirred on a rocking table

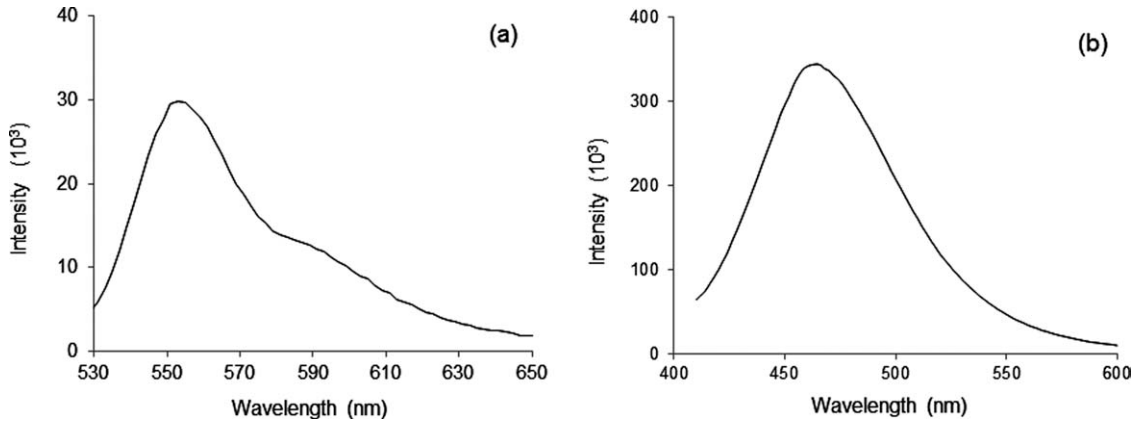

Figure 2. Florescence emission spectra of 3BII (a) and 2BII (b) in PBS buffer at pH 7.4. The concentration of the boronic acids was 0.91 m*M*.

overnight at room temperature. The Sepharose gel was finally washed with water (15 mL) for five times before it was suspended in water and stored at 4°C.

Sepharose-4BII. Sepharose-4BII was prepared following the same approach as used for Sepharose-3BII, except that boronic acid 4BII was used instead of 3BII.

Detection of Primary Amine Groups

Stock solution of naphthalene-2,3-dicarboxaldehyde (NDA) (4 m*M*) was prepared in methanol. The stock solution was diluted with 0.1*M* phosphate buffer (pH 8) to give 0.4 m*M* of NDA solution. Amine-modified Sepharose 4B (100 μL) was mixed with 500 μL of NDA solution and 500 μL of 0.1 m*M* KCN dissolved in 0.1*M* phosphate buffer. The mixture was stirred for a few minutes at room temperature. After the NDA treatment, the gel was inspected with a Nikon Eclipse 400 epifluorescence microscope fitted with a CCD camera, using excitation wavelength 418 nm and emission wavelength 460 nm.

Detection of Immobilized Alkyne Groups

The terminal alkyne groups on the modified Sepharose 4B were confirmed by fluorescent labeling of the gels with azide-modified fluorescein (FITC-N_3) using CuAAC. FITC-N_3 was prepared by reacting fluorescein isothiocyanate with excess of 11-azido-3,6,9-trioxaundecan-1-amine in DMF.[19] The alkyne-modified Sepharose 4B (100 μL) was mixed with 25 μL of FITC-N_3 solution in a mixture of 460 μL water and 40 μL methanol, followed by addition of 10 μL of 100 m*M* $CuSO_4$, 50 μL of 100 m*M* sodium ascorbate and 440 μL water. The mixture was stirred at room temperature for 48 h in dark. After labeling with FITC-N_3 and thorough washing, the fluorescence emission from the gels were measured with a QuantaMaster model C-60/2000 spectrofluorometer from Photon Technology International (Lawrenceville, NJ). To maintain a stable gel suspension, the samples were stirred with a built-in magnetic stirrer during the fluorescent measurement (excitation wavelength 494 nm).

Detection of Immobilized Boronic Acid

Stock solution of Alizarin Red S (ARS, 1 m*M*) was prepared in water, which was diluted for 10 times in PBS buffer (pH 7.4) before use. Sepharose 4B containing the immobilized boronic acid (100 μL) was added to 1 mL of the ARS solution (0.1 m*M*) and 1.9 mL of PBS buffer (pH 7.4). The mixture was stirred at room temperature for a few minutes before fluorescence emission was measured (excitation wavelength 494 nm).

Measurement of Fluorescence Response to Addition of Monosaccharides

For the fluorescence measurement, the sample volume was kept at 2 mL. The amount of the fluorogenic gels was fixed at 4 mg (wet mass), which was titrated by addition of concentrate monosaccharides. The solvent was PBS buffer (pH 7.4). The samples were stirred for 30 min at room temperature before their fluorescence spectra were recorded. The samples were stirred continuously with a built-in magnetic stirrer during the fluorescence measurement.

RESULTS AND DISCUSSION

Azide-Tagged Clickable Boronic Acids

The clickable boronic acids were synthesized by introducing terminal azide into commercially available aminophenyl boronic acids (APBA). Synthesis of the azide-tagged boronic acids was achieved in two steps, as schematically shown in Figure 1. Compared to our previously reported alkyne-tagged boronic acid,[18] the azide-tagged boronic acids designed in this work have higher water solubility and are easier to use in pure aqueous solvent.

Interestingly, boronic acid 3BII displayed unexpected fluorescence property in aqueous solution. When 3BII was dissolved in PBS buffer at pH 7.4, the solution displayed a maximum fluorescence emission at 555 nm when excited at 494 nm [Figure 2(a)]. This fluorescence was not expected because 3BII does not contain any conventional fluorophore. Boronic acid 2BII was also found fluorescent, but had a different maximum emission wavelength at 465 nm (excitation wavelength 394 nm) [Figure 2(b)]. The broad emission spectra in Figure 2(a) may be explained as a result of dimerization of the boronic acid or the formation of adducts with the phosphate ions,[20] because the relative intensity of the additional peak at 590 nm decreased dramatically when 3BII was measured at lower concentration or in pure water (data not shown). It should be noted that the fluorescence quantum yields of 3BII and 2BII, as measured using fluorescein and Coumarine 153 as standards, were only approximately 0.058 and 0.066, respectively. Nevertheless, the fluorescence emission exhibited by 3BII and 2BII was very obvious.

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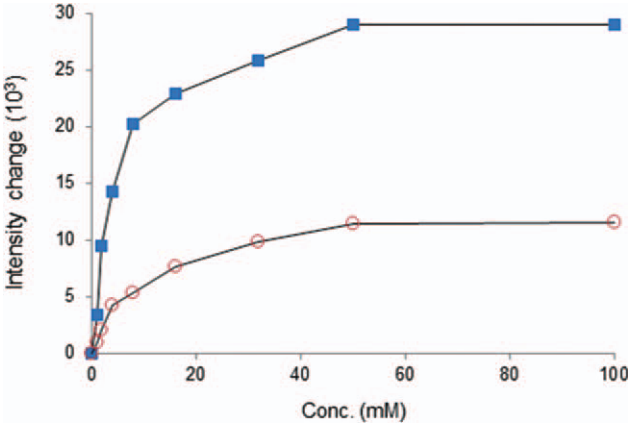

Figure 3. Fluorescence intensity of 3BII (0.91 m*M*) in response to fructose (■) and glucose (○) in PBS buffer (pH 7.4). The fluorescence intensity was measured at 555 nm using excitation wavelength at 494 nm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Under the same measurement condition, the boronic acid functionalized at the C-4 position, compound 4BII, did not show any fluorescence.

The unexpected fluorescence emission of 3BII may be explained as a result of possible dative (intra molecular) B-N bond formed between the boron and one of the nitrogen atoms in the terminal azide. Such intra molecular N-B bond may change the configuration of the boron from planar triangle to tetrahedron, which is generally considered more favorable for phenylboronic acid to bind *cis*-diol compounds.[21–23] For the same reason, binding of *cis*-diol compounds may contribute to stabilize the intra molecular N-B bond, thereby leading to alteration of

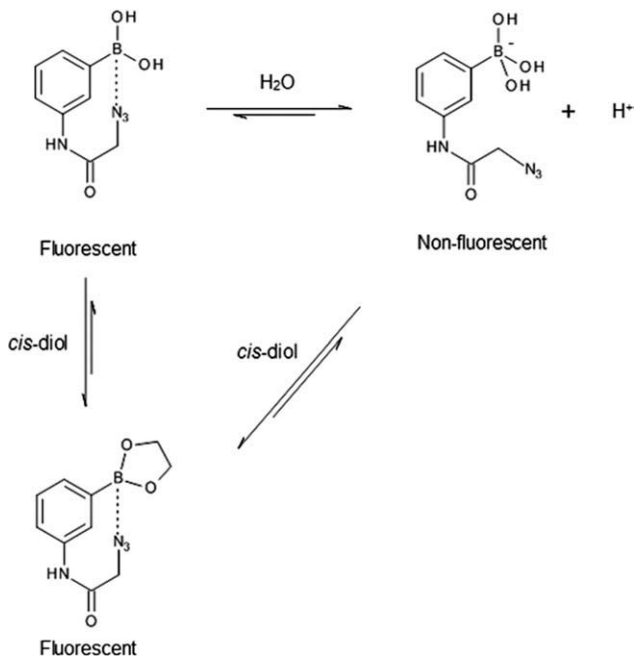

Figure 4. Hypothetical mechanism of fluorescence change in response to binding of *cis*-diol.

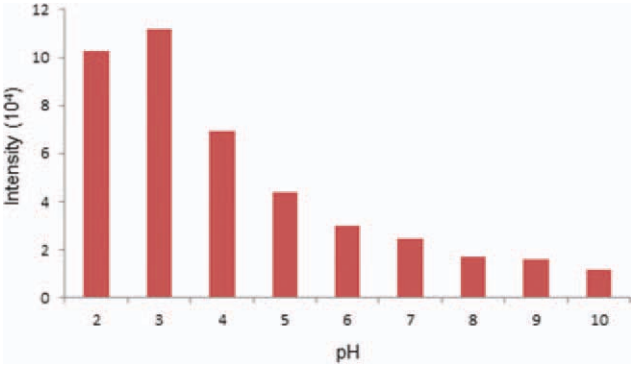

Figure 5. Influence of pH value on the fluorescence intensity of 3BII in PBS buffer (0.91 m*M*). The pH was adjusted by adding concentrate solution of HCl or NaOH immediately before the measurement. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the fluorescence emission. Indeed, addition of fructose and glucose into 3BII solution caused obvious increase of the fluorescence intensity (Figure 3).

According to the literature, phenylboronic acid in water can exist as several interchangeable coordination structures, and the proportion of the different structures can be influenced by the solution pH and the concentration of *cis*-diols added in the solution.[24] On the basis of our fluorescence measurement results and the literature information, we propose a possible mechanism to explain the fluorescence change of 3BII in response to fructose (Figure 4), where among the different solution structures of 3BII, only the structures containing the dative B-N bond can be fluorescent. According to this scheme, binding fructose or decreasing pH value have the same effect of increasing the proportion of the fluorescent boronic acid (containing the dative B-N bond), thereby leading to an overall increase of the fluorescence intensity. This mechanism was supported by the fact that the fluorescence intensity of 3BII in water increased when the pH was decreased (Figure 5).

The fluorescence emission from 2BII may be explained as a result of the intra molecular B-O bond,[25–28] as shown in Figure 1. The structure of 2BII was considered optimal for the formation of this B-N bond, therefore adding fructose in a solution of 2BII did not affect its fluorescence emission.

Preparation of Alkyne-Modified Sepharose

Terminal alkyne groups were introduced into hydrophilic Sepharose in three reaction steps (Figure 6). Sepharose 4B was first treated with epichlorohydrin under basic condition to introduce epoxy groups, which were converted into terminal amines in ammonia solution. The successful ring-opening reaction at the epoxy group through treatment in $NH_3 \cdot H_2O$ was confirmed by a fluorogenic assay,[29] in which the amine-modified Sepharose 4B reacted with naphthalene 2,3-dicarboxaldehyde (NDA), turning the colorless gel into strongly fluorescent beads (Figure 7).

The amine-modified Sepharose was reacted with propargyl chloroformate to introduce terminal alkyne groups. The

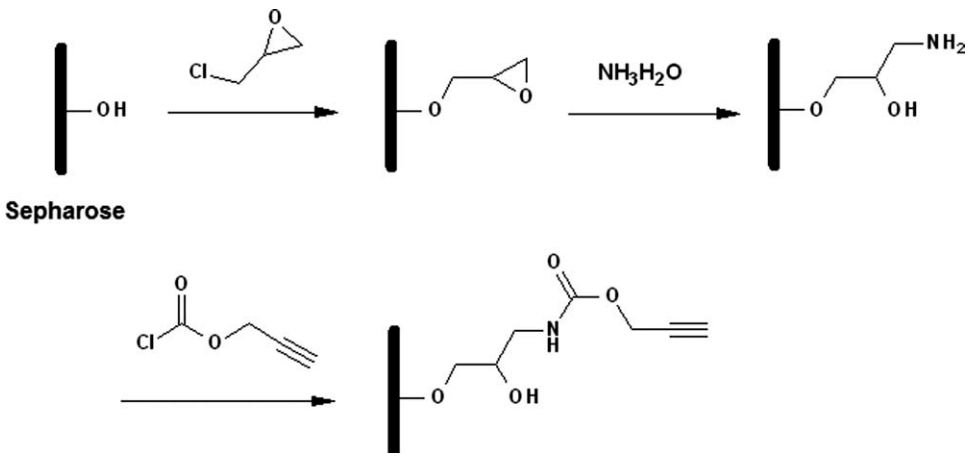

Figure 6. Modification of Sepharose 4B with terminal alkyne groups.

presence of the terminal alkyne groups in the final modified Sepharose 4B was verified by labeling the beads with azide-tagged fluorescein (FITC-N_3).[19] After labeling with FITC using CuAAC, the alkyne-modified Sepharose 4B displayed strong fluorescent emission that was characteristic of fluorescein (Figure 8), indicating that modification of Sepharose 4B with the intended terminal alkyne group was successful. The FITC labeling results also proved that CuAAC was a viable method to conjugate azide-containing molecules with the alkyne-modified Sepharose.

Immobilization of Azide-Functionalized Boronic Acids on Alkyne-Modified Sepharose

Immobilization of the azide-functionalized boronic acids was achieved using the CuAAC reaction as shown in Figure 9. This coupling reaction allowed the ligand immobilization to be easily carried out under very mild reaction condition. The boronate affinity gels obtained displayed fluorescence property very similar to the corresponding boronic acid, *i.e.* Sepharose-3BII had fluorescent emission at 555 nm when it was excited at 494 nm (*vide infra*). Because of the identical fluorescence emission of Sepharose-3BII respect to 3BII, we suggest that the same dative B-N bond remained after the azide group in 3BII was converted into the triazole structure in Sepharose-3BII.

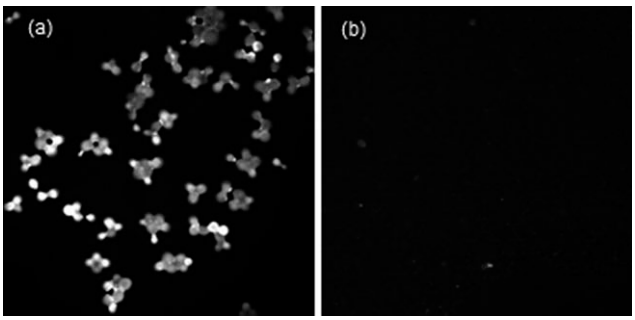

Figure 7. Fluorescent image of amine-modified Sepharose (a) and unmodified Sepharose (b). The gels were treated with the NDA reagents before the fluorescent imaging.

The two boronate affinity gels containing immobilized 3BII and 4BII (Sepharose-3BII and Sepharose-4BII) were tested with Alizarin Red S (ARS) to confirm their capability to bind *cis*-diol compounds. As a convenient fluorogenic reagent, ARS is now routinely used to study the binding between phenylboronic acids and *cis*-diol compounds.[30–32] When ARS binds *cis*-diol compounds, it generates strong fluorescence emission. This fluorogenic property of ARS is ideal for our qualitative assay of the boronate affinity gels. In our study, when Sepharose-4BII was mixed with ARS in PBS buffer (pH 7.4), the solution emitted strong fluorescence at 580 nm, indicating that Sepharose-4BII readily bound ARS at physiological pH. Upon addition of excess fructose, the fluorescence intensity at 580 nm decreased significantly, indicating that the boronate ester bond formed between the immobilized boronic acid and ARS was reversible, and the bound ARS could be replaced by fructose [Figure 10(a)]. Similar to Sepharose-4BII, when Sepharose-3BII was

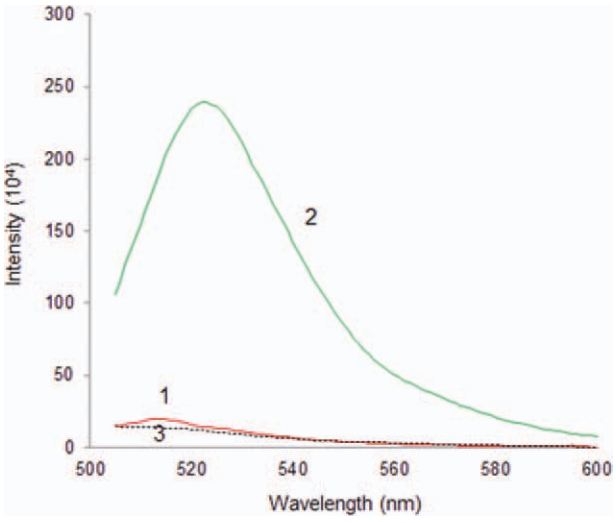

Figure 8. Fluorescence emission spectra of alkyne-modified Sepharose before (1) and after (2) being labeled with FITC-N_3. The dotted line (3) is the background collected from the unmodified Sepharose (not treated with FITC-N_3). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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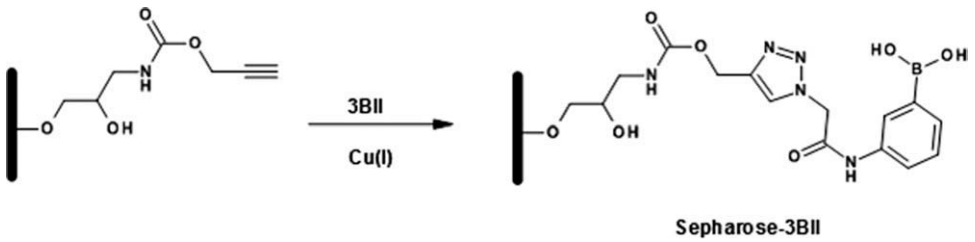

Figure 9. Immobilization of 3BII using CuAAC.

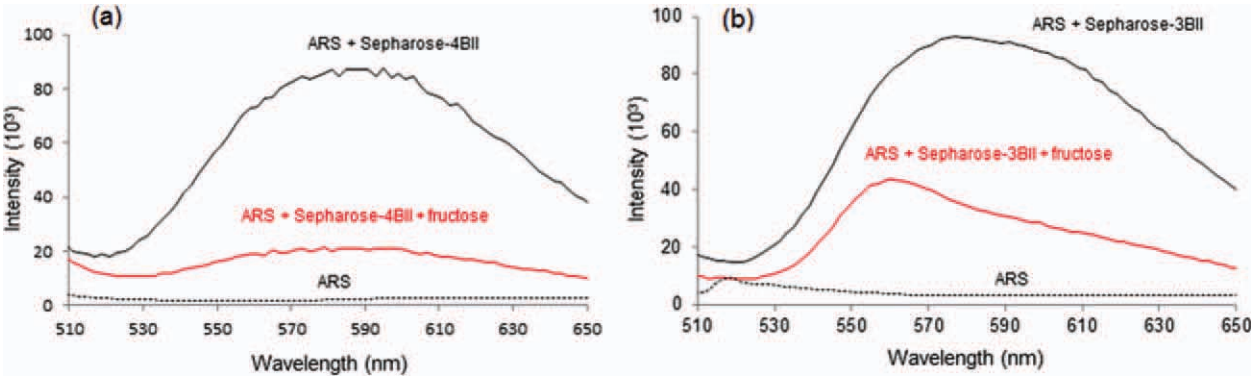

Figure 10. ARS assay of Sepharose-4BII (a) and Sepharose-3BII (b). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mixed with ARS, the solution also emitted strong fluorescence at 580 nm that is characteristic for phenylboronic acid-ARS complex. Addition of excess fructose also caused the fluorescence intensity at 580 nm to decrease, meaning that fructose effectively displaced the bound ARS from Sepharose-3BII. However, because of the intrinsic fluorescence of 3BII, the fluorescence emission of the mixture containing Sepharose-3BII, ARS and fructose did not disappear completely, and the maximum emission wavelength changed to 555 nm, which was characteristic for 3BII [Figure 10(b)].

Change of Fluorescence Emission of Sepharose-3BII in Response to Fructose and Glucose

To investigate if the interesting fluorescence response of 3BII to *cis*-diol compounds remained after the boronic acid was immobilized (which changed the terminal azide into a triazole ring), we measured the fluorescence spectra of Sepharose-3BII in PBS buffer (pH 7.4) in the presence of different amount of fructose. Figure 11(a) shows the change of the fluorescence emission when increasing amount of fructose was added into PBS buffer containing Sepharose-3BII. Clearly, after binding fructose, Sepharose-3BII exhibited dose dependent enhancement of fluorescence emission. Glucose was also found to have similar effect on the fluorescence emission of Sepharose-3BII, although the modulation of fluorescence by glucose was less significant than by fructose [Figure 11(b)]. The lower fluorescence intensity change induced by glucose may be explained by the weaker affinity between phenylboronic acid and glucose, as has been discussed in previous literature.[33] It should be noted that in this work, all the saccharide binding and the corresponding fluorescence response were observed under physiological pH conditions, which makes the clickable boronic acids and the

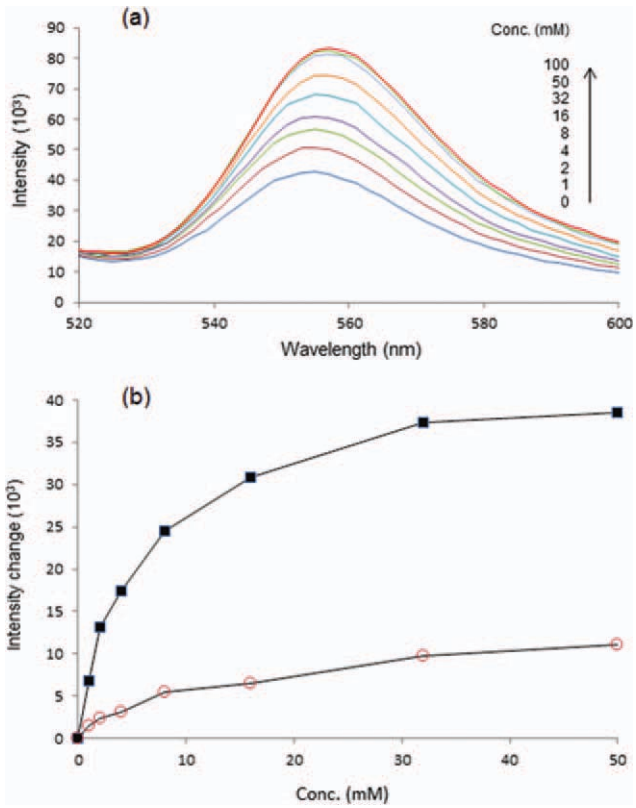

Figure 11. (a) Change of fluorescence intensity of Sepharose-3BII in response to fructose. (b) Dose-response curves of fluorescence intensity for fructose (■) and glucose (○).The conc. of Sepharose-3BII was 2 mg mL^{-1} in PBS buffer (pH 7.4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

fluorogenic boronate affinity gel suitable for separation and analysis of biological samples.

CONCLUSIONS

In this work we have developed new azide-functionalized phenylboronic acids that can be conveniently immobilized on alkyne-modified Sepharose using simple click chemistry. One of the new azide-functionalized phenylboronic acids, 3BII, displayed unexpected fluorescence emission, and its fluorescence intensity could be modulated through binding *cis*-diol compounds, *e.g.* fructose and glucose under physiological pH condition. The boronate affinity gel constructed from the two types of modular building blocks (*i.e.* the boronic acid and the supporting matrix) has both molecular binding and fluorogenic response to *cis*-diol molecules. The fact that the click-conjugated boronic acid 3BII maintained fluorogenic response to *cis*-diol compounds has interesting implications for further exploration of the unique clickable boronic acid. Using high efficiency click chemistry, we expect that the very versatile boronic acid 3BII can be easily conjugated to a large variety of alkyne-tagged molecular building blocks to tune molecular binding selectivity as well as to improve fluorescence response. We believe that the functional and modular building block and the click-conjugation scheme reported here should be very useful for further development of new affinity gels, new fluorescent assays and sensor platforms for separation and analysis of saccharides and other bioactive *cis*-diol molecules.

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