Molecular Imprinting

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Photolithographic Boronate Affinity Molecular Imprinting: A General and Facile Approach for Glycoprotein Imprinting**

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Molecularly imprinted polymers (MIPs),[1] as economical and stable mimics of antibodies and enzymes, have found important applications, such as chemical separation and catalysis. The imprinting of proteins is challenging, which is due to conformational changes during polymerization and slow mass transfer in the polymers.[2] Although several strategies, such as surface imprinting,[3] epitope imprinting,[4] and Pickering emulsions,[5] have been proposed to solve these issues, the imprinting of proteins still remains a challenge.

Glycoproteins, which occupy more than 50% of the total proteins in mammalian systems, play key roles in many biological processes, such as molecular recognition, inter- and intra-cellular signaling, and immune response. Furthermore, many glycoproteins are disease biomarkers and therapeutic targets. Therefore, the imprinting of glycoproteins is of great importance and in high demand. A variety of approaches have been proposed to imprint glycoproteins;[1c–e,6] however, a universal and simple approach for glycoprotein imprinting has not yet been reported.

Boronic acids can covalently interact with *cis*-diol-containing molecules, such as sugars, to form stable cyclic esters in an alkaline aqueous solution while the boronate esters dissociate when the environmental pH is switched to acidic.[7] This reversible binding has made boronic acids excellent affinity ligands for creating functionalized materials.[8] As such easy on/off reactivity favors the imprinting and removal of glycoprotein templates, boronic acids can be promising functional monomers for establishing a general approach for glycoprotein imprinting. However, such a possibility has never been well explored. Mosbach et al.[6a] first reported covalent imprinting of glycoproteins in which transferrin was successfully imprinted with boronate–silane; however, attempts to imprint other glycoproteins with the same approach had not been successful owing to problems such as protein precipitation. Although boronic acids have been demonstrated as important functional monomers for the covalent imprinting of small molecules,[9] most attempts[6c,d,10] towards boronic acid-based protein imprinting used *m*-aminophenylboronic acid as a polymerizable reagent, for which benefits from boronate affinity interactions for imprinting have not been demonstrated.

Herein we report a general and facile approach, photolithographic boronate affinity molecular imprinting, for the imprinting of glycoproteins. The key of the approach was boronic acid-based UV-initiated polymerization, in which a boronic acid, as the sole functional monomer, was found to play essential roles. MIP thin-layer arrays were produced using a photolithographic fabrication procedure.[11] The generality of the approach was demonstrated with the successful imprinting of five distinct glycoproteins. The approach is fast (total processing time less than 3 h) and has potential for easy mass production. More interestingly, the prepared MIP arrays exhibited several highly favorable features that are beyond normal expectations, particularly superb tolerance for interference and the applicability to a wide range of sample pH values. The feasibility for real-world applications was demonstrated with an MIP array-based enzyme-linked immunosorbent assay (ELISA) of trace α-fetoprotein (AFP) in human serum.

The principle and procedure of the approach is shown in Scheme 1. The principle relies on UV-initiated free radical polymerization between a cross-linker (such as polyethylene glycol diacrylate; PEGDA) and a functional monomer of regular boronic acid (such as 4-vinylphenylboronic acid; VPBA). The template was first mixed with the monomer in an appropriate porogen solution of pH $\geq$ 8.0. The template and the monomer self-assembled into a covalent complex owing to boronate affinity binding. Mixing of the complex with the cross-linker and an appropriate UV initiator (such as Irgacure 184) yielded a pre-polymer solution. Through UV curing for a short period (tens of seconds), the pre-polymer quickly polymerized into a polymer. Such a fast polymerization speed avoided apparent conformational change of the template. The imprinted template in the MIP was easily removed by extracting with an acidic solution, leaving behind cavities complementary to the 3D shape of the template. To make molecularly imprinted thin-layer arrays, the pre-polymer solution was first cast onto the surface of a solid substrate, such as a glass slide or a filter membrane. Attached with a mask in a designed pattern, the pre-polymer-coated substrate was then subjected to UV exposure. After the mask was removed, non-polymerized reactants under the non-transparent areas of the mask were washed away with an appropriate solution. Finally, the residual template inside the MIP was extracted out. After drying, the prepared MIP array was ready for use.

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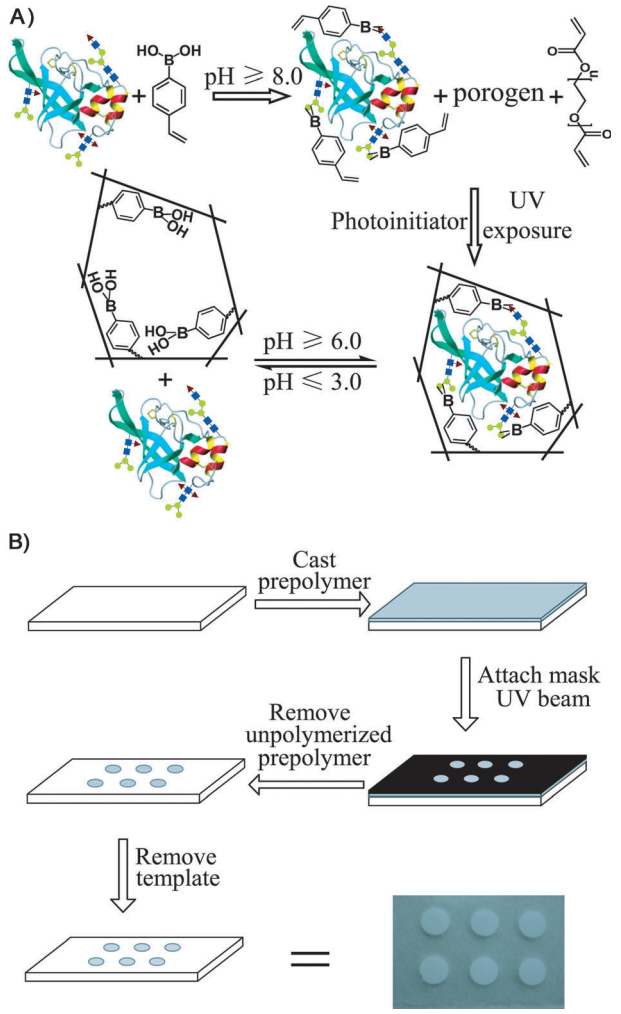

Scheme 1. The principle (A) and procedure (B) of photolithographic boronate affinity molecular imprinting.

To demonstrate the general applicability of the approach, five glycoproteins that are distinct in structure and properties were employed as template molecules in this study, including horseradish peroxidase (HRP; 9 glycosylation sites, 44 kDa, pI 3.0–9.0), ribonuclease B (RNase B; 1 glycosylation site, 15 kDa, pI 8.9), AFP (11 glycosylation sites, 67 kDa, pI 4.7, 5.3), transferrin (2 glycosylation sites, 80 kDa, pI 5.2–6.2), and anti-AFP monoclonal IgG (anti-AFP; 2 glycosylation sites, 150 kDa, pI 7.5–7.9). MIP arrays with specific affinity towards these glycoproteins have been successfully synthesized using the approach, through fine-tuning the ratios of the components in the pre-polymers. Because its detection at trace concentration can be easily implemented using colorimetric assay or chemiluminescence assay, HRP was the major template for the characterization of the properties of the prepared MIP arrays.

Two representative MIP arrays, a filter membrane-based array and a glass slide-based 96-spot array, are shown in the Supporting Information, Figure S1. The latter can be directly detected on commercial microplate readers. The MIP arrays have a macroporous structure (Supporting Information, Figure S2) and uniform thickness of about 1.8 μm (Supporting Information, Figure S3). The macroporous structure facilitated mass transfer in the MIPs. 99.4% of the cross-linker was polymerized in the MIP, while 99.7% was polymerized in a non-imprinting polymer (NIP), which was prepared without the template under otherwise identical conditions (Supporting information, Figures S4, S5). Complete removal of the imprinted template from the accessible surface of the MIP arrays took only 2 h (Supporting Information, Figure S6). As a comparison, imprinted templates were rather difficult to remove completely in bulk imprinting.[1e]

The functional monomer boronic acid was found to play essential roles. First, the presence of boronic acid was crucial in the imprinting step. MIPs generated with boronic acid showed more efficient glycoprotein incorporation and higher success rate (nearly 100%) than those without boronic acid (Supporting Information, Figure S7). Furthermore, polymerization at a basic pH, which favors boronate affinity binding, produced more efficient glycoprotein incorporation (Supporting Information, Figure S8). These results suggest that the boronic acid effectively stabilized the conformation of the templates and facilitated the formation of cavities complementary to the templates. More interestingly, beyond our expectation, the MIP arrays exhibited enhanced affinity towards to the template glycoprotein at acidic pH, at which value boronate affinity interaction is normally impossible or rather weak. NIP exhibited apparent affinity to HRP at pH 8.5 but no affinity at pH 6.0, whereas the HRP-imprinted MIP exhibited apparent affinity to HRP at pH 6.0 and permitted the detection of less HRP (Figure 1). This suggests that the boronate affinity of the boronic acid towards the target at acidic pH was dramatically enhanced by the imprinted cavities.

The unique binding mechanism is a favorable factor, making the MIPs not only applicable for a wider pH range but also tolerant for interference of competing molecules such as sugars. The two advantages were verified experimentally. As shown in Figure 2, trace HRP (10 ng mL^{-1}) in the presence of fructose or mannose (typical glycoprotein-constructing sugars) at a one million-fold higher concentration was effectively extracted by HRP-imprinted MIP arrays within

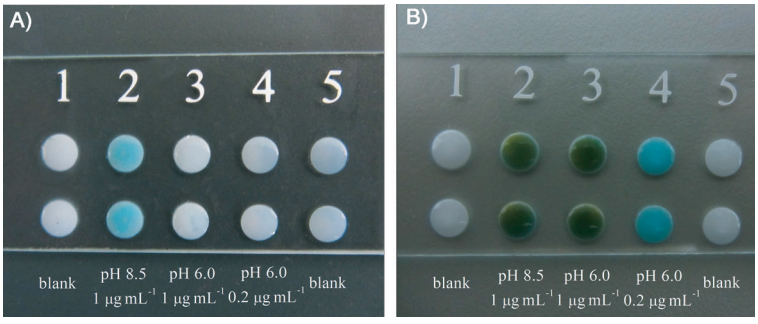

Figure 1. Comparison of the affinity of NIP (A) and HRP-imprinted MIP (B) towards the template at different pH values. The extracted HRP was stained with 3,3,5′,5′-tetramethylbenzidine dihydrochloride (TMB) (a denser color indicates that more template molecules were extracted).

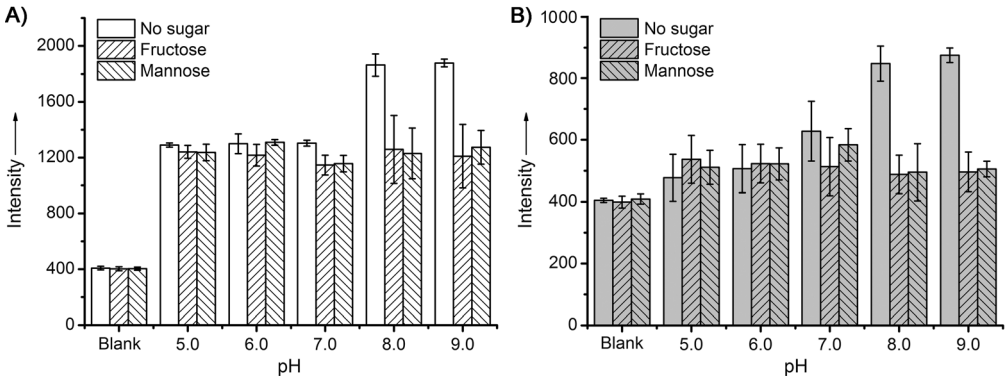

Figure 2. Comparison of the affinity of MIP (A) and NIP (B) towards the template HRP in the presence of abundant competing sugar at different sample pH values. Chemiluminescence detection; sample, 10 ng mL^{-1} HRP without or with 10 mg mL^{-1} fructose or mannose in 100 mM phosphate buffer at different pH values; blank, 100 mM phosphate buffer (pH 6.0) without or with 10 mg mL^{-1} fructose or mannose.

a pH range of 5.0 to 9.0. In comparison, the amounts of HRP extracted by NIP arrays were much lower. This broad range of binding pH greatly favors applications to real samples. The normal pH range of human blood, saliva, tears, and urine is 7.4, 6.2–7.4, 6.5–7.6, and 4.5–8.0, respectively. Conventional boronate affinity materials require a basic pH (usually $\geq$ pH 8.0) for binding and thereby need a pH adjustment step for these samples, leading to not only inconvenience in operation but also the risk of degradation of labile components. To reduce the binding pH, single boronic acids[8b,c] or molecular teams[8a,d,e] with special structures are indispensible. However, the MIP arrays synthesized with a common boronic acid worked well within a pH range of 5.0 to 9.0, which almost covers the pH range of blood, saliva, tears, and urine.

Figure 3 shows the binding isotherm of an HRP-imprinted MIP. It exhibited much stronger affinity towards the template

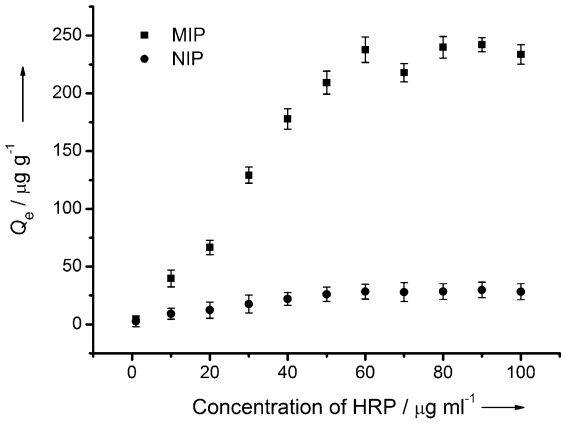

Figure 3. Binding isotherms of an HRP-imprinted array and a NIP array.

molecule as compared with a NIP. Scatchard plot analysis (Supporting Information, Figure S9) for the MIP gave a dissociation constant of 85 nM, which is comparable to conventional antibodies (usually 10^{-7}–10^{-9} M). Such a binding constant is six orders of magnitude higher than that between phenylboronic acid and typical glycoprotein-constructing sugars (approximately 10^{-2} M).[12] This explains well the superb tolerance of the MIP for the interference of abundant sugars. Using the maximum binding capacities that can be found in Figure 3 along with the experimental conditions used, the imprinting efficiency was estimated to be 41 % while the imprinting factor was 9.2. These data reveal high imprinting performance of the proposed approach.

The specificity of the MIPs imprinted with HRP, RNase B, and transferrin was evaluated in terms of their extraction capability to the templates from mixtures containing each template and two competing proteins, one non-glycoprotein and one glycoprotein. The extracted molecules were analyzed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Human serum albumin (HSA), myoglobin and ribonuclease A (RNase A) were used as competing non-glycoproteins while α1-acid glycoprotein (AGP) and HRP were used as competing glycoproteins. Leakage tests were also performed, which confirmed no leakage of residual templates from the MIPs. As shown in the Supporting Information, Figures S10–S12, the MS data reveal excellent specificity of these MIPs. The cross-reactivities were roughly estimated in terms of the peak intensities in the MS spectra. The results are summarized in Table 1. The highest

Table 1: Cross-reactivity of the MIP arrays.[a]

Protein	HRP-imprinted MIP	RNase B-imprinted MIP	Transferrin-imprinted MIP
HSA	0	–	6.3 %
AGP	0	–	–
Myoglobin	–	0	–
RNase A	–	8.8 %	–
HRP	–	–	2.0 %

[a] – not measured.

cross-reactivity (8.8 %) occurred between RNase B-imprinted MIP and RNase A. As the structural difference between RNase A and RNase B is only one glycan chain, such cross-reactivity is well acceptable.

The feasibility of the prepared MIP arrays for real-world applications was demonstrated with the measurement of AFP in human serum. AFP has been routinely used as a biomarker in clinical screening for liver cancer. Combined with chemiluminescence detection, an MIP array-based ELISA permitted the specific detection of trace AFP in serum (Figure 4). The sample consumption was only 1 μL. The limit of detection was 1 ng mL^{-1} (signal/noise ratio = 6), which meets well the requirement for early clinical diagnosis (threshold for pos-

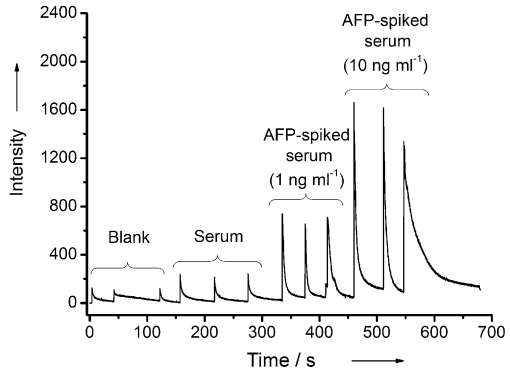

Figure 4. MIP array-based immunochemiluminescence assay of human serum samples spiked with different concentrations of AFP.

itive: 5 ng mL^{-1}). Similarly, an anti-AFP-imprinted MIP array allowed for the detection of 1 ng mL^{-1} HRP-conjugated anti-AFP spiked in serum (Supporting Information, Figure S13).

In summary, by using boronic acid-based UV-initiated polymerization and a photolithographic fabrication route, the issues impeding glycoprotein imprinting were effectively overcome and thus a universal and facile approach has been established for the imprinting of glycoproteins. The use of a boronic acid as the functional monomer played essential roles, which provided several highly attractive features that are beyond normal expectation, making the prepared MIP arrays feasible for the recognition of trace target glycoproteins in complicated real samples. We foresee rapid development and promising applications of the approach in future.

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