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Site-specific post-imprinting modification of molecularly imprinted polymer nanocavities with a modifiable functional monomer for prostate cancer biomarker recognition

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ABSTRACT

Recognition of glycans of glycoproteins using biotic materials such as antibodies is challenging due to lack of antigenicity. Polymeric materials suitable for the molecular recognition of glycoproteins have attracted considerable attention. In this study, we aimed to develop abiotic molecular materials for the recognition of prostate-specific antigen (PSA), a known biomarker for prostate cancer. We used a non-covalent bonding-based molecular imprinting technique to introduce post-imprinting poly(ethylene glycol)-based capping agent into a low-affinity recognition cavity. Details of the binding properties of these groups were investigated to optimize their affinity and selectivity for PSA. Molecularly imprinted polymers (MIPs) were prepared using a bottom-up approach based on surface-initiated atom transfer radical polymerization from a PSA-conjugated sensor chip with a functional monomer-bearing carboxy and secondary amine groups as interaction and post-imprinting modification (PIM) sites, respectively. PSA was orientationally conjugated on the sensor chip through diesters between the immobilized 3-fluorophenyl boronic acid and the cis-diol groups of PSA glucans. Treatment with the capping agent selectively inactivated low-affinity recognition cavities while protecting high-affinity cavities with the addition of a low concentration of PSA as a dynamic protection agent. The MIP thickness is critical in the present molecular imprinting, as a value of less than 5 nm can enable high selectivity. We believe that the proposed strategy based on a non-covalent molecular imprinting approach combined with a PIM-based capping treatment provides a novel method for the development of highly sensitive and selective glycoprotein recognition materials for use in biomarker sensing.

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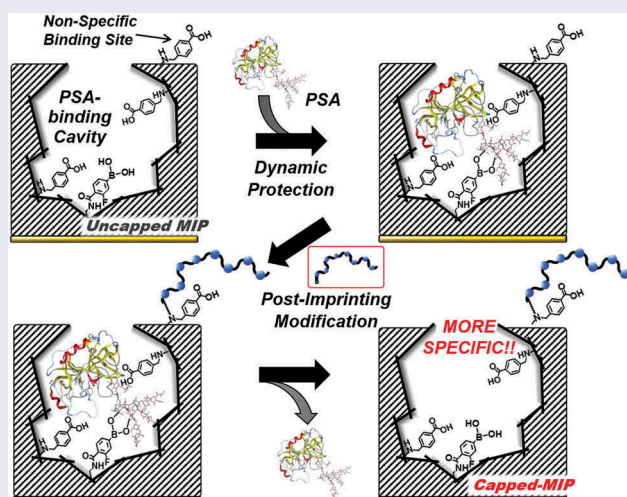
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1. Introduction

The development of molecular recognition materials is crucial for advances in a wide range of research fields, with potential uses in affinity separation, sensors, and diagnostics in medicine [1]. Natural

antibodies are powerful molecular recognition elements for various biomacromolecules and biologically active substances. However, antibodies are fragile and difficult to functionalize. In addition, natural antibodies offer disadvantages in terms of

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recognition of antigens bearing carbohydrates, as anti-carbohydrate antibodies are difficult to prepare, despite the fact that the detection of specific glycoproteins is important for various research fields due to their important biological roles in the human body [2–4]. Therefore, the development of artificial glycoprotein recognition materials is greatly needed.

Molecularly imprinted polymers (MIPs) are promising artificial molecular recognition materials, and they have been used in various applications such as sensors, diagnostics, and drug delivery. MIPs are prepared by a molecular imprinting process based on the radical co-polymerization of functional monomers interacting with target molecules to form a template in the presence of a crosslinking agent and co-monomer. Subsequent template removal leaves molecularly imprinted nanocavities complementary in size and shape to the target molecules [5–14]. Molecular imprinting processes can be divided into two types depending on how the functional monomer interacts with the template molecules: functional monomers are covalently conjugated to the template molecules in covalent-type molecular imprinting, while they interact with the template molecules via non-covalent interactions such as hydrogen bonding and electrostatic interactions in non-covalent-type molecular imprinting. In the former approach, interaction sites are introduced only at molecularly imprinted nanocavities, whereas in the latter approach, interaction sites also occur in the polymer matrix and various imprinted cavities with different affinities are constructed [15]. Thus, some non-specific binding of off-target proteins occurs in non-covalent molecular imprinting due to the presence of low-affinity imprinted cavities.

Inspired by post-translational modifications of natural proteins, the post-imprinting modification (PIM) technique has been developed as an efficient functionalization approach for the molecularly imprinted nanocavities of MIPs [16–22]. PIM enables the creation of various functional MIPs. Utilizing covalent-type molecular imprinting, the interaction sites in molecularly imprinted nanocavities can be transformed into other interaction sites using template molecules conjugated to polymerization groups with reversible linkages such as disulfides, leading to the production of MIPs bearing tunable affinities toward target molecules. In addition, fluorescent signaling groups can also be introduced into molecularly imprinted nanocavities, leading to the highly sensitive detection of target molecules with high signal-to-noise ratios.

Recently, a new application of PIM has enabled the selection of high-affinity recognition cavities in non-

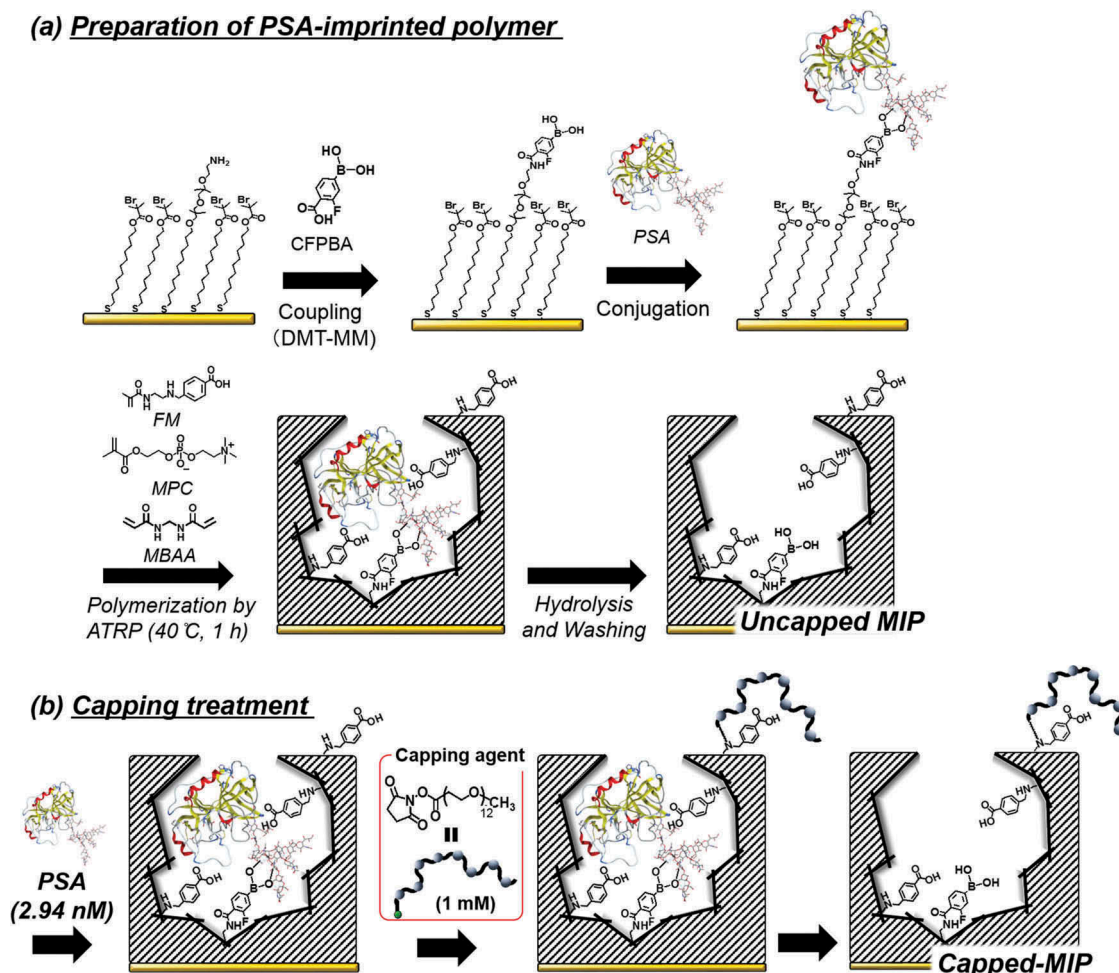
covalent-type molecular imprinting [23]. In this approach, the introduction of fluorescent signaling molecules into low-affinity recognition cavities is effectively suppressed by treatment with a saccharide-based capping agent after the dynamic protection of high-affinity recognition cavities, resulting in MIPs bearing fluorescent signaling groups at only the high-affinity recognition cavities. The selectivity has been improved compared to that of non-capped MIPs even though non-specific binding similarly occurred in both capped and non-capped MIPs, as no fluorescent signaling groups existed in low-affinity cavities due to the blocking of the PIM sites by the capping agent prior to the introduction of the fluorescent reporter molecules.

In this study, we aimed to develop a PIM based on the capping of low-affinity recognition cavities constructed by non-covalent imprinting (Scheme 1). We used a new capping agent based on poly(ethylene glycol) (PEG) to obtain high-affinity MIPs targeting the known prostate cancer biomarker glycoprotein, prostate-specific antigen (PSA; molecular weight 34 kDa, isoelectric point pI = 6.9) [24–27]. PEG, which is well known to block non-specific protein adsorption due to the steric hindrance [28–30], was adopted to be the capping agent as the saccharide-based capping agent may be too small to suppress non-specific binding of off-target proteins. The template PSA was orientationally conjugated on a surface plasmon resonance (SPR) sensor chip via the formation of diesters between 3-fluorophenyl boronic acid and cis-diols. The boronic acid groups remaining after template removal were used as interaction sites for PSA glycans. Non-covalent molecular imprinting utilizing surface-initiated atom transfer radical polymerization (SI-ATRP) to control the polymer thickness was adopted as a bottom-up approach for the synthesis of MIPs with functional monomers bearing carboxy and secondary amine groups as interaction sites for PSA and PIM sites, respectively. Furthermore, the appropriate polymer thickness was also investigated to obtain high-affinity glycoprotein MIPs. Utilizing non-covalent molecular imprinting and the PIM-based capping treatment, MIPs capable of high-affinity glycoprotein recognition were successfully developed.

2. Experimental section

2.1. Preparation of uncapped MIP (Scheme 1a) and non-imprinted polymer (NIP)

Gold-coated SPR sensor chip was washed with ethanol, and the sensor chip was immersed in ethanol (0.5 mL) containing bis[2-(2-bromoisobutyryloxy)

(a) Preparation of PSA-imprinted polymer

Scheme 1. Synthesis of molecularly imprinted polymer with conjugated PSA on the gold-coated SPR sensor chip ((a) uncapped MIP) and the following post imprinting modification with the poly(ethylene glycol)-based capping agent ((b) capped MIP) .

undecyl] disulfide (5 mM) and 11-amino-PEG₆-undecanethiol hydrochloride (2.5 mM), and incubated for 24 h at 25 °C. Then, the sensor chip was immersed in ethanol (1 mL) containing 4-carboxy-3-fluorophenylboronic acid (CFPBA, 10 mM) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM, 10 mM) at 25 °C for 24 h for the immobilization of CFPBA on the SPR sensor chip. The CFPBA-immobilized SPR sensor chip was incubated with PSA (200 µg/mL, 5.88 nM) in phosphate buffer (10 mM, pH 7.4) for 1 h at room temperature, yielding the SPR chip conjugated with PSA via cyclic diester formation.

The PSA-conjugated SPR sensor chip was immersed in 10 mM phosphate buffer (pH 7.4, 2.5 mL) dissolving 4-[2-(*N*-methacrylamido)ethylaminomethyl]benzoic acid (FM, 4.45 µmol) as a functional monomer prepared as previously reported [31], 2-methacryloyloxethyl phosphorylcholine (MPC, 75.9 µmol), *N,N'*-methylenebisacrylamide (MBAA, 8.93 µmol), CuBr₂ (1.4 µmol), 2,2'-bipyridyl (BPy, 2.8 µmol). After N₂/degas cycles, 10 mM phosphate buffer (pH 7.4, 0.1 mL) containing ascorbic acid (0.7 µmol) was added to start the polymerization at 40 °C. After various polymerization times (1 h, 2 h, and 3 h), the sensor chip

was incubated with EDTA-4Na aqueous solution (100 mM) for 30 min at 25 °C, to remove copper ions. For the removal of PSA, the sensor chip was further immersed in 10 mM glycine-HCl buffer (pH 2.5) for 30 min at 25 °C to hydrolyze the cyclic diester, 330 mM NaCl aqueous solution at 25 °C for 30 min, and 0.33 wt % aqueous solution of sodium dodecyl sulfate (SDS) at 25 °C for 30 min, resulting in the MIP thin layer (uncapped MIP). NIP was also prepared by the same procedure without the PSA conjugation on the SPR sensor chip.

2.2. Preparation of capped MIP and capped NIP via PIM (Scheme 1b)

PSA (2.94 nM) dissolved in phosphate buffer (10 mM, pH 7.4) was injected (20 µL) for uncapped MIP or NIP in the SPR sensor as a dynamic protection agent for high-affinity recognition cavities. NHS-PEG₁₂-Me (1 mM), a capping agent, dissolved in phosphate buffer (10 mM, pH 7.4) was injected for 15 min (300 µL) and then washed with aqueous solutions of glycine-HCl (10 mM, pH 2.5), 330 mM NaCl and 0.33 wt% SDS (20 µL × 3 for each step),

resulting in the capped MIP thin layer. Capped NIP was also prepared in the same manner.

2.3. Protein binding experiments by SPR

For SPR measurements, resonance unit (RU) values were monitored with phosphate buffer (10 mM, pH 7.4) as a running buffer at a flow rate of 20 $\mu\text{L}/\text{min}$. In the PSA binding experiments, PSA (final concentrations: 0, 1.47, 2.94, 7.35, and 14.7 nM) dissolved in phosphate buffer (10 mM, pH 7.4) was applied for the CFPBA-immobilized SPR sensor chip ($n = 3$). For MIP and NIP thin layers, the injected concentrations of PSA, human serum albumin (HSA), and immunoglobulin G (IgG) dissolved in phosphate buffer (10 mM, pH 7.4) were 0, 0.294, 0.735, 1.47, and 2.94 nM ($n = 4$). The injection volume was 20 μL , and the data collection point was 4.5 min after injection. Aqueous solutions of glycine-HCl (10 mM, pH 2.5), 330 mM NaCl, and 0.33 wt% SDS were used as regeneration solutions (100 μL). The amount of bound protein was calculated from the signal intensity, where 1 RU corresponds to approximately 1 pg/mm^2 of bound protein [32]. Apparent limit of detection for PSA was estimated from binding isotherms using $3S_D/m$ (m : slope of the linear part of the binding isotherm, S_D : standard deviation for a value of 0 nM PSA).

3. Results and discussion

3.1. Preparation of uncapped MIP and NIP on SPR sensor chips

4-[2-(*N*-Methacrylamido)ethylaminomethyl]benzoic acid (FM) was prepared as a functional monomer as reported previously [31]. FM was designed to have carboxy groups and methacryloyl groups as interaction sites for PSA via electrostatic interactions and polymerization groups, respectively (the chemical structure is shown in Scheme 1a). In addition, the secondary amine group can be used as a functional site for PIM. A mixed self-assembled monolayer (SAM) comprising 11-amino-PEG₆-undecanethiol hydrochloride and bis[2-(2-bromoisobutyryloxy)undecyl] disulfide was formed on a gold-coated SPR sensor chip, and CFPBA was reacted with the amine groups, with 2-bromoisobutyryl groups and CFPBA used for SI-ATRP and template PSA conjugation via a boronate ester formation reaction with the glycans of PSA, respectively (Scheme 1a). CFPBA was selected for PSA conjugation due to its appropriate pK_a (approximately 7.2) under physiological conditions, unlike unsubstituted phenyl boronic acids [33]. The conjugation of PSA on the CFPBA-immobilized substrate was confirmed by SPR measurements using phosphate buffer (10 mM, pH 7.4) as a running buffer, with an estimated affinity constant of $1.63 \times 10^8 \text{ M}^{-1}$ (Figure S1). After the conjugation of PSA at

a concentration of 200 ng/mL (5.88 nM), SI-ATRP was performed with FM, MPC, and MBAA as the functional monomer, co-monomer, and cross-linker, respectively. Subsequently, the template was removed using 10 mM glycine-HCl buffer (pH 2.5) and 330 mM NaCl/0.33 wt% SDS aqueous solution to form molecularly imprinted nanocavities in the MIP thin layer. Polymerized MPC is known as a biocompatible polymer, reducing the non-specific binding of various proteins [34,35]. The obtained thickness of the polymeric layer was estimated as 2.3 nm by X-ray reflectivity (XRR).

3.2. Binding ability of uncapped MIP

The ability of the MIP to recognize the target PSA was investigated using SPR measurements. The amount of bound PSA on the MIP thin layer as prepared (uncapped MIP) was gradually increased by increasing the initial PSA concentration, and the affinity was estimated to be approximately $9.15 \times 10^8 \text{ M}^{-1}$ (Figure 1 and S2). Furthermore, the selectivity was demonstrated using IgG (150 kDa, pI : ~8.5) and HSA (66 kDa, pI : 4.7) as reference proteins. The selectivity factor, defined as the ratio of $\Delta\text{RU}_{\text{reference}}$ to $\Delta\text{RU}_{\text{PSA}}$, was used as an index of the selectivity of PSA binding. A value < 1 indicates high PSA selectivity. However, the amount of bound IgG was greater than that of bound PSA, indicating significant non-specific binding of off-target proteins.

3.3. Effect of the capping treatment

After incubating the uncapped MIP thin layer with a low concentration of PSA (100 ng/mL, 2.94 nM) to dynamically protect high-affinity recognition cavities, a PIM capping treatment was developed using NHS-PEG₁₂-Me to cap the secondary amine group derived from FM remaining in the molecularly imprinted nanocavities (Scheme 1b). This treatment was designed to selectively inactivate the low-affinity recognition cavities, resulting in the presence of only high-affinity recognition cavities for PSA in the capped MIP thin layer. The affinity toward PSA of capped MIP was higher ($K_a = 1.24 \times 10^9 \text{ M}^{-1}$) than that of uncapped MIP, although the amount of bound PSA was reduced from 26.7 pg/mm^2 to 2.79 pg/mm^2 (Figure 1). Moreover, the affinity toward PSA of capped MIP were distinctly higher than those of capped NIP ($K_a = 9.56 \times 10^8 \text{ M}^{-1}$) (Figure 1 and S3). After the capping treatment, the selectivity for PSA was noticeably improved as demonstrated by a decrease in the non-specific binding of IgG, while non-specific binding of HSA did not improve as significantly. This may be due to HSA binding to the polymer matrix not in low-affinity recognition cavities. Further optimization of

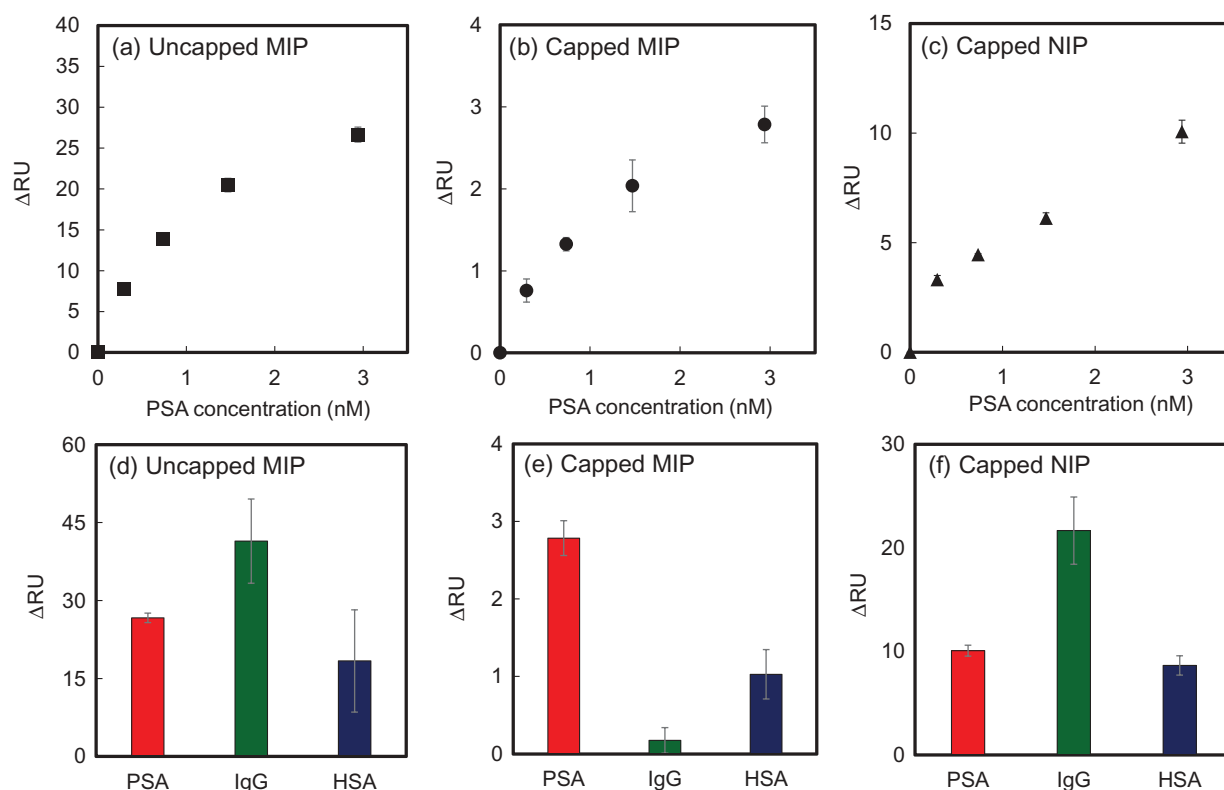


Figure 1. PSA binding activity as measured by SPR of uncapped MIP (a), capped MIP (b), and capped NIP (c). Selectivity of uncapped MIP (d), capped MIP (e), and capped NIP (f) for PSA, IgG, and HSA. MIP: molecularly imprinted polymer; NIP: non-imprinted polymer; PSA: prostate specific antigen; IgG: immunoglobulin G; HSA: human serum albumin.

polymer matrix contents and polymerization conditions would allow the non-specific binding of HSA to be reduced. These results confirm that the capping treatment using NHS-PEG₁₂-Me effectively worked to obtain a MIP thin layer bearing high-affinity and selective PSA recognition cavities. In addition, the limit of detection for PSA was estimated to be approximately 5.4 ng/mL, which is an appropriate sensitivity for the diagnosis of prostate cancer because of under 10 ng/mL which is the PSA value in human serum being strongly suspected of getting prostate cancer [36].

3.4. The effect of polymer thickness on protein recognition ability

The effect of MIP thickness on PSA recognition was further investigated. The MIP thin layer was prepared by a bottom-up approach utilizing SI-ATRP for controlled/living radical polymerization [37–41]; therefore, polymer thickness could be controlled by changing the polymerization time. Indeed, MIP thickness, as measured by XRR, increased with polymerization time (Figure S3). In addition to MIP prepared with 1 h polymerization time (film thickness: approximately 2.3 nm), two other MIP thin layers were prepared at different thicknesses with different polymerization times of 2 h (approximately 5.7 nm) and 3 h

(approximately 6.2 nm), and the PSA recognition abilities of the MIP thin layers were investigated by SPR. Significant non-specific binding of IgG and HSA was observed for uncapped MIPs prepared with 2- and 3-h polymerizations as well as the results of MIP thin layer prepared for 1 h (Figure 2 hatched bars). Even after the capping treatment using NHS-PEG₁₂-Me, the non-specific binding of IgG and HSA was not efficiently suppressed for MIPs prepared with 2- and 3-h polymerizations to detect PSA selectively (Figure 2 solid bars). It is worth noting that the amounts of bound PSA for uncapped MIPs prepared with 2- and 3-h polymerizations were apparently lower than that for the MIP prepared with a 1-h polymerization. These values were not significantly different after the capping treatment (2 h: from 6.3 pg/mm² to 6.1 pg/mm²; 3 h: from 7.6 pg/mm² to 4.9 pg/mm²), indicating that the number of recognition cavities was not altered by the capping treatment. These results suggest that the MIPs prepared with 2- and 3-h polymerizations may be too thick to effectively remove the template PSA, which has a size of approximately 4 nm. Thus, an appropriate polymer thickness relative to the size of the target protein is critical in molecular imprinting with an immobilized template and subsequent PIM based on capping treatment [42–44].

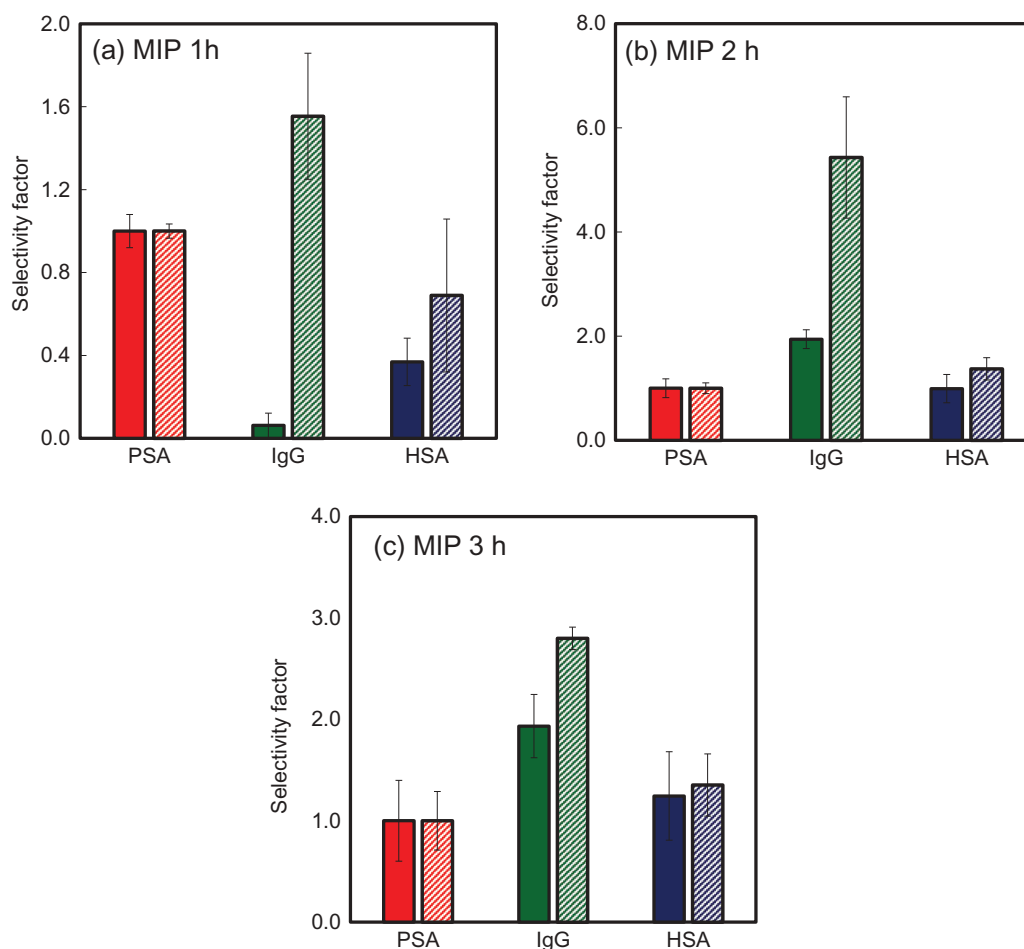


Figure 2. Selectivity of uncapped and capped MIP thin layers prepared with 1 h (a), 2 h (b), and 3 h (c) polymerizations for PSA, IgG, and HSA. Solid bars: capped MIPs; hatched bars: uncapped MIP. The polymer thickness was approximately 2.3 nm (a), 5.7 nm (b), and 6.2 nm (c), respectively. The selectivity factor is defined as the ratio of $\Delta RU_{\text{reference}}$ to ΔRU_{PSA} . MIP: molecularly imprinted polymer; PSA: prostate specific antigen; IgG: immunoglobulin G; HSA: human serum albumin.

4. Conclusions

Molecularly imprinted nanocavities targeting the prostate cancer biomarker glycoprotein PSA were successfully created via non-covalent molecular imprinting and subsequent PIM involving capping of low-affinity recognition cavities; these nanocavities possess boronic acid groups and carboxy groups as sites for interacting with carbohydrates and polypeptides, respectively. Treatment with a new PEG-based capping agent selectively inactivated low-affinity recognition cavities in the MIP thin layer while dynamically protecting high-affinity recognition cavities, resulting in high-affinity PSA-imprinted nanocavities. SI-ATRP allows for controlling the polymer thickness, and our results revealed that MIP thin layer thickness was also an essential parameter for obtaining selective PSA recognition nanocavities via non-covalent imprinting and subsequent PIMs. This proposed technology can change MIPs with moderate affinity and selectivity into MIPs with high affinity and selectivity by simple procedures

without time-consuming optimization step. Therefore, we believe that the developed approach based on non-covalent molecular imprinting and PIM is a facile and efficient synthetic route for the production of high-affinity artificial recognition materials targeting various glycoproteins.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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