Journal of Materials Chemistry B

Signalling Molecular Recognition Nanocavities with Multiple Functional Groups Prepared by Molecular Imprinting and Sequential Post-Imprinting Modifications for Prostate Cancer Biomarker Glycoprotein Detection

Journal:	*Journal of Materials Chemistry B*
Manuscript ID	TB-ART-03-2020-000685.R2
Article Type:	Paper
Date Submitted by the Author:	25-Jul-2020
Complete List of Authors:	Saeki, Tetsuro; Kobe University Takano, Eri; Kobe University, Graduate School of Engineering Sunayama, Hirobumi; Kobe University, Graduate School of Engineering Kamon, Yuri; Kobe University, Graduate School of Engineering Horikawa, Ryo; Kobe University Kitayama, Yukiya; Kobe University, Graduate School of Engineering Takeuchi, Toshifumi; Kobe University, Graduate School of Engineering

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ARTICLE

Signalling Molecular Recognition Nanocavities with Multiple Functional Groups Prepared by Molecular Imprinting and Sequential Post-Imprinting Modifications for Prostate Cancer Biomarker Glycoprotein Detection

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Tetsuro Saeki[1], Eri Takano[1], Hirobumi Sunayama[1], Yuri Kamon[1], Ryo Horikawa[1], Yukiya Kitayama[1,2] and Toshifumi Takeuchi[1,2]*

Fluorescent-signalling molecularly-imprinted nanocavities possessing orthogonal dual interaction sites for the detection of prostate cancer biomarker glycoprotein were constructed through molecular imprinting and sequential multistep post-imprinting modifications (PIMs) using a newly designed multi-functionalised PIM reagent (PIR). The PIR, possessing an interaction site and dual reaction sites for PIMs, enabled us to introduce multiple functions including interaction sites and fluorescent reporter groups in a single PIM site, leading to the sensitive fluorescent detection of target glycoproteins with a high signal-to-noise ratio. Prostate specific antigen (PSA), used as a biomarker for prostate-related diseases, was selected as a target glycoprotein. Surface-initiated atom transfer radical polymerisation from template PSA immobilised the substrate with a functional monomer possessing a phenyl boronic acid group, where the template PSA was designed to possess polymerisation groups aligned with disulphide linkage. Using the thiol groups left after removing templates, PIR could be introduced as the 1st PIM. An evaluation of the effect of crosslinking density and blocking treatment on selective detection indicated that the highly selective and sensitive detection of PSA was achieved. Furthermore, the 2nd PIM to introduce fluorescent molecules into the nanocavities led to the fluorescent detection of PSA. The new sequential PIM strategy using multi-functional PIR can potentially create various sophisticated artificial molecular recognition materials.

Introduction

Molecularly-imprinted polymers (MIPs) are promising materials capable of molecular recognition as substitutes for natural molecular recognition materials.[1-10] The molecular imprinting involves three main steps as follows: (i) complex formation between functional monomers and template molecules; (ii) polymer matrix formation around the template via radical polymerisations with comonomer and crosslinkers; (iii) template removal from the crosslinked polymers. Based on the simple procedure of molecular imprinting, MIPs generally have a simple function, whereas it has been difficult to impart precise molecular recognition properties and sophisticated functions on MIPs.

Post-imprinting modifications (PIMs), which were inspired by natural post-translational modifications, have led to a new era of molecular imprinting.[11, 12] PIMs enable us to construct sophisticated molecularly imprinted nanocavities through the precise a posteriori introduction of various functional groups. We have previously reported the in-cavity selective (re)introduction of interaction sites, leading to nanocavities with tunable affinity towards target molecules.[13-15] The site-specific introduction of fluorescent reporter molecules is one fascinating application of PIM, since the fluorescent molecules introduced in nanocavities can selectively report the binding events as a change in the fluorescent property with high signal-to-noise (S/N) ratio.[16-20] Various fluorescent probes have been reported.[21-23] Therefore, apparent specificity can be enhanced by selecting suitable fluorescent probes for the readout of binding events.

The capping treatment of low affinity binding sites was also developed as PIM after the dynamic protection of high affinity recognition cavities.[24, 25] However, an important limitation to conventional PIM while creating functional MIPs is that one PIM site and one corresponding PIM reagent (PIR) are necessary to introduce one function in molecularly-imprinted nanocavities. Thus, two orthogonal modifications of template proteins must be performed to introduce two different functions to the molecularly-imprinted nanocavities via PIM.[16]

In this study, novel sequential multistep PIMs were developed with a newly-designed PIR that enables MIPs to be multi-functionalised using one PIM site. The new PIR possesses three different functional groups including a pyridyl disulphide

[1]*Graduate School of Engineering, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan.*
[2]*Medical Device Fabrication Engineering Center, Graduate School of Engineering, Kobe University, Kobe 657-8501, Japan.*
**Corresponding author (takeuchi@gold.kobe-u.ac.jp)*
ORCID: Toshifumi Takeuchi: 0000-0002-5641-2333; Yukiya Kitayama: 0000-0002-7418-301X

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

group for the 1st PIM, a secondary amino group for the 2nd PIM, and a carboxyphenyl group as an interaction site. By using this reagent, the molecularly-imprinted nanocavities were functionalised by introducing interaction sites and fluorescent reporter groups using only one PIM site.

Herein, fluorescent-signalling MIPs capable of specific glycoprotein detection were developed using molecular imprinting with a functional monomer possessing a boronic acid group and sequential multistep PIMs with the new PIR (Figure 1). Glycoproteins have specific functions in living systems and the sugar chain structure is related to various diseases such as cancer and diabetes, as the glycosylation of proteins is related to cellular interactions, signal transduction, and protein folding.[26-29] Thus, the highly sensitive detection of glycoproteins possessing specific sugar chains is attracting great attention. However, the molecular recognition of glycoproteins possessing specific sugar chains is currently challenging, because antibodies targeting sugar chains are difficult to create. Lectins are another possible recognition material for glycoproteins, but they have low affinity and selectivity towards target sugar chains. The artificial molecular recognition materials possessing high glycoprotein affinity and selectivity have great potential for development in various research fields.[30-39] In this study, prostate specific antigen (PSA) used as a biomarker of prostate cancer was selected as a target glycoprotein.[40-44] The highly sensitive detection of PSA enabled us to demonstrate the early diagnosis of prostate-related diseases.

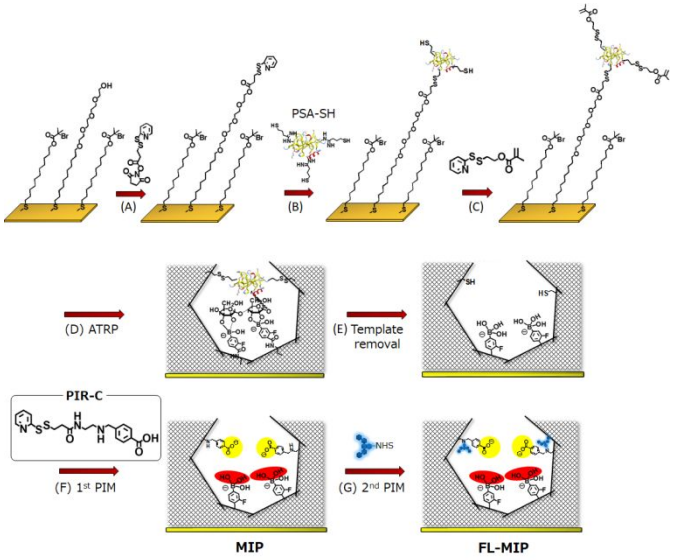

Figure 2. Synthesis procedure of molecularly-imprinted polymer (MIP) thin layer possessing orthogonal dual interaction sites and fluorescent reporting groups via molecular imprinting and sequential post-imprinting modifications (PIMs).

Results and discussion

A new PIM reagent (PIR-C) was designed to possess a thiol reactive pyridyl disulphide moiety for the 1st PIM, a secondary amine group to introduce fluorescent reporter molecules as the 2nd PIM, and a carboxyphenyl group as an interaction site for PSA (Figure 1). To achieve in-cavity selective PIM treatments, a template PSA was designed to possess a disulphide linkage and a polymerisation group. Using amino groups on the PSA, the thiol groups were introduced using 2-iminothiolane (2-IT), which can maintain the net charge of PSA even after the ring opening reaction, leading to the suppression of PSA denaturation.[45] The number of introduced thiol groups in PSA (PSA-SH) was estimated to be approximately 4.5 after the 2-IT treatment, based on the molar mass change measured by MALDI-TOF MS (Figure S1). The PSA-SH was immobilised on gold-coated glass substrates that had a mixed self-assembled monolayer comprising 2-bromoisobutyryl and pyridyl disulphide groups as initiator groups for atom transfer radical polymerisation (ATRP) and PSA-SH immobilisation sites, respectively (Figure 1-Step A and B). The successful immobilisation of PSA-SH on the substrates was monitored by surface plasmon resonance (SPR; ΔRU = approximately 131.9; Figure S2). To introduce methacryloyl groups aligned with a disulphide linkage on the PSA immobilised on the substrate, 2-(2-pyridyl)dithioethyl methacrylate was further reacted to obtain the methacryloyl PSA-immobilised substrates (Figure 1-Step C). The number of methacryloyl groups introduced on PSA-SH was estimated to be approximately 3.3; the molar mass change in PSA was evaluated using free PSA-SH (Figure S1). The secondary structure of the modified PSA was not changed by functionalisation (Figure S3).

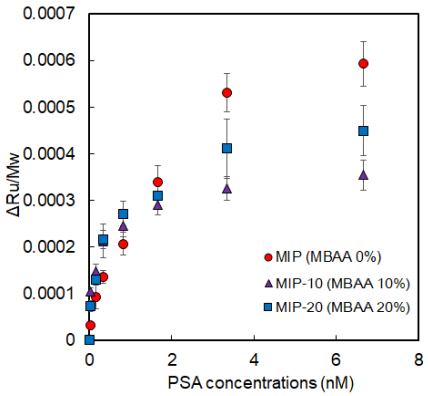

Figure 1. PSA binding isotherms of molecularly-imprinted polymer (MIP) thin layers evaluated by surface plasmon resonance. MBAA contents: 0 mol% (red circle); 10 mol% (purple triangle); 20 mol% (blue square). MBAA: *N,N′*-methylenebisacrylamide; PSA: prostate specific antigen.

Surface-initiated ATRP[46-48] was performed from the methacryloyl PSA-immobilised substrates using a functional monomer (4-(2-methacrylamidoethylcarbamoyl)-3-fluorophenylboronic acid: FMB1) possessing 3-fluorophenyl boronic acid and 2-methacryloyloxyethyl phosphorylcholine as a comonomer in 10 mM phosphate buffer containing 4 wt% DMSO (Figure 1-Step D). The 3-fluorophenyl boronic acid-based functional monomer can be used for sugar chain binding, since the groups can form boronate ester with cis-diol groups under physiological pH conditions.[49-51] After washing the polymer thin layers with tris(2-carboxyethyl)phosphine and sodium dodecyl sulphate aqueous solutions to remove the template PSA (ΔRU = approximately - 65.3; apparent template removal rate was approximately 50%; Figure S4; Figure 1-Step E), the in-cavity

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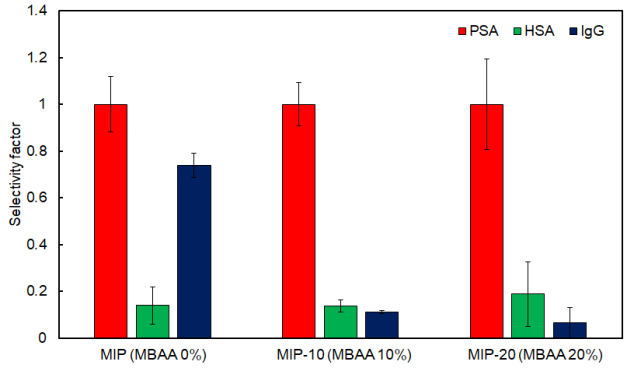

Figure 3. Selectivity tests of molecularly-imprinted polymer (MIP) thin layers evaluated by surface plasmon resonance. MBAA contents: 0 mol% (MIP); 10 mol% (MIP-10); 20 mol% (MIP-20). Protein concentration: 0.83 nM. MBAA: *N,N'*-methylenebisacrylamide; PSA: prostate specific antigen; IgG: immunoglobulin G; HSA: human serum albumin.

selective introduction of PIR-C was performed with thiol groups left in the nanocavities as the 1st PIM (Figure 1-Step F). The procedure enables the construction of molecularly-imprinted nanocavities possessing orthogonal dual interaction sites, including 3-fluorophenyl boronic acid and carboxyphenyl groups for sugar chains and proteins, respectively. The PSA-recognition property of the MIP thin layer was evaluated by SPR measurements. To investigate the selectivity of the prepared MIPs, we employed a selectivity factor, which was calculated by dividing an SPR response of a reference protein, human serum albumin (HSA) or immunoglobulin G (IgG), by that of PSA. Although high PSA affinity for MIP (K_a = 1.28 × 10^9 M^{-1}; Figure 2, red circle and Figure S5a) and low non-specific binding of HSA were observed, the significant non-specific binding of IgG was also observed (Figures 3). This might be due to the flexible non-crosslinked polymer matrix.

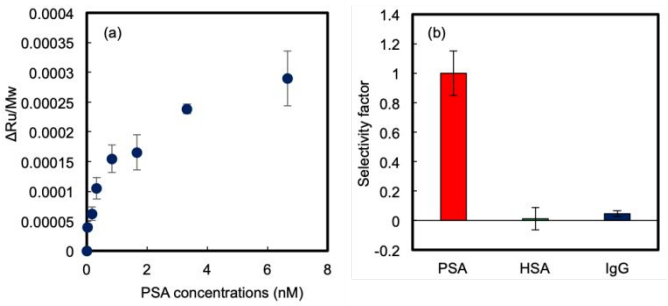

Figure 4. PSA binding isotherm (a) and selectivity test (protein concentration; 0.83 nM) (b) of Block-MIP (molecularly-imprinted polymer) thin layers evaluated by surface plasmon resonance. MBAA contents 10 mol%. PSA: prostate specific antigen; IgG: immunoglobulin G; HSA: human serum albumin MBAA: *N,N'*-methylenebisacrylamide.

Maintaining cavity shapes in the polymer matrix is of great importance for precise molecular recognition, because the multiple interactions between the nanocavity and target proteins can occur only when the shape of the molecularly-imprinted nanocavities is maintained. To increase the polymer matrix rigidity, *N,N'*-methylenebisacrylamide (MBAA) was co-polymerised as a crosslinking agent with 10 or 20 mol% of total monomers (MIP-10 and MIP-20, respectively). The PSA selectivity was significantly increased in both crosslinked MIPs compared to that for non-crosslinked MIPs (Figure 3). The highest PSA affinity was obtained for MIP-10 (K_a= 4.65 × 10^9 M^{-1}) among all MIPs (non-crosslinked MIP: K_a= 1.28 × 10^9 M^{-1}; MIP-20: K_a= 2.21 × 10^9 M^{-1}), which might be due to the optimal crosslinking density for induced fit-based protein recognition (Figures 2 and S5). Furthermore, the lowest limit of detection was also observed with MIP-10 (0.33 ng/mL), indicating that a polymer matrix with optimal crosslinking density led to the improved affinity and selectivity towards PSA.

To further suppress the non-specific binding of off-target proteins, blocking treatment was carried out for MIP-10 (Block-MIP) by injecting 1 mg/mL HSA prior to the binding experiments in SPR. The PSA selectivity was further improved, achieving less than 10% non-specific binding of off-target proteins with the blocking treatment while maintaining high affinity (K_a= 1.18 × 10^9 M^{-1}) towards PSA (Figures 4 and S5d). These results imply that less selective and/or incompletely formed binding cavities were masked by HSA.

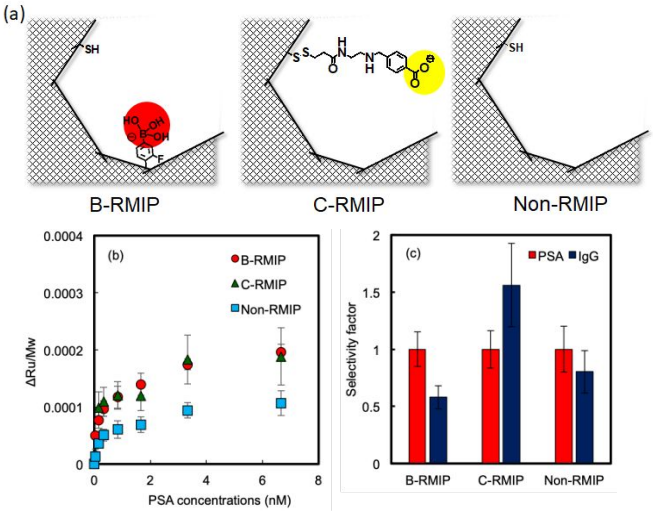

Figure 5. PSA binding isotherms (a) and selectivity tests (protein concentration; 0.83 nM) (b) of three reference molecularly-imprinted polymer (MIP) thin layers (B-RMIP, C-RMIP, Non-RMIP) evaluated by surface plasmon resonance. MBAA contents 10 mol%. B-RMIP: MIPs bearing 3-fluorophenyl boronic acid group; C-RMIP: MIPs bearing carboxyphenyl group; Non-RMIP: MIPs bearing no interaction sites. PSA: prostate specific antigen; MBAA: *N,N'*-methylenebisacrylamide.

The effect of orthogonal dual interaction sites consisting of 3-fluorophenyl boronic acid and carboxyphenyl groups was investigated in detail using three reference MIPs, specifically the MIPs bearing a 3-fluorophenyl boronic acid group (B-RMIP), carboxyphenyl group (C-RMIP), and no interaction sites (Non-RMIP) (Figure 5a). These reference MIPs were prepared with 10 mol% of MBAA and evaluated after the blocking treatment. Among the reference MIPs, the B-RMIP and C-RMIP had higher PSA adsorption capability than the Non-RMIP (Figure 5b). However, sufficient PSA selectivity was not observed for all reference MIPs, because the significant non-specific binding of IgG (selectivity factor > 0.5) was observed (Figure 5c). Furthermore, a higher amount bound and selectivity towards PSA were obtained for Block-MIP possessing dual interaction

sites than for the three reference MIPs. From these results, it can be concluded that the orthogonal dual interaction sites based on 3-fluorophenyl boronic acid and carboxyphenyl groups in the molecularly-imprinted nanocavities are necessary for precise PSA recognition with high selectivity.

Finally, to achieve the fluorescent detection of PSA, Alexa Fluor 647 was introduced as a 2nd PIM into the secondary amino groups of PIR-C that had been already introduced in the molecularly-imprinted nanocavities, yielding the fluorescent reporting MIP thin layer (FL-MIP; Figure 1-Step G). Furthermore, to increase the number of fluorescent reporting molecules introduced into the nanocavities, a functional monomer possessing phenyl boronic acid and secondary amino groups (4-(2-methacrylamidoethylaminomethyl)phenylboronic acid: FMB2) was designed and used for FL-MIP synthesis; the fluorescent molecules were also introduced into the FMB2 moiety. The fluorescent intensity derived from the introduced fluorescent molecules was confirmed after the 2nd PIM (Figure S6).

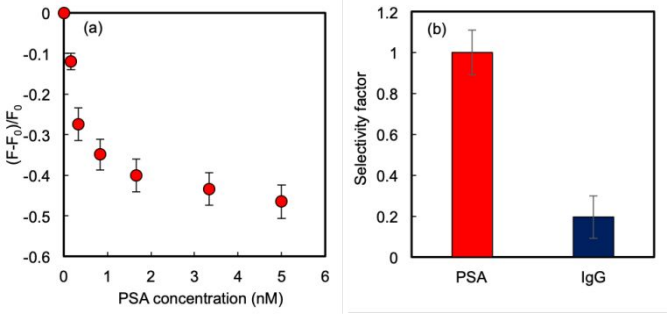

Figure 6. PSA binding isotherm (a) and selectivity test (protein concentration; 0.83 nM) (b) of FL-MIP (fluorescent reporting molecularly-imprinted polymer) thin layers evaluated by fluorescent microscopy. MBAA contents 10 mol%. PSA: prostate specific antigen; IgG: immunoglobulin G; MBAA: *N,N'*-methylenebisacrylamide.

For fluorescent detection using FL-MIPs, a fluorescent microscope possessing automatic pipetting systems was used, where the FL-MIP that formed gold-coated glass substrates was inserted into the pipetting tip, and all reaction procedures including suction, discharge, incubation, and fluorescent detection were performed automatically as programmed.[52, 53] The fluorescent intensity derived from Alexa Fluor 647 gradually decreased with an increasing PSA concentration; the decrease in fluorescent intensity might have been caused by the energy transfer from the fluorescent molecules to certain amino acid residues such as tryptophan (Figure 6a).[54] Furthermore, a low fluorescent response was obtained when IgG was incubated as a reference protein (Figure 6b). The apparent PSA affinity constant derived from the fluorescent detection using FL-MIP (K_a= 2.82 × 10^9 M^{-1}) was similar to that derived from SPR measurements with Block-MIP (K_a= 1.18 × 10^9 M^{-1}), indicating that the PSA recognition events were successfully transduced into fluorescent changes with similar affinity (Figure S5e).

The phenomenon can be rationalised as follows: (i) in the case of fluorescent detection, FL-MIPs can transduce the in-cavity binding events selectively into the change in fluorescent properties; (ii) for SPR measurements, the non-specific binding to the out-cavity regions (i.e. polymer matrix) could be negligible in Block-MIPs due to the blocking treatment. This FL-MIP exhibited a linear detection range from 5 to 150 ng/mL (Figure S7). The limit of detection estimated from the fluorescent detection (2.35 ng/mL) was high and similar to that determined by SPR (4.41 ng/mL).

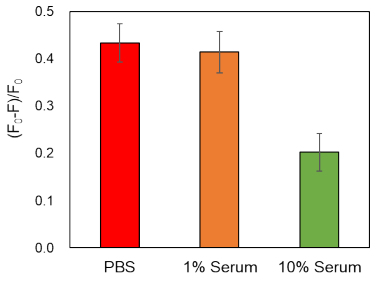

Figure 7. Effect of serum on the detection of spiked prostate specific antigen (PSA: 3.3 nM).

The effect of serum on the detection of PSA was examined Figure 7). A similar response was obtained when 3.3 nM (100 ng/mL) PSA was used to spike 100-fold diluted serum; however, the response was slightly reduced when 10-fold diluted serum was used. For further application to clinical samples, the reduction of serum-derived non-specific binding would be recommended; for example, the combined use of other blocking agents with surfactants. Compared to antibody- and aptamer-based fluorescence sensing systems for PSA previously reported,[55-57] the present system is less sensitive; however, our system could achieve reagent-less detection, unlike sandwich assays, thereby shortening the operation time to within 5 min/sample with enough sensitivity to detect PSA.

Conclusions

A new PIR was developed for multistep sequential PIMs to introduce multiple functions into the molecularly-imprinted nanocavities using one PIM site. In a regular PIM, multiple PIM sites and multiple corresponding PIRs are necessary in the nanocavity to introduce two or more functions; thus, the template molecules must be intricately modified. In contrast, the developed sequential multistep PIMs using the newly designed PIR-C can be used to create a FL-MIP by simply using a modified template glycoprotein. The optimisation of crosslinking density and blocking treatment was also investigated in detail, resulting in highly sensitive and selective PSA detection. Furthermore, the importance of the orthogonal dual interaction sites was confirmed using three reference MIPs. We hope that the rational design of PIR and the sequential multistep PIMs provides a new way to create various sophisticated MIPs.

Conflicts of interest

There are no conflicts to declare.

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Acknowledgements

This work was partially supported by Japan Society for the Promotion of Science, JSPS KAKENHI Grant Numbers JP15K14943, JP16K18300, JP18K14252, and JP18H05398, and Japan Science and Technology Agency, the Matching Planner Program MP27115663368 and MP28116808085. This work was also partially supported by the Cooperative Research Program of "Network Joint Research Center for Materials and Devices" and the Iketani Science and Technology Foundation (0301038-A). We would like to thank the late Professor Isao Karube, President at Tokyo University of Technology (Japan) for his valuable suggestions.

Associated Content

The Supporting Information is available free of charge on the website: Detailed experimental section, CD spectra, MALDI-TOF-MS spectra, SPR results, fluorescent intensity change after PIM, and curve fitting results.

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TOC Entry

Novel sequential post-imprinting modifications was demonstrated on the development of multi-functionalized molecularly imprinted polymers for a biomarker glycoprotein.