Electronic Supplementary Information for

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

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Contents

Experimental section

1. Materials

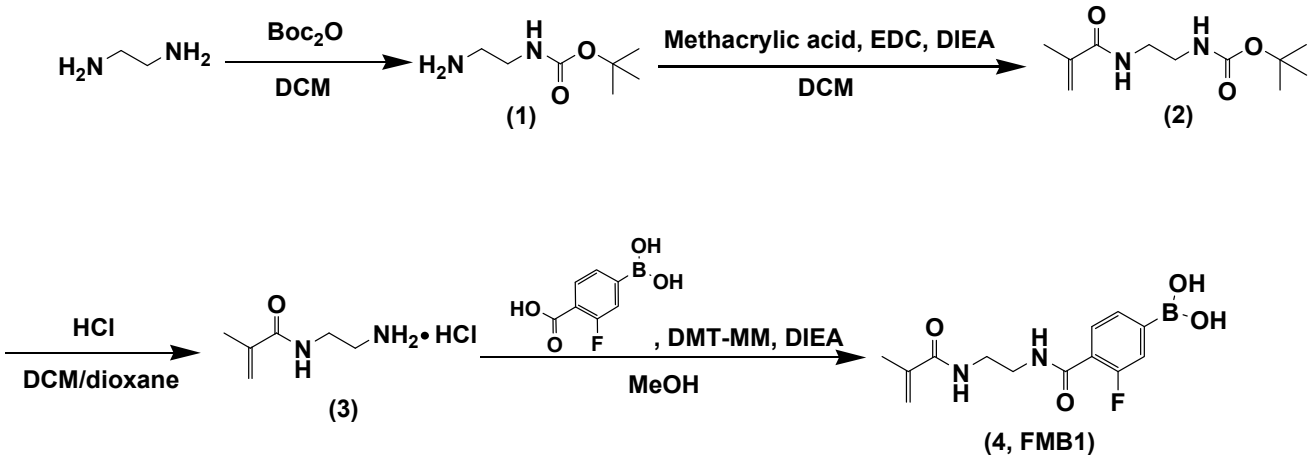
Dichloromethane (DCM), methanol (MeOH), ethanol (EtOH), ethyl acetate (EtOAc), diethyl ether, acetic acid (AcOH), sodium dihydrogen phosphate, disodium hydrogen phosphate, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), methacrylic acid (MAA), glycine, sodium azide (NaN_3), and human serum albumin (HSA) were purchased from Wako Pure Chemical Co. Ltd (Osaka, Japan). Di-*tert*-butyl dicarbonate (Boc_2O) and 4*N* HCl/dioxane were purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride *n*-hydrate (DMT-MM), *N,N*'-diisopropylethylamine (DIEA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 4-formylphenylboronic acid, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Tokyo Chemical Industries (Tokyo, Japan). Ethylenediamine, sodium carbonate (Na_2CO_3), sodium sulfate (Na_2SO_4), citric acid, *n*-hexane (Hex), 3-mercaptopropionic acid, 2,2'-bipyridyl, ethylenediaminetetraacetic acid tetrasodium salt (EDTA 4Na), triethylamine (TEA), sodium borohydride ($NaBH_4$), hydrochloric acid (HCl), and *N,N*'-methylenebisacrylamide (MBAA) were purchased from Nacalai Tesque Co. (Kyoto, Japan). 2,2'-Dipyridyldisulfide, *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 2-iminothiolane hydrochloride (2-IT), copper(I) bromide (CuBr), *p*-formylbenzoic acid, *γ*-immunoglobulin (IgG), and 11-mercaptoundecyl tetraethyleneglycol were purchased from Sigma-Aldrich (MO, USA). 4-Carboxy-3-fluorophenylboronic acid (CFPBA) was purchased from Combi-Blocks Inc. (CA, USA). 11-Sulfanyloundec-1-yl 2-bromo-2-methylpropionate was purchased from ProChimia Surfaces (Zacisze, Poland). 2-Methacryloyloxyethyl phosphorylcholine (MPC) was purchased from NOF Corporation (Tokyo, Japan). The water used in all experiments was obtained from a Millipore Milli-Q purification system. The prostate-specific antigen (PSA) was purchased from Abcam K. K. (Cambridge, UK). Alexa Fluor 647 carboxylic acid succinimidyl ester was purchased from Thermo Fisher Scientific Inc. (MA, USA). Au-coated SPR sensor chips (superficial area: 120 mm^2) were purchased from GE Healthcare Japan (Tokyo, Japan). Human serum was purchased from Jackson ImmunoResearch (PA, USA). 2-(2-Pyridyl) dithioethyl methacrylate was synthesised as in a previously reported procedure. [1]

2. Characterisation

^1H NMR, MALDI-TOF-MS, UV-Vis, and circular dichroism (CD) spectra were measured using a 300 MHz FT-NMR spectrometer (JNM-LA300 FT NMR system, JEOL Ltd., Tokyo, Japan), Voyager-DE-1000 instrument (AB SCIEX, Tokyo, Japan), V560 spectrometer (JASCO, Japan), and J-725K

spectrometer (JASCO, Japan), respectively. ^{13}C NMR spectra were recorded on a Bruker AVANCE^{TM} II 500 (Bruker Corporation, MA, USA). Surface plasmon resonance (SPR) measurements were performed using a Biacore 3000 instrument (GE Healthcare Japan). The fluorescence microscopy measurements were conducted with an automatic analysing device including tip rack, a reagent rack, an incubation port, and a computer (System Instruments Co. Ltd., Japan). For the automatic analyser, a pipette tip was designed to be suitable for substrate gold sensor chip (4.3×9.8 mm) and set in the chip rack. This automatic analysing system was demonstrated using a device capable of pipetting, incubating, washing, and detecting according to the program's input in the computer.[2, 3]

3. 4-(2-Methacrylamidoethylcarbamoyl)-3-fluorophenylboronic acid (FMB1)



Scheme S1. Synthetic of FMB1

3-1. *N*-Boc Ethylenediamine (1)

Ethylenediamine (9.21 mL, 138 mmol) was dissolved in DCM (20 mL). The solution was slowly added to another DCM solution (30 mL) containing Boc_2O (3.0 g, 13.8 mmol) in an ice bath. After 2.5 h of reaction at 0°C, the solution was washed with a Na_2CO_3 aqueous solution twice. After drying and isolating the Na_2SO_4, the solvent was vacuum-evaporated, yielding product 1.

Yield 1.30 g, 59%. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 4.84 (br, 1H), 3.20-3.14 (q, 2H), 2.82-2.75 (t, 2H), 1.45 (s, 9H)

3-2. *N*-Methacryloyl-*N*'-Boc-ethylenediamine (2)

MAA (0. 828 mL, 9.76 mmol), EDC (2.03 g, 10.6 mmol), and DIEA (1.84 mL, 10.6 mmol) were dissolved in the DCM (20 mL). Product 1 (1.3 g, 8.14 mmol) was added to the DCM solution, reacting

for 24 h at 0°C. The resulting product was washed with a citric acid aqueous solution, once, and dried with Na_2SO_4. After the vacuum evaporation of the solvent, the crude product was purified by a silica gel column chromatography (EtOAc/Hex = 50/50 - 100/0, v/v) to yield the white powder product.

Yield: 511 mg, 28%. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 6.69 (br, 1H), 5.75 (s, 1H), 5.33 (s, 1H), 4.89 (br, 1H), 3.44-3.34 (m, 4H), 1.96 (s, 3H), 1.44 (s, 9H).

3-3. *N*-Methacryloyl ethylenediamine (3)

DCM solution (20 mL) dissolving 4*N* HCl/dioxane (5.04 mL, 20.2 mmol) was added into another DCM solution containing product 2 (0.511 g, 2.24 mmol) in an ice bath, and reacted for 20 h at room temperature. The precipitate was collected and washed with diethyl ether thrice. The obtained white product was vacuum dried.

Yield: 362 mg, 98%. ^{1}H-NMR (D_2O, 300 MHz): δ = 5.66 (s, 1H), 5.39 (s, 1H), 3.48-3.44 (t, 2H), 3.09-3.05 (t, 2H), 1.83 (s, 3H)

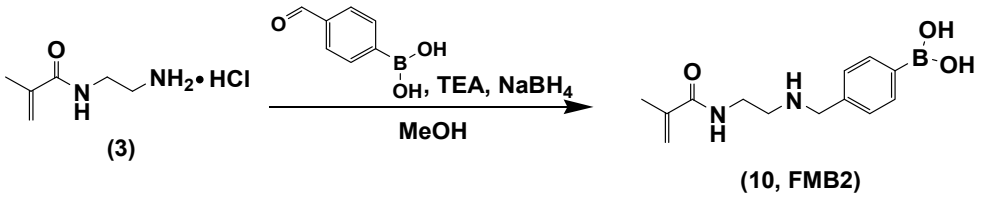
3-4. 4-(2-Methacrylamidoethylcarbamoyl)-3-fluorophenylboronic acid (FMB1) (4)

Product 3 (198 mg, 1.2 mmol), CFPBA (266 mg, 1.44 mmol), DMT-MM (433 mg, 1.57 mmol), and DIEA (419 μL, 2.41 mmol) were reacted in MeOH (20 mL) at room temperature for 3 h. After vacuum drying, the crude product was purified by silica gel column chromatography (water/MeOH = 95/5 - 50/50, v/v). The solvent was vacuum-evaporated, yielding the product as a white powder.

Yield: 271 mg, 77%. ^{1}H-NMR (CD_3OD, 300 MHz): δ = 7.72-7.67 (br, 3H), 5.72-5.37 (s, 2H), 3.57-3.46 (m, 4H), 3.34 (s, 2H), 1.94 (s, 3H)

^{13}C-NMR (125 MHz, DMSO-*d6*): δ=167.9, 164.2, 159.9, 140.1, 130.0, 129.3, 125.4, 121.1, 119.3, 38.8, 18.8

4. 4-(2-Methacrylamidoethylaminomethyl) phenylboronic acid (FMB2)



Scheme S2. Synthetic of FMB2

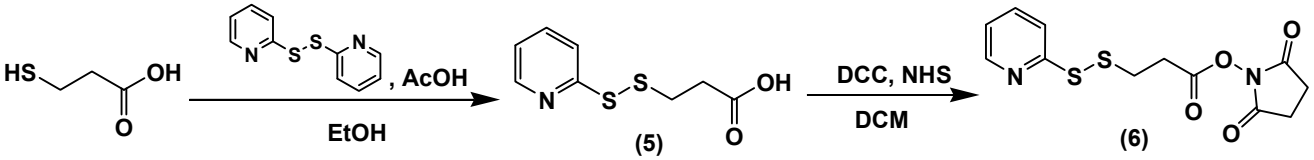
Compound 3 (470 mg, 2.85 mmol), 4-formylphenylboronic acid (450 mg, 3.00 mmol) and TEA (840 μL, 6.00 mmol) were dissolved in MeOH (15 mL) and the solution was stirred at room temperature for 30 min. Afterwards, $NaBH_4$ (360 mg, 9.00 mmol) was added to the mixture in small batches. The further stirring was conducted overnight at room temperature. The solvent was, thereafter, removed in vacuo. The residue was purified by a silica gel column chromatography (MeOH/DCM=70/30→100/0) to yield the white solid product.

Yield: 330 mg (1.26 mmol, 44 %)

^{1}H-NMR (300 MHz, D_2O): δ=7.63 (d, 2H, phenyl), 7.31 (d, 2H, phenyl), 5.70 (s, 1H, vinyl), 5.46 (s, 1H, vinyl), 3.94 (s, 2H, N-CH_2-), 3.47 (t, 2H, -CH_2-), 2.96 (br, 2H, -CH_2-), 1.91 (s, 3H, -Me)

^{13}C-NMR (125 MHz, D_2O): δ=137.8, 140.6, 134.5, 129.7, 123.15, 53.6, 48.3, 39.8, 19.5

5. 4-({2-[3-(Pyridyldithio)-propionylamino]-ethylamino}-methyl)-benzoic acid (PIR-C)



Scheme S3. Synthesis of 3-Pyridyldithio propionic acid NHS ester (6)

5-1. 3-Pyridyldithio propionic acid (5)

2,2'-Dipyridyldisulfide (2.53 g, 11.5 mmol) and AcOH (50 μL) were dissolved in EtOH (30 mL). Another EtOH solution (20 mL) containing 3-mercaptopropionic acid (500 μL, 5.75 mmol) was added to the solution and the mixture reacted for 15 min at room temperature. After vacuum evaporation of the solvent, the product was purified by the silica gel column chromatography (AcOEt/Hex = 60/40, v/v). The solution was vacuum-evaporated, yielding the white powder product.

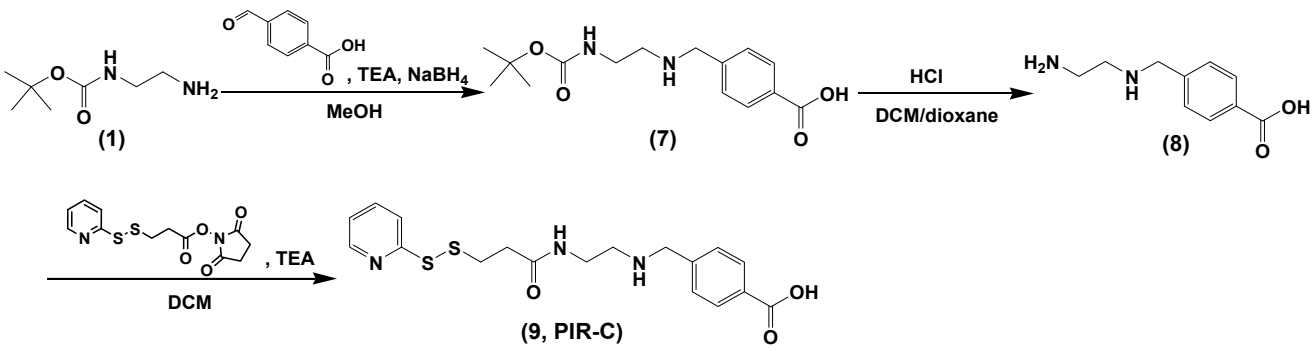
Yield 867 mg, 70%. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.50-8.47 (m, 1H), 7.70-7.60 (m, 2H), 7.18-7.14 (m, 1H), 3.10-3.06 (t, 2H). 2.83-2.78 (t, 2H)

5-2. 3-Pyridyldithio propionic acid NHS ester (6)

Compound 5 (865 mg, 4.03 mmol), DCC (998 mg, 4.84 mmol), and NHS (557 mg, 4.84 mmol) were added to a flask containing DCM (30 mL) and reacted for 3 h. The flask was surrounded by an ice bath. After filtration for removing byproduct derived from DCC, the solution was evaporated. The crude product was purified by silica gel column chromatography (AcOEt/Hex = 50/50, v/v) and the remaining solution

was vacuum-evaporated, yielding the product in the form of a white powder.

Yield: 593 g, 59%. ^{1}H-NMR ($CDCl_3$, 300 MHz): δ = 8.51-8.49 (m, 1H), 7.68-7.66 (m, 2H), 7.14-7.09 (m, 1H), 3.17-3.05 (m, 4H). 2.84 (s, 4H)



Scheme S4. Synthesis of PIR-C

5-3. 4-[(2-tert-butoxycarbonylamino-ethylamino)-methyl]-benzoic acid (7)

Product 1 (274 mg, 1.7 mmol), *p*-formylbenzoic acid (225 mg, 1.5 mmol), and TEA (420 μL, 3.0 mmol) were added to MeOH (10 mL) and reacted at room temperature for 0.5 h. After the addition of $NaBH_4$ (120 mg, 3.0 mmol), the reaction was allowed to proceed overnight at room temperature. The solution was thereafter vacuum-evaporated, and the crude product was purified by silica gel column chromatography (MeOH/DCM = 10/90 - 65/35, v/v). Thereafter, the solution was vacuum-evaporated again, yielding the product.

Yield: 419 mg, 83%.^{1}H-NMR (DMSO-d_6, 300 MHz): δ=7.80 (d, 2H), 7.27 (d, 2H), 3.67 (s, 2H), 3.01-2.97 (m, 4H), 1.36 (s, 9H)

5-4. 4-[(2-amino-ethylamino)-methyl]-benzoic acid (8)

4*N* HCl in dioxane (2.0 mL, 8.0 mmol) was added into a DCM solution containing product 7 (419 mg, 1.4 mmol) inside an ice bath and reacted overnight at room temperature. The result was washed with diethyl ether thrice. The white product obtained was, thereafter, vacuum dried.

Yield 323 mg, 100%. ^{1}H-NMR (D_2O, 300 MHz): δ=8.06 (d, 2H, phenyl), 7.60 (d, 2H, phenyl), 4.40 (s, 2H, -CH_2-), 3.50 (m, 2H, -CH_2-), 3.40 (m, 4H, -CH_2-)

5-5. 4-({2-[3-(Pyridyldithio)-propionylamino]-ethylamino}-methyl)-benzoic acid (9, PIR-C)

Product 8 (323 mg, 1.4 mmol) and TEA (420 μL, 3.0 mmol) were mixed in a DCM solution (15 mL) at

room temperature. After addition of another DCM solution (5 mL) of 3-pyridyldithio propionic acid NHS ester (468 mg, 1.5 mmol), the reaction proceeded for 2 h at room temperature. After filtration and solvent evaporation, the crude product was purified by the silica gel column chromatography (ODS silica gel, MeOH/DCM = 10/90 - 65/35, v/v). The resultant product was thereafter vacuum-evaporated, yielding the product.

Yield 280 mg, 51%. ^{1}H-NMR (DMSO-*d*$_6$, 300 MHz): δ=8.44 (d, 1H) 7.93 (br, 1H), 7.92-7.72 (m, 4H), 7.31 (d, 2H), 7.24-7.20 (m, 2H), 3.70 (s, 2H), 3.16 (q, 2H), 2.99 (t, 2H), 2.57-2.45 (m, 4H)

^{13}C-NMR (125 MHz, DMSO-*d6*): δ = 169.9, 167.6, 159.4, 149.8, 138.0, 129.3, 128.0, 121.3, 119.3, 52.4, 48.2, 38.8, 34.8, 34.3

6. Methacryloyl PSA

PSA dissolved in 50 mM PBS (5 μL. 3.93 mg/mL, 0.655 nmol, pH 7.5, 150 mM NaCl, 0.09 % NaN_3) was mixed with 2-IT and dissolved in 10 mM of phosphate buffer (35 μL, 328 nmol, pH 8.0). The reaction was performed at 4°C for 6 h. Afterwards, the PSA was washed by ultrafiltration (Amicon Ultra-0.5: 10 kDa) five times with a 10 mM phosphate buffer (pH 7.4), yielding PSA-SH (100 μL, 19.65 μg), which was mixed with 10 mM of phosphate buffer solution (pH 7.4, 131 μL) containing 2-(2-pyridyl) dithioethyl methacrylate (0.655 μmol). After the reaction was conducted overnight, at 4°C, the result was washed by ultrafiltration (Amicon Ultra-0.5: 10 kDa) thrice with 10 mM of phosphate buffer (pH 7.4), yielding methacryloyl PSA.

To estimate the number of modified thiol groups on PSA, the PSA solution (1 μL) was collected at different reaction stages and the mass of the PSA sample was quantified by MALDI-TOF-MS with sinapinic acid as a matrix. As the molar mass of the 2-IT is 102, the number of 2-IT entities introduced on PSA could be estimated by using the following equation.

$$\text{Number of 2-IT introduced in PSA-SH} = [(M_{w,\,\text{PSA-SH}}) - (M_{w,\,\text{native PSA}})] \,/\, (M_{w,\,\text{2-IT moiety}})$$

The number of introduced 2-(2-pyridyl)dithioethyl methacrylate was also determined by MALDI-TOF-MS with sinapinic acid as a matrix. As the molar mass of the 2-(2-pyridyl) dithioethyl methacrylate moiety was 144, the number of 2-(2-pyridyl) dithioethyl methacrylates introduced on methacryloyl PSA could be estimated using the following equation.

$$\text{Number of 2-(2-pyridyl)dithioethyl methacrylate introduced in the thiolated PSA} = [(M_{w,\,\text{methacryloyl PSA}}) - (M_{w,\,\text{native PSA}}) - (M_{w,\,\text{2-IT introduced}}) \times (\text{number of introduced 2-IT})] / (M_{w,\,\text{2-(2-pyridyl)dithioethyl methacrylate entities}})$$

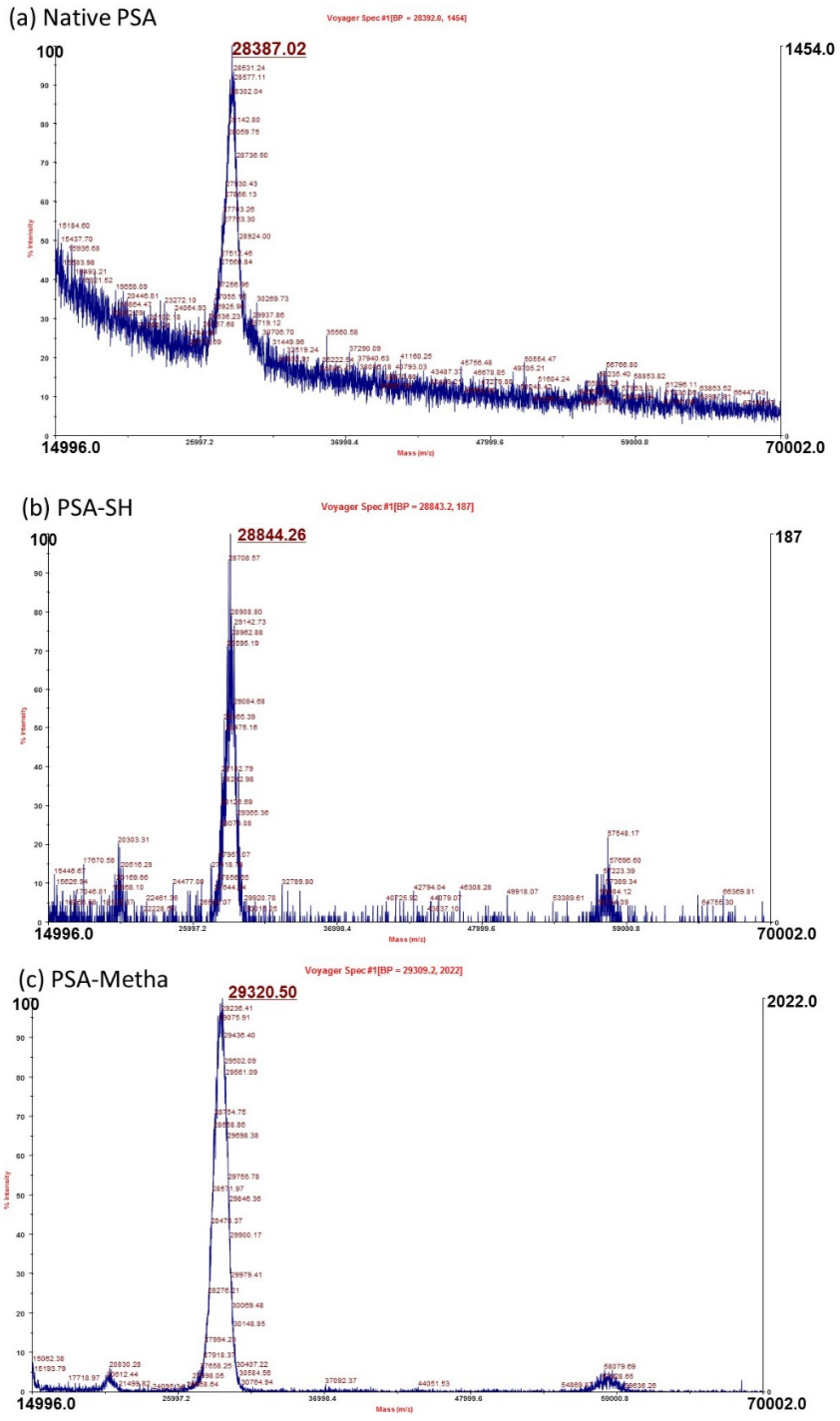


Figure S1. MALDI-TOF-MS spectra of the PSA-Native (a), PSA-SH (b), and methacryloyl PSA (c).

7. Mixed SAM

The gold-coated glass substrate was washed with EtOH. The substrate was immersed in an EtOH solution (1 mL) containing 11-mercaptoundecyl tetraethyleneglycol (0.5 mM) and 11-sulfanyloundec-1-yl 2-bromo-2-methylpropionate (0.5 mM). After 24 h of incubation at 25 °C, the substrate was washed with EtOH and dried with nitrogen gas. Afterwards, the product was incubated in a DCM solution (1 mL) of 5 mM 3-pyridyldithio propionic acid NHS ester for 24 h, at 25 °C, and the previous washing and drying steps were repeated. SPR sensor chips and gold-coated glass substrates (5 mm × 10 mm) were, thereafter, employed for SPR measurements and fluorescent detections, respectively.

8. Immobilisation of methacryloyl PSA

3-Pyridyldithio-functionalised gold-coated glass substrate was immersed in 10 mM of phosphate buffer solution containing PSA-SH (1 μg/mL, 2 mL, pH 7.4) for 1 h, at 4 °C. The reacted gold-coated glass substrate was washed with 10 mM of phosphate buffer solution (pH 7.4). Afterwards, the substrate was further immersed in a 2-(2-pyridyl) dithioethyl methacrylate (8.53 μg, 33.4 nmol) solution dissolved in 10 mM of phosphate buffer (2 mL, pH 7.4) at 4 °C overnight.

9. Template protein binding on 3-pyridyldithio-functionalised substrates

PSA-SH immobilisation of 3-pyridyldithio-functionalised gold-coated glass substrate was monitored by SPR. Resonance unit (RU) values were monitored using 10 mM phosphate buffer (pH 7.4) as a running buffer until they stabilised. PSA-SH solution dissolved in 10 mM phosphate buffer (pH 7.4) (final concentrations: 1 μg/mL) was injected with flow rate of 10 μL/min, 100 μL of injection volume, and the data collection point was 5 min after injection.

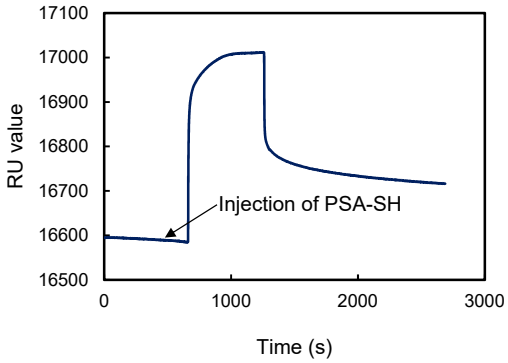


Figure S2. SPR sensorgram of PSA-SH immobilisation on a 3-pyridyldithio-functionalised gold-coated glass substrate.

10. CD spectra

Native PSA and methacryloyl PSA were dissolved in 10 mM of phosphate buffer (1 μM, 400 μL, pH 7.4). Furthermore, CD measurements were performed between 195 and 250 nm, at 25 °C.

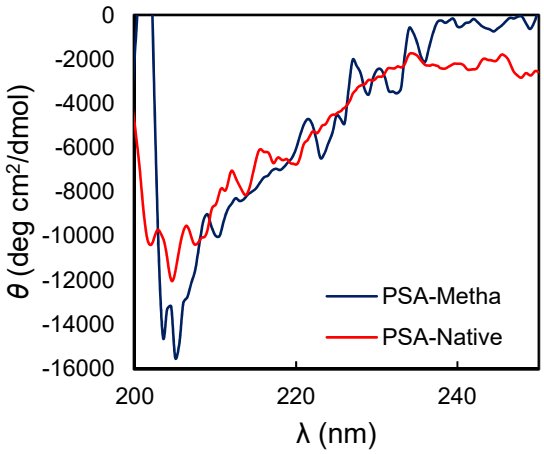


Figure S3. CD spectra of the native PSA (red) and methacryloyl PSA (blue).

11. Preparation of the MIP thin layers

MPC (48 mM) and FMB1 (2 mM) were dissolved in 9 mL of 10 mM phosphate buffer (pH 8.0) containing 4 wt% of DMSO to prepare the pre-polymerisation solution. The mixture was placed in a Schlenk flask and, after the freeze-pump-thaw process, the methacryloyl PSA-immobilised gold-coated glass substrate was added. The mixture was degassed and filled with nitrogen. For preparation of MIP thin layer without crosslinking, a solution containing CuBr (1 mM) and 2,2′-bipyridine (2 mM) in 10 mM phosphate buffer (1 mL, pH 8.0) was added to the pre-polymerisation solution to start the surface-initiated atom transfer radical polymerisation (SI-ATRP), in which the catalyst solution has been degassed by the freeze-pump-thaw process. After 1 h of the polymerisation process, conducted at 40 °C, the sensor chip was washed with pure water, and immersed in a 100 mM EDTA 4Na aqueous solution (2 mL) at 25 °C, for 30 min, to remove any residual copper ions. To remove the template PSA, the obtained polymer-coated sensor chip was immersed in a 20 mM TCEP aqueous solution (2 mL) for 15 min at 25 °C. Afterwards, the SPR sensor chip was immersed in a 0.5 wt% aqueous SDS solution (2 mL) for 15 min at 25 °C and washed with pure water.

Crosslinked MIP thin layers were prepared using a similar procedure with MIP, in which MPC (43 or 38 mM), MBAA (5 or 10 mM) and FMB1 (2 mM) dissolved by a 10 mM phosphate buffer (pH 8.0) containing 4 wt% DMSO was used as the pre-polymer solution for MIP-10 and MIP-20.

B-RMIP thin layer was also prepared by a similar procedure as MIP-10 without introducing PIR-C by 1st PIM (see Section 13), which corresponds to MPC (43 mM), MBAA (5 mM), and FMB1 (2 mM) dissolved in a 10 mM phosphate buffer (pH 8.0) containing 4 wt% DMSO, and it was used as the pre-polymerisation solution. For C-RIP and Non-RMIP thin layers, MPC (45 mM) and MBAA (5 mM) dissolved 10 mM phosphate buffer (pH 8.0) containing 4 wt% DMSO was also used as the pre-polymerisation solution.

For FL-MIP, MPC (43 mM), MBAA (5 mM), and FMB2 (2 mM) dissolved in 10 mM phosphate buffer (pH 8.0) containing 4 wt% DMSO was used as the pre-polymerisation solution.

12. SPR measurements of template PSA removal

Template PSA removal from the polymer-formed SPR substrate was monitored by SPR. 20 mM TCEP aqueous solution and 0.5 wt% SDS aqueous solution were injected to the polymer-formed SPR substrate. The flow rate, the injection volume were 20 μL/min and 100 μL, respectively, and the data collection point was 5 min after injection.

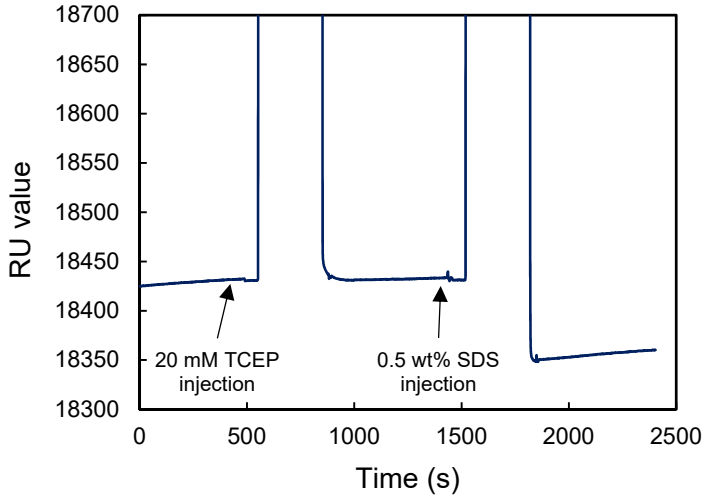


Figure S4. SPR sensorgram of the template PSA removal from the polymer thin layer using 20 mM TCEP aqueous solution and 0.5 wt% SDS aqueous solution

13. 1st PIM

To introduce interaction (carboxy phenyl groups) and fluorescent labelling sites (secondary amino groups), the obtained MIP thin layer was immersed in a 1 mM PIR-C solution and dissolved in a water/DMSO (7/3 v/v) mixture (2 mL) for 12 h, at 25 °C. Afterwards, the sensor chip was washed with pure water and dried

with nitrogen. For MIP thin layers (MBAA: 0%, 10%, and 20%), C-RMIP, and FL-MIP, the 1st PIM procedure was carried out.

14. Protein binding experiments by SPR

To obtain the Block-MIP thin layer, the blocking treatment was conducted before the protein binding experiments. The HSA solution was dissolved in a 10 mM phosphate buffer (pH 7.4) (final concentration: 1 mg/mL) and the product was injected with flow rate of 20 μL/min, and injection volume of 20 μL.

RU values were monitored using 10 mM phosphate buffer (pH 7.4) as a running buffer until the values stabilised. In the PSA binding experiments MIP-formed SPR sensor chip, the PSA solution dissolved in 10 mM phosphate buffer (pH 7.4) (final concentrations: 0, 0.033, 0.167, 0.33, 0.83, 1.67, 3.33, and 6.67 nM) was injected with flow rate of 20 μL/min, and injection volume of 20 μL, and the data collection point was 5 min after injection. SDS (0.5 wt%) and 10 mM glycine-HCl (pH 2.5) were used as regeneration solutions (100 μL) to remove adsorbed proteins from the MIP thin layer. The amount of bound protein was calculated from the signal intensity (1 RU corresponds to approximately 1 pg/mm^2 of bound protein at 150 s after protein injection).[4] Apparent limit of detection for the PSA was estimated from the binding isotherm using $3S_D/m$ (m: slope of the linear region of the binding isotherm, S_D: standard deviation for 0 ng/mL PSA).

In order to investigate the selectivity of prepared MIP thin layers, HSA and IgG solution were dissolved in a 10 mM phosphate buffer (pH 7.4) (final concentrations: 0.83 nM) and were used for binding experiments to reference proteins. Selectivity factor was calculated from the following equation:

$$selectivity\ factor = \frac{\Delta RU_{reference\ protein}}{\Delta RU_{PSA}}$$

Where $\Delta RU_{\text{reference protein}}$ and ΔRU_{PSA} are ΔRU values incubated with 0.83 nM of the reference proteins (HSA or IgG) and PSA, respectively.

15. Binding constants

Binding constants were estimated by a curve-fitting software (DeltaGraph, Nihon Poladigital, Tokyo, Japan). The fitting equation (1), which is generally used for determining the binding constant of the formation of a 1:1 complex, is shown below. K is the apparent affinity constant, H is the host, G is the guest, ΔRU_{max} and ΔF_{max} are the ΔRU and ΔF values given by the binding at the maximum amount of

guest found by fitting a theoretical curve to the raw data. Equations (1) and (2) were used for the curve fitting in order to obtain the SPR and fluorescent detection, respectively.

$$\Delta RU = \Delta RU_{max} \times \frac{1+K[G]+K[H]-\sqrt{(1+K[G]+K[H])^2-4K^2[G][H]}}{2K[H]} \quad (1)$$

$$\frac{\Delta F}{F_0} = \frac{\Delta F_{max}}{F_0} \times \frac{1+K[G]+K[H]-\sqrt{(1+K[G]+K[H])^2-4K^2[G][H]}}{2K[H]} \quad (2)$$

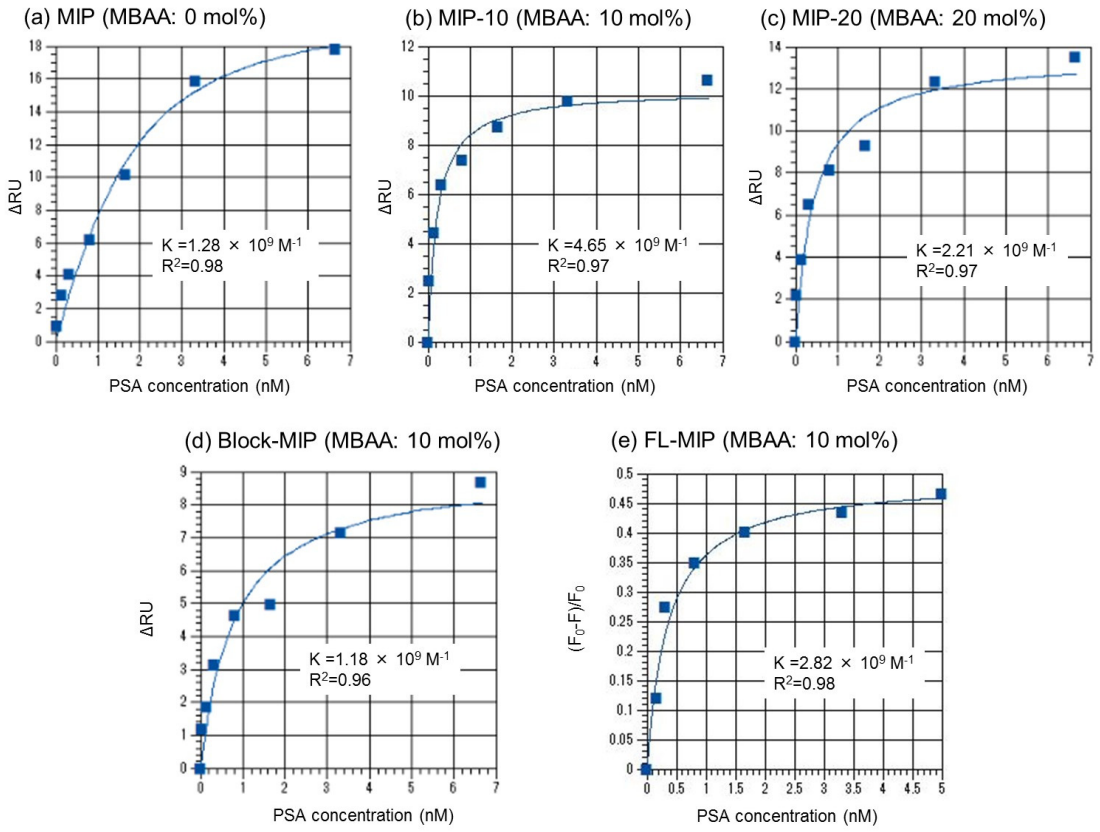


Figure S5. Apparent affinity constants (*Ka*) for the binding of PSA to MIP (MBAA: 0 mol%) (a), MIP-10 (MBAA: 10 mol%) (b), MIP-20 (MBAA: 20 mol%) (c), Block-MIP (MBAA: 10 mol%) (d), FL-MIP (MBAA: 10 mol%)

16. 2nd PIM

To introduce fluorescent reporter molecules, the PIR-C the MIP thin layer was immersed in the Alexa Fluor 647 carboxylic acid succinimidyl ester DMSO solution (50 µM, 2 mL) for 1 h, at 25 °C. The sensor chip was washed with DMSO and pure water and the substrates were further immersed in pure water for 30 min at the same temperature and in a dark environment. Then, the blocking treatment was carried out

by immersing the sensor chip in 1 mg/mL HSA dissolved in 10 mM phosphate buffer (pH 7.4) for 30 min at 25 °C. After that, the sensor chip was washed with pure water, yielding FL-MIP.

To identify that the fluorescent molecules were labelling into the molecularly imprinted nanocavities, an automatic fluorescent detection system based on a pipette tip-type biosensor, reported previously, was employed.[2, 3] The analyser consists of an automatic pipetting device, a tip rack, a reagent rack, an incubation port, a detector, and a computer. The plastic pipette tip can settle a small gold-coated glass substrate (reaction plate, 5 × 10 mm), and the tip was set at the tip rack. The device automatically demonstrated operations, such as pipetting reagents, incubation, washing, and fluorescent detection as programmed in the computer.

After the suction of the 10 mM phosphate buffer (pH 7.4) into the pipet tip setting the MIP-formed substrate, the fluorescent image was obtained from the detection port before and after introducing the fluorescent molecules. Five different ROIs were obtained in order to calculate the fluorescence intensity in each substrate. The light of the xenon lamp passed through a band-pass filter (628 ± 20 nm) and fluorescence was measured using a fluorescent microscope (Zyla SCMOS, Andor Technology, UK) with filter (692 nm ± 20 nm).

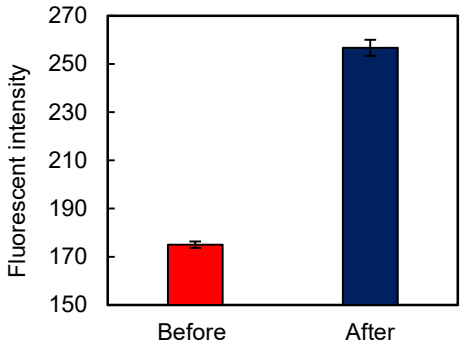


Figure S6. Fluorescent intensities before and after the introduction of Alexa Fluor 647 carboxylic acid succinimidyl ester for PIR-C introduced MIP thin layer

17. Fluorescent detection

The automatic fluorescent detection system was based on a pipette tip-type biosensor (reported previously) and was used to obtain the fluorescent detection of proteins using FL-MIP[2, 3]. The FL-MIP thin layer-immobilised substrate was incubated in a PSA solution (0, 0.033, 0.167, 0.33, 0.83, 1.67, 3.33, and 6.67 nM concentrations dissolved in 10 mM phosphate buffer (pH 7.4) and in a 10 mM phosphate

buffer (pH 7.4, 100 μL) for 1 min. After discharging the samples, 10 mM phosphate buffer (pH 7.4, 100 μL) was suctioned into the pipet tip. Afterwards, the fluorescent intensity was measured at the detection port. Ten different ROIs were obtained to calculate the fluorescence intensity in each sensor chip. F_0 and F were fluorescent intensities derived from FL-MIP before and after incubation with proteins, respectively.

In order to investigate the selectivity of the FL-MIPs and IgG solutions dissolved in 10 mM phosphate buffer (pH 7.4) (final concentrations: 0.83 nM) were used for binding experiments as the reference protein. The selectivity factor was calculated from the following equation:

$$selectivity\ factor = \frac{(F - F_0)/F_{0_{IgG}}}{(F - F_0)/F_{0_{PSA}}}$$

Where $(F\text{-}F_0)/F_{0\ IgG}$ and $(F\text{-}F_0)/F_{0\ PSA}$ were $(F\text{-}F_0)/F_0$ values incubated with 0.83 nM of IgG and PSA, respectively.

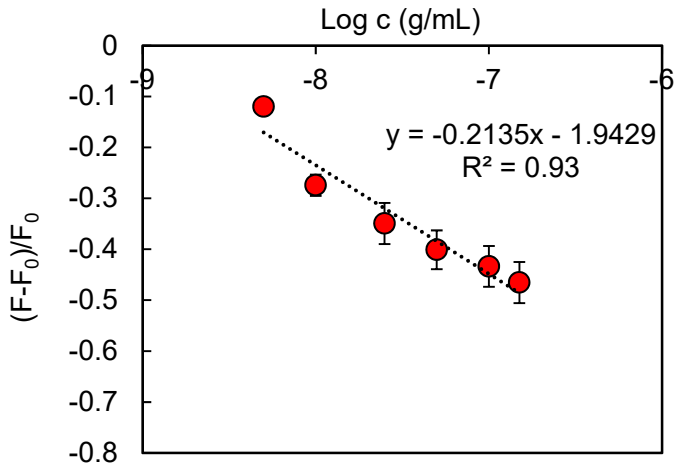


Figure S7. Fluorescent response of FL-MIP toward PSA.

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