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Molecularly Imprinted Protein Recognition Cavities Bearing Exchangeable Binding Sites for Postimprinting Site-Directed Introduction of Reporter Molecules for Readout of Binding Events

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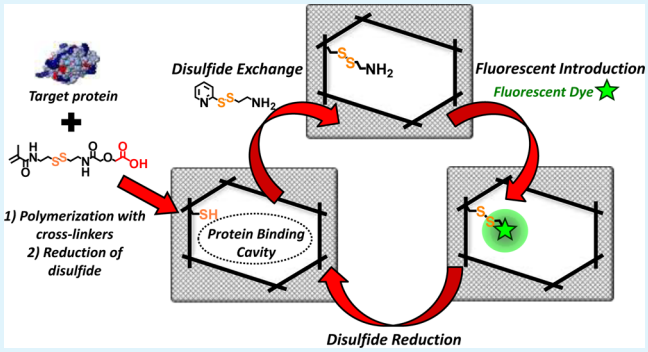
Supporting Information

ABSTRACT: Protein-imprinted cavities bearing exchangeable domains to be used for postimprinting fluorophore introduction to transform binding events into fluorescence changes were constructed in molecularly imprinted polymer (MIPs) matrixes prepared on glass substrates. Copolymerization was performed with acrylamide, *N*,*N*′-methylenebisaclylamide, and a newly designed functional group-exchangeable monomer, ({[2-(2-methacrylamido)ethyldithio]ethylcarbamoyl}-methoxy)acetic acid (MDTA), in the presence of a model basic protein, lysozyme (Lyso); MDTA can interact with Lyso and assemble close to Lyso in the resulting polymer. After removal of Lyso, followed by a disulfide reduction to cleave the (ethylcarbamoylmethoxy)acetic acid moiety from the MDTA residues, the exposed thiol groups within the imprinted cavities were modified by aminoethylpyridyldisulfide to be transformed into aminoethyl groups that function as active sites for amine-reactive fluorophores. Fluorescein isothiocyanate (FITC) was then coupled with the aminoethyl groups, yielding site specifically FITC-modified signaling imprinted cavities for Lyso binding. Because the in-cavity fluorescent labeling was achieved via a disulfide linkage, it was easy to remove, exchange, and/or replace amine-reactive fluorophores. This facilitated the screening of fluorophores to select the highest readout for binding events, replace fluorophores when photobleaching occurred, and introduce other functions. The proposed molecular imprinting process, combined with postimprinting modifications, is expected to provide an affordable route to develop multifunctional MIPs for specific detection of protein binding events.



KEYWORDS: *Molecular imprinting, synthetic receptor, fluorescence, protein sensing, postimprinting modification*

INTRODUCTION

Biomolecular recognition materials are of great importance in life sciences research, for example, in diagnosis and development of disease therapeutics. Usually, antibodies and enzymes are used as molecular recognition elements for these purposes because of their high affinity and selectivity toward target molecules. However, these molecules possess some disadvantages, including high cost, low stability, and difficulty of mass production.[1,2] Therefore, robust materials with the ability to recognize biomolecules are desirable as substitutes for such biomolecules. Molecular imprinting is a promising method for preparing artificial receptors bearing predetermined binding cavities capable of recognizing target molecules, including biomolecules, such as peptides and proteins.[3−20] Molecularly imprinted polymers (MIPs) have been prepared by copolymerization of a template molecule and functional monomers in the presence of cross-linkers. After removal of the template molecule, binding cavities of a size and shape complementary to the template molecule were generated in the polymer matrixes.

MIPs with fluorescent properties have attracted attention for detecting binding events, owing to their high sensitivity and simplicity of operation.[21] Previously, various fluorescent MIPs have been prepared using fluorescent functional monomers.[22−25] These reports showed that the prepared fluorescent MIPs could selectively transduce target binding events into fluorescent changes. However, when using fluorescent monomers for preparation of MIPs, especially those prepared using a noncovalent imprinting process, the obtained MIPs often show high background fluorescence because of the presence of randomly positioned fluorescent functional monomer residues in the polymer matrixes.

Recently, we have developed a new method to introduce fluorophores within imprinted binding cavities using postimprinting modifications (PIMs), which are in-cavity chemical modifications to introduce additional functions after the construction of specific binding cavities in MIPs.[26−31] Site-directed introduction of fluorophores into the binding cavities by PIMs is a promising method to decrease the background

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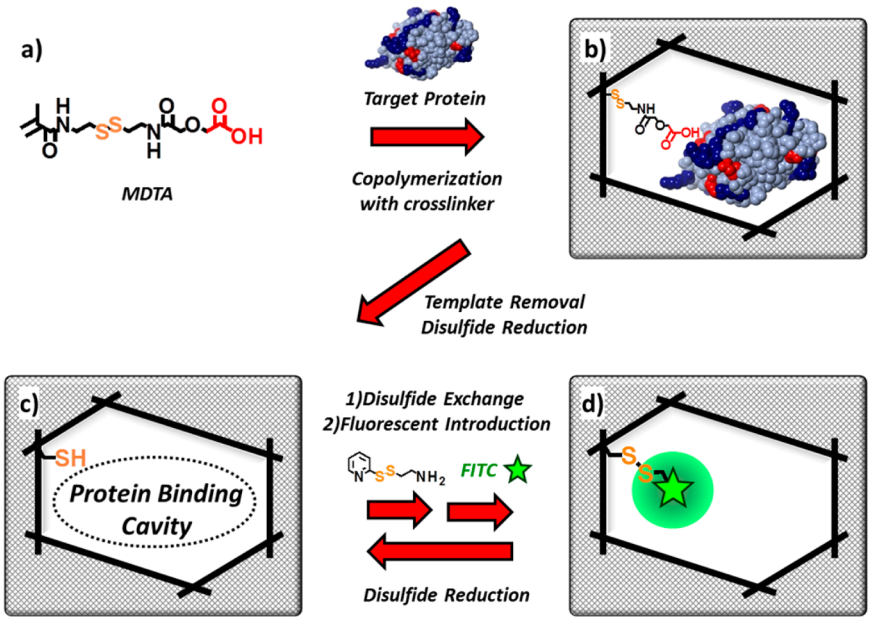

Figure 1. Schematic illustration of a lysozyme-imprinted polymer preparation combined with postimprinting modifications: (a) chemical structure of MDTA, (b) protein-imprinted polymer prepared using MDTA, (c) binding cavity created by reduction of the disulfide linkage, and (d) fluorophore introduction to an amino group by reformation of the disulfide linkage by APDS.

signal derived from the emissions caused by fluorophore residues randomly located outside the imprinted binding cavities.[32−34] Furthermore, combined with a capping process to hinder functional monomer residues located in low-affinity binding cavities, fluorophores were selectively introduced into high-affinity binding cavities, resulting in selective fluorescent detection of target proteins.[34] Although the PIMs described above are advantageous, these fluorophores are normally labeled into polymer matrixes by covalent bonding; therefore, it is always difficult to exchange or replace these fluorophores for optimization of sensitivity and selectivity.

To address this issue, we designed a functional monomer bearing an exchangeable moiety in which a disulfide linkage was assembled in the monomer that can be cleaved by reduction and formed by reaction with pyridyldisulfide compounds. Here, we synthesized a new monomer bearing a polymerizable methacryl group, a carboxylic acid group for interaction with a target protein, and a disulfide linkage as an exchangeable moiety for introducing a fluorophore, ({[2-(2-methacrylamido)-ethyldithio]ethylcarbamoyl}methoxy)acetic acid (MDTA, Figure 1a). Lysozyme (Lyso) was employed as a model protein in this study, and Lyso-imprinted polymers (Lyso-IPs) were prepared on glass substrates by copolymerization with MDTA, using acrylamide as a functional monomer and *N*,*N*′-methylenebis(acrylamide) (MBAA) as a cross-linker, in the presence of Lyso (Figure 1b). In the first PIM, after the removal of Lyso, resulting in the creation of the Lyso binding cavities, the residual (ethylcarbamoylmethoxy)acetic acid moiety within the cavities was removed by reduction (Figure 1c). In the second PIM, the disulfide linkage was reformed using aminoethylpyridyldisulfide (APDS) to introduce aminoethyl groups (Figure 1d), followed by treatment with fluorescein isothiocyanate (FITC) to label the amino groups within the cavities, in the third PIM. The exchangeability and tunability of the prepared MIPs were evaluated by taking fluorescent measurements to demonstrate the effectiveness of the proposed method.

EXPERIMENTAL SECTION

Materials. *N*,*N*′-Diisopropylethylamine (DIEA), triethylamine (TEA), sodium chloroacetate, sodium *N*,*N*′-diethyldithiocarbamate, ribonuclease A (RNase A), sodium chloride, sodium sulfate, citric acid, sodium hydrogen carbonate, sulfonic acid, hydrogen peroxide (30% H_2O_2), diethyl ether, methanol, and dichloromethane were purchased from Nacalai Tesque (Kyoto, Japan). Methacryloyl chloride, acrylamide, *N*,*N*′-methylenebis(acrylamide) (MBAA), sodium dihydrogen phosphate dihydrate (NaH_2PO_4), sodium dodecyl sulfate (SDS), acetic acid, *n*-hexane, ethyl acetate (EtOAc), and tris(hydroxymethyl)aminomethane (Tris) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Di-*tert*-butyldicarbonate and 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide, hydrochloride (EDC·HCl) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). Rhodamine B isothiocyanate, diglycolic anhydride, lysozyme (Lyso), and human serum albumin (HSA) were purchased from Sigma-Aldrich (USA). Hydrochloric acid (HCl, 4 M in dioxane), 2,2′-dipyridyl disulfide, tetraethylene glycol, cystamine dihydrochloride, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), and cysteamine were purchased from Tokyo Chemical Industry (Tokyo, Japan). 3-Methacryloxypropyltrimethoxysilane was purchased from Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Fluorescein isothiocyanate (FITC) was purchased from DOJINDO Laboratories (Kumamoto, Japan). Dichloromethane was distilled prior to use.

Preparation of Designed Functional Monomer, MDTA (4). *Preparation of N-tert-Butoxycarbonyl Cystamine (1).* Compound **1** was prepared by a modified method reported by Jacobson et al.[35] Di-*tert*-butyldicarbonate (900 mg, 4.0 mmol) and triethylamine (1.68 mL, 12 mmol) were added to a methanolic solution (25 mL) containing cystamine dihydrochloride (916.7 mg, 4.2 mmol). After 3 h of incubation at room temperature, the solvent was evaporated, and 1 M NaH_2PO_4 was added (20 mL, pH 4.2). The aqueous phase was washed

with diethyl ether to remove di-*tert*-Boc-cystamine as a byproduct. The aqueous solution was basified to pH 9 with 1 M NaOH and extracted with EtOAc (10 mL × 2). The combined organic phases were dried over Na_2SO_4 and evaporated to yield **1** (435.8 mg, 43%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.92 (br, 1H, carbamide), 3.45 (t, 2H, $-CH_2-$, J = 3.1), 3.01 (t, 2H, $-CH_2-$, J = 3.1), 2.79 (q, 4H, $-CH_2-CH_2-$, J = 6.0), 1.45 (s, 9H, *t*-Bu).

*Preparation of N-tert-Butoxycarbonyl-N′-methacryloyl-cystamine (**2**).* Methacryloyl chloride (193 μL, 2.00 mmol) was dissolved in CH_2Cl_2 (10 mL) and was added to CH_2Cl_2 (15 mL) containing **1** (435.8 mg, 1.73 mmol) and DIEA (450 μL, 3.00 mmol). The reaction mixture was stirred at 0 °C for 1 h, and an additional 5 h of incubation at room temperature. The mixture was washed with saturated $NaHCO_{3aq}$, saturated citric $acid_{aq}$, and brine, and then the combined organic phases were dried over Na_2SO_4 and evaporated. The obtained crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 2/3 to 1/1) to yield **2** (432.3 mg, 78.2%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.45 (br, 1H, amide), 5.75 (s, 1H, vinyl), 5.35 (s, 1H, vinyl), 4.97 (br, 1H, carbamide), 3.67 (q, 2H, F$-CH_2-$, J = 6.1), 3.46 (m, 2H, $-CH_2-$), 2.89 (t, 2H, $-CH_2-$, J = 6.1), 2.80 (q, 2H, $-CH_2-$, J = 6.0), 1.97 (s, 3H, methyl), 1.44 (s, 9H, *t*-Bu).

*Preparation of N-Methacryloyl Cystamine Hydrochloride (**3**).* Compound **2** (100 mg, 0.31 mmol) was dissolved in CH_2Cl_2 at 0 °C, and then 1.0 mL of 4 M HCl/dioxane (4.00 mmol) was added to the solution and stirred for 12 h. The hydroscopic salt was filtered and washed with diethyl ether to obtain the product as a white solid (quant.). ^{1}H NMR (300 MHz, *d*-DMSO): δ 8.18 (br, 1H, amide), 7.99 (br, 3H, $-NH_3Cl$), 5.67 (s, 1H, vinyl), 5.35 (s, 1H, vinyl), 3.41 (q, 2H, $-CH_2-$, J = 6.1), 3.08 (t, 2H, $-CH_2-$), 2.94 (t, 2H, $-CH_2-$, J = 6.1), 2.85 (q, 2H, $-CH_2-$, J = 6.0), 1.85 (s, 3H, methyl).

*Preparation of the Designed Functional Monomer, MDTA (**4**).* Compound **3** (79.4 mg, 0.31 mmol) and TEA (140 μL, 1.00 mmol) were dissolved in CH_2Cl_2, and then diglycolic anhydride (58 mg, 0.50 mmol) was added to the mixture and stirred for 1 h at 0 °C. After an additional 3 h of incubation at room temperature, the mixture was washed with saturated citric $acid_{aq}$ and brine. The combined organic phases were dried over Na_2SO_4 and evaporated to yield **4** (90%). ^{1}H NMR (300 MHz, *d*-DMSO): δ 8.10 (br, 1H, amide), 8.03 (br, 1H, amide), 5.66 (s, 1H, vinyl), 5.33 (s, 1H, vinyl), 4.11 (s, 2H, $-CH_2-$), 3.96 (s, 2H, $-CH_2-$), 3.42 (t, 4H, $-CH_2-$ CH_2-, J = 6.1), 2.85 (q, 4H, $-CH_2-$ CH_2-, J = 6.0), 1.84 (s, 3H, methyl). MALDI TOF-MS (Matrix; CHCA): calculated for $C_{12}H_{21}N_2O_5S_2$, 337.0886 $(M + H)^+$; found, 337.7946.

Preparation of Aminoethylpyridyldisulfide Hydrochloride (APDS, 5). Compound **5** was prepared by a modified method reported by van der Viles et al.[36] 2,2′-Dipyridyl disulfide (2.21 g, 10.0 mmol) was dissolved in 20 mL of methanol and 0.8 mL of acetic acid. To this solution was added within 30 min a solution of 2-aminoethanethiol hydrochloride (0.57 g, 5.0 mmol) dissolved in 10 mL of methanol. The reaction was stirred under argon for 24 h at room temperature. After evaporation of the solvent, the residual oil was washed twice with 20 mL of diethyl ether. The crude compound was dissolved in 5 mL of methanol, and the product was precipitated twice with 25 mL of diethyl ether. Then the crude product was dissolved in pure water and basified to pH 9 by 1 M NaOH and extracted with ethyl acetate (10 mL × 4). The combined organic phases were dried over Na_2SO_4 and evaporated. The obtained residue was dissolved in CH_2Cl_2 and 1 mL of 4 N HCl/dioxane was added. Then precipitate was collected to yield **5** (301 mg, 54.7%). ^{1}H NMR (300 MHz, D_2O): δ 8.40 (d, 1H, pyridinyl, J = 6.0), 7.99 (t, 1H, pyridinyl, J = 6.1), 7.84 (d, 1H, pyridinyl, J = 8.1), 7.41 (t, 1H, pyridinyl, J = 4.2), 3.22 (t, 2H, $-CH_2-$, J = 6.6), 2.97 (t, 2H, $-CH_2-$, J = 6.3).

Preparation of Hydrophilic Initiator (7). *Preparation of N,N-Diethyldithiocarbamideacetic Acid (**6**).* Compound **6** was prepared by a modified method reported by Tsujii and Kawaguchi.[37,38] Sodium chloroacetate (466 mg, 4 mmol) and sodium *N*,*N*-diethyldithiocarbamate (900.6 mg, 4 mmol) were dissolved in 10 mL of water. The reaction mixture was shielded from light and magnetically stirred for 2 days. The mixture was acidified to below pH 4 by 1 M HCl_{aq} for precipitation of the product. The precipitate was filtered and dried in vacuo to yield **6** (412 mg, 50%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.18 (s, 2H, $-CH_2-$), 4.03 (q, 2H, $-CH_2-$, J = 7.5), 3.78 (q, 2H, $-CH_2-$, J = 7.5), 1.34 (t, 3H, $-CH_3$, J = 6.0), 1.30 (t, 3H, $-CH_3$, J = 6.3).

*Preparation of the Hydrophilic Initiator (**7**).* Tetraethylene glycol (310 μL, 2.0 mmol), **6** (207 mg, 1.0 mmol), and TEA (280 μL, 2.0 mmol) were dissolved in CH_2Cl_2. Then EDC·HCl (286.5 mg, 1.5 mmol) was added to the mixture and stirred for 1 h at 0 °C. After an additional 12 h of incubation at room temperature, the mixture was washed with brine. The combined organic phases were dried over Na_2SO_4 and evaporated. The crude product was purified by silica gel column chromatography using an automated preparative chromatography system with an initial condition of ethyl acetate/hexane = 3/2 (v/v) (YAMAZEN, SCIENCE Inc., Japan), to yield **7** as clear oil (109.4 mg, 28.5%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.33 (t, 2H, $-CH_2-$, J = 4.8), 4.19 (s, 2H,), 4.03 (q, 2H, $-CH_2-$, J = 6.9), 3.8−3.6 (m, 16H, $-CH_2-CH_2-O-$, $-CH_2-$), 1.33 (t, 3H, methyl, J = 7.2), 1.26 (t, 3H, methyl, J = 7.2).

Preparation of Lysozyme-Imprinted Polymer (Lyso-IP) Film. *Methacrylation of Glass Substrates.* A glass substrate (9.7 mm × 11.7 mm) was immersed in piranha solution (30% H_2O_2:conc. H_2SO_4 = 1:3) for 15 min and washed with pure water. After the substrate was placed in a UV$-O_3$ cleaner (UV.TC.110, Bioforce Nanosciences, USA) for 20 min, the glass substrate was immersed in 1% 3-methacryloxy propyl trimethoxysilane solution containing 1% AcOH for 12 h. The substrate was washed with ethanol and baked at 110 °C for 1 min.

Preparation of Lysozyme-Imprinted Polymers (Lyso-IPs) on the Methacrylated Glass Substrates. Lysozyme (1.0 μmol), MDTA (2 μmol), acrylamide (58 μmol), MBAA (20 μmol), and the prepared photo initiator (**7**) (1% mol/mol with monomers) were dissolved in 1.0 mL of 0.1 M Tris/HCl buffer (pH 7.4), and N_2 gas was slowly bubbled through this solution for 5 min. A silicon rubber having a square hole (L 8.0 × W 6.0 × D 1.0 mm) was placed on the methacrylated glass substrate to make a shallow well, and 60 μL of the prepolymerization solution was dropped into the well and then sealed by a trimethylchlorosilane-treated cover glass chip. Polymerization was conducted under UV irradiation at 4 °C for 2 min (365 nm, 260 mW × 6, KeyChem-Lumino, YMC Co., Ltd. Japan), and after polymerization, the substrate was washed with pure water, methanol, 1 M $NaCl_{aq}$, and 0.5% (w/v) SDS solution to remove Lyso.

Postimprinting Modifications. *Cleavage of the Disulfide Linkage in Lyso-IP Thin Films.* For cleavage of the disulfide

linkage, the obtained polymer thin film prepared on glass substrate was immersed in 50 mM TCEP (in 0.1 M phosphate buffer, pH 6.3) solution for 12 h at room temperature, and then the glass substrate was washed with pure water.

Reformation of the Disulfide Linkage in Lyso-IP Thin Films. The disulfide-cleaved Lyso-IP thin film on the glass substrate was immersed in 10 mM APDS (**5**) solution (in phosphate buffer, pH 5) to introduce aminoethyl group in the Lyso-imprinted cavities via reformation of the disulfide linkage. To confirm the disulfide formation, absorbance at 343 nm in the supernatant was measured for the detection of 2-thiopyridone as a byproduct of the disulfide formation.

Postimprinting Introduction of FITC to Lyso-IP Thin Films. FITC (1 mM) dissolved in 0.1 M carbonate buffer (pH 9.2, 20 μL) was dropped onto the aminoethylated Lyso-IP thin film on the grass substrate, and after 30 min, the substrate was washed with pure water, acetone, and MeOH successively.

Fluorophores Exchange Test. The fluorescein-introduced Lyso-IP thin film was immersed in TCEP solution for the cleavage of the disulfide linkage. After the TCEP treatment, fluorescence was measured (λ_{ex}: 485 nm) to confirm the removal of fluorescein moiety. The film was then immersed in the APDS solution to introduce the aminoethyl groups again, and the FITC solution was dropped onto the film to reintroduce fluorescein moiety. This procedure was repeated in triplicate.

Readout of Binding Behavior by Fluorescent Measurements. The FITC-treated Lyso-IP thin film on the glass substrate was placed in 10 mM Tris/HCl buffer (pH 7.4), and fluorescence was measured (λ_{ex}: 485 nm/λ_{Em}: 520 nm, 25 °C. 1 cm cell) by an Hitachi F-2500 spectrofluorometer (Hitachi, Japan) with various concentrations of Lyso (0–3.0 μM). Selectivity tests were performed by incubation with 1 μM of HSA, and RNase A.

RESULTS AND DISCUSSION

When amine-reactive fluorophores are adopted for labeling within molecularly imprinted cavities to yield a reporter function for binding events, the amino groups should be located there; an effort has been made to develop fluorescent MIPs prepared using an amine-containing functional monomer.[32] However, it appears to be difficult to maintain a stable interaction between the amine-containing functional monomers and basic proteins during the molecular imprinting process due to electrostatic repulsion; thus, the use of such amine-containing functional monomers toward basic proteins may result in low-affinity binding.

Here, we designed a functional monomer that has an exchangeable interaction site capable of binding to basic proteins during the molecular imprinting process and after the creation of protein-imprinted cavities; the interaction site can be replaced by an amino group for site-specific labeling with amine-reactive fluorophores. In this context, MDTA was synthesized, which has an alignment with a methacryl group for radical polymerization, a cleavable disulfide linker, and a carboxy group for interacting with basic target proteins (Figure 1a). Because disulfide bonds are formed reversibly under mild conditions, disulfide exchange reactions can be used to replace the original functionality with any functionality. Because its carboxylate moiety is negatively charged at neutral pH, MDTA can maintain an interaction with basic proteins during polymerization as was the case for an acidic functional monomer commonly used for MIPs toward basic proteins. Following the molecular imprinting process, the carboxyl group can be replaced with an aminoethyl group by a disulfide exchange reaction using APDS, which is used as the labeling site for amine-reactive fluorophores.

Lyso was used as a model target protein and Lyso-IP thin films (Lyso-IPs) were prepared on methacrylated glass substrates by photoinitiated radical polymerization of a prepolymerization mixture containing MDTA, acrylamide as a functional monomer, and MBAA as a cross-linker, and the prepared photoinitiator. Following polymerization, Lyso was removed and the disulfide linkages of the MDTA residues were reduced by TCEP, yielding free thiol residues within the Lyso binding cavities. The exposed thiol residues were treated with APDS, reforming the disulfide linkages to introduce the aminoethyl groups as the fluorophore-labeling sites. The quantity of reformed disulfide linkages was estimated to be 0.5 nmol/mm^2, calculated using the results of UV–vis spectra (Figure S1 of the Supporting Information) derived from 2-thiopyridone, which is a byproduct of the disulfide exchange reaction between a thiol group and a pyridyldithio group.[39] Next, FITC was introduced as a reporter molecule for readout of protein binding events. The obtained Lyso-IP showed fluorescence emission at 520 nm with an excitation wavelength of 485 nm, which could be derived from the labeled fluorescein residues in Lyso-IP. Thus, it was confirmed that fluorescein was successfully introduced into the amino group located on the MDTA residues within the imprinted cavity.

To examine exchangeability, repetitive cleavage and formation of the disulfide linkage in Lyso-IP were performed. The fluorescence intensity derived from the fluorescein residues was reduced by ca. 65% when the disulfide was cleaved by TCEP (Figure 2). Three consecutive aminoethylations by APDS and

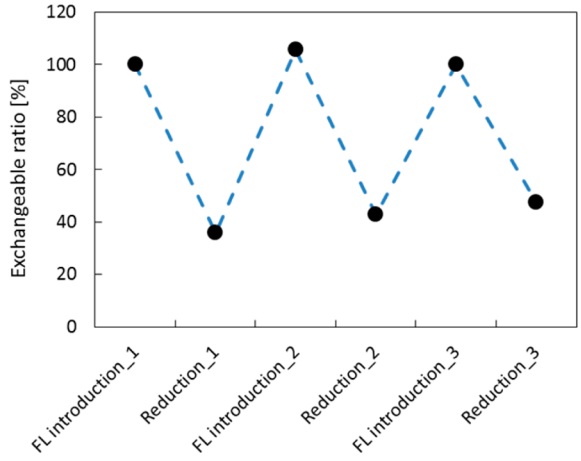

Figure 2. Repetitive removal and introduction of the FITC moiety by the PIMs.

subsequent FITC treatment reproducibly recovered the fluorescence, indicating that the prepared Lyso-IP is capable of reversible introduction of a fluorophore via disulfide linkage. The exchanging and tuning activities of Lyso-IP were further confirmed using other amine-reactive fluorophores, DBD-F (λ_{ex}: 450 nm) and rhodamine B isothiocyanate (λ_{ex}: 530 nm), in the same manner. Fluorescence intensities at 530 and 575 nm, derived from DBD-F and rhodamine B, respectively, were observed (see Figure S2), indicating that these fluorophores were successfully introduced by the three-step PIMs, including the disulfide linkage reduction, the aminoethyl group

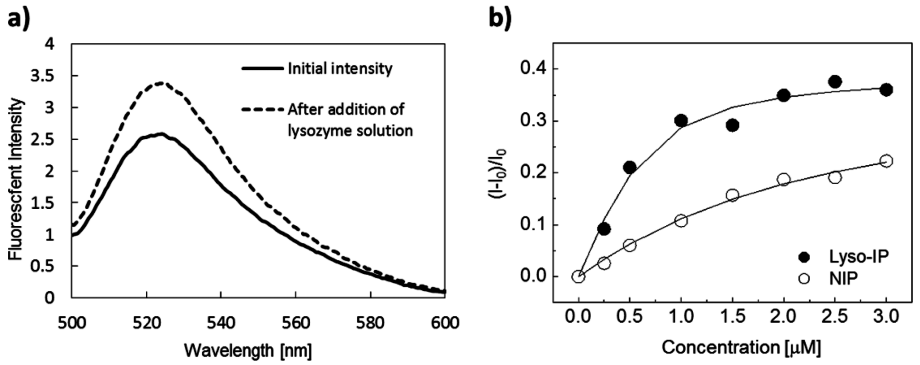

Figure 3. (a) Changes in fluorescence spectra after addition of Lyso (conc. 1.0 μM) (Ex: 485 nm). (b) Relative fluorescence changes of Lyso-IP and NIP with addition of Lyso solution.

introduction, and the fluorophore labeling. Therefore, it appears that a diverse range of amine-reactive fluorophores can be used as fluorescent reporter molecules for the detection of protein binding events.

The relative change in fluorescent intensity increased with the addition of Lyso (Figure 3a) and the value was saturable (Figure 3b), indicating that Lyso can be bound to the limited number of binding sites in the imprinted cavities labeled by FITC. To confirm that Lyso binding can be read out by observing fluorescence change, surface plasmon resonance (SPR) measurements were performed of Lyso-IP on gold-coated SPR sensor chips. A change in SPR response occurred due to a change in the surface permittivity, which is influenced by binding events. From the SPR data, the binding constant was estimated to be 2.26×10^6 M^{-1} with binding based on 1:1 stoichiometry using curve-fitting software (Figure S3).

The apparent binding constant was also estimated from observed fluorescent change (Figure 3b) and was calculated to be 3.14×10^6 M^{-1} (Lyso-IP), which is consistent with that obtained from the SPR data, suggesting that Lyso binding events can be successfully transduced into fluorescence change. These figures are comparable to previously reported Lyso-imprinted materials[32,40−42] but are still lower than those of natural antibodies by 1−3 orders of magnitude.[43] For the NIP, the binding constant was estimated to be 2.70×10^5 M^{-1}, revealing that the affinity of Lyso-IP is enhanced by the imprinting process and the designed functional monomer interacted with Lyso during polymerization as expected.

The selectivity of Lyso-IP was evaluated using HSA (66 kDa, *p*I = 4.8) and RNase A (13.7 kDa, *p*I = 9.6) as reference proteins. HSA and RNase A both yielded less fluorescent change than Lyso (Figure 4). The statistical significance between the data set of Lyso binding and those of the reference proteins in Lyso-IP was confirmed by a *t* test in which the rejection region was set at 0.05. HSA is known to bear hydrophobic domains, leading to the assumption that HSA binding may provide a hydrophobic environment around the fluorescein residues, resulting in the enhancement of fluorescent intensity.[44,45] Nevertheless, HSA showed lower response than Lyso. RNase A contains positively charged amino groups, which may interact nonspecifically with acrylamide-based polymers due to their weak hydrogen bonding and ion-exchange abilities. These results indicate that specific binding cavities were created in Lyso-IP by the imprinting process and that the PIMs strategy was successfully used here to introduce fluorophore into the cavities.

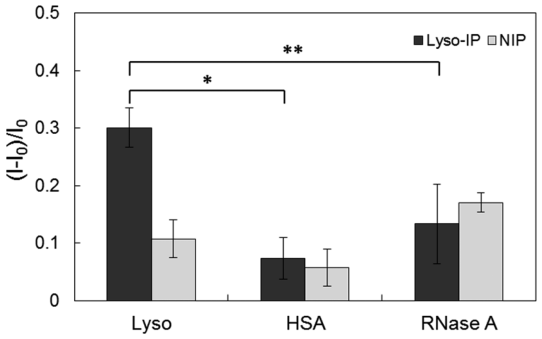

Figure 4. Selectivity of Lyso-IP and NIP for the tested proteins (1 μM). *t* test: *, ** < 0.05 (n = 3).

CONCLUSION

A newly designed functional group-exchangeable monomer, MDTA, containing a disulfide linkage for reversible introduction of the aminoethyl group that was the labeling site for amine-reactive fluorophores within the imprinted cavities was synthesized, and fluorescent Lyso-IP was prepared using three-step PIMs. The prepared fluorescent MIPs were able to transduce the binding events into fluorescence changes, and the fluorescent response was confirmed to be selective toward the target protein, Lyso. It was demonstrated that different functional groups can be combined with the present disulfide-based monomer, yielding new insight into protein-imprinted polymer preparation strategy. Conventional fluorophore conjugation into a binding cavity by covalent bonding is difficult to exchange or replace these fluorophores for optimization of sensitivity and selectivity. Therefore, the present technique using a functional group-exchangeable monomer in conjunction with PIMs will provide a novel method to develop protein recognition materials possessing specific signal transduction activity.

ASSOCIATED CONTENT

Supporting Information

UV−vis spectra of 10 mM APDS solution before and after incubation of MIP film in Figure S1: Fluorescent spectra of MIP films labeled by DBD-F or rhodamine B isothiocyanate in Figure S2. SPR data in Figure S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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