Electronic Supplementary Information

Molecularly Imprinted Polymers Prepared Using Protein-Conjugated Cleavable Monomers Followed by Site-Specific Post-Imprinting Introduction of Fluorescent Reporter Molecules

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Experimental Section

Materials

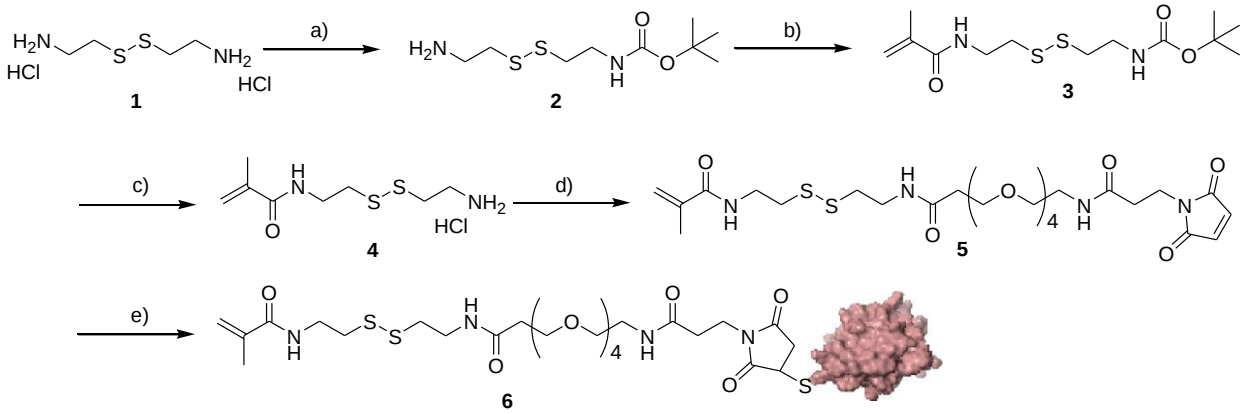
N,*N*′-Diisopropylethylamine (DIEA), triethylamine (TEA), *N*,*N*′-diethyldithiocarbamate, glutaric anhydride, sodium chloride, citric acid, sodium carbonate, ribonuclease A (RNase A), cytochrome c (Cyt), human serum albumin (HSA), hemoglobin (Hb), trichloromethane ($CHCl_3$), and dimethyl sulfoxide (DMSO) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Acrylamide, *N*,*N*′-methylenebisacrylamide, methacryloyl chloride, 2-({2-[bis(carboxymethyl)amino]ethyl}(carboxymethyl)amino) acetic acid (EDTA), acetic acid, hexane, acetone, acetonitrile (CH_3CN), ethanol (EtOH), diethyl ether (Et_2O), *N*,*N*-dimethylacetamide (DMA), methanol (MeOH), ethyl acetate (AcOEt), and dichloromethane (CH_2Cl_2) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cystamine dihydrochloride, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), β-mercaptoethanol, *N*-(9-acridinyl)maleimide and tris(2-carboxyethyl)phosphine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Sodium chloroacetate and bis(acryloyl)cystamine were purchased from Sigma-Aldrich Co. (St. Louis, USA). Di-*tert*-butyl dicarbonate and 4N hydrogen chloride in 1,4-dioxane (4N HCl/dioxane) were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). *N*-Hydroxysuccinimidyl-15-(3-maleimidopropionyl)-amido-4,7,10,13-tetraoxapentadecanoate (MAL-dPEG$_4$-NHS ester) was purchased from Toyobo Co., Ltd. (Osaka, Japan). Cy^{TM}5 Maleimide Mono-reactive Dye 5-Packs (Cy5 maleimide), Au-coated SPR sensor chips and a Vivaspin 20 instrument were purchased from GE Healthcare (Tokyo, Japan). One vial of Cy5 maleimide was dissolved in of 50 μL DMF prior to use. Immunoglobulin G (IgG) was purchased from MP Biomedicals LLC (USA), and a Float-A-Lyzer G2 (MWCO: 8–10 kD) was purchased from Spectrum® Laboratories, Inc. (USA).

Apparatus

^{1}H NMR spectra were measured using a 300 Hz FT-NMR apparatus (JNM-LA300 FT NMR system, JEOL Ltd., Tokyo, Japan). MALDI-TOF mass spectra were measured using a MALDI-TOF MS apparatus (Voyager-DE^{TM}-1000, AB SCIEX, Tokyo, Japan). Circular dichroic spectra were measured using a circular dichroism spectrometer (J-725, JASCO Corporation, Tokyo, Japan). Fluorescence spectra were recorded on a

fluorescence spectrophotometer (F-2500, Hitachi High-Technologies Corporation, Tokyo, Japan). Surface plasmon resonance (SPR) measurements were performed on a Biacore 3000 (GE Healthcare Japan, Tokyo, Japan).

Syntheses of maleimide-type cleavable monomer and template molecule 6 (Scheme 1)



Scheme 1. Syntheses of maleimide-type cleavable monomer **5** and **template molecule 6**: a) di-*tert*-butyl dicarbonate/triethylamine, MeOH, 0°C, 6 h, 41.7%; b) methacryloyl chloride/*N*,*N*-diisopropylethylamine, CH_2Cl_2, 0°C, 24 h, 90.7%; c) HCl/dioxane, CH_2Cl_2, 0°C, 24 h, 100%; d) MAL-dPEG$_4$-NHS ester/*N*,*N*-diisopropylethylamine, DMA, r.t., 12 h, 39.3%; e) Cyt, 10 mM phosphate buffer (pH 7.3), 4°C, 2days.

N-*tert*-Butoxycarbonyl cystamine (**2**)

Cystamine dihydrochloride (2.252 g, 10.0 mmol) and triethylamine (4.18 mL, 30.0 mmol) were dissolved in MeOH (30 mL) and added to a methanolic solution (20 mL) of di-*tert*-butyl dicarbonate (1.091 g, 5.00 mmol). The reaction mixture was stirred for 6 h at 0°C, after which the solution was evaporated, and 1 M NaH_2PO_4 was added to the residue. The aqueous solution was washed with diethyl ether to remove di-*t*-Boc-cystamine. The aqueous solution was adjusted to pH 9 by addition of 1 M NaOH, and the solution was extracted with AcOEt. The organic phase was dried over $MgSO_4$ and then evaporated. The resulting yellow oil was dried *in vacuo* to give **2**. Yield: 0.526 g (41.7%). TLC: R_f 0.35 (MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.97 (b, 1H, NH-Boc), 3.47 (q, 2H, C(=O)-NHCH_2), 3.03 (t, 2H, CH_2NH_2), 2.80 (q, 4H, CH_2SSCH_2), 1.46 (s, 9H, CH_3).

N-*tert*-Butoxycarbonyl-*N*'-methacryloylcystamine (**3**)

Compound **2** (0.576 g, 2.28 mmol) and *N*,*N*-diisopropylethylamine (0.79 mL, 4.56 mmol) were dissolved in CH_2Cl_2 (20 mL) and added to methacryloyl chloride (0.29 mL, 2.96 mmol) dissolved in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 24 h at 0°C, after which the reaction solution was washed with brine. The organic phase was evaporated and purified by silica gel column chromatography (eluent: hexane:AcOEt = 1:1). Compound **3** was obtained as a yellow solid. Yield: 0.634 g (90.7%). TLC: R_f 0.40 (hexane:AcOEt = 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δδ 6.52 (b, 1H, C(=O)-NH), 5.73 5.33 (s, 2H, H_2C=C(CH_3)C(=O)-), 5.03 (b, 1H, NH-Boc), 3.62 (q, 2H,

C(=O)-NHCH$_2$), 3.43 (q, 2H, CH$_2$NH-Boc), 2.86 (t, 2H, C(=O)-NHCH$_2$CH$_2$S), 2.79 (t, 2H, SCH$_2$CH$_2$NH-Boc), 1.96 (s, 3H, H$_2$C=C(CH$_3$)C(=O)-), 1.46 (s, 9H, CH$_3$).

N-Methacryloylcystamine (**4**)

Compound **3** (0.304 g, 0.99 mmol), dissolved in CH_2Cl_2 (40 mL), was added to 4 N HCl/dioxane (0.745 mL, 2.98 mmol) in CH_2Cl_2 (20 mL), and stirred for 24 h at 0°C. The solution was evaporated to give the crude product as a white solid, which was washed with diethyl ether and dried *in vacuo*. Yield: 0.257 g (100%). ^{1}H NMR (300 MHz, (CD$_3$)$_2$SO): δ 8.19 (b, 1H, C(=O)-NH), 8.10 (b, 2H, NH$_2$), 5.76 5.68 (s, 2H, H$_2$C=C(CH$_3$)C(=O)-), 3.43 (q, 2H, C(=O)-NHCH$_2$), 3.08 (quint., 2H, CH$_2$NH$_2$), 2.96 (t, 2H, C(=O)-NHCH$_2$CH$_2$S), 2.86 (t, 2H, SCH$_2$CH$_2$NH$_2$), 1.86 (s, 3H, H$_2$C=C(CH$_3$)C(=O)-).

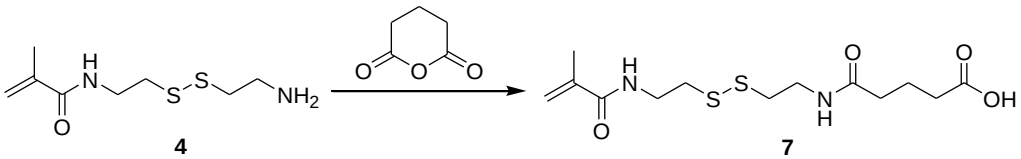
Maleimide-type cleavable monomer for conjugated template (**5**)

Compound **4** (0.028 g, 0.107 mmol) and *N*,*N*-diisopropylethylamine (0.019 mL) were dissolved in DMA (10 mL), after which MAL-dPEG$_4$-NHS ester (0.050 g, 0.097 mmol) was added to the solution. The reaction mixture was stirred for 12 h at room temperature and evaporated *in vacuo*. The residue was dissolved in $CHCl_3$ and washed with brine, and the organic phase was dried over $MgSO_4$ and evaporated. The resulting yellow oil was dried *in vacuo* to give **5**. Yield: 0.026 g (39.3%). TLC: R_f 0.24 ($CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.07 (b, 1H, CH$_2$C(=O)-NH(CH$_2$CH$_2$O)$_4$), 6.69 (s, 2H, CH=CH of maleimide), 6.63 (b, 1H, CH$_2$NHC(=O)-(CH$_2$CH$_2$O)$_4$), 6.57 (b, 1H, H$_2$C=C(CH$_3$)C(=O)-NHCH$_2$), 5.75, 5.35 (s, 2H, H$_2$C=C(CH$_3$)C(=O)-), 3.84 (t, 2H, CH$_2$N(CO)$_2$), 3.73 (t, 2H, CH$_2$C(=O)-NH(CH$_2$CH$_2$O)$_4$), 3.62 (t, 16H, NHC(=O)-CH$_2$(CH$_2$OCH$_2$)$_4$), 3.54 (m, 2H, CH$_2$NHC(=O)-(CH$_2$CH$_2$O)$_4$), 3.41 (q, 2H, H$_2$C=C(CH$_3$)C(=O)-NHCH$_2$), 2.86 (m, 4H, CH$_2$SSCH$_2$), 2.50 (m, 4H, C(=O)-CH$_2$(CH$_2$OCH$_2$)$_4$CH$_2$C(=O)-), 1.97 (s, 3H, H$_2$C=C(CH$_3$)C(=O)-). MS: 642.19 [M+Na].

Synthesis of template molecule; Cyt-conjugated cleavable monomer (**6**)

Native Cyt (11.7 mg, 0.95 μmol) and **5** (12 mg, 19.4 μmol) were dissolved in 10 mM phosphate buffer at pH 7.3 and stirred for 2 days at 4°C. The reaction mixture was dialyzed for 2 days in 10 mM phosphate buffer at pH 7.3 using a Float-A-Lyzer G2 device (MWCO: 8–10 kD) to remove unreacted monomers. The dialyzed solution was ultra-filtered using a Vivaspin 20 (4000 rpm, 10 min) and lyophilized to give **6**.

Carboxylic acid-type cleavable monomer (**7**)

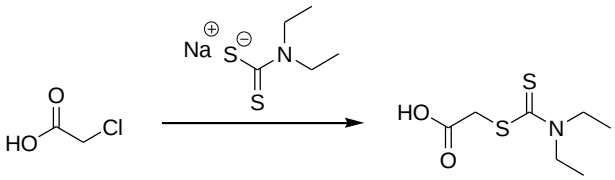


4 **7**

Compound **4** (0.128 g, 0.500 mmol) and *N*,*N*-diisopropylethylamine (0.174 mL, 1.00 mmol) were dissolved in CH_2Cl_2 (20 mL), and the solution was added to a CH_2Cl_2 solution (20 mL) of glutaric anhydride (0.074 g, 0.65

mmol) at 0°C. The reaction mixture was stirred for 1.5 h and then evaporated, and the residue was dissolved in AcOEt and washed with aqueous citric acid and brine. The organic phase was evaporated to precipitate a white solid, which was washed with AcOEt and dried *in vacuo* to give **7**. Yield: 0.042 g (25.2%). ^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 12.06 (s, 1H, COOH), 8.13 (s, 1H, $H_2C=C(CH_3)C(=O)$-NH), 8.03 (s, 1H, NHC(=O)-CH_2CH_2) 5.67, 5.35 (s, 2H, $H_2C=C(CH_3)C(=O)$-), 3.40 (m, 4H, $CH_2CH_2SSCH_2CH_2$), 2.80 (m, 4H, CH_2SSCH_2), 2.21 (t, 2H, CH_2COOH), 2.10 (t, 2H, $CH_2CH_2CH_2COOH$), 1.85 (s, 3H, $H_2C=C(CH_3)C(=O)$-), 1.70 (t, 2H, CH_2CH_2COOH).

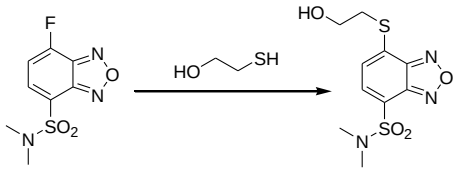
Preparation of water-soluble initiator



A water-soluble initiator was prepared by modification of a method reported by Tsuji and Kawaguchi.[1] Sodium chloroacetate (466 mg, 4 mmol) and sodium *N*,*N*-diethyldithiocarbamate (900.6 mg, 4 mmol) were dissolved in 10 mL of water, and the reaction mixture was stirred for 2 days at room temperature with light shielding. HCl was then added to the solution to precipitate the product, which was filtered and dried in vacuo. TLC: R_f 0.75 (MeOH). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.18 (s, 2H, CH_2COOH), 4.03 (q, 2H, NCH_2CH_3), 3.78 (q, 2H, NCH_2CH_3), 1.31 (quint., 6H, CH_3).

[1](a) T. Sato, S. Tsuji and H. Kawagauchi, *Ind. Eng. Chem. Res.*, 2008, **47**, 6358-6361. (b) S. Tsuji and H. Kawagauchi, *Macromolecules*, 2006, **39**, 4338-4344.

Preparation of 7-(2-hydroxyethylsulfanyl)-benzo[1,2,5]oxadiazole-4-sulfonic acid dimethylamide (coupling reaction between DBD-F and β-mercaptoethanol)



DBD-F (24.5 mg, 0.1 mmol) and sodium carbonate (31.8 mg, 0.3 mmol) were dissolved in CH_3CN (10 mL) and added to β-mercaptoethanol (42 μL, 0.6 mmol), and the reaction mixture was stirred for 2 h at 50°C. The solvent was removed by evaporation and the residue was dissolved in AcOEt. The organic phase was washed with brine and then evaporated and purified by silica gel column chromatography (eluent: hexane:AcOEt = 1:2) to give the product as a white solid. Yield: 27.0 mg (89.0%). TLC: R_f 0.53 (AcOEt). ^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 7.92 (d, 1H, C^5H of benzofurazane), 7.51 (d, 1H, C^6H of benzofurazane), 5.17 (t, 1H, OH), 3.78 (q, 2H, CH_2OH), 3.40 (t, 2H, CH_2S), 2.78 (s, 6H, NCH_3). MS: 327.94 [M+Na].

Preparation of glass substrate for introduction of polymerizable groups

A glass substrate (9.7 × 11.7 mm, Matsunami Glass Co., Ltd., Japan) was washed with methanol and pure water, and placed under UV-O_3 light for 30 min. The cleaned glass substrate was immersed in 1% 3-methacryloxypropyltrimethoxysilane aqueous solution (containing 1% acetic acid). After 24 h, the glass substrate was washed with ethanol and pure water and placed on a hot plate at 110°C for 1 min.

Preparation of Cyt-imprinted polymer using 6 (MIP), Cyt-imprinted polymer using 7 (comp-MIP) and non-imprinted polymer (NIP)

Pre-polymerization solutions for MIP, comp-MIP and NIP were prepared, as shown in Table 1, by dissolving each compound in 2.5 mL of 10 mM phosphate buffer (pH 7.3). The 3-methacryloxypropyltrimethoxysilane-modified glass substrate was covered by a silicone rubber sheet (9.7 × 11.7 mm) with a shallow well (6.0 × 9.0 mm), and 55 μL of the pre-polymerization solution was placed in the well, which was then sealed by a cover glass (24 × 24 mm) that had been treated with trimethylchlorosilane. To initiate polymerization, the reaction mixture was irradiated with LED light (365 nm, 250 mW, ThermoStage-AS, YMC Co. Ltd., Kyoto, Japan) from the bottom of the substrate for 1 h at 4°C. After polymerization, the resulting polymeric film was washed with ethanol and pure water.

Table 1. Recipe for pre-polymerization solution.[a]

	Cleavable monomer (0.83 μmol)	Functional monomer (27 μmol)	Cross-linker (114.6 μmol)
MIP	Cyt-conjugated-monomer **6**	Acrylamide	*N*,*N*′-methylenebis acrylamide
Comp-MIP	Monomer **7** + Cyt[b]		
NIP	Monomer **4**		

[a] All compounds were dissolved in 2.5 mL of 10 mM phosphate buffer (pH 7.3).

[b] Cyt (0.83 μmol) was added as a template to the pre-polymerization solution of comp-MIP.

Introduction of a fluorescent reporter molecule into the polymeric thin film by post-imprinting treatment

For disulfide linkage reduction accompanied by removal of Cyt, each polymeric thin film was immersed in 10 mM tris(2-carboxyethyl)phosphine solution (5 mL) for 4 h, followed by washing with pure water. The exposed thiol group in the polymeric film was allowed to react with thiol-reactive fluorescent dye (DBD-F). The polymeric thin film was immersed in 5 mM DBD-F solution (acetonitrile:borate buffer containing 1% EDTA = 1:1) and allowed to stand for 1 h at 50°C. After the reaction, the film was washed with methanol and pure water. *N*-(9-acridinyl)maleimide and Cy5 maleimide were also applied to the post-imprinting treatment. The polymeric thin film, in which thiol group was exposed by the same procedure for the DBD-F introduction, was immersed in 40 mM *N*-(9-acridinyl)maleimide solution (acetone : 100 mM borate buffer (pH 9.3) containing 1% EDTA = 1:1)

for 1h at room temperature. Similarly, the thiol-exposed film was immersed in 400 μL of 10 mM phosphate buffer (pH 7.3) dissolving Cy5 maleimide that was prepared by the addition of Cy5 maleimide dissolved in DMF (50 μL), and allowed to stand for 12 h at room temperature. After the reactions, each film was washed with methanol and pure water.

Fluorescence titration of proteins with prepared polymer films

MIP, comp-MIP and NIP, in which DBD-F was introduced by post-imprinting treatment, were placed in a cuvette (AS ONE Corporation, Osaka, Japan, 12.5 × 12.5 × 45 mm) containing 10 mM phosphate buffer (pH 7.3, 3 mL). The fluorescence intensity at 537 nm (Ex: 410 nm) was measured after addition of 20 μL of 30 μM Cyt solution. Hb, RNase A, HSA, and IgG were used as controls. The final concentration of each protein was varied from 0 to 1.0 μM. In the same way, MIPs, in which either *N*-(9-acridinyl)maleimide or Cy5 maleimide was introduced, were subjected to fluorescence titration of Cyt. Fluorescence intensities at 439 nm (Ex: 360 nm) and 668 nm (Ex: 650 nm) were measured for *N*-(9-acridinyl)maleimide and Cy5 maleimide, respectively.

Fluorescence titration of proteins with DBD-F conjugated with 2-mercaptoethanol (DBD-F derivative)

The DBD-F derivative (1.74 μmol, 0.53 mg) was dissolved in DMSO (58 μL) to give a 30 μM DBD-F solution, of which 20 μL was added to a cuvette filled with 10 mM phosphate buffer (pH 7.3, 3 mL). The fluorescence intensity was measured after addition of 20 μL of a 30 μM solution of each protein (Cyt, Hb, RNase A, HSA, and IgG). The final concentration of each protein was varied from 0 to 1.0 μM.

Preparation of polymeric thin film on Au substrate for SPR measurements

A gold sensor chip was washed with ethanol and water and placed under UV-O_3 light for 30 min. The sensor chip was immersed in a 5 mM ethanolic solution of bis(acryloyl)cystamine. After 24 h, the chip was washed with ethanol and pure water. Polymeric thin films of MIP and NIP were prepared on the Au sensor chip using the same manner of preparation as for the glass substrate. These films were then immersed in 10 mM tris(2-carboxyethyl)phosphine solution (5 mL) for disulfide linkage reduction. After 4 h, the films were allowed to react with DBD-F (in a 1:1 solution of acetonitrile and 100 mM borate buffer (pH 9.3) containing 1% EDTA) for 1 h at 50°C, followed by washing with methanol and pure water.

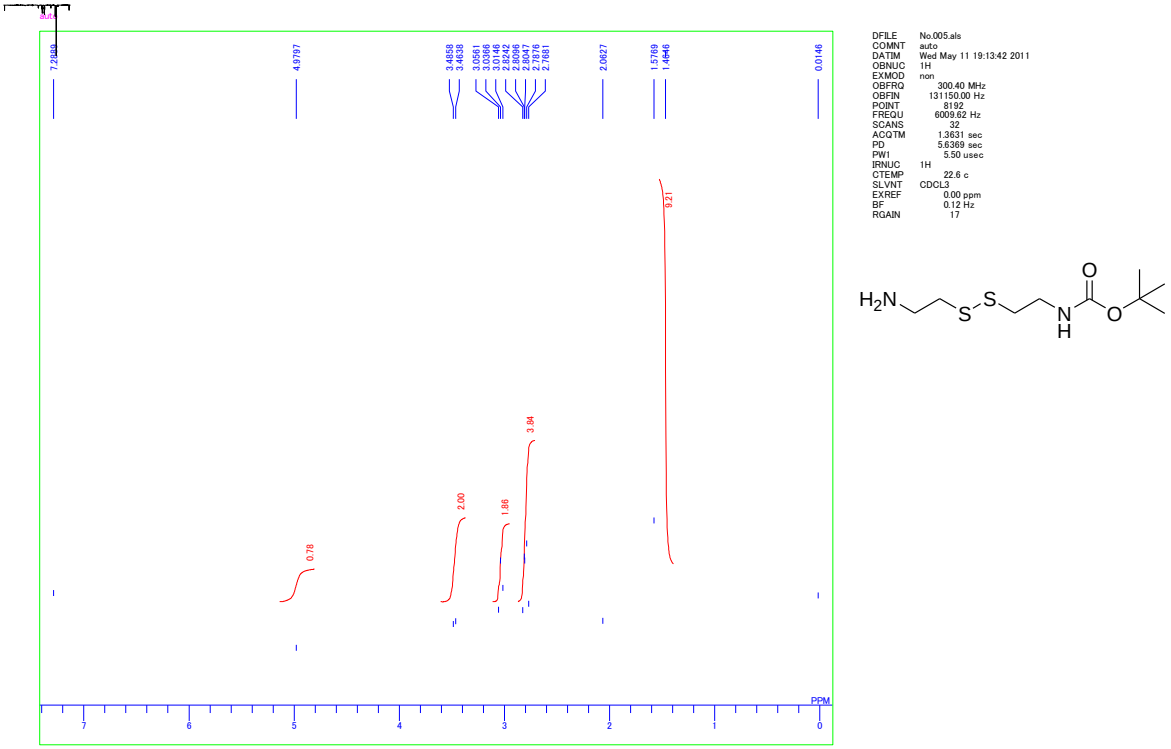
SPR measurements

Binding experiments for MIP and NIP prepared on the Au chip were performed at 25°C. Cyt, Hb, RNase A, HSA, and IgG, dissolved in 10 mM phosphate buffer (pH 7.3), were used for the experiments. The measurement conditions were as follows. Running buffer: 10 mM phosphate buffer (pH 7.3); flow rate: 20 μL/min; injection volume: 20 μL; regeneration solution: 10 mM phosphate buffer containing 1 M NaCl and 0.3% SDS aq. with 200 mM NaCl (flow rate: 30 μL/min, 30 s). The amounts of the bound proteins were calculated from the signal intensity (measured in resonance units, RU) 120 s after the end of each sample injection period. The concentration of each

protein was varied from 0 to 1.0 µM. Binding isotherms were drawn using ΔRU values for each protein concentration.

NMR charts

N-tert- Butoxycarbonyl cystamine, **2**



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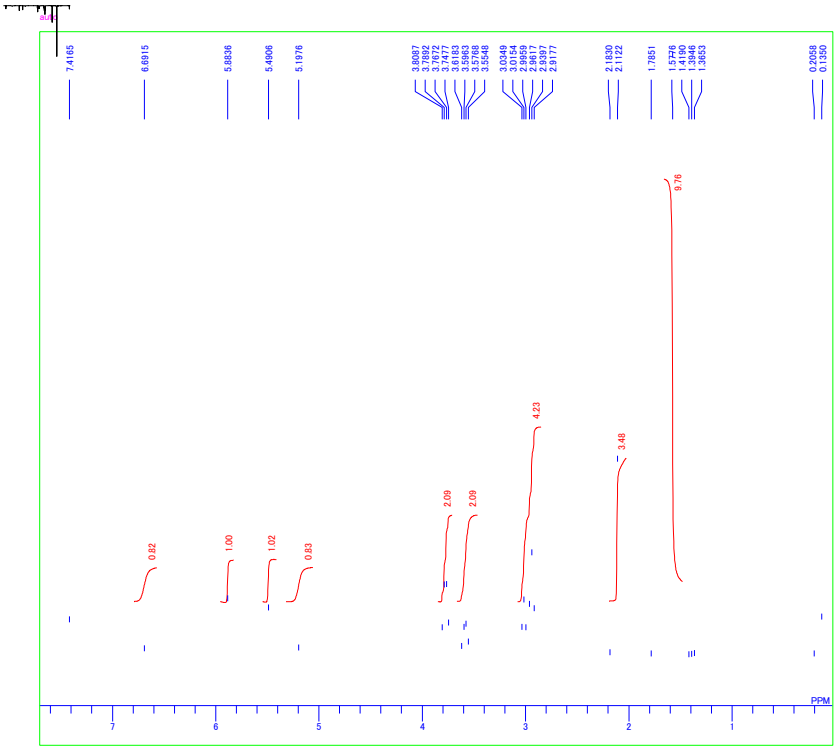
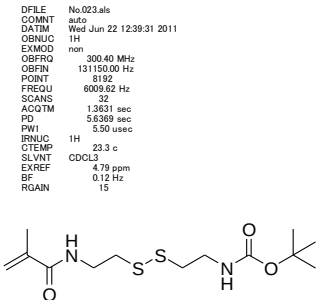
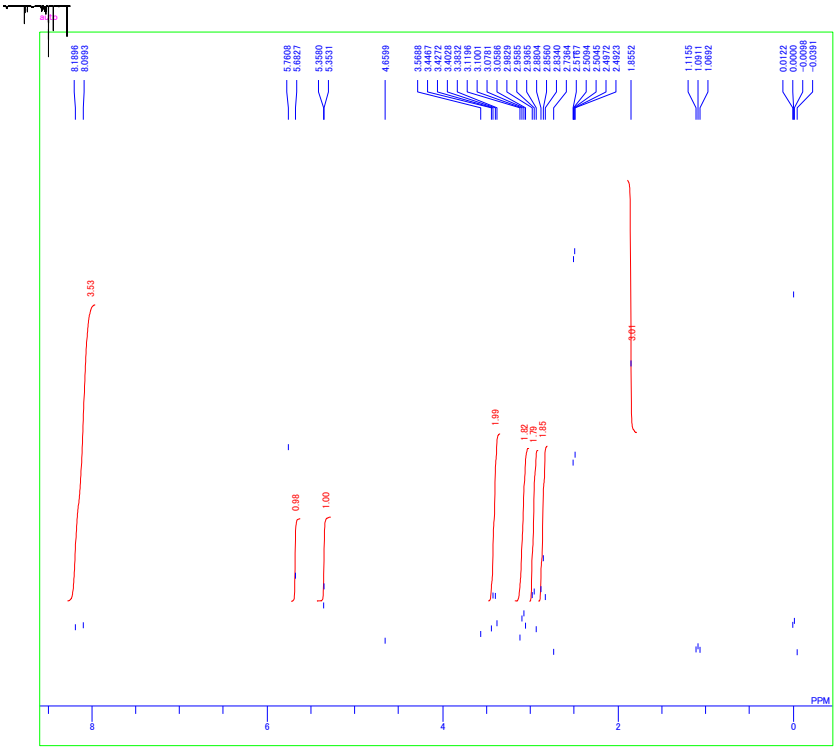
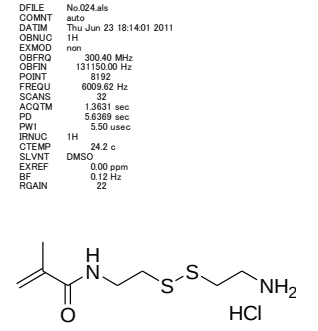
N-tert-Butoxycarbonyl-*N'*-methacryloylcystamine, **3**



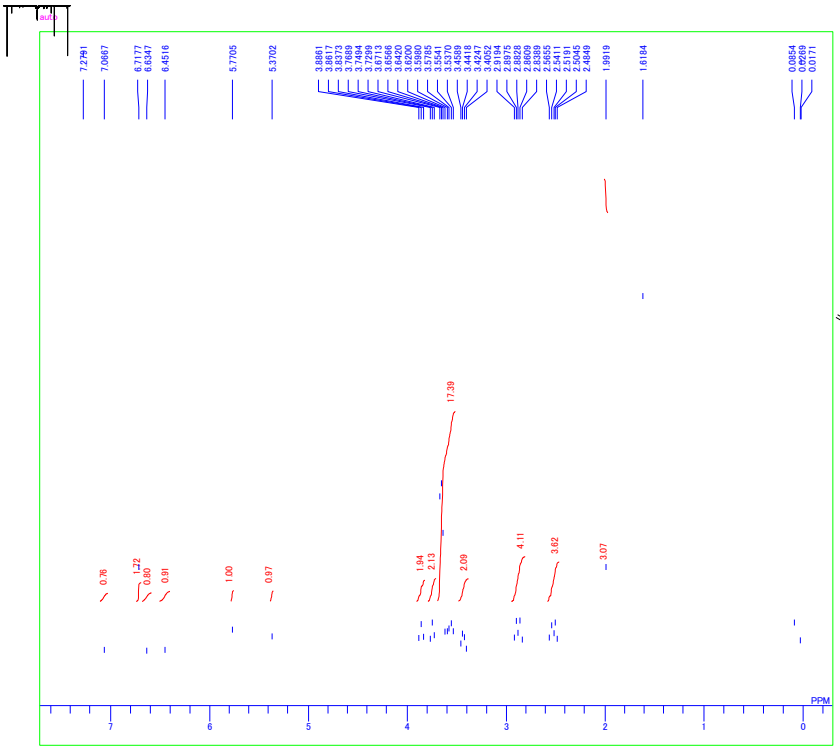


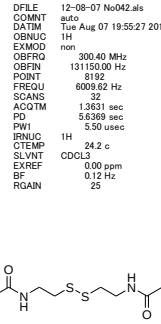
N-Methacryloylcystamine, **4**

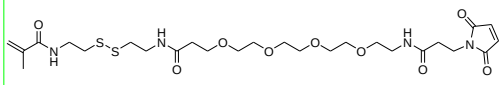




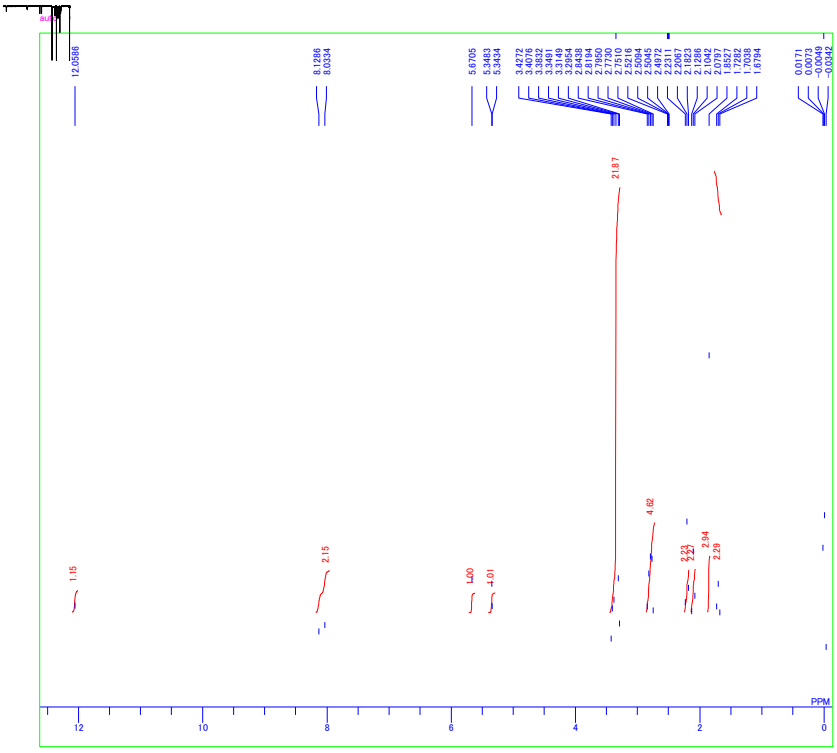
Maleimide-type cleavable monomer, **5**

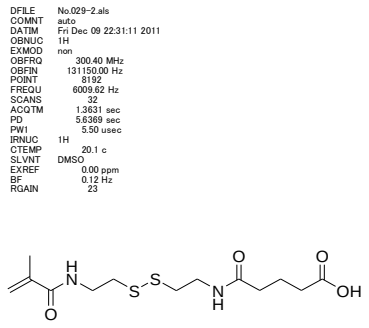




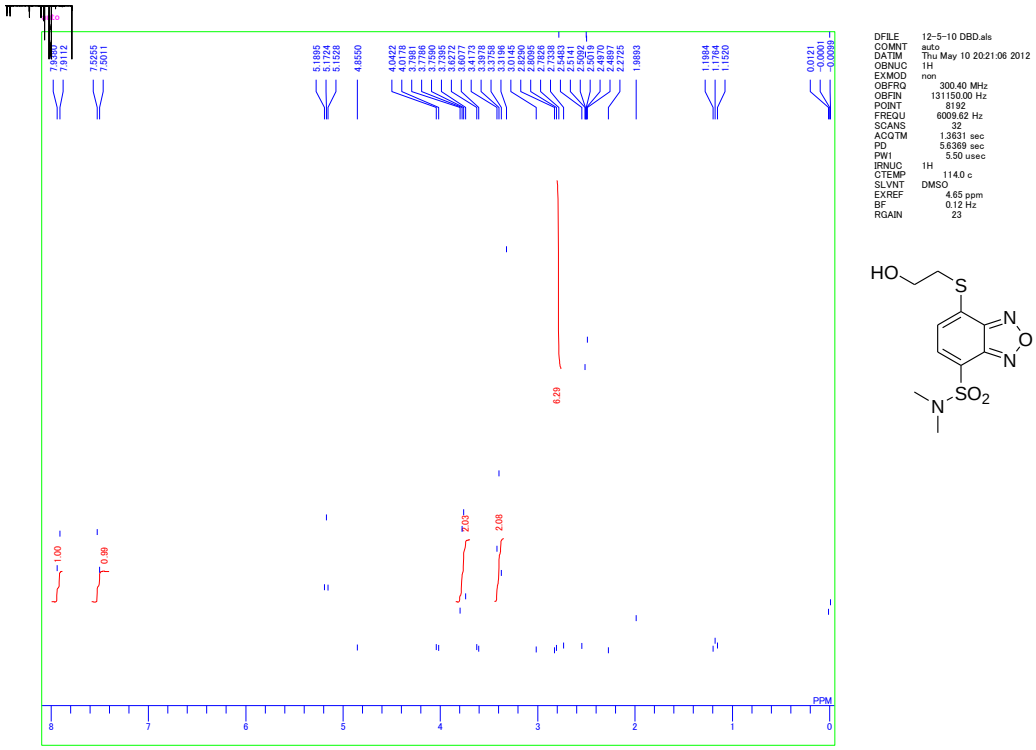


Carboxylic acid-type cleavable monomer, **7**





7-(2-Hydroxy-ethylsulfanyl)-benzo[1,2,5]oxadiazole-4-sulfonic acid dimethylamide



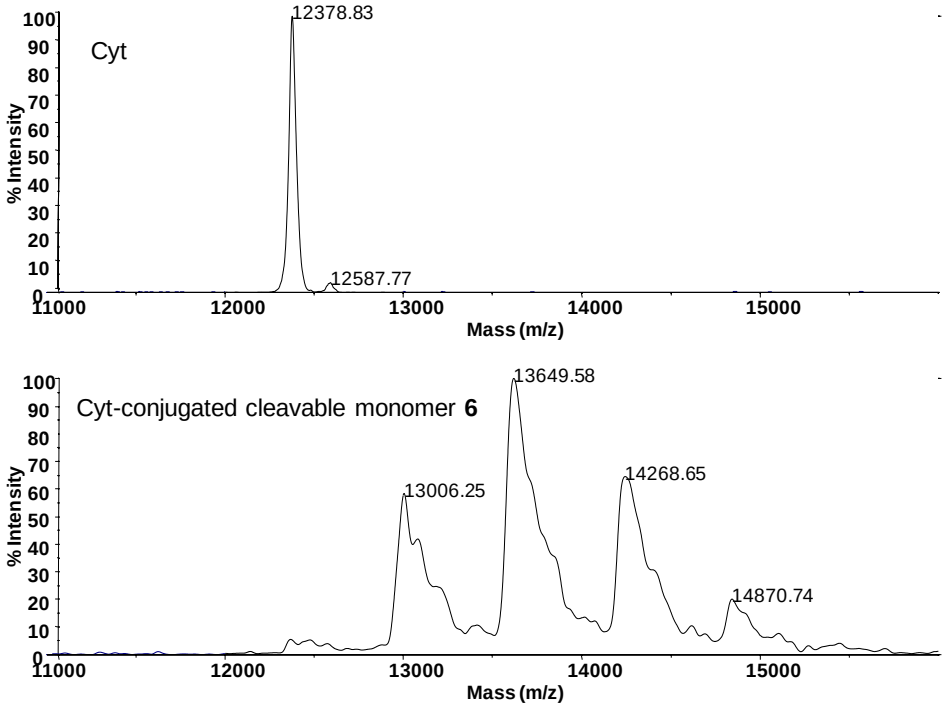
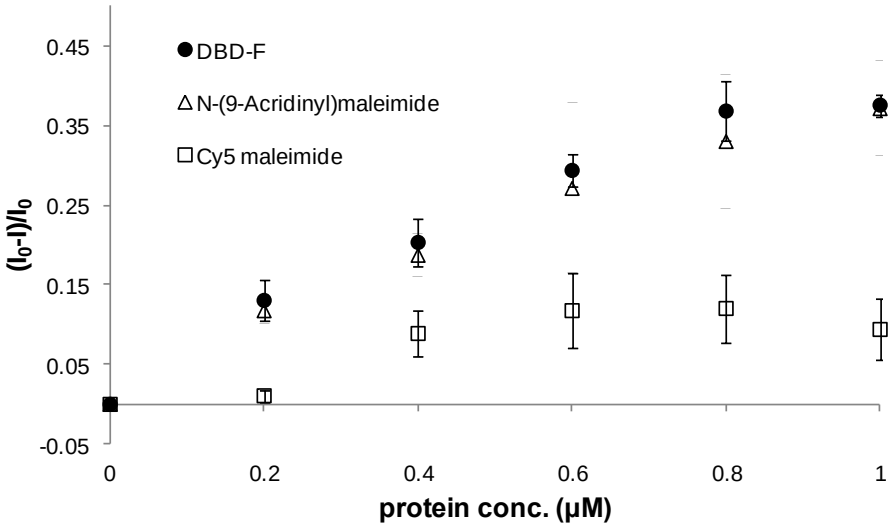
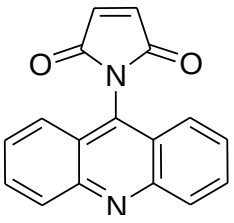


Fig. SI-1. Mass spectra of (a) native Cyt and (b) Cyt-conjugated cleavable monomer **6**.

(a)



(b)



(c)

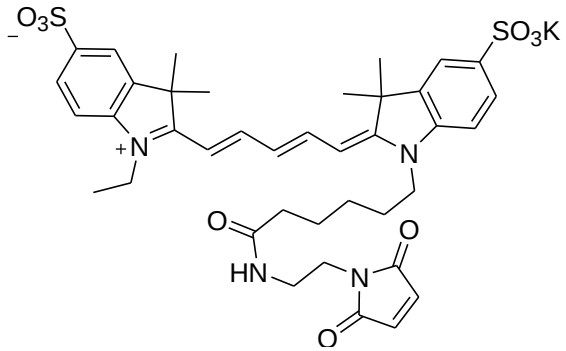


Fig. SI-2. (a) Relative fluorescence change at 537 nm (Ex: 410 nm) of MIPs modified modified by DBD-F (closed circle), *N*-(9-acridinyl)maleimide (open triangle) and Cy5 maleimide (open square) after the addition of Cyt in 10mM phosphate buffer (pH 7.3). (b) Chemical structire of *N*-(9-acridinyl)maleimide and (c) Cy5 maleimide.

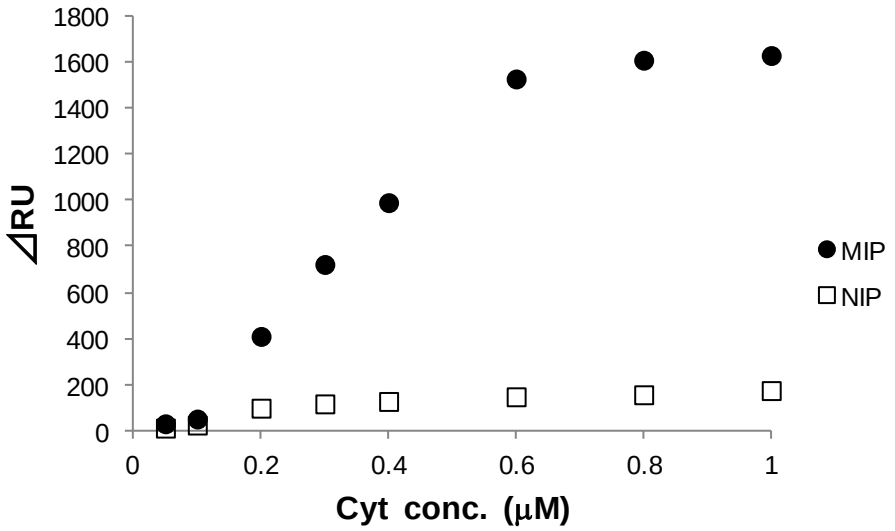


Fig. SI-3. Binding isotherms of Cyt with MIP (closed circle) and NIP (open square), measured by SPR in 10 mM phosphate buffer (pH 7.3).

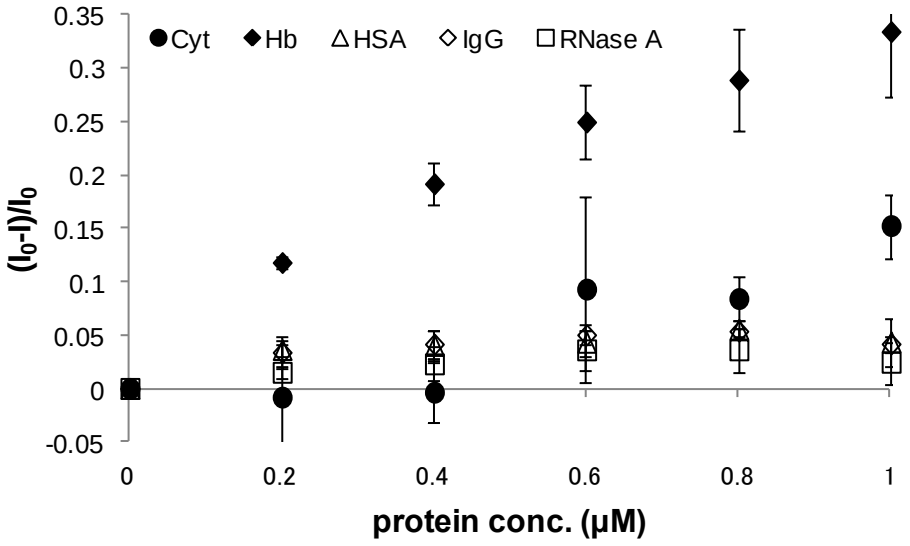


Fig. SI-4. Relative fluorescence change at 537 nm (Ex: 410 nm) of NIP after the addition of each protein [Cyt (closed circle), Hb (closed diamond), HSA (open triangle), IgG (open diamond), and RNase A (open square)] in a 10mM phosphate buffer (pH 7.3).

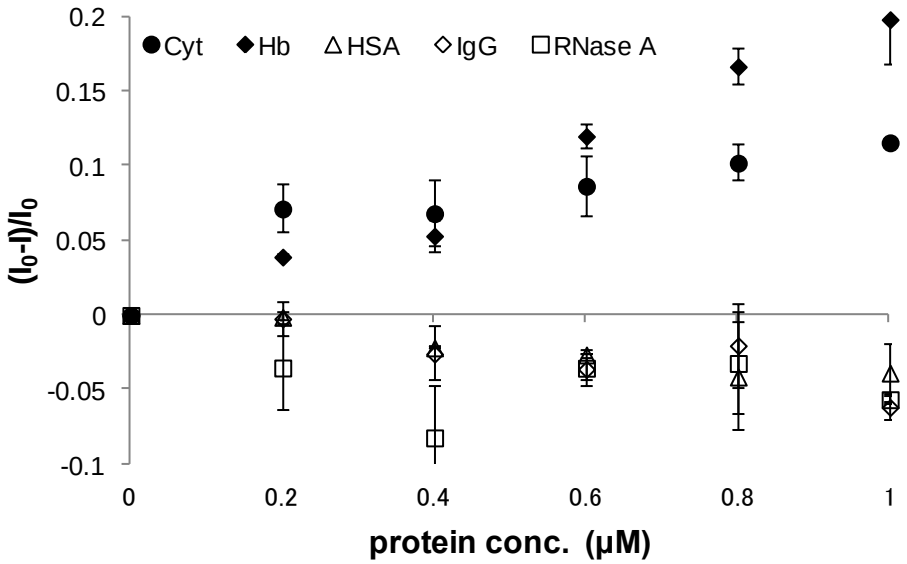


Fig. SI-5. Relative fluorescence change at 537 nm (Ex: 410 nm) of a DBD-F derivative conjugated with 2-mercaptoethanol (7-(2-hydroxy-ethylsulfanyl)-benzo[1,2,5]oxadiazole-4-sulfonic acid dimethylamide) after the addition of each protein [Cyt (closed circle), Hb (closed diamond), HSA(open triangle), IgG (open diamond), and RNase A (open square)] in a 10mM phosphate buffer (pH 7.3).