Electronic Supplementary Information

Preparation of Molecularly Imprinted Polymers for the Recognition of Proteins via the Generation of Peptide-Fragment Binding Sites by Semi-Covalent Imprinting and Enzymatic Digestion

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EXPERIMENTAL SECTION

Materials

Dichloromethane (DCM), tetrahydrofuran (THF), diethylether, methanol, ethanol, trimethylchlorosilane, hydrochloric acid (HCl), sulfuric acid, 30% hydrogen peroxide solution, magnesium sulfate ($MgSO_4$), potassium carbonate, sodium chloride (NaCl), triethylamine, acetic acid, acetonitrile, trifluoroacetic acid (TFA), and sinapinic acid were purchased from Nacalai Tesque (Kyoto, Japan). 2,2'Azobis{2-methyl-*N*-[bis(hydroxymethyl)-2-hydroxyethyl] propylamide} (VA-080), pyridine, potassium hydroxide (KOH), trehalose dihydrate, acrylic acid, cytochrome-c (Cyt), tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acryloyl chloride and *N*-acryloylsuccinimide were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Polyethyleneglycol 2000 (PEG 2000), (3-aminopropyl)triethoxysilane (APTES), *N*-hydroxysuccinimide, *N,N'*-bis(acryloyl)cystamine and lysozyme (Lyso) were purchased from Sigma-Aldrich Co. LLC. (USA). Coomassie Plus Assay Kit was purchased from Thermo Fisher Scientific Inc. (USA). THF, pyridine and triethylamine were distilled prior to use.

Apparatus

Circular dichroism (CD) spectra were measured using J-725 (JASCO Corporation, Tokyo, Japan). ^{1}H-NMR spectra were measured using 300 MHz FT-NMR, JNM-LA300 (JEOL Ltd., Tokyo, Japan). MALDI-TOF/MS were measured using Voyager-DE-1000 (AB SCIEX, Tokyo, Japan). A desktop programmable dispensing robot, SHOT-mini 200s

(Musashi Engineering Inc., Tokyo, Japan) was used for the small volume of liquid handling. Absorbance was measured using a V-550 UV/VIS spectrophotometer (JASCO, Tokyo, Japan). Surface plasmon resonance (SPR) measurements were performed using Biacore 3000 (GE Healthcare Japan, Tokyo, Japan).

Synthesis of 6,6'-diacryloyl-trehalose (DAT) and 6-monoacrylhepta(trimethylsilyl)trehalose (MAT)

*Synthesis of Octa(trimethylsilyl)trehalose (compound **1**)*

Octa(trimethylsilyl)trehalose (compound **1**) was synthesized by dissolving trehalose dihydrate (5 g, 14.61 mmol) in pyridine (80 mL). Trimethylchlorosilane (37.5 mL, 292 mmol) was then added in a dropwise manner, to the cooled solution and incubated for 2 h. Solutions were cooled, in this and other instances, by keeping them in an ice bath. After the incubation period, water was added to quench the reaction, and the solution was washed with diethyl ether. The oil phase was dried using $MgSO_4$ and evaporated *in vacuo* (11.13 g, yield: 83%).

*Synthesis of Hexa(trimethylsilyl)trehalose (compound **2**)*

Hexa(trimethylsilyl)trehalose (compound **2**) was synthesized by dissolving compound **1** (11.13 g, 12.11 mmol) in methanol (30 mL) and adding calcium bicarbonate (250 mg, 1.81 mmol) to the cooled solution, followed by a 3 h incubation period. Following the incubation, the cooled solution was neutralized by the addition of diluted HCl. The solvent was evaporated *in vacuo*, and a white solid was obtained. The solid was dissolved in DCM and washed with brine. The oil phase was dried by $MgSO_4$ and evaporated *in vacuo*, yielding a white solid (7.02 g, yield: 75%).

Synthesis of DAT and MAT

Compound **2** (7.02 g, 9.07 mmol) was dissolved in dry THF (80 mL), and triethylamine (5.7 mL, 40 mmol) was added to the cooled solution. Acryloyl chloride (2.58 mL, 30 mmol), dissolved in dry THF (10 mL), was then added dropwise to the cooled solution and incubated for 4 h. After filtration, the solvent was evaporated *in vacuo*. The obtained yellow oil was dissolved in DCM and washed with brine and purified using silica-gel column chromatography (DCM:hexane = 7:1, v/v), yielding two different white solids, namely, compounds **3** (1.22 g, yield: 15%) and **4** (0.74 g, yield: 10 %).

Compound **3** (1.22 g, 1.38 mmol) was dissolved in methanol (100 mL) followed by the addition of water (5 mL) and acetic acid (3 mL) to the cooled solution, to remove the

protecting group. The solvent was evaporated *in vacuo*, yielding the white solid, DAT (559.1 mg, yield: 90%).

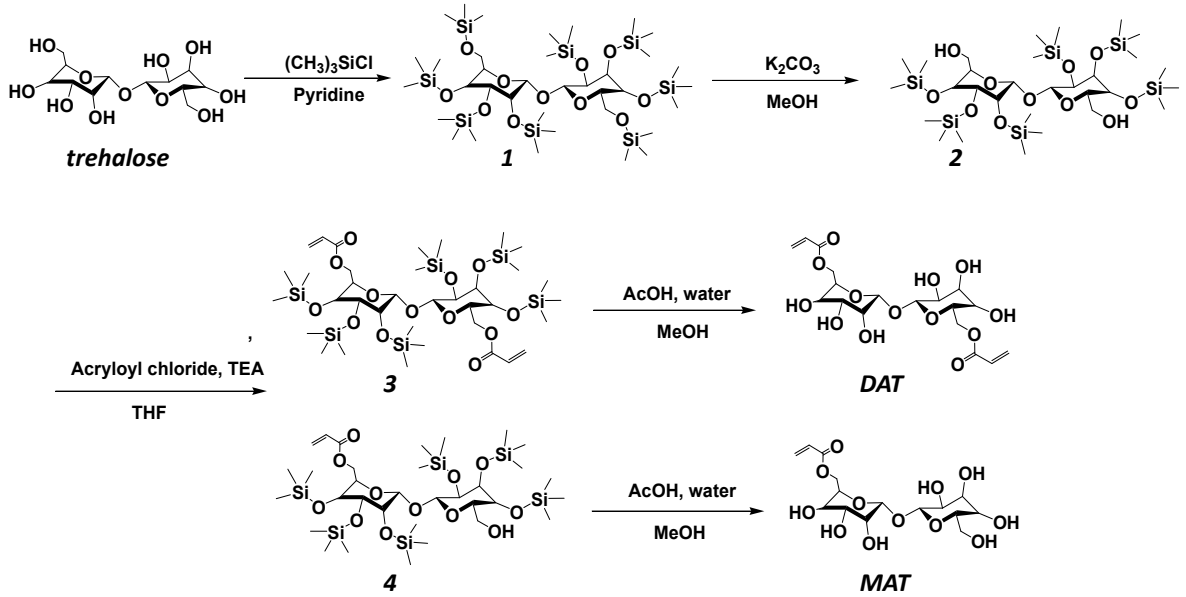
^{1}H-NMR (300 MHz, D_2O): δ=6.27(d, 2H, *J*=17.6 Hz, vinyl), 6.03(dd, 2H, *J*=16.9, 10.3 Hz, vinyl), 5.82(d, 2H, *J*=10.3 Hz, vinyl), 4.95(d, 2H, *J*=3.7 Hz, trehalose-H_1), 4.30(d, 2H, *J*=11.0 Hz, trehalose-H_6), 4.20(dd, 2H, *J*=12.1, 4.8 Hz, trehalose-H_6), 3.88(dd, 2H, *J*=10.3, 2.2 Hz, trehalose-H_5), 3.66(t, 2H, *J*=9.5 Hz, trehalose-H_2), 3.46(dd, 2H, *J*=10.3, 3.7 Hz, trehalose-H_3), 3.34(t, 2H, *J*=9.5 Hz, trehalose-H_4),

MALDI TOF-MS (Matrix; CHCA): calculated for C18H26NaO13: 473.17 $(M+Na)^+$, Found: 473.70.

Compound **4** (760 mg, 0.88 mmol) was dissolved in cooled methanol (100 mL), and then water (5 mL) and acetic acid (3 mL) were added to remove the protecting group. The solvent was evaporated *in vacuo*, yielding MAT (288.1 mg, yield: 82%).

^{1}H-NMR (300 MHz, D_2O): δ=6.30(d, 1H, *J*=17.6 Hz, vinyl), 6.06(dd, 1H, *J*=17.2, 10.6 Hz, vinyl), 5.85(d, 1H, *J*=10.3 Hz, vinyl), 5.00(dd, 2H, *J*=9.9, 4.0 Hz, trehalose-H_1), 4.33(d, 1H, *J*=12.1 Hz, trehalose-H_6), 4.22(dd, 2H, *J*=12.1, 4.8 Hz, trehalose-H_6), 3.9(m, 1H, trehalose-H_5), 3.72-3.24(m, 9H, trehalose).

MALDI TOF-MS (Matrix; CHCA): calculated forC15H24NaO12: 419.12 $(M+Na)^+$, Found: 419.07.



Scheme 1. Synthesis of DAT and MAT.

Preparation of 3, 5, and 10 molar ratio equivalents of N-acryloyl succinimide to Cyt (3, 5,

and 10acrylated Cyt)

Cyt (2 μmol) was dissolved in 7 mL of a 10 mM HEPES buffer (pH 7.4) and 3 mL of *N*-acryloyl succinimide (made up as 6, 10, or 20 μmol in the HEPES buffer containing 5% (v/v) DMSO) was added to the cooled solution. Following a 1 h incubation at room temperature, the solution was dialyzed by a Float-A-Lyzer (Spectra/Por, MWCO: 8–10 kDa) for 12 h at 4°C. The molecular weight of each acrylated Cyt was determined by MALDI TOF MS using sinapinic acid, dissolved in water/acetonitrile (75:25 v/v), as a matrix.

Preparation of acrylated glass substrates

A glass substrate was immersed in a piranha solution (concentrated sulfuric acid:30% hydrogen peroxide solution = 3:1) for 20 min, followed by an argon plasma soft etching treatment at 5 mA, for 30 s (SEDE-GE, Meiwafosis, Tokyo, Japan). The treated glass substrate was immersed in a 1% APTES ethanolic solution containing 5% (v/v) water for 1 h. After washing with ethanol, the substrate was baked for 1 h at 90°C, followed by immersion in 5 mM *N*-acryloyl succinimide dissolved in DMSO for 2 h, and washing it with methanol and water.

Synthesis of thin films of molecularly imprinted polymers (MIPs) and a non-imprinted polymer (NIP) on acrylated glass substrates

The compounds MAT, DAT, acrylated Cyt (3acrylated Cyt or 5acrylated Cyt) (1 nmol) and VA-080 (2 mol % monomer species) were each dissolved in a 10 mM Tris-HCl buffer, pH 7.4 (10 μL), to obtain pre-polymerization solutions. A PDMS plate (length, 26 mm; width, 18 mm; height, 5 mm), with 35 holes (Φ 1.25 mm, 5 × 7 holes), was placed on the acrylated glass substrate to make 35 wells. The pre-polymerization solutions (2 μL) were dispensed into each well by a desktop programmable dispensing robot (SHOT-mini 200s), followed by covering the PDMS plate with a glass substrate of the same size. The polymerization was carried out for 15 min at 4°C by photo-irradiation (365 nm, 3.5 V, 1600 mW, Key Chem-Lumino, YMC CO., LTD). After the polymerization, the PDMS plate was removed and the spotted glass plate was washed with 0.5 % SDS_{aq}, 1 M $NaCl_{aq}$, and water, then dried *in vacuo*. The following pepsin digestion was carried out for 24 h at 37 °C. After the digestion of Cyt, the glass plate was washed sequentially with 10 mM HCl, 0.5 wt. % SDS_{aq}, 1 M $NaCl_{aq}$, and water.

A molecularly imprinted polymers, prepared with native Cyt as the template molecule

(free Cyt-MIP) was synthesized by same protocol using acrylic acid (3 nmol) as a functional monomer, and the corresponding NIP was prepared in the same manner as free Cyt-MIPs without the use of Cyt.

Effect of cross-linker contents on the pepsin digestion

Three cross-linker contents (50 %, 25 %, and 5 % DAT) of Cyt-MIPs were prepared with acrylated Cyt (10 nmol) and VA-080 (2 mol%) dissolved in 10 mM Tris-HCl buffer pH 7.4 (10 μL), where the ratios of DAT to MAT were changed to be 10 μmol/ 10μmol, 5 μmol/ 15 μmol, and 1 μmol/19 μmol, respectively. The following polymerization procedure was the same as the previous section. The degree of the Cyt removal was determined by Coomassie Plus Assay Kit (Thermo Fisher Scientific Inc.). After 30 min incubation with the Coomassie reagent, absorbance on the substrates were measured.

Binding Experiments by using MALDI TOF MS

The same PDMS plate, which was used in the polymerization protocol, was reused by placing it on the MIPs- spotted and NIPs-spotted substrate. The test protein solutions (3 μL, 2.5 μM), *i.e.*, Cyt and lysozyme (Lyso), made up in 10 mM Tris-HCl buffer (pH 7.4), were dispensed into the wells by SHOT-mini 200s, and a glass substrate of the same size was placed on the PDMS plate. After incubation for 30 min, the wells were washed with 10 mM Tris-HCl buffer, pH 7.4 (10 μL), four times by SHOT-mini 200s. The matrix solution for MALDI TOF MS measurements (sinapinic acid (1 μL, 10 mM) in water/acetonitrile (75:25 v/v) containing PEG (MW: 2000, 0.05 mg/mL) was dispensed into the wells and then dried *in vacuo*. For quantification, MALDI TOF MS spectra were measured between 12300 and 12500 (m/z) for Cyt, 14150 and 14350 (m/z) for Lys, and 1500.1 and 2500.1 for PEG 2000, respectively, and total intensities of the measured range were plotted in Figure 5. Normalized intensity was defined as a ratio of measured values of Cyt or Lys to that of PEG 2000.

Synthesis of MIP thin film on SPR sensor chips and SPR measurements

A gold-coated SPR sensor chip (GE Healthcare Japan, Tokyo, Japan) was cleaned by UV-O_3 treatment for 30 min. The chip was then immersed in *N,N'*-bis(acryloyl)cystamine (5 mM in ethanol), and after 4 h, the chip was washed with ethanol and pure water. MAT (15 μmol), DAT (5 μmol), 3acrylated Cyt (1 nmol) and VA-080 (2 mol % monomer species) were dissolved in 10 mM Tris-HCl buffer, pH 7.4 (10 μL), to obtain pre-polymerization solution.

The solution (5 μL) was dropped on the chip and then covered by a cover glass. The polymerization was carried out for 15 min at 4°C by photo-irradiation (365 nm, 3.5 V, 1600 mW, Key Chem-Lumino, YMC CO., LTD). After the polymerization, the chip was washed with 0.5 % SDS_{aq}, 1 M $NaCl_{aq}$, and water, then dried *in vacuo*. The following pepsin digestion was carried out for 24 h at 37 °C. After the digestion of Cyt, the chip was washed sequentially with 10 mM HCl, 0.5 wt. % SDS_{aq}, 1 M $NaCl_{aq}$, and water.

Cyt and Lyso (2.5 μM) were dissolved in 10mM Tris-HCl buffer pH 7.4. The measurement conditions were as follows; running buffer: 10mM Tris-HCl buffer pH 7.4; flow rate: 20 μL/min; injection volume: 20 μL; regeneration solution: 1M $NaCl_{aq}$ (30 μl/min, 30 s), and 0.3 wt% SDS_{aq} (30 μl/min, 30 s).

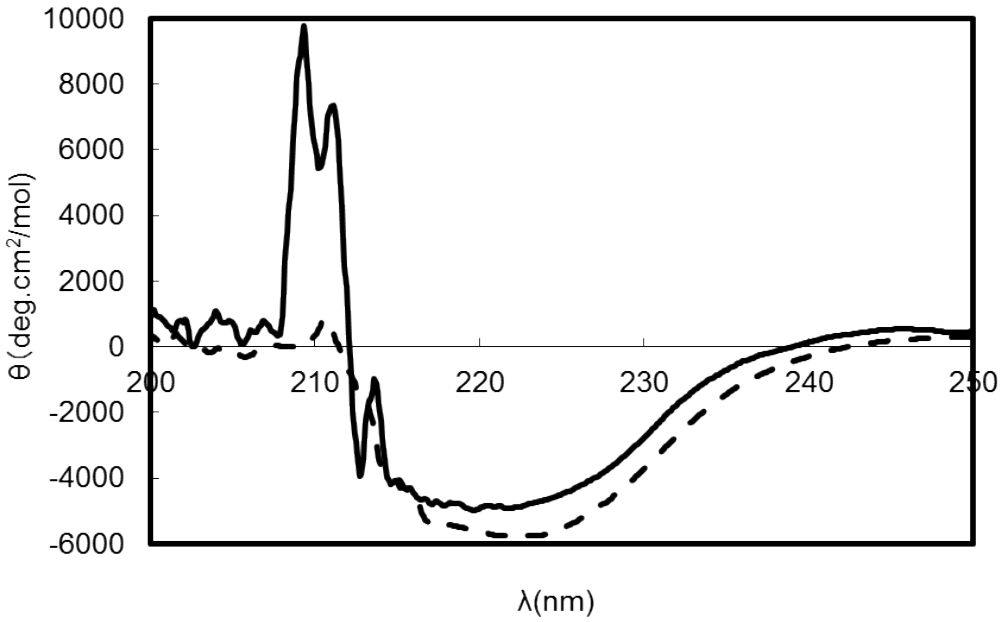


Figure S1. Circular dichroism spectra of native Cyt (broken line) and 10acrylated Cyt (solid line)

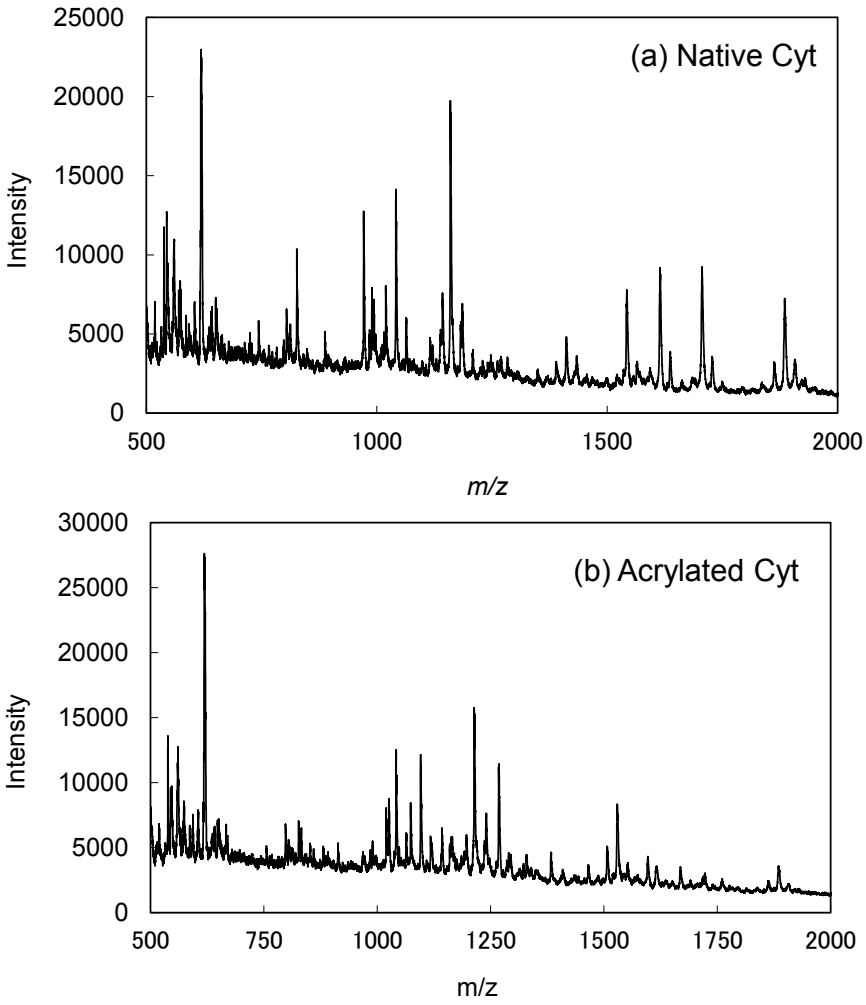


Figure S2 MALDI TOF MS spectra of native Cyt (a) and 10acrylated Cyt (b) after the digestion by pepsin at 37 °C.

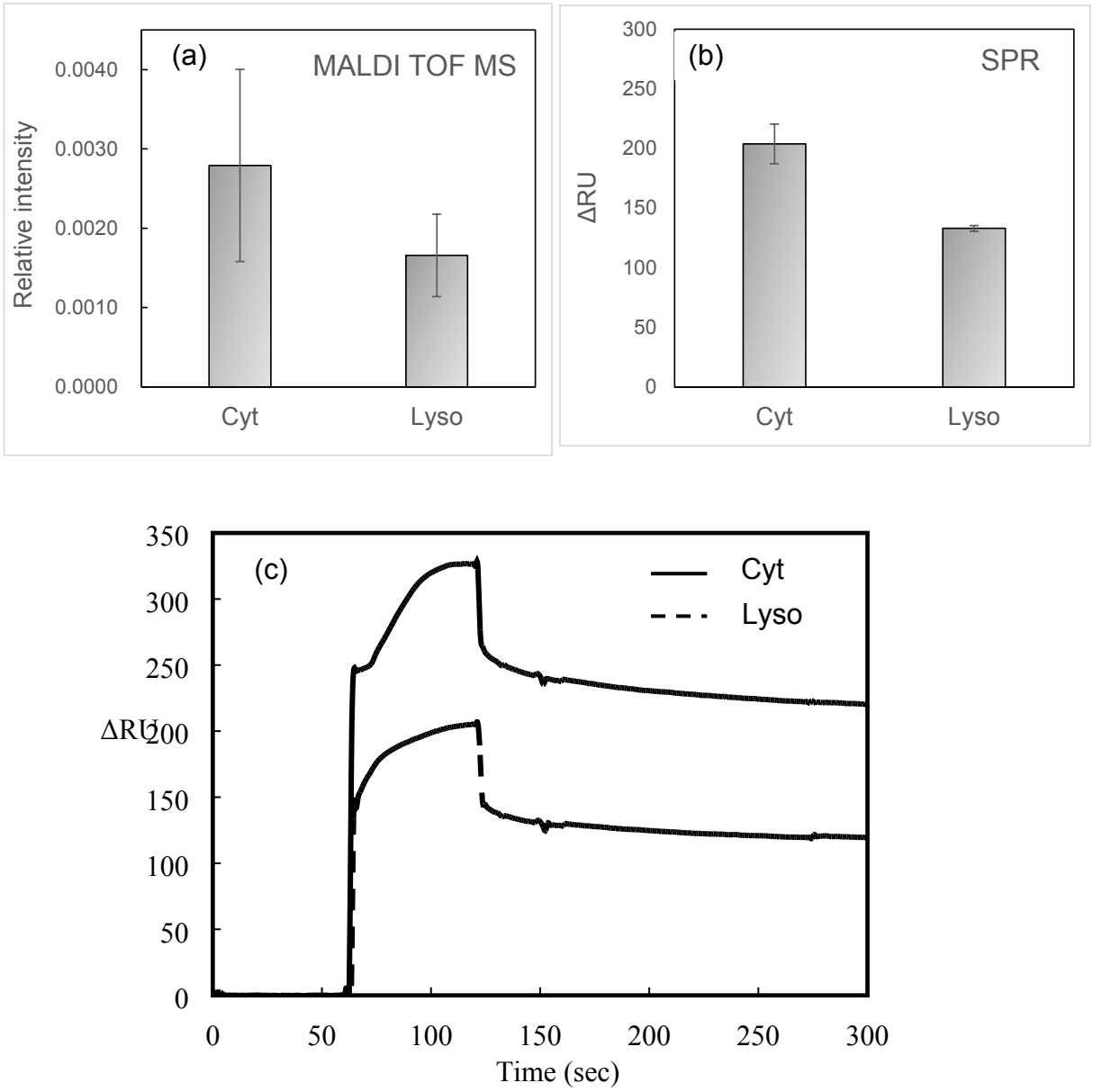


Figure S3 Comparison of the data from MALDI TOF MS (a) and SPR (b) for the binding of 2.5 μM Cyt and Lyso in 10 mM Tris-HCl buffer (pH 7.4) toward 3acrylated Cyt-MIP, where (c) shows the SPR sensorgrams for Cyt and Lyso.