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Thermo-responsive Protein Adsorbing Materials for Purifying Pharmaceutical Protein on Exposed Charging Surface

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Experimental Information

Materials

N-isopropylacrylamide (IPAAm) was kindly provided by Kohjin (Tokyo, Japan) and recrystallized from *n*-hexane. *n*-Butyl methacrylate (BMA) was obtained from Wako Pure Chemicals Industries (Osaka), and *tert*-Butylacrylate (tBA), purchased from Tokyo Kasei Kogyo (Tokyo), were purified by distillation at 35 °C (3 mmHg) and 51 °C (58 mmHg), respectively. CuCl and $CuCl_2$ were purchased from Wako Pure Chemicals. Tris(2-aminoethyl)amine (TREN) was purchased from Acros Organics (Pittsburg, PA, USA). Formaldehyde, formic acid, and sodium hydroxide were purchased from Wako Pure Chemicals. Tris(2-(*N*,*N*-dimethylamino)ethyl)amine (Me_6TREN) was synthesized from TREN, according to the previous reports[1] . Silica beads (the average diameter: 5 μm, the pore size: 300 Å, the specific surface area: 100 m^2/g) were purchased from Chemco Scientific (Osaka). Hydrochloric acid, methanesulfonic acid, hydrofluoric acid, and ethylenediamine-*N*, *N*, *N*', *N*'-tetraacetic acid disodium salt dehydrate (EDTA•2Na) were purchased from Wako Pure Chemicals. 2-(*m/p*-Chloromethylphenyl)ethyltrichlorosilane was obtained from ShinEtsu Chemical Industry (Tokyo). 2-Propanol (HPLC grade), dichloromethane, and toluene (dehydrate) were purchased from Wako Pure Chemicals. Catecholamines and steroids were purchased from Wako Pure Chemicals, and angiotensin peptides were purchased from Sigma Chemicals (St. Louis, MO). Water used in this study was Milli-Q water prepared by an ultrapure water purification system, synthesis A10, Millipore (Billerica, MA)) unless otherwise mentioned.

Preparation of ATRP Initiator Immobilized Silica Beads

2-(*m/p*-Chloromethylphenyl) ethyltrichlorosilane as an ATRP-initiator modified silica were prepared according to the previous reports.[2, 3] First, silica beads were washed with concentrated

hydrochloric acid for 3 h at 90 °C, then rinsed with a large amount of distilled water repeatedly until the washing water pH became neutral, followed by thorough drying in a vacuum oven at 110 °C for 18 h. Formation of silane layers comprising the ATRP initiator on silica surfaces was performed as follows: silica beads (15.1 g) were placed into a round-bottom flask and humidified at 60% relative humidity for 4.0 h, followed by the addition of 3.53 mL of 2-(*m/p*-chloromethylphenyl)ethyltrichlorosilane in 302 mL of dried toluene. Nitrogen gas was flowed over the reaction mixture for the first 5 min as HCl gas evolved and then sealed. The reaction proceeded at room temperature for overnight with continuous stirring. ATRP initiator-immobilized silica beads were collected by filtration and extensively rinsed with toluene, methanol, dichloromethane, and acetone, and dried in a vacuum oven at 110 °C.

Surface Modification of Silica Beads with Anionic Terpolymer by ATRP

Anionic copolymer brushes composed of IPAAm, AAc, and BMA were prepared on the ATRP-initiator immobilized silica beads by ATRP. First, the copolymer brushes composed of IPAAm, tBA, and BMA were prepared by the surface initiated ATRP from silica. Typical preparation procedure was as follows: the total monomer concentration was set at 1 mol/L with the following monomer composition in feed: IPAAm (4.57 g, 40.4 mmol), tBA (0.055 g, 0.43 mmol), and BMA (0.31 g, 2.15 mmol) (the monomer composition: tBA 1mol% and BMA 5mol%) were dissolved in 42.8 mL of 2-propanol, and deoxygenated by nitrogen gas bubbling for 30 min. The feed composition of tBA (Anionic monomer with protective group) was changed to 0mol%, 1mol%, or 2mol% (Table 1). Hydrophobic monomer, BMA, composition in feed was set at 5% regardless of tBA composition. CuCl (84.7 mg, 0.86 mmol), $CuCl_2$ (11.5 mg, 0.086 mmol) and Me_6TREN (0.22 g, 0.959 mmol) were added under nitrogen atmosphere, and the solution was stirred for 20 min to form CuCl/$CuCl_2$/Me_6TREN catalyst system. ATRP initiator-immobilized silica beads (1.0 g) were placed into a clean dry 50 mL glass vessel. Both monomer solution and the silica beads were placed into a glove bag purged with dry nitrogen gas by repeated vacuum and nitrogen flush (three times). The monomer solution was then poured into the glass vessel containing the silica beads and sealed under nitrogen. The ATRP reaction proceeded for 4 h at 25 °C under continuous shaking on a shaker (SN-M40S) (NISSIN, Tokyo). Terpolymer-grafted silica beads were washed by ultrasonication in acetone for 30 min followed by centrifugation to remove unreacted monomers and ungrafted Terpolymers. This washing process by ultrasonication was repeated twice. Copolymer-grafted silica beads were further washed by sequential centrifugation and resuspension in methanol, 50 mM EDTA solution, and finally with Milli-Q water. Modified silica beads were filtered and rinsed with Milli-Q water and acetone, and dried in a high vacuum oven at 50 °C for 5 h. After drying, the deprotection of *tert*-butylacrylate to acrylic acid was performed by immersing the silica beads into 5% methanesulfonic acid in dichloromethane at 25 °C for 1 h. The anionic Terpolymer brush grafted silica beads were filtered and rinsed with dichloromethane and acetone, and dried in a high vacuum oven at 50 °C for 5 h.

Characterization of Anionic Terpolymer Grafted Silica Beads

In order to determine the amount of ATRP-initiator and grafted terpolymer on the silica beads, the silica beads were subject to elemental analysis using a CHN elemental analyzer (PE 2400 series II CHNS/O analyzer) (PerkinElmer, Waltham, MA, USA). ATRP-initiator and terpolymer (milligrams per square meter) on silica beads was calculated by the following equations:

$$\text{ATRP-initiator} = \frac{\%C_I}{\%C_I(calcd)\times\left(1-\%C_I/\%C_I(calcd)\right)\times S} \quad \text{(S1)}$$

$$\text{Grafted terpolymer} = \frac{\%C_C}{\%C_C(calcd)\times\left(1-\%C_C/\%C_C(calcd)-\%C_I/\%C_I(calcd)\right)\times S} \quad \text{(S2)}$$

where *%C* is the percent carbon increase as determined by elemental analysis, *%C(calcd)* is the calculated weight percent of carbon in initiator or terpolymers, *S* is the specific surface area of the silica beads in square meters per gram (the manufacture's data: 100 m^2/g), and the subscripts *I* and *C* denote initiator and terpolymer, respectively.

Grafted terpolymer on the silica bead surfaces was also retrieved and analyzed by gel permeation chromatography (GPC) for determining both the molecular weight and polydispersity index (PDI). Terpolymer grafted silica bead surfaces were treated with concentrated hydrofluoric acid for 3 h, and the solution was neutralized by the addition of sodium carbonate. The solution was filtered and dialyzed against Milli-Q water using a dialysis membrane [Spectra/Por standard regenerated cellulose dialysis membrane, Molecular Weight Cut Off (MWCO): 1000] (Spectrum Laboratories, Rancho Dominguez, CA) for 3 days with daily water changed, and the terpolymer was recovered by freeze-drying. Number-average molecular weights and PDI values of the polymer were determined using a GPC system (the columns: TSKgel G3000H and TSKgel G4000H) (Tosoh, Tokyo, Japan) controlled with an SC-8020 software. A calibration curve was obtained using poly(ethylene glycol) standards. The flow rate was 1.0 mL/min. The mobile phase was *N*,*N*-dimethylformamide (DMF) containing 50 mmol/L LiCl, and the column temperature was controlled at 45 °C using a column oven (CO-8020) (Tosoh), and the elution profiles were monitored by a refractometer (RI-8022) (Tosoh). Graft density of coplymer on the silica beads surfaces was estimated using the follow equation:

$$\text{Graft density} = \frac{m_C \cdot N_A}{M_n} \quad \text{(S3)}$$

where m_c is the amount of grafted terpolymer on the silica bead surfaces per square meter (g/m^2), N_A is Avogadro's number, and M_n is the number average molecular weight of the grafted terpolymer.

Table S.1. Characterization of P(IPAAm-*co*-AAc-*co*-BMA) grafted silica beads.

Code [a]	Elemental composition (%) [b]			Immobilized initiator density ($\mu mol/m^2$)	Grafted polymer density (mg/m^2)	Mn [c]	Mw/Mn [c]	Polymer brush density ($chains/nm^2$)
	C	H	N					
Initiator-immobilized silica	4.5	0.4	0.1	4.56				
IAB-0B	17.2	1.6	2.2		2.66	12,400	2.48	0.13
IAB-1B	17.2	1.8	2.2		2.68	n.d. [d]	n.d.	n.d.
IAB-2B	17.7	2.1	2.3		2.81	n.d.	n.d.	n.d.

a) Abbreviated as IAB-xB where x represents the feed composition of tBA (AAc). B in xB denotes the "brush"
b) Determined by elemental analysis (n = 3).
c) Determined by GPC using DMF containing 50 mmol/L LiCl.
d) Not determined.

Temperature Modulated Elution of Basic Analyte

P(IPAAm-*co*-AAc-*co*-BMA) grafted silica beads were packed into a stainless steel column (4.6 mm i.d. x 50 mm). A slurry of terpolymer-grafted silica beads in water/methanol mixed solvents (1:1) was poured into a slurry reservoir (TOSOH, Tokyo) connected to a stainless steel column. Water/methanol mixed solvent (1:1) was flowed through the slurry reservoir using an HPLC pump (PU-980) (JASCO) at 350 kg/cm^2 for 1 h, followed by equilibration with Milli-Q water for at least 12 h. Terpolymer-grafted bead-packed columns were connected to an HPLC system (PU-980 and UV-970) (JASCO) controlled by a personal computer with a Borwin analysis software version 1.21 (JASCO). PB (66.7mM, pH 7.0) was used as a mobile phase. Thermoresponsive elution behavior for lysozyme and egg-white was monitored at 220 nm with a flow rate of 1.0 mL/min. Column temperature was controlled with a deviation of ± 0.1 °C using a thermostated water bath (RE206) (Lauda, Lauda-Königshofen).

Basic analytes having amino groups, catecholamine derivatives (adrenaline, and dopamine) were used for obtaining chromatograms at concentration of 1.0 mg/mL with 2.7 mg/mL Na_2SO_3 for preventing sample oxidization. Phosphate buffer (PB) (66.7 mmol/L, pH 7.0) was used as a mobile phase. Thermoresponsive elution behavior for catecholamine derivatives was monitored at 254 nm with a flow rate of 1.0 mL/min. Column temperature was controlled with a deviation of ± 0.1 °C using a thermostated water bath (RE206) (Lauda, Lauda-Königshofen).

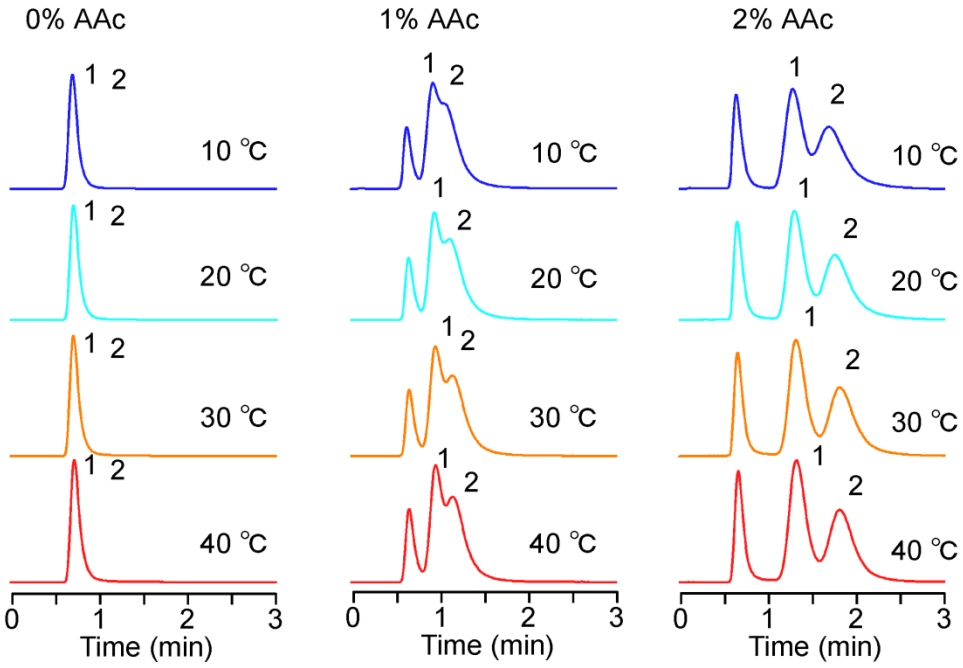

Fig. S.1 HPLC chromatograms of catecholamine derivatives separated with poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-butyl methacrylate) grafted silica beads as a stationary phase at various temperatures using 66.7 mmol/L phosphate buffer (pH 7.0) as a mobile phase. The peaks No. 1, and No. 2 are adrenaline and dopamine, respectively.

Recovery Rate of Adsorbed Protein

Recovery rate of adsorbed lysozyme was estimated from the peak area of eluted lysozyme. Sample lysozyme solution (0.5 mg/mL) was prepared with phosphate buffer (PB) (66.7 mmol/L, pH 7.0). Elution behavior of lysozyme was monitored at 220 nm with a flow rate of 1.0 mL/min. Recovery rate was calculated from the ratio of the peak area of eluted lysozyme from 1% AAc column to that without connecting column. Lysozyme solution was injected at 30 °C, and lysozyme was adsorbed on 1% AAc column. Subsequently, adsorbed lysozyme was eluted by changing column temperature from 30 °C to 10 °C. The control values of peak area were measured without connecting columns. Data from three separate experiments, expressed as mean ± SD.

Table S.2. Peak area and recovery rate of eluted lysozyme by reducing column temperature.

Experimental number	Peak area		Recovery rate
	Control [a)]	Eluted lysozyme [b)]	(%)
1	4646700	4462300	
2	4415000	4306100	
3	4563600	4361500	
Averaged	4541800 ± 117400	4376600 ± 79100	96.4

a) Measured without connecting column.
b) Measured from the peak area of eluted lysozyme by changing column temperature.

Confirmation of Structure of Lysozyme

Structure change of lysozyme after 1% AAc column separation process, thermally modulated adsorption and desorption, was investigated by circular dichroism (CD) spectroscopy. The spectra of the eluted lysozyme from the column and the control were shown in Fig S.2(a) and S.2(b), respectively. Table S.2 shows composition of secondary structure (alpha helix, beta sheet, turn, and random coil) of lysozyme before and after column separation. These data indicated that there was practical no change in the lysozyme structure before and after column separation.

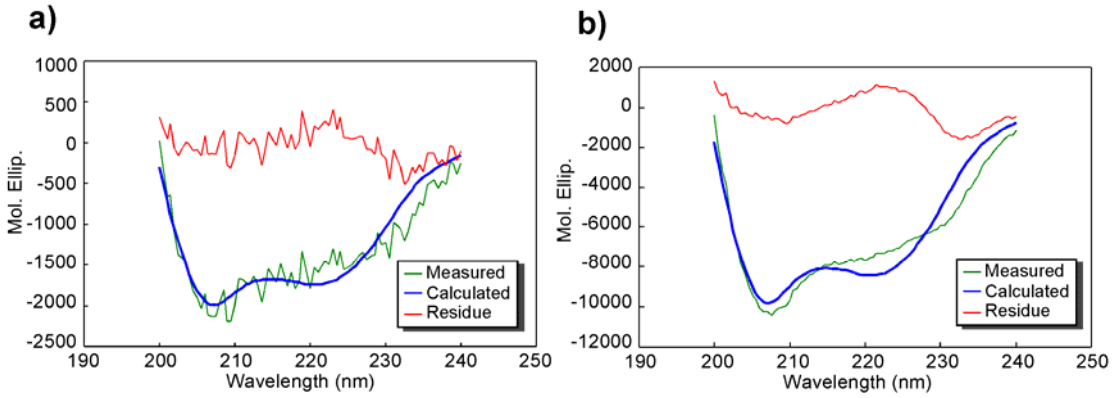

Fig. S.2. CD spectra of (a) eluted lysozyme from 1% AAc column and (b) before column separation.

Table S.3. Secondary structure of lysozyme

	Alpha helix (%)	Beta sheet (%)	Turn and Random coil (%)
Control	51	18	31
Recovered [a)]	49	20	31

a) Eluted lysozyme from 1% AAc column through temperature modulation.

Synthesis and Characterization of Anionic Terpolymer

To characterize the anionic terpolymer, P(IPAAm-*co*-AAc-*co*-BMA) products with various feed ratios (the monomer composition in feed: tBA 0mol%, 1mol%, or 2mol% with BMA 5%) were synthesized by solution-phase ATRP in the similar conditions as the terpolymer grafting onto silica bead surfaces. Terpolymerization was performed by the same protocol as grafting terpolymer onto silica except that α-chloro-*p*-xylene (53.4 mg, 380 μmol) was added as an initiator in the reaction solution instead of silica beads. After the terpolymerization, the solution was dialyzed against EDTA solution using the dialysis membrane for 3 days with changing EDTA solution every day, followed by dialysis against Milli-Q water for 2 days, and the terpolymer was obtained by lyophilization. Deprotection of *tert*-butyl acrylate to acrylic acid was performed by immersing the silica beads into 5% methanesulfonic acid in dichloromethane at 25 °C for 1 h. The anionic terpolymer was obtained by reprecipitation through dropping the solution including anionic terpolymer into diethylether, and the precipitate was dried in vacuum at 25 °C for 3 h. The obtained terpolymer was dialyzed and lyophilized for removing methanesulfonic acid.

Prepared terpolymer, P(IPAAm-*co*-tBA-*co*-BMA), was analyzed by the GPC system to determine both the molecular weight and PDI. Phase transitions of the anionic terpolymer solutions in pure water were observed by optical transmittance changes. Solutions of P(IPAAm-*co*-AAc-*co*-BMA) containing various amounts of AAc were prepared using 66.7 mmol/L PB at pH 7.0 (10 mg/mL). Optical transmittance changes of the terpolymer solutions were monitored at 600 nm by a UV/visible spectrometer (V-530) (JASCO, Tokyo). The sample cuvette was thermostated with a Peltier-effect cell holder (EHC-477) (JASCO) with a heating rate of 0.10 °C/min. The LCST was defined as the temperature at 90% transmittance of solution. BMA content in the terpolymers was determined by ^{1}H-NMR (^{UNITY}INOVA 400MHz spectrometer) (Varian, CA) using chloroform-d containing 0.03v/v% tetramethylsilane as a solvent. AAc content in the terpolymer was determined by acid–base titration in water at 4 °C with N_2 gas bubbling. Apparent dissociation constants p*K'a* of the terpolymers in a 66.7 mmol/L KCl solution were determined by titration using the following Henderson–Hasselbalch equation.

$$pK'_a = pH - \log\frac{\alpha}{1-\alpha} \quad \text{(S4)}$$

where α is the degree of dissociation for carboxyl groups. Experimental details of pK'_a measurement procedure was as follows: Terpolymer (100 mg) was dissolved in 20 mL of distilled water containing 66.7 mmol/L KCl. A half of carboxyl groups in terpolymer were dissociated stoichiometrically by adding 0.05 mmol/L NaOH aq, resulting in α of 0.5. According to Eq. (S4), the relationship between pH and pK'_a at α = 0.5 represents:

$$pK'_a = pH \quad \text{(S5)}$$

pH values of the terpolymer solution as pK'_a was measured by pH-meter with vigorous stirring and at various temperatures.

Table S.4 Characterization of P(IPAAm-*co*-AAc-*co*-BMA) anionic terpolymer.

Code [a]	IPAAm/tBA(AAc)/BMA (molar ratio)		Mn[c]	Mw/Mn [c]	LCST [d]
	In feed	In terpolymer [b]			
IAB-0	95.0/0/5.0	90.4 / 0 / 9.63	6000	1.22	14.4
IAB-1	94.0/1.0/5.0	88.8 / 2.52 / 8.70	7900	1.33	24.1
IAB-2	93.0/2.0/5.0	87.7 / 3.70 / 8.64	8100	1.35	26.1

a) Abbreviated as IAB-x where x represents the feed composition of tBA (AAc) in the terpolymer
b) Determined by acid-base titration (n = 3) and ^{1}H-NMR measurement.
c) Measured by GPC using DMF containing 50 mmol/L LiCl with PEG standards.
Mw/Mn and Mn of IAB terpolymer were measured before the deprotection for avoiding the peak tailing.
d) Defined as the temperature at 90% transmittance at 600 nm in 66.7 mmol/L phosphate buffer solution (pH 7.0).

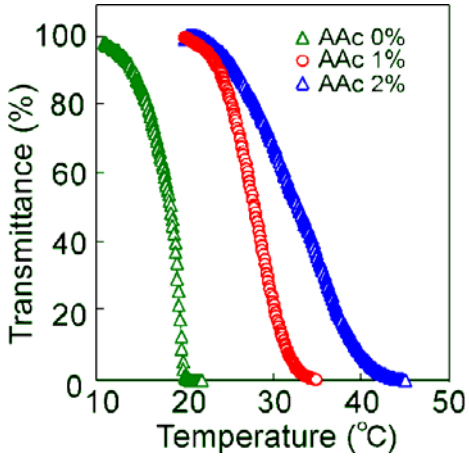

Fig. S.3 Phase transition profiles of poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-butyl methacrylate) determined by optical turbidity/transmittance change at 600 nm in 66.7 mmol/L phosphate buffer (pH 7.0) by a UV/visible spectrometer. Solutions of terpolymer containing various amounts of AAc were prepared using 66.7 mmol/L phosphate buffer at pH 7.0 (10 mg/mL). The sample cuvette was thermostated with a Peltier-effect cell holder with a heating rate of 0.10 °C/min. The lower critical solution temperature (LCST) was defined as the temperature at 90% transmittance of solution.

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