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Regulation of Protein Binding toward a Ligand on Chromatographic Matrixes by Masking and Forced-Releasing Effects Using Thermoresponsive Polymer

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A novel concept of affinity regulation based on masking and forced-releasing effects using a thermoresponsive polymer was elucidated. Affinity chromatographic matrixes were prepared using either poly(glycidyl methacrylate-*co*-ethyleneglycol dimethacrylate) or poly(glycidyl methacrylate-*co*-triethyleneglycol dimethacrylate) beads immobilized with ligand molecule, Cibacron Blue F3G-A (CB), together with poly(*N*-isopropylacrylamide) (PIPAAm), a polymer with a cloud point of 32 °C. Two different lengths of spacer molecules were used for the immobilization of CB while maintaining the PIPAAm size constant. Chromatographic analyses using bovine serum albumin as a model protein showed a clear correlation between spacer length and binding capacity at temperatures lower than the lower critical solution temperature (LCST) of PIPAAm. The binding capacity under the LCST was significantly reduced only when the calculated spacer length was shorter than the mean size of the extended PIPAAm. Furthermore, the adsorbed protein could be desorbed (released) from the matrix surface by lowering the temperature to below the LCST while maintaining other factors such as pH and ion strength. Selective recovery of human albumin from human sera was demonstrated using this newly developed thermoresponsive affinity column.

Poly(*N*-isopropylacrylamide) (PIPAAm), one of the extensively studied thermoresponsive polymers, exhibits a thermoreversible phase transition in aqueous solution at 32 °C, a lower critical solution temperature (LCST).[1] The PIPAAm chain hydrates to form an extended chain conformation below the LCST, while it dehydrates to form a shrunken globule structure above the LCST. Utilizing the unique characters of PIPAAm, it has been widely applied for drug delivery systems,[2,3] cultured cell-detachable substrates for tissue engineering,[4,5] and a modifier of reusable catalysts for chemical reactions.[6] It has also been used as novel chromatographic surfaces with a constant aqueous mobile phase, on which the surface property changes from hydrophilic to hydrophobic by temperature.[7,8] Another important application field is temperature-induced regulation of molecular recognition events. Ding et al.[9] immobilized thermoresponsive polymers in the vicinity of genetically engineered protein binding sites and demonstrated that the binding abilities abated as temperature increased higher than LCST. The mechanism they suggested was the induced structural changes in the region of the binding site due to the dehydration of the polymer. Although this concept was confirmed to work, it cannot be generally applicable since site-specific modification to a specific residue vicinity of the binding site with a thermoresponsive polymer is a very demanding task.

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(1) Heskins, M.; Guillet, J. E. *J. Macromol. Sci., Chem.* **1968**, *2*, 1441−1455.
(2) Yoshida, R.; Sakai, K.; Okano, T.; Sakurai, Y.; Bae, Y. H.; Kim, S. W. *J. Biomater. Sci., Polym. Ed.* **1991**, *3*, 155−162.
(3) Chung, J. E.; Yokoyama, M.; Yamato, M.; Aoyagi, T.; Sakurai, Y.; Okano, T. *J. Controlled Release* **1999**, *62*, 115−127.
(4) Yamada, N.; Okano, T.; Sakai, H.; Karikusa, F.; Sawasaki, Y.; Sakurai, Y. *Makromol. Chem., Rapid Commun.* **1990**, *11*, 571−576.
(5) Kikuchi, A.; Okuhara, M.; Karikusa, F.; Sakurai, Y.; Okano, T. *J. Biomater. Sci., Polym. Ed.* **1998**, *9*, 1331−1348.

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Affinity modulation by the polymer chain can also be seen in nature. For example, one of the functions of proteoglycan was suggested to mask epitopes on the cell surface with its glycosaminoglycan chains.[10] It is important to clarify whether this masking effect can be mimicked by synthetic polymers and be regulated by temperature. A further challenge will be to clarify whether forced release of the bound molecule can be induced by temperature change. Galaev et al.[11] observed shielding of Blue Sepharose with noncovalently bound poly(*N*-vinylcaprolactam), which is also a thermoresponsive polymer with a LCST of 38 °C, made it possible to elute the majority of bound lactate dehydrogenase (LDH) by a temperature decrease as the only eluting factor. In this case, however, they concluded that the relatively weak ($K_d \approx 1.8$ μM) but augmentable affinity due to the multivalent interaction between the polymer chain and Cibacron Blue F3G-A (CB), a ligand immobilized onto Blue Sepharose, was the key for the efficient displacement of the bound LDH.

In the current study, we investigated the possibility of temperature-controllable molecular recognition based on the mechanisms of masking and forced-releasing effects using thermoresponsive polymers by considering both the polymer size and spacer length for the ligand immobilization. We selected CB as the ligand molecule and PIPAAm as the thermoresponsive polymer. CB is a well-known ligand for the purification of albumin and also a variety of enzymes that require adenylic cofactors such as NAD^+, $NADP^+$, and ATP.[12,13] CB and PIPAAm were covalently immobilized onto the base matrix, and binding activities toward bovine serum albumin (BSA) were measured under different temperatures. A clear correlation between spacer length and binding capacity at a lower temperature than LCST was observed, suggesting that the intended masking and forced-releasing effects worked. Finally, the concept of temperature-induced affinity regulation was applied for the purification of human serum albumin (HSA), which is one of the most important proteins in clinical use,[14] from sera.

EXPERIMENTAL SECTION

Reagents. *N*-Isopropylacrylamide (IPAAm) was obtained from Wako Pure Chemicals (Osaka, Japan) and was purified by recrystallization from hexane. 2,2′-Azobis(isobutyronitrile) (AIBN) was obtained from Kanto Chemicals (Tokyo, Japan) and purified by recrystallization from methanol. Benzoyl peroxide, 3-mercaptopropionic acid, glycidyl methacrylate, and ethyleneglycol dimethacrylate (Wako Pure Chemicals) were purified by distillation. BSA, HSA, and human sera lyophilized powder were obtained from Sigma (St. Louis, MO). CB was obtained from Fluka (Buchs, Switzerland). 1,3-Butadiene diepoxide and triethyleneglycol dimethacrylate were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Ethylene glycol diglycidyl ether was a kind gift from Kyoeisya Chemicals (Osaka, Japan). Protein standard markers (α_2-macroglobulin, β-galactosidase, transferrin, glutamic dehyrogenase) for electrophoresis were purchased from Amersham Biosciences. The micro-BCA protein assay kit was obtained from Pierce (Rockford, IL). Other reagents were obtained from Wako Pure Chemicals and were used without further purification.

Preparation of Carboxyl-Terminated PIPAAm. A carboxyl-terminated PIPAAm at one end was prepared by radical telomerization using a thiol compound as a telogen.[15] IPAAm (0.17 mol), AIBN (0.7 mmol), and 3-mercaptopropionic acid (7.4 mmol) were dissolved in tetrahydrofuran (200 mL). The reaction mixture was degassed by repeated freeze−thaw cycles, and then a glass ampule was sealed under reduced pressure. Polymerization was proceeded at 70 °C for 2 h. The reaction mixture was poured into an excess volume of diethyl ether to precipitate the polymer. The number-average molecular weight (M_n) of the obtained polymer was 1430 ($M_w/M_n = 3.8$) as determined by gel permeation chromatography (TSKgel α-3000 column) at 40 °C using *N*, *N*-dimethylformamide containing 0.1% trifluoroacetic acid as an eluent. PIPAAm with different molecular weight ($M_n = 2490$, $M_w/M_n = 2.1$) was obtained by the same procedure with minor changes as follows: IPAAm (0.20 mol), BPO (20 μmol), 3-mercaptopropionic acid (0.2 mmol), and tetrahydrofuran (200 mL). Temperature and reaction time were 60 °C and 1 h, respectively.

Calculation of the Hydrodynamic Size of PIPAAm. The correlation function between the size and molecular weight of PIPAAm was obtained from the Stokes radius values of PIPAAm ($M_n = 11\,200$ (∼3.3 nm) and 27 400 (∼4.6 nm)) reported previously[16] and the calculated size of the IPAAm trimmer ($M_w = 341.5$). The IPAAm trimmer was built using the builder module of the program SYBYL (Tripos Inc., St Louis, MO). The trimmer was then minimized using the mmff94 force field,[17] and the largest distance between two non-hydrogen atoms was calculated to be ∼1.0 nm. The hydrodynamic sizes of PIPAAms used in this study were then calculated using the quadratic correlation function derived from the above information.

Preparation of Aminated Polymethacrylate Beads. Polymethacrylate beads were prepared using the membrane emulsification method.[18] Membrane emulsification equipment was obtained from Ise Chemicals (Tokyo, Japan). A mixture of glycidyl methacrylate (6.2 mL), ethyleneglycol dimethacrylate (3.8 mL), toluene (14.3 mL), dodecane (0.8 mL), and AIBN (0.1 g) was added into aqueous solution containing 2 wt % poly(vinyl alcohol) (DP = 2000, saponification degree, 78−82 mol %) through a Shirasu porous glass membrane of 1.95-μm pore size under nitrogen pressure. The obtained emulsion was heated at 70 °C for 6 h under a nitrogen atmosphere with stirring. The resulting polymethacrylate beads were washed with methanol and tetrahy-

(6) Matsukata, M.,; Takei, Y.; Aoki, T.; Sanui, K.; Ogata, N.; Sakurai, Y.; Okano, T. *J. Biochem*. **1994**, *116*, 682−686.
(7) Kanazawa, H.; Yamamoto, K.; Matsushima, Y.; Takai, N.; Kikuchi, A.; Sakurai, Y.; Okano, T. *Anal. Chem*. **1996**, *68*, 100−105.
(8) Yakushiji, T.; Sakai, K.; Kikuchi, A.; Aoyagi, T.; Sakurai, Y.; Okano, T. *Anal. Chem*. **1999**, *71*, 1125−1130.
(9) Ding, Z.; Long, C. J.; Hayashi, Y.; Bulmus, E. V.; Hoffman, A. S.; Stayton, P. S. *Bioconjugate Chem*. **1999**, *10*, 395−400.
(10) Hinrichs, U.; Rutteman, G. R.; Nederbragt, H. *Br. J. Cancer* **1999**, *80*, 1359−65.
(11) Galaev, I. Yu.; Warrol, C.; Mattiasson, B. *J. Chromatogr., A* **1994**, *684*, 37−43.
(12) Travis, J.; Pannell, R. *Clin. Chim. Acta* **1973**, *49*, 49−52.
(13) Allary, M.; Saint-Blancard, J.; Boschetti, E.; Girot, P. *Bioseparation* **1991**, *2*, 167−175.
(14) Peters, T., Jr. *All About Albumin: Biochemistry, Genetics and Medical Applications*; Academic Press: New York, 1995.
(15) Takei, Y. G.; Aoki, T.; Sanui, K.; Ogata, N.; Okano, T.; Sakurai, Y. *Bioconjugate Chem*. **1993**, *4*, 42−46.
(16) Tiktopulo, E. I.; Uversky, V. N.; Lushchik, V. B.; Klenin, S. I.; Bychkova, V. E.; Ptitsyn, O. B. *Macromolecules* **1995**, *28*, 7519−7524.
(17) Mastryukov, V. S.; Chen, K.; Yang, L. R.; Allinger, N. L. *Theochem* **1993**, *99*, 199−204.
(18) Omi, S. *Colloids Surfaces A: Physicochem. Eng. Aspects.* **1996**, *109*, 97−107.

drofuran by a repeated sedimentation−redispersion process and were quantitatively yielded. The average particle diameter of the polymethacrylate beads was 12.5 μm (coefficient of variation, 12.4%) as measured from a optical microscopic photograph. The specific surface area of the porous polymethacrylate beads was 30 m^2/g of beads as determined by the nitrogen adsorption method. The polymethacrylate beads with epoxy groups were suspended into aqueous solution containing 1 wt % 1,6-diaminohexane (DAH) under 30 °C for 2 h, and the polymethacrylate beads with primary amino groups were obtained. The primary amino groups were 178 μmol/g of beads as determined from precipitation titration.[19] The titration was performed as follows; the polymethacrylate beads were immersed into 1.0 N HCl solution for a few minutes and were washed vigorously by distilled water, to transform amino groups to the HCl salt. Subsequently, the polymethacrylate beads were soaked in 1.0 N HNO_3 solution. The HCl salt was exchanged into a HNO_3 salt. The solution containing HCl was retrieved by filtration, and the chloride ion was titrated with 0.1 N $AgNO_3$ solutions using K_2CrO_4 as an indicator.

Modification of Aminated Polymethacrylate Beads with PIPAAm (PIPAAm Matrix). Terminal carboxyl groups on the PIPAAm molecules were activated with *N*-hydroxysuccinimide using *N*,*N*′-dicyclohexylcarbodiimide as a condensing agent (mole ratio 1:1.1:1.1) in tetrahydrofuran. The active esterified PIPAAm was recovered by precipitation from diethyl ether. The active esterified PIPAAm (4.5 g, 3.1 mmol) was dissolved in acetonitrile (75 mL) and reacted with the aminated polymethacrylate beads (4.5 g, primary amino groups, 0.8 mmol) for 12 h at room temperature. A portion of the obtained polymethacrylate beads with PIPAAm was suspended in acetonitrile containing 10 vol % acetic anhydride to deactivate residual amino groups. This type of PIPAAm-modified beads is abbreviated as PIPAAm matrix. The organic contents of the PIPAAm matrix were determined from an increase in the N atom after the immobilization, which was measured by elemental analysis (Carlo Erba, model 1106 and EA-1108). Immobilized PIPAAm was confirmed to be 2.9 wt %.

Modification of PIPAAm Matrix with CB (Matrixes A−C). Residual amino groups on the PIPAAm-modified polymethacrylate beads without acetic anhydride treatment (0.70 g) were reacted with either 1,3-butadiene diepoxide (BDDE, 0.09 g, 1.0 mmol) or ethylene glycol diglycidyl ether (EGDGE, 0.21 g, 1.1 mmol), respectively, in acetonitrile (10 mL). After deactivation of the residual amino groups by reaction with acetic anhydride, the introduced epoxy groups on the beads (0.70 g) were reacted with 6-aminohexyl-CB (0.89 g, 0.7 mmol) in alkaline aqueous solution (10 mL, pH 11) at room temperature for 3 h. 6-Aminohexyl-CB was synthesized according to the previously reported method.[20] Thus, two types of polymethacrylate beads grafted with PIPAAm and CB were prepared: CB on BDDE as spacer (matrix A) or CB on EGDGE as spacer (matrix B). Using the preparation method of matrix A, polymethacrylate beads grafted with PIPAAm and CB were prepared using PIPAAm with a different molecular weight (M_n 2490, M_w/M_n 2.1) (matrix C). Polymethacrylate beads used for matrix C were prepared from a mixture of glycidyl methacrylate (3.3 mL), triethyleneglycol dimethacrylate (2.9 mL), and toluene (9.4 mL).

Calculation of Spacer Lengths. Spacers for matrixes A and B are regarded as 3-aza-2, 4-dihydroxy-1, 9-nonanediamine (BDDE reacted with DAH) and 9-aza-3,6-dioxa-1, 8-dihydroxy-1, 5-diaminopentadecane (EGDGE reacted with DAH), respectively. The calculations of the sizes were performed by the same procedure used for the calculation of IPAAm trimer.

Modification of Aminated Polymethacrylate Beads with only CB (CB Matrix). The aminated polymethacrylate beads (0.5 g, primary amino groups, 89 μmol) reacted with BDDE (0.09 g, 1.0 mmol) in acetonitrile (10 mL). After deactivating the residual amino groups by acetic anhydride, the obtained beads were reacted with 6-aminohexyl-CB (2.5 g, 2.0 mmol) in alkaline aqueous solution (20 mL, pH 11) at room temperature for 3 h.

Determination of Immobilized CB Concentration. Determination of CB contents on each matrix was done according to the previously reported method.[21] Briefly, each modified matrix (30−40 mg) was added to 7 M NaOH (3 mL) and heated to 90 °C for 3 days to completely solubilize the polymethacrylate matrix beads. The resulting solution was adjusted to pH 7 with HCl, and absorbance at 620 nm was noted. The amount of the immobilized CB was calculated using a molar extinction coefficient of 13 600 $L \cdot mol^{-1} \cdot cm^{-1}$ for CB at 620 nm[21].

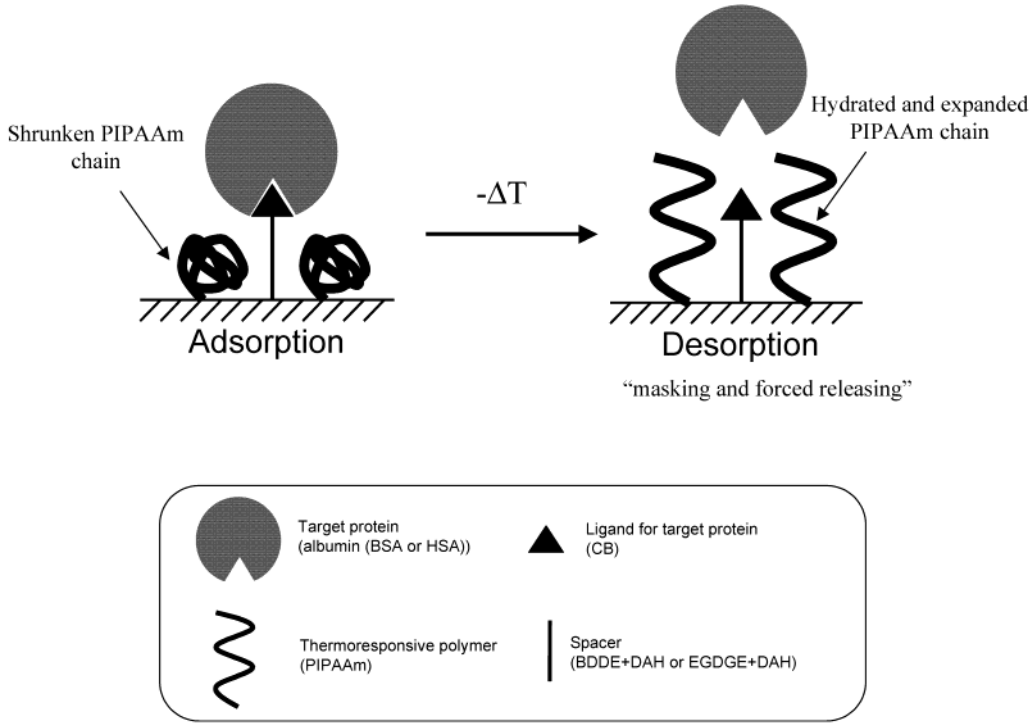
Measurement of Albumin Binding Capacity. Each matrix was separately packed into a stainless steel column (30 mm × 4.6 mm id.) by a slurry technique. Chromatography was performed using a Shimadzu LC-10AD pump, a Rheodyne 7125 valve loop injector, and a Shimadzu SPD-10AV UV detector. The mobile phase was 0.01 M phosphate buffer at pH 6.6 and was eluted with a flow rate of 0.2 mL/min. The column temperature was controlled with thermostated water bath connected to the column jacket within ±0.1 °C. A 20-μL aliquot of BSA solution (2.5 mg/mL) was applied to the column, and column effluent was collected. The binding capacity was determined by measuring the amount of applied BSA and eluted BSA using the micro-BCA protein assay kit.

Spectral Titration for the Interaction Analysis between PIPAAm and CB. The spectral titration was performed at 20 °C. Sample and reference cuvettes each containing a solution of 10 μM CB in 67 mM phosphate buffer (pH 7.0) were placed in a Hitachi U-3010 spectrophotometer. An 11 mM PIPAAm solution (1−5 μL) was added to the sample cuvette (final concentration of PIPAAm in the sample cuvette was 26 μM). Difference spectra were produced in the range of 400−800 nm.

Purification of HSA from Crude Sera. A 100-μL sample of human sera lyophilized powder dissolved in 5 mM Tris-HCl buffer (pH 8.6) + 5 mM NaCl solution (0.5 mg/mL) was injected onto a column packed with matrix C at 37 °C. The temperature decreased to 20 °C after 15 min, and fractions eluted from the column were collected. The eluted fraction was applied to SDS−PAGE with 3−12% linear gradient slab gel together with a protein standard marker following the procedure reported by Laemmli.[22] Other measurement conditions were as follows: flow rate, 0.2 mL/min; column size, 4.6 mm i.d. × 30 mm; detection, 280 nm; mobile phase, 5 mM Tris-HCl buffer (pH 8.6) + 5 mM NaCl. The

(19) Guenther, W. B. *Quantitative Chemistry: Measurements and Equilibrium*; Addison-Wesley Publishing Co.: Redding, MA, 1968; Chapter 23.
(20) Lowe, C. R.; Glad, M.; P−O. Larsson, P.-O.; Ohlson, S.; Small, D. A. P.; Atkinson, T.; Mosbach, K. *J. Chromatogr.* **1981**, *215*, 303−316.
(21) Dean, P. D.; Watson, D. H. *J. Chromatogr.* **1979**, *165*, 301−319.
(22) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.

Scheme 1. Concept of the Temperature-Controllable Molecular Recognition Based on Masking and Forced-Releasing Effects by Thermoresponsive Polymer



difference in pH of analytical conditions between BSA and HSA experiments is based upon the fact that they have different pH-dependency profiles as reported by Leatherbarrow and Dean.[23]

RESULTS AND DISCUSSION

Preparation of Chromatographic Matrixes Modified with PIPAAm or CB. The conceptual view of the temperature-controllable molecular recognition based on masking and forced-releasing effects by the thermoresponsive polymer is illustrated in Scheme 1. PIPAAm and the ligand were independently immobilized onto the matrix surface. Binding partners in solution might easily access to the immobilized ligand when the temperature is higher than LCST and the ligand is exposed, while it becomes difficult when it is sheltered under extended polymer chains at a temperature lower than the LCST (masking effect). The bound protein molecules at higher temperatures than LCST could be forced out when the polymer chains extend through hydration at a temperature lower than LCST (forced-releasing function).

On the basis of this concept, the design of matrixes modified with PIPAAm/CB was performed by considering the size of PIPAAm and the spacer lengths for the CB immobilization. As shown in Figure 1, aminated polymethacryl beads were first modified with PIPAAm whose mean M_n was 1430 (PIPAAm matrix). Either BDDE or EGDGE was then introduced onto the matrix. The 6-aminohexyl CB was finally attached onto the matrix via the epoxide group (matrixes A and B). Therefore, the spacers for matrixes A and B are regarded as 3-aza-2,4-dihydroxy-1,9-nonanediamine (BDDE reacted with DAH) and 9-aza-3,6-dioxa-1,8-dihydroxy-1,5-diaminopentadecane (EGDGE reacted with DAH), respectively. The length of each spacer was calculated to be 1.2 and 2.0 nm, respectively (see Experimental Section). The size of the extended PIPAAm (M_n = 1430) was estimated to be ~1.7 nm from the correlation function determined from a previously reported values[16] (see Experimental Section). Considering that the hydrodynamic size of the PIPAAm chain was reported to shrink by about half above the LCST, which corresponds to a $\sim^1/_{10}$-fold hydrodynamic volume change,[16,24] matrixes A and B may be a good combination for the feasibility study of this concept.

The immobilized PIPAAm concentration was calculated to be 2.9 wt % by elemental analysis, and specific surface area of polymer matrix was calculated to be 30 m^2/g by the nitrogen adsorption method. These values give the number of PIPAAm molecules per 10 nm^2 of the matrix surface to be ~4.0, which means the average distance between PIPAAms was ~1.7 nm. That the distance was quite comparable with the size of the extended PIPAAm further supports the validity of the employed experimental design. The CB contents of matrixes A and B were comparable. The detailed properties of each matrix are summarized in Table 1.

Effect of Temperature on the Binding Capacity. The effect of temperature on the binding capacity was compared among four matrixes (PIPAAm matrix, CB matrix, matrixes A and B) by elution profile analyses using columns packed with those matrixes. As shown in Figure 2, adsorption of BSA toward PIPAAm matrix was quite small regardless of temperature change. Considering that the surface of the PIPAAm matrix is exposed by either PIPAAm or base matrix, this observation suggests that the bindings of BSA toward not only PIPAAm but also base matrixes were quite small under the temperature range employed.

The binding capacities measured at 40 °C for CB matrix and matrixes A and B were 27.8, 17.3, and 19.5 $\mu g/mL$, respectively.

(23) Leatherbarrow, R. J.; Dean, P. D. G. *Biochem. J.* **1980**, *189*, 27−34.

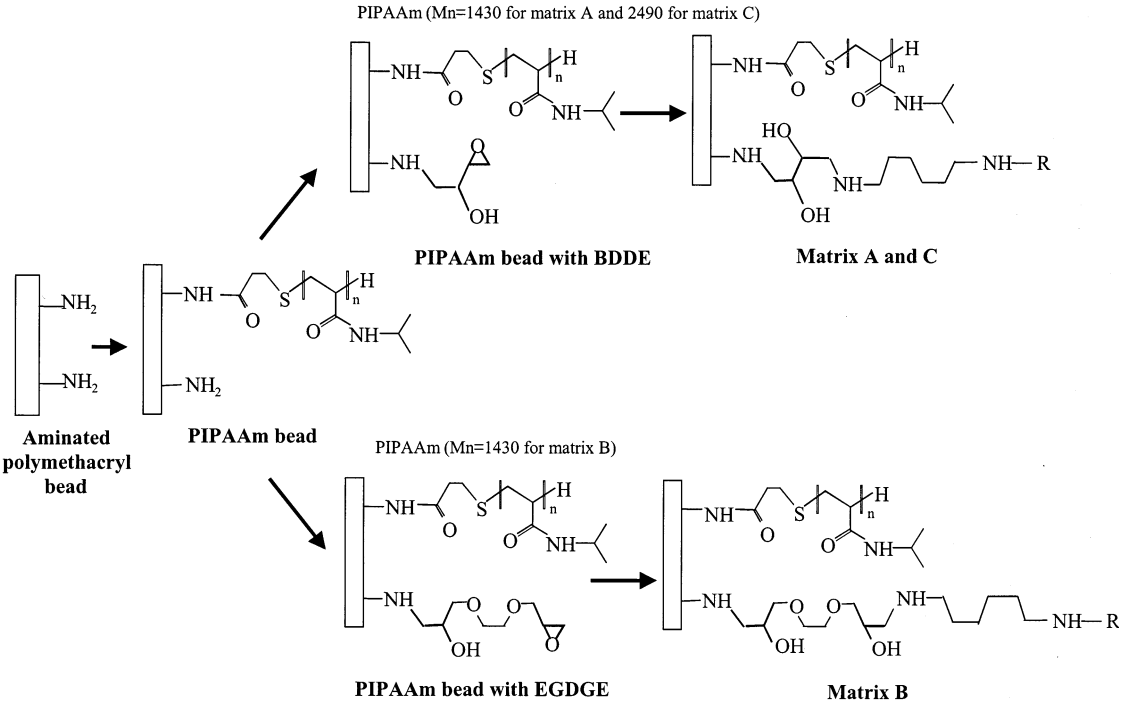


Figure 1. Synthetic procedure of affinity column matrixes.

Table 1. Properties of Matrixes A and B, PIPAAm Beads, and CB Matrix

	matrix A	matrix B	PIPAAm matrix	CB matrix
		Base Matrix		
particle size (μm)	12.5	12.5	12.5	12.5
amt of amino groups (μmol)	178	178	178	178
surface area (m^2/g)	30	30	30	30
		PIPAAm		
M_n	1430 (n = 12-13)	1430 (n = 12-13)	1430 (n = 12-13)	
diam of extended PIPAAm[a] (nm)	~1.7	~1.7	~1.7	
concn of polymer[b] (wt %)	2.9	2.9	2.9	
		Spacer		
types of spacer	BDDE+DAH	EGDGE+DAH		BDDE+DAH
spacer length[c] (nm)	~1.2	~2.0		~1.2
		CB Contents		
concn of CB (μmol)	0.19	0.21		0.34

[a] The size of the extended PIPAAm (M_n = 1430) was estimated to be ~1.7 nm from the correlation function determined from a previously reported values[16] (see details in Experimental Section). [b] Concentration of immobilized polymer was determined from the increase of CHN after immobilization of the polymer. [c] Length of compounds obtained from 1,6-diamonohexane and epoxicide (BDDE or EGDGE) as a spacer.

Note that this ratio is almost identical to the estimated CB content ratio (Table 1), suggesting that the adsorption of BSA to each matrix is specific toward immobilized CB. The binding capacity of the CB matrix decreased linearly as the temperature decreased. This is consistent with the previous observation from the adsorption isotherm studies of HSA binding to immobilized CB.[25] The trend that binding capacity decreased as the temperature decreased was also true for matrixes A and B; however, the slope at temperatures lower than 30 °C was significantly steeper only for matrix A.

No interaction was observed between CB and PIPAAm in the spectral titration analysis (data not shown). It was therefore unlikely that weak but augmentable affinity due to the multivalent interaction between the polymer chain and CB was the basis for the observed steep temperature dependency as proposed by Galaev et al.[11] The base matrix and size/concentration of immobilized PIPAAm are exactly the same between matrixes A and B. The concentration of CB is also quite comparable (Table 1). Therefore, the observed difference between matrixes A and B in binding capacity at a temperature lower than the LCST should be attributed to the spacer molecules used to introduce CB onto the base matrix. Considering the sizes of PIPAAm and spacer molecules, the intended masking effect by hydrated PIPAAm functioned. Though a portion of BSA was still observed to bind even at 20 °C, this may be attributable to the heterogeneity in

(24) Wu, C.; Zhou, S. *Macromolecules* **1995**, *28*, 5388−5390.

(25) Finette, G. M. S.; Mao, Q.-M.; Hearn, M. T. W. *J. Chromatogr., A* **1997**, *763*, 71−90.

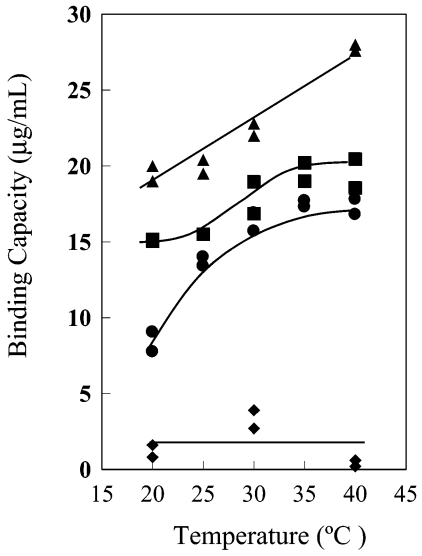


Figure 2. Effect of temperature on the binding capacity of BSA on PIPAAm matrix (◆), CB matrix (▲), matrix A (●), and matrix B (■). The duplicate experimental data are presented on the figure.

immobilizing ligand/PIPAAm or in the size of PIPAAm, suggesting that further optimization of matrix preparation is required.

Release of Adsorbed BSA by Step Temperature Gradient. Now that the intended masking effect was confirmed to work, the next step was to see whether the forced release of bound protein could be induced by temperature change. BSA was injected over a column packed with matrix A at 40 °C. The flow was interrupted and the column was cooled to 20 °C and maintained for 20 min. Pumping was then restarted and column eluent was fractionated. Elution profile was compared with the result obtained from the column packed with CB matrix. As shown in Figure 3a, the temperature change allowed elution of very small amounts of BSA from the CB matrix-packed column. Typical elution condition of albumins from the CB-immobilized column requires drastic changes in salt concentration, pH, or both. We confirmed that ~16 h of incubation was needed for a complete elution of the bound BSA from the CB matrix-packed column by temperature elution alone (data not shown). By sharp contrast, the same temperature change allowed effective elution of BSA from the matrix A-packed column (Figure 3b). These results suggest that the efficient recovery observed with the matrix A-packed column was not due to the intrinsic nature of the temperature dependency of the interaction between CB and BSA, but bound BSA was actively forced out by the existence of

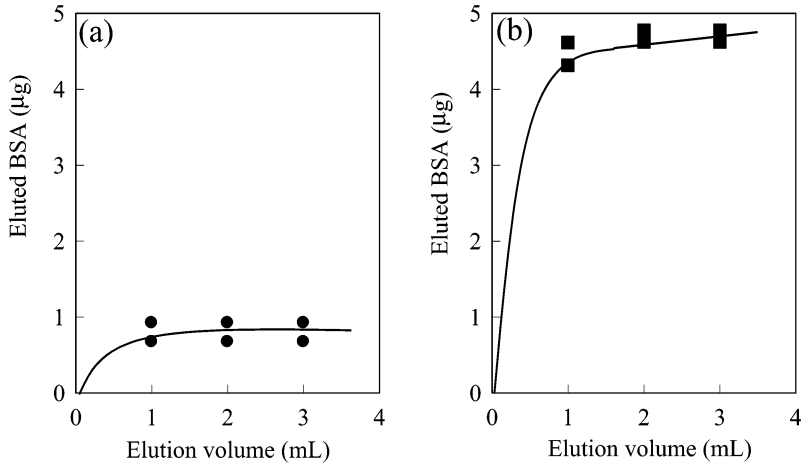


Figure 3. Cumulative release of bound BSA from columns packed with CB matrix (a) and matrix A (b) by a step temperature gradient. The duplicate experimental data are presented on the figure.

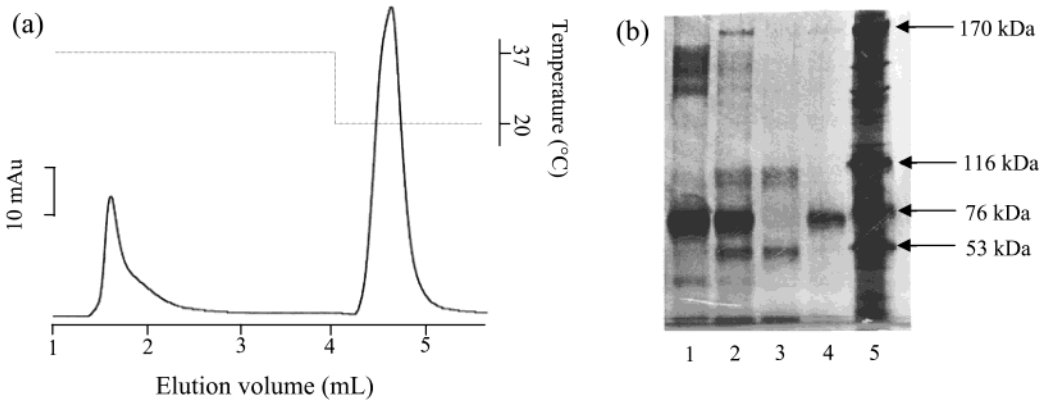


Figure 4. (a) Elution profile of human sera on the matrix C-packed column by controlling temperature. Applied sample, 100 μL of human sera (0.5 mg/mL). Mobile phase, 5 mM Tris-HCl buffer (pH 8.6) containing 5 mM NaCl. Detection and flow rate were UV 280 nm and 0.2 mL/min, respectively. (b) SDS−PAGE of HSA profiled from crude human sera. Lanes: (1) commercially available HSA, (2) human sera, (3) eluted proteins at 37 °C, (4) eluted protein at 20 °C, (5) molecular weight standards (α_2-macroglobulin (170 000), β-galactosidase (116 000), transferrin (76 000), and glutamic dehyrogenase (53 000)).

hydrating PIPAAm and eventually prevented BSA from rebinding to the CB molecules.

Purification of HSA from Crude Sera by a Step Temperature Gradient. The concept of temperature-induced affinity regulation described here was finally applied to the purification of HSA from crude human sera. Matrix C was specifically prepared to increase the capacity by increasing the immobilized amount of CB and to make the masking and forced-releasing effects more efficiently by making the PIPAAm chain relatively larger than matrix A. The CB content of matrix C was 1.8 μmol/g of beads, which is almost 10-fold higher than that of matrix A. The PIPAAm used for matrix C had an M_n of 2490, and the calculated extended size was 2.4 nm.

Figure 4a shows the chromatographic behavior of the matrix C-packed column when crude human sera were injected at 37 °C and temperature elution was performed. SDS−PAGE analysis revealed that the elution fraction at 20 °C contained an almost solitary band at 70 kDa, which corresponds to the molecular weight of HSA (Figure 4b, lane 4), while the flow-through fraction did not (Figure 4b, lane 3). It demonstrated that HSA was adsorbed onto the gel at 37 °C, was separated from the other components present in human sera, and was recovered successively by changing temperature as the only variables.

The dissociation constants (K_d) of HSA and BSA against CB have been reported to be 0.55-43.5 and 2.0−196 μM, respectively, depending on the methodologies including equilibrium dialysis,[26] batch adsorption,[27] affinity electrophoresis,[28] and frontal analysis.[29] It was indicated earlier that the ligands for affinity chromatography typically have an affinity for the binding substances in the range of 0.01−100 μM.[30] The K_ds for albumins−CB interactions are well within this range, suggesting that the proposed concepts may be applicable for many other interactions. How these effects work for those interactions having lower K_d would be the next important subject.

In conclusion, we elucidated the possibility of affinity modulation by masking and forced-releasing effects using thermoresponsive polymer. It was revealed that not only the masking effect but also the forced-releasing effect were possible to attain by designing the immobilization conditions (e.g., density and sizes of spacer molecule and polymer). Since PIPAAm is covalently attached onto the chromatographic beads, the loss of the temperature-responsive property by repeated use can be neglected. The concept is clear and preparation of chromatographic beads would be fairly easy; therefore, it should be applicable to many other molecular interactions.

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(26) Ling, T. G. I.; Mattiasson, B. *J. Chromatogr.* **1982**, *252*, 159−166.
(27) Horstmann, B. J.; Kenney, C. N.; Chase, H. A. *J. Chromatogr.* **1986**, *361*, 179−190.
(28) Metcalf, E. C.; Crow, B.; Dean, P. D. G. *Biochem. J.* **1981**, *199*, 465−470.
(29) Anspach, F. B.; Johnston, A.; Wirth, H.-J.; Unger, K. K.; Hearn, T. W. *J. Chromatogr.* **1990**, *499*, 103−124.
(30) Scopes, R. K. *Protein Purification: Principles and Practice*; Springer-Verlag, New York 1987.