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Affinity Chromatography with Collapsibly Tethered Ligands

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We introduce a novel affinity chromatography mode in which affinity ligands are secured to the media surface via collapsible tethers. In traditional affinity chromatography, the immobilized ligands act passively, and their local concentration is static. In collapsibly tethered affinity chromatography, the ligand can move dynamically in response to external stimuli, a design that enables marked changes in both the local concentration of the ligand and its surrounding environment without exchange of solvent. Using the thermoresponsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) as a scaffold for ligand and hapten attachment, we were able to achieve controlled mobility and microenvironment alteration of the affinity ligand *Ricinus communis* agglutinin (RCA_{120}). The glycoprotein target, asialotransferrin, was loaded onto a column in which PIPAAm was partially substituted with both RCA_{120} and lactose. At 5 °C, the column retained the glycoprotein, but released most (95%) of the asialotransferrin upon warming to 30 °C. This temperature-induced elution was much greater than can be explained by temperature dependency of sugar recognition by RCA_{120}. The simplest explanation is that upon thermally induced dehydration and collapse of the PIPAAm chains, coimmobilized RCA_{120} ligand and lactose hapten are brought into closer proximity to each other, enabling immobilized lactose to displace affinity-bound asislotransferrin from the immobilized RCA_{120} lectin.

Affinity chromatography has been used widely in biomedical research and biotechnology.[1] It is based on molecular recognition where one recognition partner is immobilized on a base matrix, and soluble target molecules can be retained from a crude mixture. The target molecule can then be released and recovered in a functional form. The basis of elution is to reduce the affinity between immobilized ligand and analyte. This is most often accomplished by use of a bond-breaking buffer (i.e., by changing pH, ionic strength, and solvent) or by use of competitive elution.[2] In any case, the diffusion of immobilized ligand is always strictly limited, and therefore, the local concentration of immobilized ligand is constant. The environment surrounding the immobilized ligand changes to modulate the ligand−analyte affinity.

An important question is whether similar elution can be induced if the immobilized ligand physically moves to produce changes in its local concentration and surrounding environment. Despite numerous elution studies, the concept of such a locally mobile ligand has never been described.

In the present stud,y we utilized poly(*N*-isopropylacrylamide) (PIPAAm),[3−6] a well-investigated thermoresponsive polymer, as a microactuator to induce significant environmental changes of immobilized ligand. Thermoresponsive polymers exhibit a thermoreversible phase transition in aqueous solution at a lower critical solution temperature (LCST). The polymer chain hydrates to form an extended chain conformation below the LCST, whereas it dehydrates to form a shrunken globule structure above the LCST. The hydrodynamic size of the PIPAAm chain is reported to shrink by about one-half above the LCST, which corresponds to an ∼10-fold hydrodynamic volume reduction.[7,8] It was, therefore, expected to provide a unique system in which local ligand concentrations could be regulated dynamically by external stimuli. To our knowledge, no such attempts have been previously described, though thermoresponsive polymers have been used for the development of new separation systems coupled with traditional separation techniques (e.g., precipitation,[9] aqueous two-phase system,[10] chromatography[11]).

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We employed immobilized lectin affinity chromatography[12,13] as a model system, using *Ricinus communis* agglutinin (RCA_{120}). Immobilized-RCA_{120} affinity chromatography is widely used for the fractionation and characterization of glycopeptides and oligosaccharides.[14,15] RCA_{120} binds specifically to nonreducing-end galactose residues.[16] Competitive elution using lactose, a hapten sugar, is generally used to elute the target molecule from the RCA_{120}-immobilized column. In this study, we introduced both RCA_{120} and lactose onto a column matrix via PIPAAm and investigated the effects of controlled mobility and microenvironment alteration on the binding behavior of immobilized lectin with target.

EXPERIMENTAL SECTION

Materials. *N*-Isopropylacrylamide (IPAAm; Wako Pure Chemicals, Osaka, Japan) was purified by recrystallization from a toluene−hexane mixture and dried at room temperature in vacuo. 2-Aminoethanethiol (Wako Pure Chemicals, Osaka, Japan) was distilled under low pressure. Acryloyl chloride, *N*-hydroxysuccinimide, isopropylamine, triethylamine, 2,2′-azobis(isobutyronitrile) (AIBN), lactose, tetrahydrofuran (THF), *N*,*N*-dimethylformamide (DMF), toluene, hexane, ethanolamine, hexylamine, and ethylenediamine were purchased from Wako Pure Chemicals. Human transferrin and sialidase from *Arthrobacter ureafaciens* were obtained from Sigma Chemical Co. (St. Louis, MO). Caster bean lectin RCA_{120} (CAS 172304-66-4) was obtained from Honen Corp. (Tokyo, Japan). A HiTrap NHS-activated HP column (1 mL; 0.7 × 2.5 cm) was obtained from Amersham Biosciences (Buckinghamshire, U.K.).

Preparation of Asialotransferrin (AST). Enzymatic digestion of transferrin by sialidase was performed according to the method described previously.[17] Briefly, human transferrin (25 nmol) was incubated with *A. ureafaciens* sialidase in 1 mL of 50 mM sodium citrate buffer (pH 5.0) at 37 °C for 8 h.

Synthesis of *N*-(Acryloyloxy)succinimide. Acryloyl chloride (33.4 g, 30 mL, 369 mmol) was added dropwise to a stirred solution of *N*-hydroxysuccinimide (42.5 g, 369 mmol) and triethylamine (41.0 g, 56.5 mL, 409 mmol, 1.1 equiv) in $CHCl_3$ (300 mL, 1.23 M) at 0 °C on an ice bath. The solution was allowed to stir for 3 h at 0 °C, and then it was washed with water (2 × 300 mL) and dried over $MgSO_4$. The product was recrystallized from a solution of ethyl acetate/hexane (1:1) to yield 46.1 g (213 mmol) of colorless crystal with 72% yield (mp 69 °C).

Synthesis of β-Lactosylamine. Lactosylamine was synthesized according to a modification of the method of Lihkosherstov et al.[18] Briefly, lactose monohydrate (1.0 g, 3.0 mmol) was dissolved in 50 mL of saturated ammonium hydrogen carbonate. Additional solid ammonium hydrogen carbonate was then added to maintain a saturated state during the reaction. The mixture was stirred at 37 °C for 24 h and then desalted by direct lyophilization. Lyophilized β-lactosylamine was stored at −20 °C.

Synthesis of Poly[*N*-(acryloyloxy)succinimide] (PNAS). A mixture of *N*-(acryloyloxy)succinimide (2.21 g, 13.0 mmol) and AIBN (14 mg, 0.007 molar equivalent) in benzene (100 mL) was heated at 60 °C for 24 h. A white precipitate was formed after cooling the solution to room temperature. This precipitate was filtered and washed four times with tetrahydrofuran (THF, 20 mL). Drying in vacuo yielded poly[*N*-(acryloyloxy)succinimide] (PNAS) (2.16 g, 12.7 mmol, 98% yield) as a white solid. The polymer was taken up in dry THF (300 mL), stirred vigorously overnight, filtered, and dried in vacuo. The molecular weight of PNAS was determined by GPC (as poly(acrylic acid) (PAA)) after complete hydrosis by treatment of a PNAS solution with 6 N HCl at 100 °C for 24 h. GPC analysis of PAA was performed on a Shimadzu LC-10AD with a Waters Ultrahydrogel linear column in water at 40 °C. The following standards were used: PAA of M_w 130 kD, 390 kD, and 1100 kD. The M_w of PAA determined thereby was 121 000, and the degree of polymerization was 1680.

Polymerization of IPAAm. PIPAAm was prepared by radical polymerization of IPAAm in THF. A mixture of IPAAm (20.0 g, 177 mmol), 2-aminoethanethiol hydrochloride (201 mg, 1.77 mmol), and AIBN (290 mg, 1.77 mmol) in THF (200 mL) was degassed by subjecting it to freeze−thaw cycles, and the sample containing the mixture was sealed under reduced pressure. Then the mixture was heated at 70 °C for 2 h. After evaporation of the solvent, the reaction mixture was poured into diethyl ether to precipitate the polymer. The polymer was further purified by repeated precipitation from THF poured into diethyl ether. The LCST of PIPAAm was 32 °C, which agreed well with the previously reported value.[3] The molecular weight (M_n) of the obtained PIPAAm was determined to be 4160 ($M_w/M_n = 2.2$) by GPC (TSKgel α-3000 column) at 40 °C using DMF as an eluent.

Partial Substitution of PNAS. Isopropylamine (157.4 mg, 2.66 mmol, 90 mol % relative to the succinimide groups of the PNAS) was added to a stirred solution of PNAS (0.5 g, 2.99 mmol) in DMF (3 mL) at room temperature. The solution was allowed to stir for 24 h, and then dried in vacuo. The product was redissolved in THF and poured into diethyl ether to precipitate the polymer, yielding a white powder designated PIPAAm-90 ("90" indicates the molar ratio of substituted isopropylamine) in quantitative yield. The same procedure, adjusted for the appropriate amount of isopropylamine, was used for the other substitution ratios described below.

Preparation of RCA_{120} and Lactose Co-Immobilized Polymer. Since attachment of hydrophilic lactose residues to the PIPAAm-90 polymer will alter the LCST, their effect must be balanced by additional of hydrophobic residues. For this purpose, we chose hexylamine. β-Lactosylamine (0.2 mg, 1 mol % relative to the succinimide groups of the PNAS) and hexylamine (0.5 mol % relative to the succinimide groups of the PNAS) were added to a solution of RCA_{120} (5 mg in phosphate-buffered saline [PBS], pH 7.2) at 4 °C, and the mixture was left for 30 min at 4 °C. Then, to this premixed solution, a solution of PIPAAm-90 (10 mg) in 0.2 mL of PBS was added, and the mixture was left for 12 h at 4 °C. This reaction product was applied directly for subsequent immobilization onto the column resin, as described below.

Modification of Sepharose Resin. Short spacer arms were attached to an *N*-hydroxysuccinimide HiTrap NHS-activated HR column (1 mL) of highly cross linked agarose-based Sepharose

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resin by reaction with ethylenediamine solution. Subsequently, the NHS polymer derivatized with RCA_{120} and lactose as described above was added to the column at 4 °C and allowed to attach to the spacer arms. The procedures for coupling, washing, and measuring of coupling efficiency were carried out according to the protocol provided by the manufacturer. Briefly, the coupling efficiency was determined by 280-nm UV monitoring of *N*-hydroxysuccunimide elution, which is released during the coupling reaction. The resulting column (column P) contained RCA_{120} lectin along with lactose haptens attached independently onto PIPAAm chains, which were in turn attached to the agarose-based resin. The column was stored at 4 °C until use.

The conventional immobilized-RCA_{120}−lectin column (column R) was prepared by direct injection of RCA_{120} solution onto a HiTrap NHS-activated HR column. The amount of immobilized RCA_{120} was estimated to be 40.1 nmol by the method described above. A column with RCA_{120} and PIPAAm immobilized independently (column I) was prepared by sequential injection of RCA_{120} and amino-terminated PIPPAm onto the NHS-activated column. The amounts of immobilized RCA_{120} and PIPAAm were estimated to be 40.5 and 457.7 nmol, respectively, by the method described above.

Measurement of Polymer LCST. The LCST of all polymers was determined as the temperature resulting in a 50% decrease in optical transmittance of polymer aqueous solutions (1 wt %), as measured at 500 nm using a Shimadzu UV-240 spectrophotometer.

Chromatography. Chromatography was performed using a Shimadzu LC-10AD pump and SPD-10AD UV detector (280 nm). The mobile phase was 0.01 M PBS, pH 7.4. The flow rate through the various HiTrap columns was held constant at 1.0 mL/min. Column temperature was controlled by submersion in water baths held at the specified temperatures using thermostated circulators (CL-80F, TAITEC, Tokyo, Japan).

Elution of Asialotransferrin by Temperature Changes. Twenty microliters of a 2.0 mg/mL asialotransferrin solution was injected onto the column at 5 °C, and subsequent elution of the protein was performed by rapid transfer of the column into a second water bath set to 20, 30, or 40 °C. Following 13 min of elution at the specified temperature, residual adsorbed materials were eluted with a linear gradient of 0−50 mM lactose in 0.01 M PBS buffer, pH 7.4. The amount of eluted glycoprotein was estimated from the area of the UV (280 nm) absorbance peak.

RESULTS AND DISCUSSION

Design and Preparation of a Matrix with Collapsibly Tethered Ligands. A galactose-specific lectin, RCA_{120}, and its hapten sugar, lactose, were selected for incorporation onto PIPAAm as a model system, and the complex was attached onto Sepharose beads. Though copolymers of IPAAm can be synthesized easily by copolymerization with other component(s),[19] it is generally difficult to achieve homogeneous polymerization, since the rate constant of polymerization for each component can be quite different.[20] Here, we strove for homogeneous incorporation of RCA_{120} and lactose in order to simplify the system and its evaluation.

Table 1. Typical Recovery Ratios of Asialotransferrin from Columns R, P, and I by Sequential Thermal and Competitive Elution

column	elution temperature (°C)	recovery ratio[a] thermal elution (%)	recovery ratio[a] lactose elution (%)
R	20	10.9	89.1
R	30	23.6	76.4
R	40	35.7	64.3
P	20	92.7	7.3
P	30	94.8	5.2
P	40	95.1	4.9
I	20	9.1	90.9
I	30	23.9	76.1
I	40	31.1	68.9

[a] The recovery ratios were calculated on the basis of the areas of the UV absorbance peaks observed upon thermal and subsequent competitive elution during the experiments shown in Figures 2 and 3.

We employed a strategy of sequential substitution reactions onto poly[*N*-(acryloyloxy)succinimide] (PNAS), which was itself synthesized via homopolymerization of *N*-(acryloyloxy)succinimide. Modification of the PNAS side chain was achieved by sequential reactions with isopropylamine and then a mixture of RCA_{120} and β-lactosylamine (Figure 1). The effect of the isopropylamine substitution ratio on the solubility of the otherwise water-insoluble PNAS was investigated by varying the feed ratio from 50 to 90 mol %. The substitution reaction proceeded quantitatively, and we found that 90 mol % substitution sufficed to make this polymer water-soluble. This partially substituted, water-soluble, temperature-responsive PIPAAm-90 was then successfully reacted with the mixture of RCA_{120} and β-lactosylamine (0.07, and 1.0 mol %). The number of molecules introduced onto each polymer main chain was calculated to be 1.2 and 16.8 for RCA_{120} and lactose, respectively. Since RCA_{120} possesses two carbohydrate-binding sites per molecule,[21] this comes to a 7-fold excess of hapten to binding site. Finally, the LCST-controlled polymer was immobilized onto an NHS-activated HiTrap column, as described in the Experimental Section.

Intrinsic Temperature Dependence of Sugar Recognition by RCA_{120}. Interactions between lectins and carbohydrates are often affected by temperature. Therefore, it was essential to clarify the intrinsic temperature-dependence of sugar-recognition by RCA_{120} under the conditions employed in the current study.

The chromatographic behavior of AST, whose major carbohydrate structure is asialobiantennary oligosaccharides,[22] was evaluated using a traditional immobilized RCA_{120} column. We prepared this column by immobilizing RCA_{120} onto an NHS-activated HiTrap column (column R). After injection of AST (0.5 nmol, 20 μL) at 5 °C, the temperature was increased to 20, 30, or 40 °C, and the extent of AST leakage from the column was monitored. Following each chromatographic analysis, all remaining bound species were eluted with an excess of lactose. As shown in Figure 2, temperature-dependent AST elution was observed at all temperatures tested, and it tended to increase with temperature. The quantitative values of recovery at each step are summarized in Table 1. Recoveries with temperature elution at 20, 30, and 40

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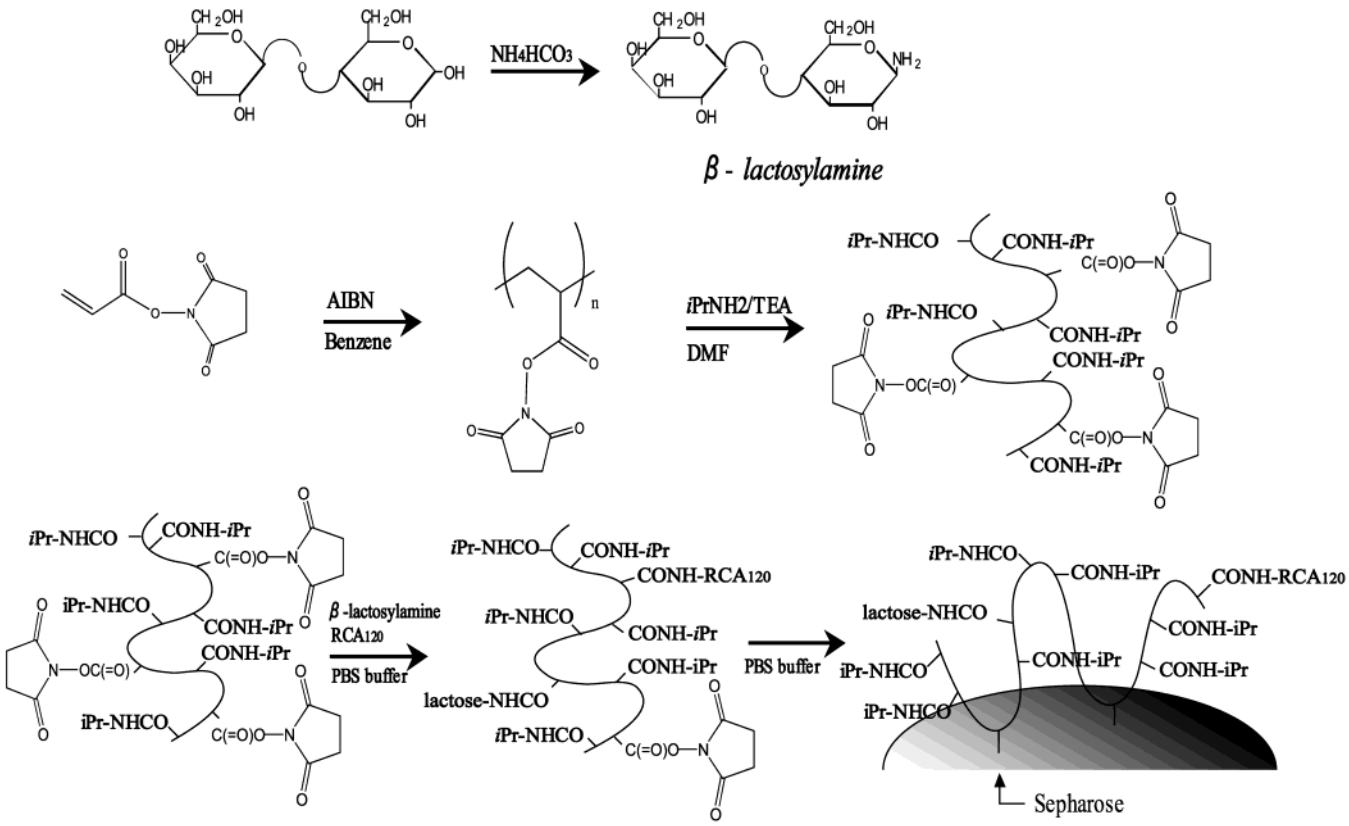

Figure 1. Synthetic procedure for preparation and immobilization of collapsibly tethered ligands onto a chromatographic matrix (column P).

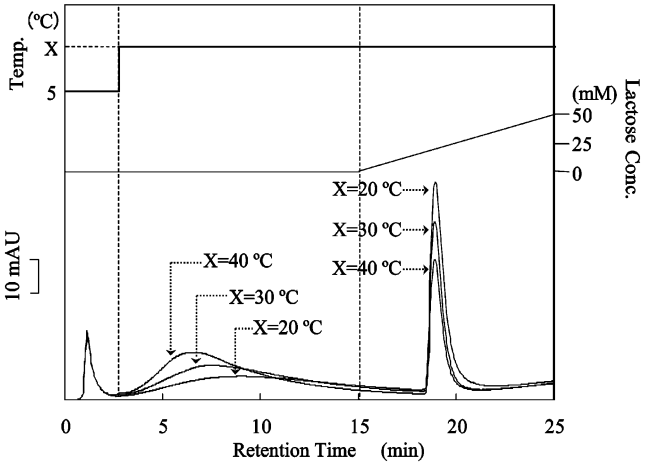

Figure 2. Chromatograms showing the intrinsic temperature dependence of sugar recognition by RCA_{120}. Flow rates were held constant at 1 mL/min. Asialotransferrin (2.0 mg/mL, 20 μL) was injected into an immobilized RCA_{120} column (1 mL) at 5 °C. Two minutes after injection, the temperature was shifted to 20, 30, or 40 °C for 13 min. Finally, residual adsorbed material was eluted with a linear (0−50 mM) lactose gradient.

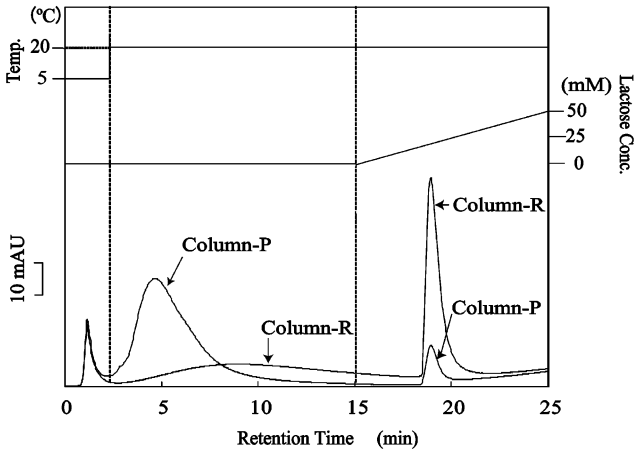

Figure 3. Elution profile comparison between columns P and R. Asialotransferrin was injected and eluted as in Figure 2, except that the same temperature shift to 20 °C was applied to both columns.

°C were calculated to be 10.9, 23.6, and 35.7%, respectively. This is consistent with the previous finding that the binding efficiency of RCA_{120} decreases slightly with increasing temperature.[23] As is generally true for carbohydrate−protein interactions, hydrogen bonding is the primary interaction force between RCA_{120} and carbohydrate.[24] The observed temperature dependence of elution is consistent with such interaction. On the other hand, the temperatures tested were insufficient to elute the majority of bound molecules.

Feasibility Study of Collapsibly Tethered Ligand Affinity Chromatography. Twenty microliters of AST (40 μg, 0.5 nmol) was injected onto column P at 5 °C, and 2 min later, the temperature was shifted to 20, 30, or 40 °C for 13 min. Each test was terminated by lactose elution of residual bound material. A 15 °C temperature shift (5 → 20 °C) eluted most of the AST (Figure 3). As summarized in Table 1, 93−95% of bound AST was

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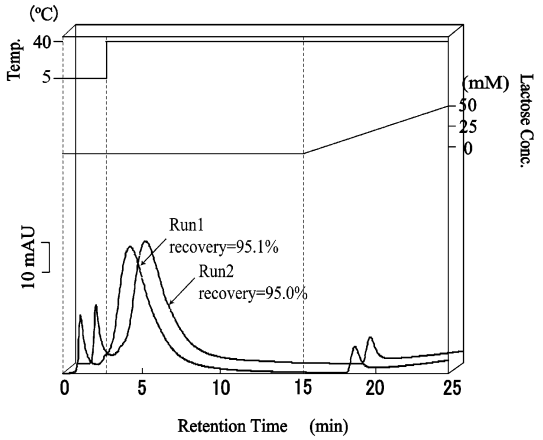

Figure 4. Chromatograms of two successive analyses on column P. The analytical conditions are the same as described in Figure 3.

eluted by temperature shift alone. This high recovery is remarkable, as compared with ordinary immobilized RCA_{120} affinity chromatography (column R). As shown in Figure 4, two successive analyses gave essentially the same chromatographic behaviors, demonstrating that all of the covalently bound components were stable under these chromatographic conditions and that response to external stimulus (temperature shift) is reversible.

To examine the effect of the introduction of PIPAAm itself on the interaction of AST with immobilized RCA_{120}, a column was prepared on which RCA_{120} and PIPAAm were independently immobilized (column I). The difference between columns I and R is that the former contains PIPAAm, but the latter does not. As indicated in the Table 1, the amount of AST recovered by temperature elution was almost the same or even less than that obtained with column R using the same injection and elution protocol. This result indicated that the effect of PIPAAm introduction on the interaction between AST and RCA_{120} is negligible.

We have previously observed thermoresponsive affinity regulation in dye-affinity chromatography through a column in which PIPAAm and Cibacron blue F3GA had been independently incorporated.[25] The interaction of Cibacron blue with proteins was regulated by masking and steric expulsion mediated by PIPAAm. That these effects were not observed with column I is most easily explained by the molecular sizes of RCA_{120} and PIPAAm employed. Although the crystal structure of RCA_{120} has not been reported, it consists of two ricin-like heterodimers, and these subunits share >80% sequence homology between the two lectins. Crystallographic analysis of ricin indicated the largest distance between two amino acid residues on the heterodimeric protein to be ~80 Å.[26] The estimated size of extended PIPAAm (M_n = 4160) based upon correlation function determined from previously reported values[25] is 56 Å.

On the basis of the above discussions, the greatly enhanced temperature elution observed with column P should be attributable to extrinsically promoted environmental change(s). Considering that the temperature shift was the sole stimulus applied, the 10-fold reduction in hydrodynamic volume reported for PIPAAm above its LCST[4,5] most likely played a key role in this phenomenon. The dramatic increase in local concentrations of immobilized ligands would alter the local environment for the two immobilized molecular species. As a consequence, the immobilized lactose hapten will compete more effectively with AST for binding to RCA_{120} (Figure 5). In effect, local movement of the immobilized ligands alters their environment and in lieu of injection of affinity-modulating or competing buffers.

Although the present study was limited to the examination of one combination of lectin and its hapten sugar, the concept of collapsibly tethered ligands was successfully demonstrated. Simple thermal modulation to achieve elution provides significant advantages over ordinary elution conditions, which require manipulation of the liquid mobile phase (i.e. pH, ionic strength or solute content). Since the required temperature change is an extremely mild elution condition, damage to biomolecules is minimized. The fact that the eluted fraction contains neither displacement agents nor salt makes the eluent very clean, providing great benefits for any subsequent assays. One promising application lies in the field of proteome research, in which such prefractionation techniques based, for example, on attached glycan moieties could remove or select a subset of proteins. The extremely mild and clean nature of the described technique is ideal for use in conjunction with other modes of chromatography and mass spectrometry.

Affinity regulation based on this concept would be applicable to other lectins as well as other specific protein−protein, drug−protein or nucleic acid−protein interactions when an appropriate hapten-like molecule is available.

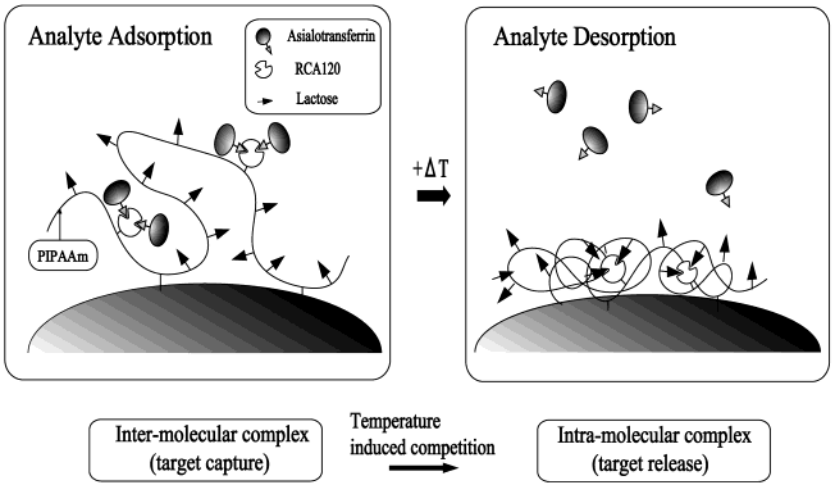

Figure 5. Schematic diagram of temperature-modulated affinity control.

CONCLUSIONS

A novel concept of affinity chromatography modulated by collapsibly tethered ligands was proposed and demonstrated. In the current study, temperature-responsive PIPAAm was used as a scaffold for the coimmobilization of RCA_{120} and lactose. This architecture enabled facile thermal manipulation of the mobility and microenvironment of the immobilized ligands. The designed column could reversibly bind asialotransferrin in a purely temperature-controlled manner. It was suggested that environmental change surrounding the immobilized ligand could be brought about by temperature shifts in which local movements of ligand and hapten altered their effective local concentrations. This simple concept is widely applicable to any case in which hapten-like molecules are available for a target.

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