Focusing Review

Development of Novel Thermally Responsible Separation Systems using Functional Polymers

Hideko Kanazawa

Faculty of Pharmacy, Keio University

1−5−30 Shibakoen, Minato−ku, Tokyo 105−8512, JAPAN

Abstract

We have been investigating a new concept of chromatography, a temperature−responsive chromatography, using a temperature−responsive poly (*N*−isopropyl acrylamide) (PNIPAAm) modified surface for the packing materials of chromatography. PNIPAAm−grafted surfaces showed temperature−responsive hydrophilic−hydrophobic surface−property alterations. Using these features, PNIPAAm and related temperature−responsive polymers have been used to generate temperature−sensitive stationary phases for chromatographic systems. We modified several different functional polymers to silica beads, including temperature and pH−responsive polymers. Temperature−dependent chromatographic interactions with soluble analytes were modulated by changing the grafted PNIPAAm chain property with a constant aqueous media as the mobile phase. These temperature−responsive stationary phases may be useful for method development as an extra tool to optimize the selectivity by adjusting the temperature. The applications of thermally responsive columns have been demonstrated for separations in the HPLC mode. Temperature−responsive chromatography is performed with an aqueous mobile phase without using an organic solvent, such as methanol or acetonitrile, which are used in reverse−phase LC. We achieved temperature−dependent separations of steroids and amino acids using only water as a mobile phase on the PNIPAAm−modified column. Temperature−responsive chromatography could achieve the separation of proteins under mild elution conditions, such as a physiological condition. Thus, temperature−responsive chromatography which can change the properties for HPLC by changing only the column temperature have advantages in maintaining the biological activity of proteins and in reduced pollution from an organic solvent. The preparation of these stationary phases and their many smart applications are reviewed here.

Key Words: Poly (*N*−isopropylacrylamide); temperature−responsive chromatography; functional polymer; environmental−responsive polymer.

1. Introduction

Stimuli−responsive polymers respond with large property changes to small physical or chemical stimuli. These functional polymers are known as “smart”, “intelligent”, “stimuli−responsive”, or “environmentally sensitive” polymers. They can take many forms: they may be dissolved in aqueous solution, adsorbed or grafted on aqueous−solid interfaces, or cross−linked in the form of hydrogels. Many different stimuli have been investigated, such as pH [1, 2], temperature [3−5], and light [5−7]. Typically, when a functional polymer’s critical response is stimulated, the polymer in aqueous solution will show a sudden onset of turbidity as it phase−separates; a surface−grafted functional polymer will collapse, converting the interface from hydrophilic to hydrophobic. These polymers have been widely investigated for drug−delivery systems [8, 9], cell culture substrates [10, 11], bioconjugates [12], and tissue engineering for regenerative medicine [13−15].

Correspondence: Faculty of Pharmacy, Keio University, 1−5−30 Shibakoen, Minato−ku, Tokyo 105−8512, JAPAN
Phone: +81−3−5400−2657,
Fax: +81−3−5400−1378
E−mail: kanazawa−hd@ pha.keio.ac.jp

There are many polymers that exhibit thermally induced precipitation, and the polymer that we have studied most extensively is poly (*N*−isopropyl acrylamide), PNIPAAm. This polymer is soluble in water below 32℃, and it precipitates sharply as the temperature is raised above 32℃ [16−18]. This spontaneous process is endothermic, and is therefore driven by a gain in entropy associated with the release of bound water molecules. The precipitation temperature is called the lower critical solution temperature (LCST). By altering the copolymer composition we could control the LCST. If the NIPAAm monomer is copolymerized with more hydrophilic monomers, LCST increases. In contrast, if the NIPAAm monomer is copolymerized with more hydrophobic monomers, such as n−butyl methacrylate (BMA), LCST decreases.

Using these features, we have been investigating PNIPAAm and related temperature−responsive polymers used to generate thermally responsible stationary phases (Fig. 1) over the last 15 years [19−41, 49]. Our major activity in this area has been to modify temperature−sensitive polymers to packing material surfaces, including terminally [19−26, 28−33], cross−linked [27], and created polymer brush [34−38]. These studies are the focus of this review. Temperature is known to play a significant role in biomolecules and chiral separation, but its influence on the separation of small molecules in conventional reverse−phase liquid chromatography (RPLC) is much less important. We have modified several different functional polymers to silica beads, including temperature− and pH−responsive polymers. These temperature−responsive stationary phases are useful in method development as an extra tool to optimize the selectivity by adjusting the temperature. This system is highly useful to control both the stationary phase function and the properties for HPLC by changing only the column temperature, with advantages in maintaining the biological activity of proteins and in reduced pollution from organic solvents commonly used in RPLC. The preparation of these stationary phases and their many smart applications are reviewed here.

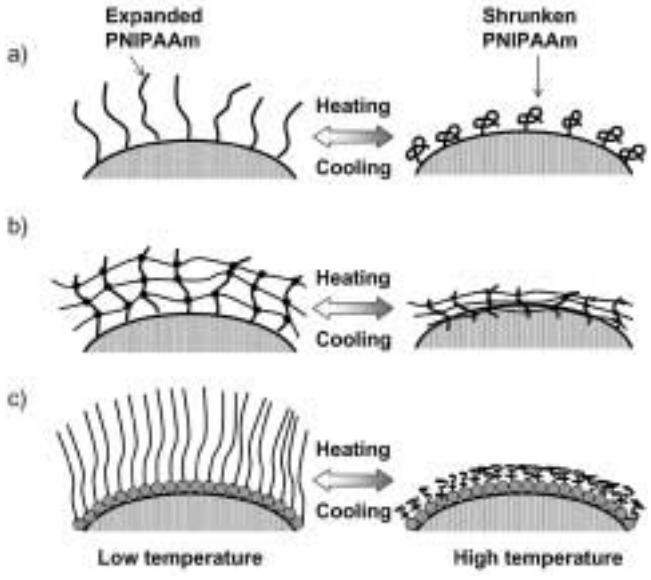


Figure 1. Schematic illustration of a various grafting methods of PNIPAAm to the silica surface; a) terminally modified; b) hydrogel modified; c) polymer brush by ATRP.

2. Synthesis of Polymer−modified surface

We have proposed three types of PNIPAAm grafting methods on silica surfaces to prepare chromatographic stationary phases (Fig. 1). First, PNIPAAm−grafted silica beads were activated using standard ester−amine coupling “grafting to” methods (Fig.1−a). We have utilized chain−transfer free−radical polymerization to synthesize polymers with one functional end group, which can then be derivatized to form a reactive group that can be grafted to the supporting materials. The synthesis of PNIPAAm was carried out by radical polymerization using 3−mercaptopropionic acid (MPA) as a chain−transfer agent, and grafted to an aminopropyl silica support by activated ester−amine coupling [23]. We have also copolymerized NIPAAm with reactive comonomers to yield a random copolymer, which was then grafted to a silica surface (Fig.2) [21]. This grafting method has the advantage of controlled molecular weights of grafted PNIPAAm chains by adjusting the relative ratios of monomers to the chain transfer agent in bulk polymerization, and purification to a pure polymer. Additionally, it also has the advantage of characterizing the properties of synthetic polymers.

A second “grafting from” method for PNIPAAm uses a surface−immobilized azo−initiator and cross−linker to prepare polymer layers with conventional radical polymerization (Fig.1−b) [27]. The PNIPAAm gel−immobilized surface described here showed a slightly lower transition temperature than that of the PNIPAAm terminally grafted surface. We observed a drastic and reversible surface hydrophilic/hydrophobic property alteration for

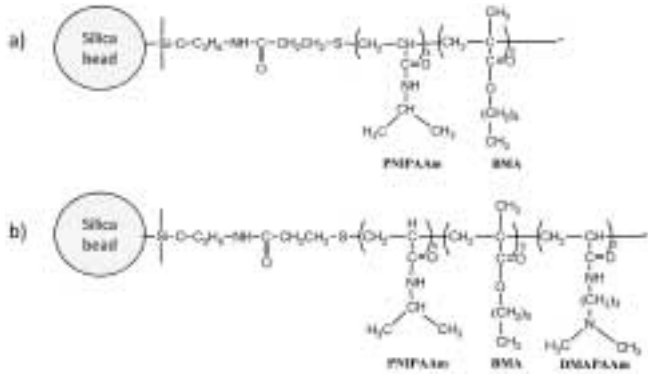


Figure 2. The structural formulas of copolymer modified silica; a) PNIPAAm−*co*−BMA modified silica, b) poly (NIPAAm−*co*−BMA−*co*−DMAPAAm) modified silica.

PNIPAAm−terminally grafted surfaces due to rapid changes in the polymer hydration state around the polymer's transition temperature [19, 28−30]. Compared with a terminally grafted surface, the restricted hydrodynamic motion of a PNIPAAm segment in grafted hydrogel was considered to be due to restricted conformational transitions. The hydrogel−modified method for preparing thermally responsive packing materials was relatively simple and easy compared with the PNIPAAm terminally grafted stationary phase. Additionally, a polymer layer formed on silica beads showed resistance to an alkaline solution [27].

A third "grafting from" method for PNIPAAm is high−density PNIPAAm brushes on silica−bead surfaces using a surface−immobilized atom transfer radical polymerization (ATRP) initiator (Fig.1 −c). Recently, controlled free−radical polymerization techniques have been utilized to synthesize polymers. Especially, an ATRP is an attractive polymer grafting method, because it enables the preparation of surfaces with dense polymer brushes from surface−immobilized ATRP initiators compared to the polymer brush surfaces prepared by conventional radical polymerizations. Thus, ATRP could produce a distinct polymer grafted layer from HPLC silica surfaces [34−38].

Yakushiji et al. [42] reported temperature−dependent wettability changes for PNIPAAm hydrogel−modified surfaces by aqueous dynamic contact−angle measurements. The graft configuration of PNIPAAm produced from different grafting methods greatly influences temperature−dependent aqueous wettability changes. Okano et al. [43] reported that the molecular mobility and density of a PNIPAAm chain are greatly influenced by a difference in the construction structure of PNIPAAm modified surfaces due to the temperature. It is considered that it maintains high mobility by fixing the PNIPAAm molecule at the end on the modified terminal, and quickly responds to any temperature change.

3. Temperature−responsive Chromatography

Using a column packed with PNIPAAm−modified silica, HPLC separation of steroids was carried out by changing the temperature. Fig. 3 shows typical chromatograms of steroids on a PNIPAAm terminally modified column at a variety of temperatures. The retarded retention times were observed with increasing temperature. With increasing temperature, an increased interaction between the solutes and PNIPAAm−grafted surfaces of the stationary phases was observed. It was considered that a hydrophobic interaction exists between the steroids and the PNIPAAm−modified column. In contrast, in the common RPLC system, the opposite behavior of the decreased retention times was observed with increasing temperature. A temperature−dependent resolution was achieved using only water as a mobile phase on the PNIPAAm−modified column. A drastic and reversible property alteration of PNIPAAm−

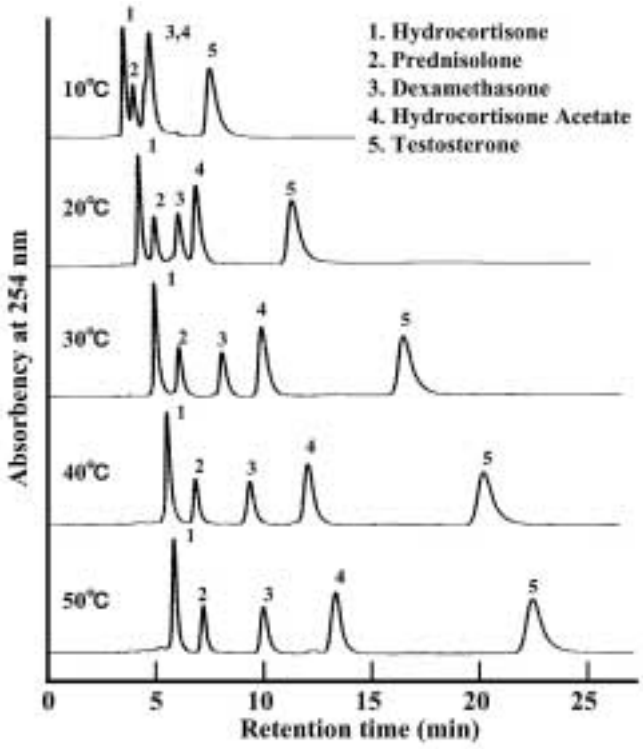


Figure 3. Temperature−dependent retention profile of the steroids using water as a mobile phase. HPLC conditions: flow rate, 1.0 mL/min; detection, UV at 254 nm; eluent, MilliQ−water; injection volume, 20μL; column, PNIPAAm −*co*−BMA 5% terminally modified silica (5μm, 4.6 mm I.D. × 150 mm).

terminally grafted surfaces from hydrophilic to hydrophobic is due to rapid changes in the polymer hydration state around the polymer's transition temperature [44]. As shown in Fig. 4, the retention of steroids exhibits a linear relationship with the log *P* values (the partition coefficients in a 1−octanol/water system). The log *P* values for each steroid are 1.61 for hydrocortisone, 1.62 for prednisolone, 1.83 for dexamethasone, 2.30 for hydrocortisone acetate, and 3.32 for testosterone. The more hydrophobic steroids showed longer retention times. These results indicate that a stronger hydrophobic interaction is the primary driving force for partitioning steroids into PNIPAAm−modified matrices. Since the phase transition of PNIPAAm results from the stability of hydrophobic groups along the polymer chain in aqueous media, the LCST for the polymer should decrease with increasing polymer hydrophobicity [21]. By altering the copolymer composition we could control the LCST and the hydrophobicity of the surface of the stationary phases, and thus the elution time of the temperature−responsive chromatography.

The plot of the retention factor (ln k) versus the reciprocal temperature ($1/T$) normally shows a linear regression, with the slope representing the enthalpy change involved for the retention

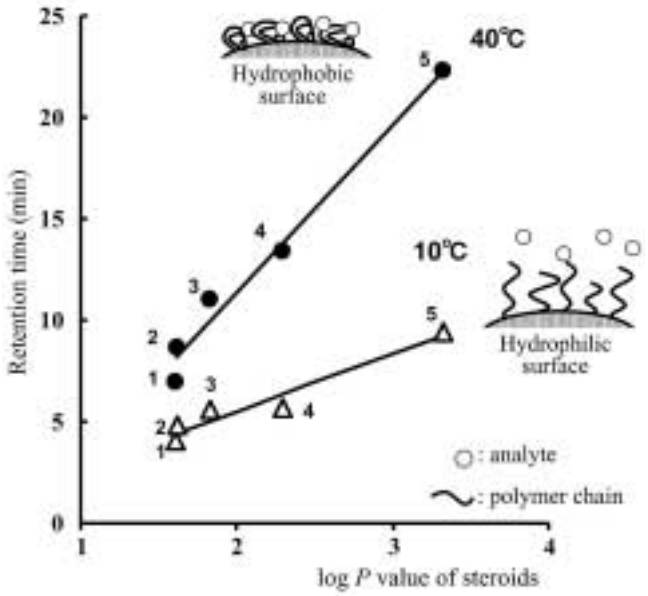


Figure 4. Effects of the log *P* value of steroids on the retention times. Column, PNIPAAm−modified column (5μm, 4.6 mm I.D. × 150 mm); samples: 1, hydrocortisone; 2, prednisolone; 3, dexamethasone; 4, hydrocortisone acetate; 5, testosterone. HPLC conditions; detection, UV at 254 nm; eluent, pure water; flow rate, 1.0 mL/min.

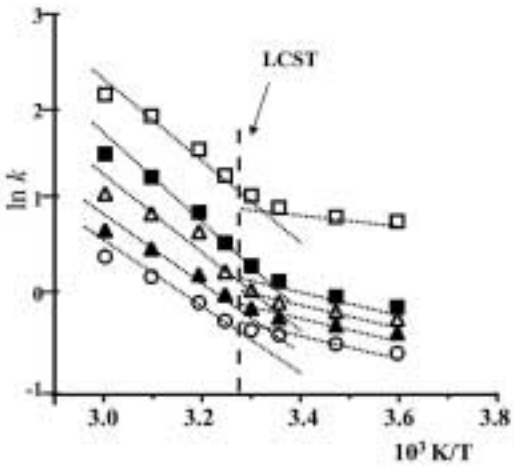


Figure 5. The effects of temperature on the retention factor of the steroids on the PNIPAAm−modified column. HPLC conditions are the same as those in Figure 3. Samples: ○, hydrocortisone; ▲, prednisolone; △, dexamethasone; ■, hydrocortisone acetate; □, testosterone.

reaction. Fig. 5 shows the plot for the steroids on the PNIPAAm−modified column. The slope of the plots on the PNIPAAm−modified column is negative, opposite to those observed for conventional chromatography. This provides evidence that the interaction between steroids and temperature−responsive surfaces becomes stronger at elevated temperature. Additionally, linearity in the plots is commonly observed for commercially available RPLC columns under standard chromatographic conditions with these narrow temperature ranges. On the PNIPAAm−modified column, however, a deviation from linearity was found between the ln *k* values and $1/T$. The slope of the plots of each analyte on the PNIPAAm−modified column changed markedly at the LCST boundary. It was indicated changing the retention mechanism at the temperature. This corresponds to a phase transition of the polymer modified on the surface.

4. Dual temperature− and pH−responsive Chromatography

There have been several reports concerning the pH dependence of LCST. Hoffman et al. [45] examined the LCST of a copolymer of NIPAAm with AAc, and showed that the LCST increased with an increase of the pH of the buffer solutions used. Kim et al. [46] reported on the pH sensitivity of the LCST of a copolymer containing, diethylaminoethyl methacrylate (DEAEMA). Also, Kobayashi [47, 48] made an examination on the effect of the charge density for a temperature change by measuring p*K*a and the Zeta−potential. Regarding the p*K*a of the copolymer of NIPAAm, BMA and *N*, *N*−dimethylaminopropylacrylamide (DMAPAAm), at high temperature, dehydration of the NIPAAm isopropyl groups occurs, as well as an enhanced deprotonation of the amino group. The surface charge density as well as the hydrophobicity group was shown to be altered by changing the temperature. These polymers responded to both the temperature and the pH. Control of the surface charge density became possible with structural changes of the polymer chain by changing the temperature. These facts show that it was expected to control different interactions by hydrophobic and electrostatic interactions, by changing only the temperature.

The analysis of adenosine nucleotides (Adenosine−5'−monophosphate; AMP, adenosine−5'−diphosphate; ADP and adenosine−5'−triphosphate; ATP) was proposed, utilizing a pH− and temperature−responsive polymer, of poly (NIPAAm−*co*−BMA−*co*−DMAPAAm) as the stationary phase (Fig. 2−b). As shown in Fig. 6, the separations of nucleotides with a difference in the number of anionic phosphate units were achieved under isocratic conditions. The retention times increased with increasing numbers of the phosphate units at the column temperature studied. The mobile phases of pH 3.0 and 4.5 were examined; the p*K*a values of the nucleotides fell between the pH values. These results indicated that a column modified with poly (NIPAAm−*co*−BMA−*co*−DMAPAAm) can respond to both the temperature and the pH to modulate the retention of charged compounds using an aqueous mobile phase. The nucleotides were retained at pH 4.5. The results are explained in terms of the electrostatic interaction. At lower temperature, the polymer chain is hydrated, and hence the electrostatic interaction between the dissociated adenosine phosphates and the anion−exchange group should increase. The retention times were retarded

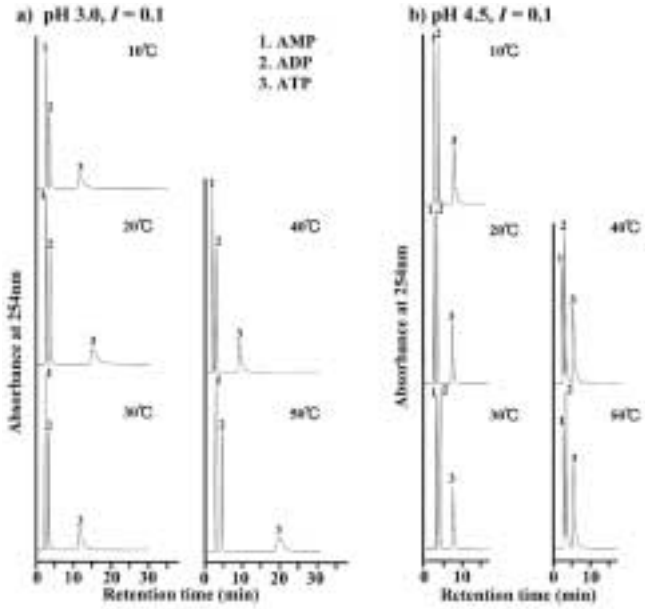


Figure 6. Chromatograms of adenosine nucleotides (AMP, ADP, and ATP) on poly (NIPAAm−*co*−BMA−*co*−DMA-PAAm) modified silica column. HPLC conditions: flow rate, 1.0 mL/min; eluent, phosphate/citrate buffer.

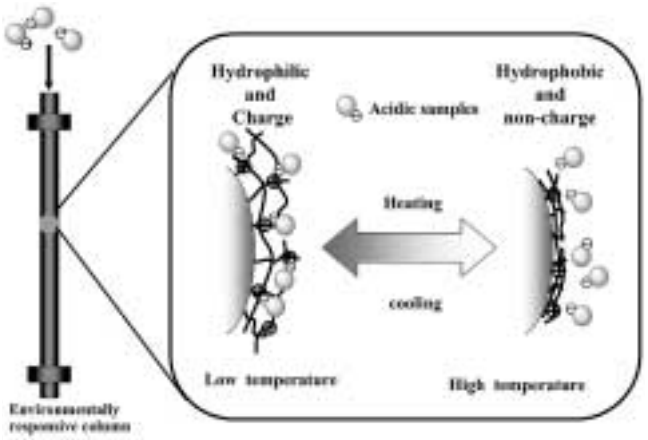


Figure 7. Schematic illustration of dual pH− and temperature−responsive chromatography.

with increasing temperature at pH 3.0. Because the adenosine phosphates are in undissociated forms under these conditions, increasing of the retention by the hydrophobic interaction was confirmed by raising the temperature. The property of the surface of the copolymer grafted stationary phase altered from hydrophilic to hydrophobic and from charged to non−charged by changes in the temperature and the pH, respectively. In addition, it is possible to expose and hide ion−exchange groups on the polymer chain surface by changing the temperature. These phenomena result from changes in the charge and the hydrophobicity of the pH− and temperature−responsive polymer on the stationary surface by controlling the temperature (Fig.7). Dual pH− and temperature−responsive chromatography would be very useful for the separation of biomacromolecules and their purification as well as nucleotides.

We also prepared dual temperature− and pH−responsive polymers, poly (*N*−isopropylacrylamide−*co*−acrylic acid−*co*−*N*−tert−butylacrylamide), poly (NIPAAm−*co*−AAc−*co*−tBAAm) or poly (*N*−isopropylacrylamide−*co*−carboxy methacrylic acid−*co*−*N*−*tert*−butylacrylamide), poly (NIPAAm−*co*−CNIPAAm−*co*−tBAAm), grafted on silica beads as column packing materials for a cation−exchange and thermally responsive chromatography [31, 32]. The stationary phase showed simultaneous changes in the temperature−responsive surface charge density and hydrophobicity by the incorporation of anionic AAc and hydrophobic tBAAm into NIPAAm sequences. Thermally responsive polymer property alterations were confirmed by temperature−responsive phase transition and shifts in the apparent p*K*a values.

Electrostatic and hydrophobic interactions could be modulated simultaneously with the temperature in an aqueous mobile phase. The present separation system would have potential applications in separation science.

5. Temperature programming on PNIPAAm modified silica column

We investigated the effect of PNIPAAm on the surface of a stationary phase on separation based on changes in the retention time with a temperature step gradient [20, 29, and 30]. As the temperature changed, the surface property of the stationary phase switched from hydrophilic to hydrophobic. The retention on the polymer−modified stationary phase remarkably changed upon changing the temperature. A PNIPAAm−modified surface of the stationary phase exhibited temperature−controlled hydrophilic−hydrophobic changes. Drastic and reversible surface hydrophilic−hydrophobic property alterations for PNIPAAm grafted surfaces should be due to rapid changes in the polymer hydration state around the polymer's transition temperature. With a single mobile phase of water, and by controlling the external temperature, it was possible to obtain an effect similar to the solvent gradient. Therefore, it was considered that it might become possible to separate those compounds for which separation has been difficult until now only in the temperature program. A solvent gradient elution−like effect could be achieved with a single mobile phase by programmed temperature changes during chromatographic runs on HPLC columns packed with thermoresponsive polymer modified silica. Fig. 8−c shows a typical chromatogram of a linear temperature gradient. Optimization of the analysis was attempted regarding two components, levonorgestrel (log P = 2.871) and ethinylestradiol (log P = 4.017) of an oral contraceptive using a temperature gradient. Those components that are not metabolized in the urine of a woman remained. As a result, it can efflux to rivers, causing

water pollution that effects fishes and animals that inhabit the rivers. It was determined using a PNIPAAm terminally modified column in which the surface property changes were rapid. The chromatograms of levonorgestrel and ethinylestradiol of an oral contraceptive that was obtained at 15℃, 40℃ and with a temperature gradient are shown in Fig. 8. For levonorgestrel with a lower hydrophobicity, the retention time hardly changed at 15℃ and 40℃. However, for ethinylestradiol with a higher hydrophobicity, the retention time increased too much, and influenced the hydrophobic interaction at 40℃. To move strongly retained components of the oral contraceptive and to optimize the analysis, we used a temperature−programming technique. With a single mobile phase of water and by controlling the external temperature from 40℃ to 15℃, the analytical time was reduced (Fig.8−c). An excellent resolution of the oral contraceptive was achieved using a temperature gradient. This was caused because the PNIPAAm−modified surface property of the stationary phase changed to hydrophilic at a decreased temperature, and the hydrophobic interaction between the component and the stationary phase was decreased. Additionally, this fact indicated the effectiveness of 'thermally−responsive' and 'thermally−reversible' property alterations of a PNIPAAm−modified surface. This system should be highly useful to control the function and property of the stationary phase for HPLC by only changing the temperature with an aqueous solvent.

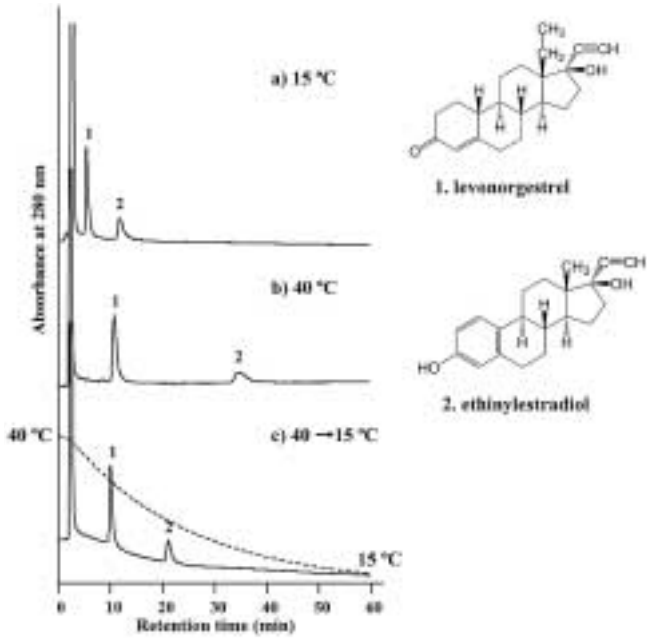


Figure 8. Effects of temperature changes on the retention times of levonorgestrel and ethinylestradiol. HPLC conditions: flow rate, 1.0 mL/min; eluent, water; detection, UV at 280 nm; column, PNIPAAm−modified column; temperature gradient speed, 25℃/60 min.

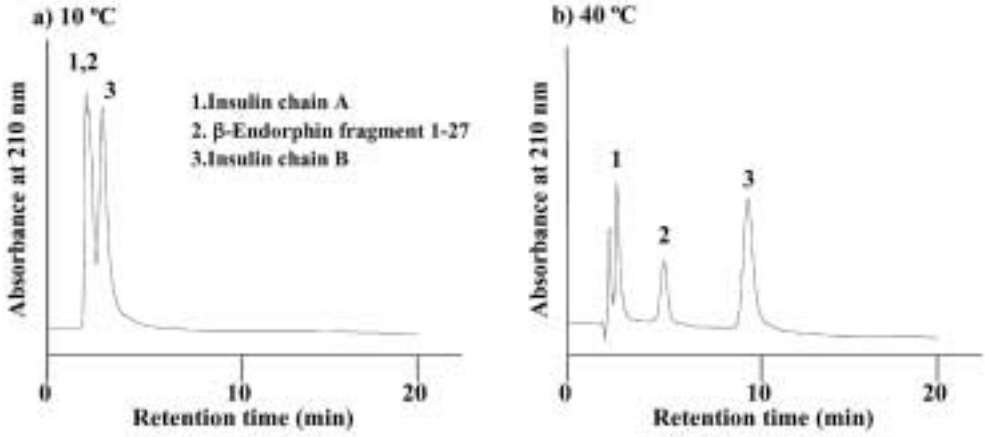
6. Application to bio−separations

Biomolecules, such as peptides and proteins, are currently separated by RPLC, ion−exchange chromatography systems and their combinations. RPLC is one of the most widely used chromatographic techniques in the separation, purification and study of peptides. The most common mobile phase used in the RPLC of peptides and proteins involves an aqueous acetonitrile solution including 0.1% (10 mM) trifluoroacetic acid (TFA). Denaturation and a loss of biological activity are unavoidable consequences of the elution process. Thus, these conditions should be avoided when working with most proteins.

Temperature−responsive chromatography was applied to the separation of peptides and proteins. As shown in Fig. 9, the separation of a mixture of three peptides, insulin chains A and B and β−endorphin fragment 1−27 was achieved by changing the column temperature with a 0.9% NaCl aqueous solution as the sole eluent. The elution order of the three peptides should reflect their hydrophobic properties. The retention times of peptides increased with increasing the temperature. The property of the surface of the stationary phase was altered from hydrophilic to hydrophobic. The results provide evidence that the interaction between peptides and temperature−responsive surfaces becomes stronger at elevated temperature. We confirmed that the method was also applicable for the separation of both low−molecular−weight proteins, such as ribonuclease and chymotrypsinogen, and high−molecular−weight proteins, such as ovalbumin, catalase, and bovine serum albumin. The temperature−dependent retention profiles of protein were observed on thermally−responsive columns [20, 41].

Recently, we designed pH− and temperature−responsive ternary co−polymers of NIPAAm introduced 2−dimethylaminoethyl methacrylate (DMAEMA), which is a cationic monomer as an ion−exchange group and BMA as a hydrophobic monomer. A cationic thermoresponsive hydrogel grafted surface would produce an alterable stationary phase with both thermally regulated hydrophobicity and charge density for the separation of bioactive compounds. We investigated the separation of human serum albumin (HSA) on a poly (NIPAAm−*co*−DMAEMA−*co*−BMA) hydrogel modified column. HSA was retained on the column with water as a mobile phase at 40℃, and eluted by changing from water to a 0.9% NaCl aqueous solution. The recovery of HSA was good [33]. The results indicated that the present chromatographic system could modulate the adsorption and desorption of albumin from the stationary phase by changing water to 0.9% NaCl.

Temperature−responsive chromatography could achieve the separation of proteins under mild elution conditions, such as a physiological condition. In a conventional hydrophobic interaction chromatography, the separations were performed using a starting mobile phase of high ionic strength to promote hydrophobic bind-



Peptides	Amino acid sequence
Insulin chain A (M.W.2531)	Gly-Ile-Val-Glu-Gln-Cys(SO3H)-Cys(SO3H)-Ala-Ser-Val-Cys(SO3H)-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys(SO3H)-Asn
Insulin chain B (M.W.3022)	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr
β-Endorphin 1-27 (M.W.3495)	Phe-Val-Asn-Gln-His-Leu-Cys(SO3H)-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys(SO3H)-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

Figure 9. Chromatograms of peptides using poly (NIPAAm−*co*−BMA 5%) modified column. Hydrophobic amino acids were indicated underline. HPLC conditions: flow rate, 1.0 mL/min; detection, UV at 210 nm; eluent, 0.9 w/v% NaCl aqueous solution; injection volume, 10μL; column, PNIPAAm−*co*−BMA 5% terminally modified Silica.

ing followed by a gradient to a decreased the salt concentration. The problem of requiring desalting also occurs from the use of a large amount of salt. We successfully achieved the separation of lysozyme without a loss of bioactivity by temperature−responsive chromatography [33]. We studied the preparative separation of lysozyme on a PNIPAAm hydrogel modified column. The recovery of lysozyme from the column was 100%. The lysozyme fractions were collected and used for measuring the bacteriolytic activity of lysozyme. After solvent removal, the activity of lysozyme was measured. The activities of lysozyme collected by temperature−responsive chromatography were approximately the same as that of the lysozyme standard (control proteins) [33]. In contrast, the activities of lysozyme collected by RPLC were reduced as compared to that of the control lysozyme. The electrostatic and hydrophobic interactions could be modulated simultaneously with the temperature in an aqueous mobile phase, and thus the separation system would have potential applications in the separation of biomolecules.

7. Conclusion

We developed a novel chromatographic system in which the surface properties and function of the HPLC stationary phase are controlled by external temperature changes without changing the mobile−phase composition. By altering the copolymer composition, we could control the LCST of the polymer and the hydrophobicity of the surface of the stationary phases, and thus the elution time of temperature−responsive chromatography. We succeeded to demonstrate hydrophobic interaction chromatography−like separation selectivity by simply changing the column temperature with pure water as a mobile phase. The ability of the proposed thermoresponsive polymer−modified stationary phase to separate the solutes without using an organic solvent is advantageous from the point of view of keeping the biological activity, environmental reasons, and mobile−phase reagent cost.

We also designed and synthesized a thermo−responsive polymer carrying an amino acid ester residue as a side chain. The poly (acryloyl−L−proline methyl ester)−modified stationary phase showed greater affinity for hydrophobic aromatic amino acids than the PNIPAAm−modified surface [49]. We are currently conducting studies to utilize a thermo−responsive polymer carrying amino acid ester residue for the chiral stationary phase.

I believe that the proposed temperature−responsive chromatography is likely to be useful for diverse applications in biotechnology including protein purification and bio−separations.

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