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Thermoresponsive-polymer-based materials for temperature-modulated bioanalysis and bioseparations

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Many bioactive compounds, pharmaceutical proteins, and therapeutic cells are used in medical treatments. Methods for the effective purification of such compounds that retain their activities are greatly needed. This review article describes various types of materials based on thermoresponsive polymers for bioanalysis and bioseparations, *e.g.*, thermoresponsive chromatography and thermally modulated cell separation. Poly(*N*-isopropylacrylamide) (PIPAAm) and its derivatives show temperature-dependent hydrophilic/hydrophobic alterations and conformational changes of their polymer chains in response to external temperature changes. These intrinsic thermoresponsive properties are used to induce thermally modulated interactions between a PIPAAm-modified stationary phase and analytes, or thermally modulated cell adhesion and detachment, enabling modulation of the separation driving force by changing the external temperature. These separation techniques are potentially useful for various applications in biotechnology and biomedicine, because separation can be achieved by simply changing the external temperature, without using reagents that damage and deactivate biological compounds, proteins, and cells.

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1. Introduction

In biomedical engineering, many bioactive compounds, pharmaceutical proteins, and therapeutic cells are used in medical treatments.[1–4] Methods for effective separation of such compounds are becoming increasingly important. Various techniques have been developed for effectively analyzing and separating

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bioactive compounds, such as chromatography and cell-sorting systems. These separation and purification techniques have been important in the development of bioengineering and biomedical engineering; for example, chromatography is a powerful separation and analytical tool for bioactive compounds and proteins, and is used in blood analysis for diagnosis, and for purification of pharmaceutical products.[5–8] Ordinary chromatographic systems such as reversed-phase chromatography or ion-exchange chromatography consist of a stationary phase (the packing material) and a mobile phase. Analytes in the mobile phase interact with the stationary phase, and the differences among the interactions between each analyte and the stationary phase lead to different elution times and therefore separation. Hydrophobic or electrostatic interactions between the stationary phase and analytes are modulated by adding an organic solvent or increasing the salt concentration in the mobile phase. Many bioactive compounds have been analyzed using chromatographic systems, and separation protocols for various compounds and proteins have been investigated systematically.

Cell-separation techniques such as fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) have also been developed.[9–15] These cell-separation (cell-sorting) systems are based on differences among cell markers. Fluorescent-labeled antibodies or magnetic beads have been used to modify cell surfaces through specific antibody reactions with cell markers. The targeted cells are sorted based on different fluorescent or magnetic particle modification, leading to precise cell separation. Cell-sorting systems have been important in developing cell biology, tissue engineering, and regenerative medicine.

However, there are problems with using such techniques for separating biorelated compounds; for example, chromatographic techniques such as reversed-phase chromatography and ion-exchange chromatography, which use organic solvents and salts in the mobile phase, can deactivate bioactive compounds and proteins. Also, organic solvent or salt removal after chromatographic separation is required for further analyte purification. In the case of cell separation, the fluorescent or magnetic beads must be detached from the cell surfaces, because the fluorescent or magnetic beads used to modify the cell surfaces would cause problems after transplantation into the human body.

Stimuli-responsive polymers and their modified surfaces have attracted much attention in materials science.[16–23] In particular, the thermoresponsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) is widely used in biomedical applications such as thermally modulated drug and gene deliveries,[24–26] thermoresponsive bioconjugated proteins and enzymes,[27,28] biosensors and actuators,[29–31] and thermoresponsive cell culture substrates for fabricating cell sheets.[32–36] This polymer shows thermoresponsive hydrophilic/hydrophobic changes across its lower critical solution temperature (LCST), 32 °C,[37] which is near the human-body temperature. Several investigators have used thermoresponsive polymer properties in bioseparations. A typical application is thermoresponsive chromatography. Such systems use a thermoresponsive-polymer-modified stationary phase and an all-aqueous mobile phase. Bioactive compounds, peptides, and proteins have been separated through the hydrophobic interactions between analytes and a thermoresponsive-polymer-modified stationary phase. The hydrophobic interaction can be modulated by changing the external temperature, because the hydrophobicity of the thermoresponsive polymer in the stationary phase changes. Unlike conventional reversed-phase chromatography, it is unnecessary to add an organic solvent to the mobile phase to modulate the interactions between analytes and the stationary phase, therefore deactivation of biorelated analytes and environmental pollution by organic solvent wastes are avoided.

Thermoresponsive polymers are also used for cell separation without cell-sorting systems such as FACS or MACS. Different cells have different adhesion or detachment properties on thermoresponsive-polymer-modified surfaces. These properties can also be modulated based on the properties of the grafted thermoresponsive polymer, *e.g.*, graft chain length, graft density, and graft amount. Such interactions between thermoresponsive polymers and cells have been investigated for use in simplified cell-separation techniques without cell–surface modification.

In this review, we describe various bioanalytical and bioseparation techniques based on thermoresponsive polymers, especially PIPAAm and its copolymers. Thermoresponsive chromatographic techniques for separating bioactive compounds and proteins while retaining their biological activities, and cell-separation techniques that do not require cell–surface modification are reviewed. We focused on these two topics to limit the length of the review; however, there are other bioanalytical and bioseparation techniques such as those using thermoresponsive-polymer-modified membranes,[38–40] magnetic beads,[41,42] and microfluidic techniques.[40,43–45]

2. Thermoresponsive chromatography matrices

Chromatography is one of the most useful and successful separation techniques. High-performance liquid chromatography, in particular, is widely used for analyzing biorelated compounds, peptides, and proteins. Ordinary liquid chromatography (reversed-phase chromatography) uses organic solvents for eluting analytes. However, some analytes such as proteins are deactivated by organic solvents. Furthermore, the use of organic solvents leads to environmental problems, because large amounts of organic waste solutions need to be treated. Thermoresponsive chromatography using thermoresponsive polymers was developed to overcome such problems. Thermoresponsive chromatography uses a thermoresponsive-polymer-modified stationary phase, usually silica beads or polymer beads (Fig. 1). Table 1 summarizes the various types of thermoresponsive chromatography columns. The hydrophobic properties of thermoresponsive polymers alter when the external temperature is changed. The hydrophobicity of the stationary phase can therefore be controlled by changing the external temperature, leading to changes in the interactions between

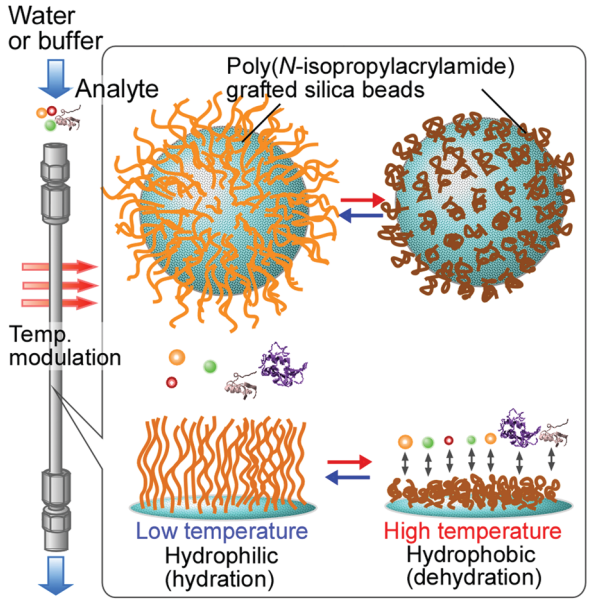

Fig. 1 Concept of thermoresponsive chromatography using stationary phase modified with thermoresponsive polymer.

analytes and the stationary phase. Such thermal modulation of interactions avoids analyte deactivation by organic solvents and reduces pollution from waste organic solvents. Thermoresponsive chromatography is a new type of tool for separating and analyzing bioactive compounds and biorelated peptides and proteins while retaining their biological activities. The separation efficiency of thermoresponsive chromatography is influenced by the PIPAAm-grafting configuration on the stationary phase. Various methods for the modification of PIPAAm and controlling the PIPAAm-grafting configuration on silica bead surfaces have been investigated.

An early type of thermoresponsive chromatography stationary phase was developed by modifying silica bead surfaces with PIPAAm *via* a coupling reaction (Fig. 2a).[46] PIPAAm of molecular weight 4400 with carboxyl terminal groups was synthesized using radical polymerization, and then the terminal carboxyl groups were activated with *N*-hydroxysuccinimide using dicyclohexylcarbodiimide. The terminally activated PIPAAm was reacted with aminopropyl silica beads to prepare modified silica beads. The prepared silica beads were packed into a stainless-steel column and the elution of hydrophobic steroids from the column was investigated. At 5 °C, all the steroids were eluted as almost one peak. With increasing temperature, the retention times of the steroids were prolonged and baseline separation was achieved at 35 °C and above (Fig. 3).[46] This is attributed to enhanced hydrophobic interactions between the PIPAAm-modified silica bead surfaces and steroids at elevated temperatures. These results indicate that interactions between the stationary phase and analytes were modulated by changing the column temperature. Thermoresponsive chromatographic systems can therefore be used for analyte separation using an all-aqueous mobile phase, without organic solvents.

The amount of PIPAAm on the silica bead surfaces was increased by preparing silica beads modified with a PIPAAm hydrogel, using an immobilized azo initiator and *N,N′*-methylenebisacrylamide as a crosslinker (Fig. 2b).[47] A thin PIPAAm hydrogel layer was formed on the silica bead surfaces. The steroid retention times on these beads packed in a column were longer, because of the larger amount of PIPAAm on the silica bead surfaces compared that on the silica bead surfaces with terminally modified PIPAAm. Also, the peak widths were wider than in the case of the beads with terminally modified PIPAAm, because the steroid molecules diffuse into the PIPAAm hydrogel layer, leading to uneven elution of steroids and wider peaks. The wider peak resulted in a poorer separation performance, although the PIPAAm hydrogel layer gave strong hydrophobic properties and long analyte retention times.

A large amount of PIPAAm was also grafted on silica bead surfaces through surface-initiated atom-transfer radical polymerization (ATRP) (Fig. 2c). ATRP is a controlled living radical polymerization method that enables accurate control of the polymer chain length. Dense polymer brushes can also be formed on substrates.[48–54] A large amount of PIPAAm can therefore be grafted on silica bead surfaces. Silica beads grafted with dense PIPAAm brushes with various chain lengths were prepared by changing the ATRP reaction time.[55] The amount of PIPAAm on the silica beads was almost ten times greater than that on the beads prepared by modification with a PIPAAm hydrogel layer. The steroid retention times on a column packed with these beads were long, because of strong hydrophobic interactions between the steroids and the large amount of PIPAAm on the silica beads. However, although a long retention time was observed, the peaks were wider than those for beads treated with terminally modified PIPAAm beads.[46] This is because the steroid molecules diffuse into the brush layer, leading to uneven elution of analytes.

A comparison of three types of PIPAAm-modified beads, *i.e.*, linear terminally modified PIPAAm, PIPAAm hydrogel, and PIPAAm brushes, showed that the steroid peaks obtained using the column with linear-PIPAAm-modified beads were sharper than those obtained using the others. In contrast, a PIPAAm brush bead column showed strong hydrophobic interactions with steroids, enabling steroid separation using a short column. PIPAAm brushes are also useful for separation of relatively hydrophilic analytes because of their strong hydrophobic properties. The type of beads used depends on the application.

ATRP can be used to modulate the PIPAAm brush density by diluting the initiator density on the substrate.[56,57] 2-(*m,p*-Chloromethylphenyl)ethyltrichlorosilane, which is an ATRP initiator, and a coadsorber, *i.e.*, phenylethyltrichlorosilane, were reacted with silica bead surfaces *via* coupling reactions by changing their contents.[56] PIPAAm brushes of various densities were prepared by surface-initiated ATRP using different initiator densities. When these beads were used as packing materials, long retention times were observed with dilute PIPAAm brushes. This is because the steroid molecules interact with exposed phenethyl groups on the PIPAAm-grafting interface.

The hydrophobicity at the PIPAAm-grafting interface was modulated by changing the coadsorbed silane-coupling agent.[58] The use of a hydrophilic coadsorbed silane-coupling agent

Table 1 Summary of thermoresponsive chromatography columns

Polymer	Graft configuration (modification method)	Base materials (diameter, pore diameter)	Column length	Analytes	Remarks	Column performance	Ref.
PIPAAm	Linear polymer (coupling reaction)	Aminopropyl silica beads (5 μm, 12 nm)	150 mm × 4.6 mm i.d.	Steroids	Narrow steroid peaks	Five steroids separated in 20 min at 35 °C Retention of testosterone was 18 min at 35 °C	46
PIPAAm	Hydrogel (radical polymerization)	Aminopropyl silica beads (5 μm, 12 nm)	150 mm × 4.6 mm i.d.	Steroids	Wide peaks, relatively long analyte retention times	Retention time of testosterone was 110 min at 50 °C	47
PIPAAm	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Steroids	Large amount of PIPAAm on surface, strong hydrophobic interactions	Retention time of dexamethasone was 35 min at 40 °C	55
PIPAAm	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Steroids	Modulation of brush density, interactions with base materials of diluted brushes	Retention time of dexamethasone was approximately 15 min at 40 °C	56
PIPAAm	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Steroids	Modulation of graft interface polarity	Retention times of hydrocortisone acetate were 4 min and 30 min on hydrophilic and hydrophobic graft interfaces, respectively	58
PIPAAm	Linear polymer (RAFT polymerization and coupling reaction)	Aminopropyl silica beads (5 μm, 12 nm)	100 mm × 2.1 mm i.d.	Steroids	Modulation of analyte interactions by terminal group of PIPAAm	Retention factors of testosterone were 8 and 2 on hydrophobic and hydrophilic terminal groups, respectively	63
P(IPAAm-*co*-BMA)	Linear polymer (coupling reaction)	Aminopropyl silica beads (5 μm, 12 nm)	150 mm × 4.6 mm i.d.	Steroids, insulin fragment, phenylthiohydantoin (PTH)–amino acid, propofol	Enhanced hydrophobic interactions, lowering of LCST, separation of hydrophilic analytes	Retention time of testosterone was 60 min at 40 °C Retention time of PTH–Trp was 45 min at 35 °C Retention time of propofol was 20 min at 20 °C	64–66
P(IPAAm-*co*-BMA)	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Benzoic acids	Enhanced hydrophobic interactions, lowering of LCST	Retention time of ethyl benzoate was 25 min at 25 °C.	67
P(IPAAm-*co*-Nap)	Linear polymer (coupling reaction)	Aminopropyl silica beads (3 μm)	100 mm × 2.1 mm i.d.	Naphthalene, 1,5-dinitronaphthalene and steroids	Selective retention of aromatic compounds	Retention times of naphthalene and 1,5-dinitronaphthalene were 5.5 and 7.6 min, respectively, at 25 °C	68
P(NIPAAm-*co*-Phe-OMe), P(NIPAm-*co*-Trp-OMe)	Linear polymer (coupling reaction)	Aminopropyl silica beads (3 μm)	100 mm × 2.1 mm i.d.	Aromatic amino acids, and steroids	Retention through hydrophobic, π–π, and hydrogen-bonding interactions	Retention factor of β-estradiol was 50	69
P(IPAAm-*co*-AAc), P(IPAAm-*co*-AAc-*co*-*t*BAAm),	Hydrogel (radical polymerization)	Aminopropyl silica beads (5 μm, 12 nm)	150 mm × 4.6 mm i.d.	Catecholamine, angiotensin	Basic analyte retention through electrostatic and hydrophobic interactions	Retention time of tyramine was 20 min at pH 7.0	72–74
P(IPAAm-*co*- DMAPAAm-*co*-BMA)	Hydrogel (radical polymerization)	Aminopropyl silica beads (5 μm, 12 nm)	150 mm × 4.6 mm i.d.	Adenosine nucleotides, oligo nucleotides, phospho-tyrosine, phosphopeptide, non-steroidal anti-inflammatory drugs (NSAIDs)	Acidic analyte retention through electrostatic and hydrophobic interactions	Retention time of ATP was 100 min at 10 °C Oligonucleotide separation was achieved within 10 min Retention time of phospho-tyrosine was 30 min at 10 °C Retention factor of naproxen was 55 at 10 °C	75–78
P(IPAAm-*co*-DMAEMA)	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Adenosine nucleotides	Strong electrostatic interactions	Retention time of ATP was 350 min at 10 °C	79

Table 1 (*continued*)

Polymer	Graft configuration (modification method)	Base materials (diameter, pore diameter)	Column length	Analytes	Remarks	Column performance	Ref.
P(IPAAm-*co*-AAc-*co*-*t*BAAm)	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Catecholamine, angiotensin	Strong electrostatic and hydrophobic interactions	Retention time of tyramine was 8 min at 10 °C. Retention time of angiotensin III was 10 min at 30 °C.	80
P(IPAAm-*co*-DMAPAAm-*co*-*t*BAAm)	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Albumin, γ-globulin	Acidic protein adsorption	HSA adsorption capacity on column was 16 mg per column at 40 °C	81
P(IPAAm-*co*-AAc-*co*-BMA)	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Lysozyme	Basic protein adsorption	Lysozyme adsorption capacity on column was 1.30 mg per column at 40 °C	82
P(IPAAm-*co*-APTAC-*co*-*t*BAAm)	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Acidic proteins	Strong basic properties	Adsorption and desorption of fibrinogen, human serum albumin, ovalbumin, and trypsin inhibitor	83
P(IPAAm-*co*-AMPS-*co*-*t*BAAm)	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Basic proteins	Strong acidic properties	Adsorption and desorption of α-chymotrypsinogen A, lysozyme, and papain	84
PAPATC-*b*-PIPAAm	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Milk serum proteins	Stronger basic properties than random copolymer brushes	Adsorption and desorption of α-lactalbumin and β-lactoglobulin.	85
P*t*BAAm-*b*-PIPAAm	Polymer brush (si-ATRP)	Silica beads (5 μm, 30 nm)	50 mm × 4.6 mm i.d.	Steroids	High stability	Retention time was maintained with continuously flowing pH 10.0 mobile phase for 54 h	86
PIPAAm	Polymer brush (si-ATRP)	Polystyrene beads (10 μm, 25 nm)	150 mm × 4.6 mm i.d.	Insulin, angiotensin, bradykinin	High stability	Insulin peptides was separated at 50 °C.	87, 88
P(IPAAm-*co*-*t*BAAm)	Polymer brush (si-ATRP)	Poly (hydroxymethacrylate) beads (10 μm, 25 nm)	150 mm × 4.6 mm i.d.	Insulin	High stability	The resolution between insulin chain A and chain B was 1.8 at 40 °C	89
PIPAAm	Linear polymer (coupling reaction)	Polymethacrylate beads (12.5 μm)	30 mm × 4.6 mm i.d.	Albumin, human serum	Affinity modulation	Binding capacity of HSA was 20 μg mL^{-1} at 40 °C.	90
PIPAAm with RCA_{120} and lactose	Linear polymer (coupling reaction with polymer side chain)	Sepharose beads	—	Asialotransferrin	Affinity modulation	Thermal elution 95.1% was performed at 40 °C.	91
PIPAAm	Linear polymer (coupling reaction)	Glass beads (pore diameter: 15.6,17.1, 23.7, and 40.8 nm)	200 mm × 7.0 mm i.d.	Dextran	Thermal modulation of GPC mode	Elution time was changed 30 s across the LCST.	92
PIPAAm	Linear, cross linking (polymerization with polystyrene particle)	Polystyrene beads	150 mm × 4.6 mm i.d.	Dextran	Thermal modulation of GPC mode	Temperature-dependent elution volume change was observed using cyclohexanol as porogen	93
PIPAAm	Polymer brush (si-ATRP)	Monolithic silica (mesopore diameter 30 nm)	50 mm × 3.2 mm i.d.	Steroids	Short retention time with high resolution	Five steroids were separated within 5 min	97
P(IPAAm-*co*-BMA)	Polymer brush (si-ATRP)	Monolithic silica (mesopore diameter 30 nm)	50 mm × 3.2 mm i.d.	Benzoic acid	Short retention time with high resolution	Benzoic acid was separated within 8 min.	98
P(IPAAm-*co*-DMAEMA-*co*-*t*BAAm)	Polymer brush (si-ATRP)	Monolithic silica (mesopore diameter 30 nm)	50 mm × 3.2 mm i.d.	Adenosine nucleotides	Short retention time with high resolution	Baseline separation of adenosine nucleotides was performed with 4 min	99

Table 1 (*continued*)

Polymer	Graft configuration (modification method)	Base materials (diameter, pore diameter)	Column length	Analytes	Remarks	Column performance	Ref.
P(IPAAm-*co*-AAc-*co*-*t*BAAm)	Polymer brush (si-ATRP)	Monolithic silica (mesopore diameter 30 nm)	50 mm × 3.2 mm i.d.	Catecholamine and angiotensin	Short retention time with high resolution	Catecholeamine was separated within 5 min. Angiotensin peptides was separated within 5 min.	100
PIPAAm	Polymer brush (si-ATRP)	Fused silica capillaries	1 m × 50 μm i.d.	Steroids	Short retention time with low amount of mobile phase	Cortisone and testosterone was separated within 10 min.	103
PBMA-*b*-PIPAAm	Polymer brush (si-ATRP)	Fused silica capillaries	1 m × 50 μm i.d.	Steroids	Short retention time with low amount of mobile phase	Retention factor of testosterone was 4.5 at 50 °C.	104

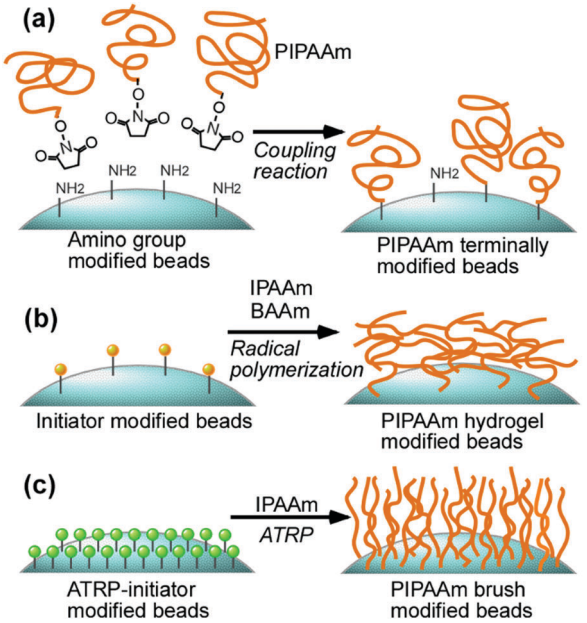

Fig. 2 Various types of thermoresponsive-polymer-modified beads: (a) PIPAAm terminally modified beads prepared *via* coupling reaction; (b) PIPAAm-hydrogel-modified beads prepared *via* radical polymerization; and (c) PIPAAm-brush-modified beads prepared *via* surface-initiated ATRP.

significantly shortened the retention times and clear changes in the hydrophobicity and retention times were observed around the LCST. In contrast, when a hydrophobic silane-coupling agent was used as a coadsorber, the retention time increased gradually with increasing temperature. The difference between the retention times is attributed to the difference in the PIPAAm hydration properties. The steroid peaks obtained using hydrophilic PIPAAm-graft interface beads were sharper than those obtained using other beads. PIPAAm brushes with hydrophilic graft interfaces therefore provide effective bead surfaces for obtaining sharp steroid peaks.

The PIPAAm-grafting configuration on the silica beads surfaces is also influenced by the ATRP reaction solvent. Three reaction solvents, *i.e.*, *N,N*-dimethylformamide, 2-propanol, and water, were investigated as appropriate solvents for PIPAAm grafting.[59] Among the prepared beads, those with PIPAAm brushes prepared using 2-propanol gave narrow steroid peaks, *i.e.*, high separation efficiency, because less pore clogging occurs when 2-propanol is used for PIPAAm grafting on silica beads surfaces than in the cases of DMF and water.[59]

Previous reports indicated that the PIPAAm hydration properties are influenced by the hydrophobicity of the PIPAAm terminal groups.[60–62] Silica bead surfaces were modified with thermoresponsive polymers with various terminal functional groups, and the beads were investigated as thermoresponsive chromatography matrices.[63] PIPAAm with two types of terminal group, *i.e.*, maleimide and dodecyl groups, were synthesized using reversible addition–fragmentation chain-transfer polymerization, and immobilized on silica beads. The steroid retention times with silica beads modified with PIPAAm with hydrophobic dodecyl groups were longer than those for PIPAAm with

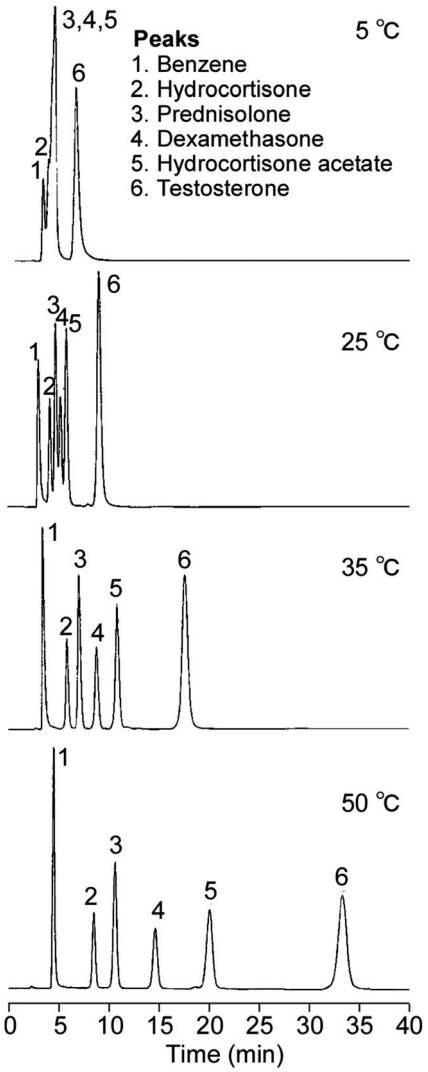

Fig. 3 Chromatograms of hydrophobic steroids separated using column packed with PIPAAm-modified silica beads. Adapted from ref. 46. Copyright 1996, American Chemical Society.

maleimide groups. This shows that the terminal groups of the PIPAAm chains affect the analyte retention profiles.[63]

3. Thermoresponsive chromatography with hydrophobic and aromatic groups

Copolymerization of a monomer containing hydrophobic groups with a thermoresponsive polymer is an effective approach for modulating the polymer hydrophobicity and LCST. Highly hydrophobic thermoresponsive chromatography matrices were prepared by incorporation of *n*-butyl methacrylate (BMA) in PIPAAm. Silica beads were modified with the copolymer, *i.e.*, P(IPAAm-*co*-BMA), *via* a coupling reaction (Fig. 4).[64] The steroid retention times on a column packed with copolymer-modified beads were longer than those on a column packed with beads modified with the PIPAAm homopolymer. This is because hydrophobic interactions between the copolymer and steroids are

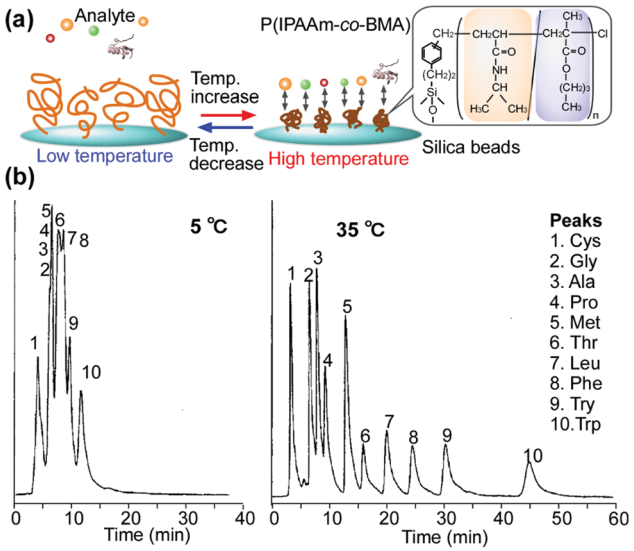

Fig. 4 Hydrophobized thermoresponsive chromatography for strong hydrophobic interactions: (a) concept of thermoresponsive hydrophobic chromatography and (b) chromatograms of PTH–amino acid separation using column packed with P(IPAAm-*co*-BMA)-modified silica beads. Adapted from ref. 65. Copyright 2000, American Chemical Society.

enhanced by BMA incorporation into the copolymer. The same column achieved separation of a peptide mixture, *i.e.*, insulin chain A, insulin chain B, and β-endorphin fragment 1–27, as a result of strong hydrophobic interactions. Similarly, a column packed with P(IPAAm-*co*-BMA)-modified silica beads was used to analyze a phenylthiohydantoin (PTH)–amino acid mixture using pure water as the mobile phase (Fig. 4b).[65] Quantification of propofol in serum was also achieved.[66]

The hydrophobicity can be further increased by producing thermoresponsive polymer P(IPAAm-*co*-BMA) brushes by copolymerization of IPAAm and BMA through surface-initiated ATRP. A column packed with P(IPAAm-*co*-BMA) brush beads showed strong interactions with analytes, and separated benzoic acids and phenol, which are relatively hydrophilic analytes.[67] These results show that incorporation of hydrophobic monomers such as BMA is an effective approach for strengthening thermally modulated hydrophobic interactions. The PIPAAm hydrophobicity also increased with increasing salt concentration in the mobile phase as a result of enhanced dehydration.[46] However, incorporation of BMA into PIPAAm has the following several merits. It does not increase the salt concentration in the mobile phase, which would make desalination after analyte separation necessary. Also, an excessively high salt concentration denatures proteins. BMA incorporation into PIPAAm-modified beads does not result in a high salt concentration in the mobile phase. The other advantage is that it lowers the LCST. Incorporation of BMA reduces the LCST of the copolymer,[64] and therefore lowers the effective temperature region for property changes. Bio-related analyte separations can therefore be performed at lower temperatures, avoiding thermal deactivation of analytes.

A thermoresponsive polymer with aromatic moieties, *i.e.*, P(IPAAm-*co*-*N*-acryloyl-3-(2-naphthyl)-L-alanine methyl ester),

was used for selective retention of aromatic compounds.[68] The polymer-modified stationary phase was used to observe the elution behaviors of aromatic steroids (estradiol and ethynylestradiol) and non-aromatic steroids (norethisterone and norgestrel); longer retention times were observed for the aromatic steroids than for the non-aromatic steroids, because aromatic steroids interact with naphthylalanine moieties through π–π interactions. *N*-Acrlyloyl aromatic amino acid methyl esters such as phenylalanine and tryptophan methyl esters were also incorporated into PIPAAm and the copolymer was used to modify silica beads.[69] When the silica beads were used as a packing material, the retention times of aromatic steroids and aromatic amino acids increased, because of hydrophobic π–π interactions and hydrogen bonding.[69] These results show that incorporation of aromatic moieties into PIPAAm is an effective approach to selectively retain aromatic compounds through π–π interactions.

4. Thermoresponsive ion-exchange chromatography

Ion-exchange chromatography is an effective separation tool for ionic biomolecules. In conventional ion-exchange chromatography, the stationary phase is modified with ionic functional groups, and Ionic compounds interact with the stationary phase through electrostatic interactions. The interactions can therefore be modulated by changing the ion concentration (ionic strength) of the mobile phase. However, modulation of the ion concentration of the mobile phase is complicated. Also, desalination is required after analyte separation. To overcome these problems, thermoresponsive ion-exchange chromatography using copolymers of PIPAAm and ionic monomers has been developed (Fig. 5). Thermoresponsive ionic copolymers prepared by random copolymerization of IPAAm and other ionic monomers show thermally modulated charge-density changes. This is attributed to hydrophobicity changes in the vicinity of ionic group in the copolymer.[70,71] These effects can be used to control the electrostatic interactions between the thermoresponsive ionic copolymer and analytes.

Early types of thermoresponsive ion-exchange chromatography were developed using copolymers of IPAAm and acrylic acid, *i.e.*, (AAc)(P(IPAAm-*co*-AAc))[72] or P(IPAAm-*co*-AAc-*co*-*tert*-butyl acrylamide) (*t*BAAm).[73] Hydrogels of these thermoresponsive ionic copolymers were prepared on silica bead surfaces. Use of these beads as packing materials enabled the separation of catecholamine derivatives at elevated temperatures through both electrostatic and hydrophobic interactions. The same copolymer-modified beads were used to separate angiotensin subtypes by changing the column temperature.[74]

Thermoresponsive cationic chromatography (thermoresponsive anion-exchange chromatography) was developed by introducing cationic groups into thermoresponsive polymers. The cationic monomer *N*,*N*-dimethylaminopropylacrylamide (DMAPAAm) was copolymerized with IPAAm and BMA, and silica beads modified with the copolymer hydrogel were used as the packing material for separating adenosine nucleotides [adenosine 5′-monophosphate (AMP), adenosine 5′-diphosphate (ADP), and adenosine 5′-triphosphate (ATP)].[75] Long adenosine nucleotide retention times were observed at low temperatures because the electrostatic interactions between the cationic copolymer and analytes were strong. The retention times of these analytes decreased with increasing temperature, because the decreased charge density on the copolymer weakened the electrostatic interactions. The same polymer-modified silica beads were used as the stationary phase in the separation of oligonucleotides[76,77] and analysis of non-steroidal anti-inflammatory drugs.[78]

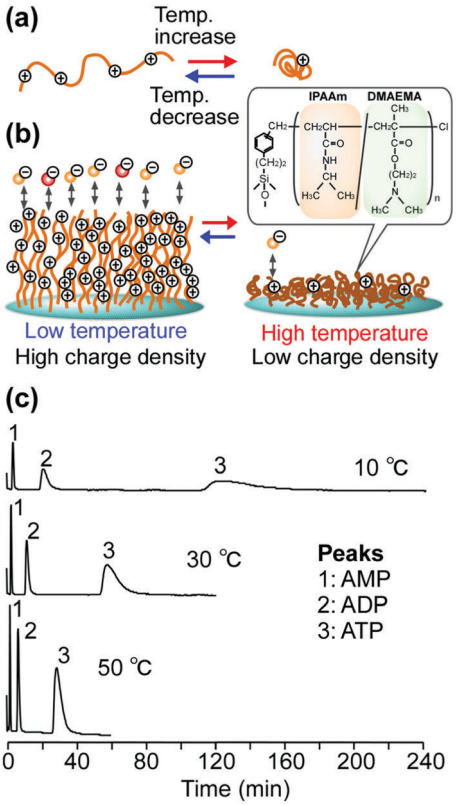

Fig. 5 Thermoresponsive ion-exchange chromatography for separation of ionic biomolecules: (a) temperature-modulated charge-density changes; (b) concept of thermoresponsive ion-exchange chromatography; and (c) chromatograms of adenosine nucleotides separated using column packed with P(IPAAm-*co*-DMAEMA)-modified silica beads. Adapted from ref. 79. Copyright 2008, American Chemical Society.

A comparison of columns packed with beads modified with thermoresponsive cationic and anionic copolymers showed that the retention on a cationic DMAPAAm copolymer clearly differed from that on an anionic AAc copolymer. This is because adenosine nucleotides are hydrophilic and acidic. Hydrophobic interactions between adenosine nucleotides and the copolymer therefore have little effect on retention. Catecholamine is slightly more hydrophobic than adenosine nucleotides, therefore hydrophobic interactions with catecholamine increases its retention time on the column. However, electrostatic interactions with catecholamine decrease with increasing temperature because the copolymer acidity decreases, leading to a decrease in the retention time. These factors compensate for each other, therefore there is little change in the retention time with changing temperature.

The thermoresponsive ion-exchange matrices were further improved by forming thermoresponsive ionic polymer brushes on silica bead surfaces. 2-(Dimethylamino)ethyl methacrylate (DMAEMA) was used as the cationic monomer to prepare P(IPAAm-*co*-DMAEMA) brushes for silica bead modification. The retention times of adenosine nucleotides on the bead-packed column were long (Fig. 5b and c),[79] but a step-temperature gradient significantly shortened the analysis time.[79] This is because the strong electrostatic interactions between the copolymer brushes with a high charge density and the adenosine nucleotides were modulated by changing the temperature. Silica beads modified with P(IPAAm-*co*-AAc-*co*-*t*BAAm) brushes through surface-initiated ATRP effectively separated basic biomolecules.[80] A column packed with the copolymer-brush-modified beads gave effective separation of catecholamine and angiotensin.[80]

5. Thermoresponsive adsorption chromatography for protein purification

Proteins can be separated using beads modified with ionic copolymer brushes by modulating protein adsorption on, and desorption from, the copolymer brushes. Brushes of a cationic copolymer, *i.e.*, P(IPAAm-*co*-DMAPAAm-*co*-*t*BAAm), were formed on silica bead surfaces, and used as column-packing materials (Fig. 6a).[81] The elution of two proteins, *i.e.*, human serum albumin (HSA) and γ-globulin, from the packed column was achieved by changing the concentration of the phosphate buffer (PB, pH 7) solution used as the mobile phase. When dilute PB (16.7 mM) was used, HSA was not eluted at high temperatures, but γ-globulin was eluted at all temperatures. This is because acidic HSA [isoelectric point (pI): 5.2] was adsorbed on the thermoresponsive cationic copolymer brushes through both electrostatic and hydrophobic interactions. When a high concentration of PB (66.7 mM) was used, HSA adsorption was not observed even at high temperatures, indicating that electrostatic interactions between the cationic copolymer and proteins are dominant in protein adsorption. The intrinsic protein adsorption and desorption properties of the copolymer brushes were used to achieve separation of these proteins. A mixture of HSA and γ-globulin was loaded onto the column at 40 °C; only HSA was adsorbed on the thermoresponsive cationic copolymer brushes and γ-globulin was eluted. At 10 °C, the adsorbed HSA was eluted from the column, enabling separation of the two proteins simply by changing the column temperature (Fig. 6a).[81]

The basic protein lysozyme was also purified using thermoresponsive anionic P(IPAAm-*co*-AAc-*co*-BMA) brushes (Fig. 6b).[82] Copolymer brushes were prepared on silica bead surfaces by surface-initiated ATRP of IPAAm, BMA, and *tert*-butyl acrylate (*t*BA), which is an AAc protecting group. The *tert*-butyl group of *t*BA was then removed and P(IPAAm-*co*-AAc-*co*-BMA) was formed on the silica bead surfaces. Lysozyme was adsorbed on the copolymer-brush-modified beads at high temperatures, because of electrostatic interactions between basic lysozyme (pI: 11.4) and the anionic copolymer brushes. Hydrophobic interactions

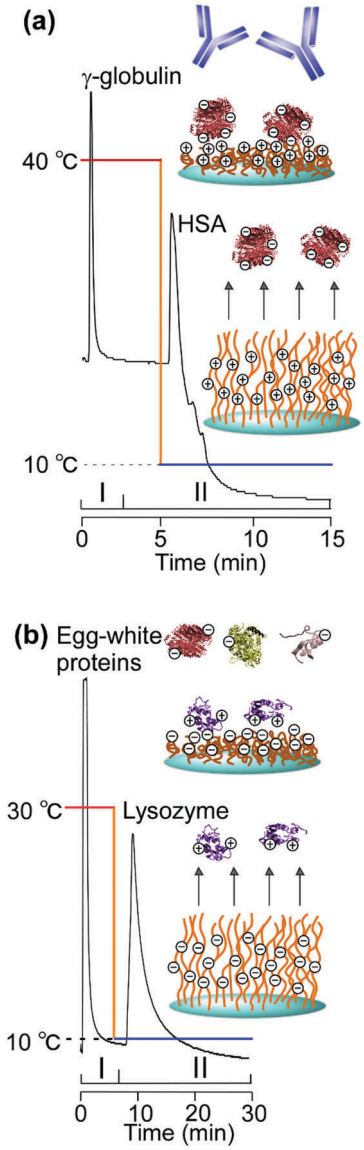

Fig. 6 Thermoresponsive protein adsorption chromatography for protein purification: (a) beads modified with thermoresponsive cationic copolymer brushes for separation of HSA and γ-globulin. Adapted from ref. 81. Copyright 2011, Elsevier. (b) Beads modified with thermoresponsive anionic copolymer brushes for purification of lysozyme. Adapted from ref. 82. Copyright 2011, Royal Society of Chemistry.

also contribute to protein adsorption. The properties of the copolymer brushes were used to achieve lysozyme purification from egg-white proteins. Egg-white proteins were injected into a column packed with copolymer-brush-modified beads at 30 °C. Only lysozyme was adsorbed on the anionic copolymer brushes and the other egg-white proteins were eluted. Then the column temperature was reduced to 10 °C, and the adsorbed lysozyme was desorbed and eluted, because the copolymer brushes were hydrated and hydrophobic interactions were weakened by reducing the temperature. Lysozyme can therefore be purified from egg-white proteins simply by changing the column temperature (Fig. 6b).[82]

The protein adsorption efficiency is attributed to electrostatic and hydrophobic interactions. The introduction of strong

ionic groups into thermoresponsive copolymers is therefore an effective approach to improving the column separation efficiency. Strong cationic groups were introduced by copolymerization of 3-acrylamidopropyltrimethylammonium chloride (APTAC), which has a quaternary amine group and is a strong amine, with a thermoresponsive copolymer.[83] The strongly cationic copolymer P(IPAAm-*co*-APTAC-*co*-*t*BAAm) brushes were grafted onto silica bead surfaces through surface-initiated ATRP. Protein adsorption on these beads in a packed column was stronger than that on silica beads grafted with P(IPAAm-*co*-DMAPAAm-*co*-*t*BAAm) brushes, which contain tertiary amino groups. Thermally modulated adsorption and desorption of fibrinogen, ovalbumin, a trypsin inhibitor, and HSA was achieved using these beads in a packed column.[83]

Sulfonic acid groups, which are strong acid groups, were incorporated by copolymerization of 2-acrylamido-2-methylpropanesulfonic acid (AMPS), IPAAm, and *t*BAAm. Thermoresponsive strongly anionic copolymer P(IPAAm-*co*-AMPS-*co*-*t*BAAm) brushes were grafted onto silica bead surfaces through surface-initiated ATRP.[84] Thermally modulated adsorption and desorption of basic proteins, *i.e.*, α-chymotrypsinogen A, lysozyme, and papain, were performed using silica beads modified with the thermoresponsive strongly anionic copolymer brushes.

Block copolymer brushes with a cationic poly(3-acrylamidopropyltrimethylammonium chloride) (PAPTAC) bottom layer and PIPAAm brush upper layer are effective for protein adsorption and desorption.[85] PAPATC-*b*-PIPAAm brushes were prepared on silica bead surfaces through two-step ATRP, *i.e.*, grafting of PAPTAC brushes on the silica bead surfaces and then grating of PIPAAm brushes onto the PAPTAC brush layer. Thermally modulated adsorption of α-lactalbumin and β-lactoglobulin on PAPTAC-*b*-PIPAAm brushes was performed, and separation of milk serum proteins was achieved simply by changing the temperature.[85]

The protein adsorption properties of these thermoresponsive adsorption chromatography systems depend on the electrostatic properties of the targeted proteins. The adsorption properties of the targeted proteins would be affected by the ion concentration and pH of the mobile phase.

6. Stability of thermoresponsive chromatography matrices

Most thermoresponsive chromatography matrices are prepared using silica-based materials (silica beads and monolithic silica). However, silica-based materials are hydrolyzed on continuous contact with an aqueous mobile phase, leading to cleavage of the grafted polymer from the silica surfaces. The stability of the silica bead surfaces was increased by grafting brushes of a block copolymer, *i.e.*, P*t*BAAm-*b*-PIPAAm, on the silica bead surfaces.[86] A column packed with beads modified with P*t*BAAm-*b*-PIPAAm brushes was stable against alkaline mobile phases (pH 8–10), because the hydrophobic base layer of the copolymer brushes provided hydrophobicity at the grafting interface, which prevented silica hydrolysis.[86]

Polymer-based materials such as polystyrene beads[87,88] and poly(hydroxymethacrylate) (PHMA) beads[89] have been investigated as highly stability thermoresponsive chromatography matrices. The polystyrene beads were chloromethylated to introduce ATRP-initiating groups *via* a Friedel–Crafts reaction. Surface-initiated ATRP was than performed. The PIPAAm-brush-modified beads were used to separate insulin fragments[87] and angiotensin subtypes[88] through hydrophobic interactions. In the case of the PHMA beads, 2-bromoisobutyl bromide was reacted with the hydroxyl groups to introduce ATRP-initiating groups. Hydrophobized thermoresponsive copolymer P(IPAAm-*co*-*t*BAAm) brushes were then grafted on the bead surfaces. These beads achieved effective separation of insulin fragments, and of a mixture of ovalbumin and aprotinin, based on a column temperature gradient.[89]

A comparison of silica-based and polymer-based materials for thermoresponsive chromatography showed that the separation efficiencies of silica-based materials are higher than those of polymer-based materials because of the uniform size and evenly distributed initiation (grafting) points on the surfaces of silica-based materials. However, polymer-based materials are more stable than silica-based materials; the performances of silica-based materials deteriorate over excessively long periods of use and under strongly alkaline conditions. These two types of material therefore have advantages and disadvantages and should be used for appropriate purposes.

7. Thermoresponsive affinity chromatography

PIPAAm shows not only temperature-dependent hydrophobicity changes but also thermoresponsive coil–globule transitions. The PIPAAm chains are extended below the LCST because of hydration of PIPAAm. Above the LCST, the PIPAAm chains dehydrate and shrink. The specific properties of PIPAAm are used in thermoresponsive affinity chromatography. Thermoresponsive affinity chromatography based on conformational changes of thermoresponsive polymers has been investigated.[90,91] PIPAAm and cibacron blue F3G-A (CB), which is a ligand for albumin, were separately immobilized on polymethacrylate bead surfaces (Fig. 7).[90] At high temperatures, PIPAAm shrank and CB was exposed, leading to binding of HSA through the affinity between CB and albumin. The PIPAAm became hydrated and expanded with decreasing temperature, leading to masking of the CB molecules by PIPAAm and release of the albumin bound on CB. Thermally modulated affinity changes were used for albumin purification of human serum proteins.[90]

Ricinus communis agglutinin (RCA120), which is a ligand for asialotransferrin, and lactose were coimmobilized on PIPAAm chains, and the PIPAAm chains were immobilized on sepharose beads.[91] At low temperatures, asialotransferrin reacted with the RCA120 ligand. The PIPAAm chains shrank with increasing temperature, and RCA120 and lactose in the PIPAAm chain interacted, leading to reduced affinity between RCA120 and asialotransferrin. This is because the distance between RCA120

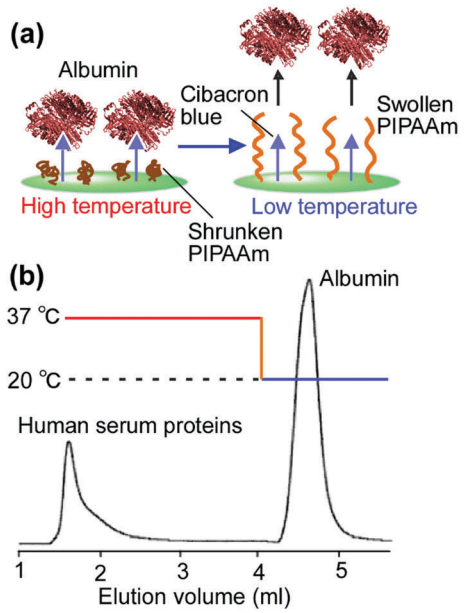

Fig. 7 Thermoresponsive affinity chromatography: (a) concept of thermoresponsive affinity chromatography for purification of albumin and (b) purification of albumin from human serum using thermoresponsive affinity chromatography column. Adapted from ref. 90. Copyright 2002, American Chemical Society.

and lactose decreased because of PIPAAm shrinkage. Asialotransferrin was therefore purified by simply changing the temperature to thermally modulate the affinity between RCA120 and asialotransferrin.[91]

The selectivity of thermoresponsive affinity chromatography is higher than that of thermoresponsive adsorption chromatography in protein purification. However, precise control of the PIPAAm chain length on the bead surfaces is needed to modulate the affinity interactions.

8. Thermoresponsive gel-permeation chromatography

The swelling and shrinking behaviors of PIPAAm are also used in thermoresponsive gel-permeation (size-exclusion) chromatography. PIPAAm was grafted on porous glass beads *via* a coupling reaction between the activated chain ends of PIPAAm and amino groups of the glass bead surfaces.[92] The PIPAAm-grafted glass beads were packed in a column and used as a gel-permeation chromatography matrix. Below the LCST of PIPAAm, *i.e.*, 32 °C, the elution times of dextrans of various molecular weights were shorter than those above the LCST. At lower temperatures, the grafted PIPAAm was swollen and extended, preventing dextran diffusion inside the pores of the glass beads. Above the LCST, the grafted PIPAAm on the glass beads dehydrated and shrank, and dextran diffused into the pores, leading to longer elution times. The elution times of dextran molecules can therefore be changed by changing the column temperature.[92]

The PIPAAm modification sites on porous beads are important for effective separation. Two types of PIPAAm-grafted polystyrene beads were prepared using cyclohexanol or toluene as porogen agents in PIPAAm polymerization in water.[93] When cyclohexanol was used, the PIPAAm was grafted on the bead surfaces inside and outside the pores, because polymerization occurred inside the pores. In contrast, when toluene was used, PIPAAm was grafted only on the external bead surfaces, because polymerization did not occur inside the pores. The elution profile of dextran was observed using these prepared beads as gel-permeation chromatography matrices. When the beads prepared with cyclohexanol were used, the dextran elution time above the LCST was longer than that below the LCST. However, the opposite results were observed using the beads prepared with toluene, *i.e.*, the elution time above the LCST was shorter than that below the LCST, and the same results were observed using an unmodified column. These results show that temperature-dependent diffusion of analytes into the pores of porous beads can be performed by grafting PIPAAm inside the pores.[93]

In thermoresponsive gel-permeation chromatography, the diffusion of analytes into pores is controlled by PIPAAm extension and shrinking, leading to retention time changes. However, the changes in analyte retention times are small compared with those in thermoresponsive chromatography based on the changes in hydrophobic interactions with changing temperature.

9. Thermoresponsive chromatography for rapid separations

In recent years, monolithic silica or polymer columns have been attracting attention as alternatives to bead-packed columns, because a monolithic column has a three-dimensionally interconnected skeleton structure, leading to low permeability of the mobile phase and a shorter analyte diffusion path length.[94–96] A monolithic silica column is therefore an effective base material for a thermoresponsive chromatography column. A PIPAAm-brush-modified monolithic silica rod column was prepared by surface-initiated ATRP (Fig. 8a).[97] The prepared monolithic column enabled high-resolution separation of a mixture of steroids with short separation times. This is attributed to the high linear velocity of the mobile phase and the reduced path lengths of analytes in the monolithic silica column (Fig. 8b). A PIPAAm-modified monolithic silica rod column is therefore an effective thermoresponsive column and achieves rapid analyte separation.[97]

Monolithic silica modified with hydrophobized thermoresponsive polymer P(IPAAm-*co*-BMA) brushes rapidly and effectively separated benzoic acids and phenol.[98] A mixture of insulin fragments, *i.e.*, insulin chain A, insulin chain B, and insulin, was separated using a P(IPAAm-*co*-BMA)-brush-modified monolithic silica column.[98]

The analysis time for thermoresponsive ion-exchange chromatography can be significantly reduced by using a monolithic silica rod column; for example, the separation of an adenosine

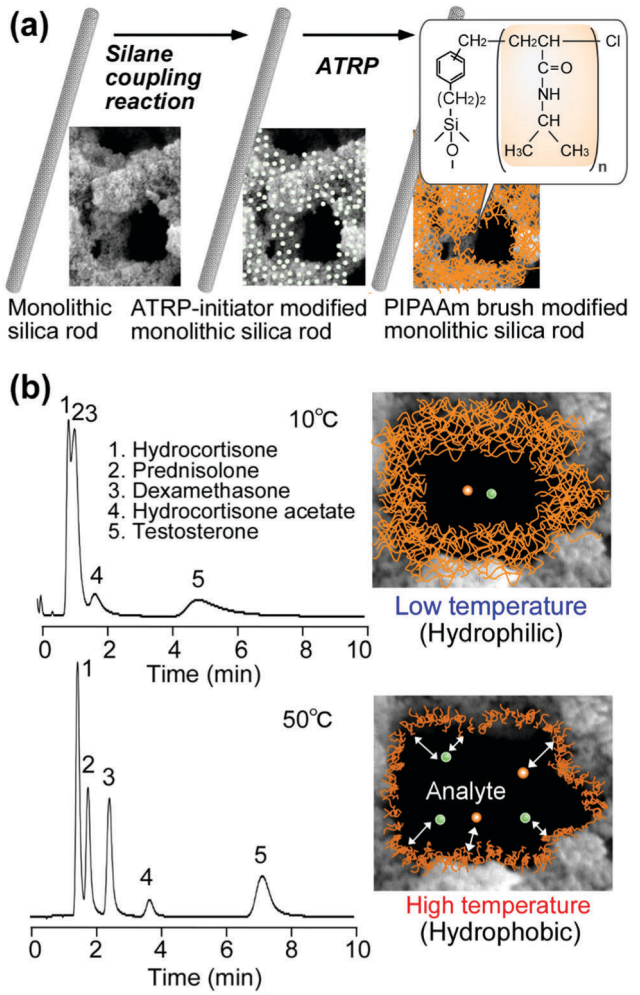

Fig. 8 Thermoresponsive monolithic silica rod for high-speed separation: (a) scheme for preparation of monolithic silica rod and (b) chromatogram of hydrophobic steroids, and thermally modulated interactions between PIPAAm brushes and analytes in monolithic silica rod. Adapted from ref. 97. Copyright 2011, American Chemical Society.

nucleotide mixture, *i.e.*, AMP, ADP, and ATP, on a column packed with beads modified with thermoresponsive cationic copolymer P(IPAAm-*co*-DMAEMA-*co*-*t*BAAm) brushes requires a long separation time (approximately 100 min). In contrast, using a monolithic silica rod column modified with the same thermoresponsive cationic copolymer brushes achieves separation of the adenosine nucleotide mixture in 5 min.[99] Separation of a mixture of catecholamine derivatives and angiotensin subtypes on a monolithic silica rod column modified with thermoresponsive anionic copolymer P(IPAAm-*co*-AAc-*co*-*t*BAAm) brushes was faster than that using a column packed with beads modified with the same copolymer.[100]

Capillary chromatography columns are also effective stationary phases for shortening analysis times and reducing the amounts of samples and mobile phases.[101,102] Thermoresponsive-polymer-modified capillary chromatography has therefore also been investigated.[103,104] A capillary inner wall was modified with PIPAAm brushes through surface-initiated ATRP, and the elution behaviors of two steroids, *i.e.*, cortisone and testosterone, on the column were observed. The steroids gave narrow, sharp peaks, with short retention times (within 10 min), at a low mobile phase flow rate (5 μL min^{-1}).[103] A capillary inner surface was also modified with block copolymer PBMA-*b*-PIPAAm brushes. The steroid retention times on the PBMA-*b*-PIPAAm-brush-modified capillaries were longer than those on PIPAAm-modified capillaries, indicating that PBMA-*b*-PIPAAm brushes separate biomolecules through strong hydrophobic interactions.[104] These results indicate that thermoresponsive-polymer-brush-modified capillaries would be useful for rapid separation of biomolecules using a small amount of mobile phase.

The analyte separation times for thermoresponsive monolithic silica and capillary silica columns are shorter than those for bead-packed columns. The analyte separation efficiencies of thermoresponsive monolithic silica columns are higher than those of capillary silica columns, although capillary columns have the merit of reducing the amount of mobile phase. The method used therefore depends on the application.

10. Cell-separating materials based on thermoresponsive polymers

Recently, cell-based regenerative medicine using therapeutic cells has attracted attention. In these therapies, cells are transplanted into patients by direct injection of cell suspensions or transplantation of tissues prefabricated *in vitro*. Effective cell-purification methods are essential for use of these therapeutic cells. Various cell-separation methods have been developed based on the recognition of cell surface markers, *e.g.*, FACS and MACS. These techniques are used for precise cell separation. However, these cell-separation methods require modification of cell surfaces and complicated apparatus. Simple cell-separation methods that do not involve labeling of cell surfaces are preferable. Various cell-separation methods using thermoresponsive polymers have been developed, and are summarized in Table 2.

A concise cell-separation method using an aqueous two-phase system and PIPAAm-modified antibodies was proposed.[105,106] PIPAAm was modified with anti-CD34 antibodies specific for KG-1 by introducing vinyl groups into the antibodies, and subsequent copolymerization with IPAAm monomer. The PIPAAm-modified antibodies were used to perform specific separation of KG-1 cells from Jurkat cells in an aqueous two-phase system consisting of an upper polyethylene glycol (PEG) phase and a bottom dextran phase. When the mixture of KG-1 cells and Jurkat cells was added to the two-phase system, above 80% of the KG-1 cells were obtained from the upper PEG solution phase, but Jurkat cells were scarcely partitioned into the PEG phase, because the PIPAAm-modified antibodies tended to partition into the upper PEG solution phase. The results indicate that PIPAAm-modified antibodies enable specific cell partitioning using a relatively simple method.

Table 2 Summary of thermoresponsive cell separations

Polymer	Graft configuration	Separation materials	Cell separation mechanism	Target cells	Performance on cell separations	Ref.
PIPAAm	Linear polymer (radical polymerization)	PIPAAm modified antibody	Partitioning to aqueous two-phase system	KG-1	More than 80% of KG-1 cells were partitioned	105
PIPAAm	Hydrogel (radical polymerization)	PIPAAm cryogel (macro porous gel) with ligand	Affinity for adhesion and mechanical compression for detachment	*Escherichia coli* and yeast cells	Cells (37%) were detached by compression of cryogel	107
PIPAAm	Linear polymer (plasma-induced polymerization)	Polypropylene membrane	Affinity with adsorbed antibody	CD80-, CD86-transfected cells	Enrichment of CD80 (72%) or CD-86 cells (66%); cells detached with antibody	109
PIPAAm	Linear polymer (plasma-induced polymerization)	Polypropylene membrane	Affinity with adsorbed antibody	KG-1a	Enrichment of Kg-1a cells to 85%; cells detached with antibody	110
PIPAAm	Polymer brush (si-ATRP)	PIPAAm modified glass beads	Difference between cell adhesive properties	Lymphocyte (T cell and B cell)	T cells showed strong adhesive properties; more than 70% of B cells were recovered, and 15% of T cells were recovered.	111
PIPAAm	Polymer brush (si-ATRP)	PIPAAm modified glass plate	Difference between detachment rates from PIPAAm brushes	HUVEC, HSMM	Prompt detachment of HUVEC was observed; HUVEC (76%) and HSMM (24%) were obtained at 20 °C in 30 min	113
P(IPAAm-*co*-BMA)	Polymer brush (si-ATRP)	Copolymer modified glass plate	Difference between effective detachment temperature from copolymer brushes	HUVEC, NHDF	Cell separation by multi-step temperature change; NHDF (79.4%) was obtained at 10 °C, and HUVEC (75.6%) was obtained at 20 °C.	114
P(IPAAm-*co*-DMAPAAm-*co*-*t*BAAm))	Polymer brush (si-ATRP)	Copolymer modified glass plate	Cell adhesion with cationic properties	hbmMSC	Effective adhesion of hbmMSC on cationic polymer brushes hbmMSC (96.1%) was obtained at 20 °C.	116

Also, a PIPAAm cryogel enabled simple and concise temperature-modulated cell separation.[107,108] The PIPAAm cryogel was prepared by crosslinking polymerization under semi-frozen conditions. Specific Cu(II)–iminodiacetate ligands for *Escherichia coli* cells were bound on the PIPAAm cryogel. The *E. coli* cells were bound to the cryogel surfaces. At 25 °C, 65% of the bound cells were eluted with 0.2 mol L^{-1} imidazole buffer solution. In contrast, using the same buffer heated to 40 °C, 85% of the bound cells were recovered, because the affinity between the cells and the ligand was reduced by disruption of the pore wall surfaces of the PIPAAm cryogel, induced by PIPAAm aggregation.

Other types of cell separations using PIPAAm-grafted polypropylene (PP) membranes and adsorbed antibodies have been proposed.[109] PIPAAm-grafted PP membranes were prepared by plasma-induced polymerization. Anti-mouse CD80 monoclonal antibodies (CD80 mAb) or CD86 monoclonal antibodies (CD86 mAb) were adsorbed on the PIPAAm-grafted membrane by incubation of the antibody solution. Adsorption of CD80 cells on the PP membrane with adsorbed CD80 mAb antibodies was better than adsorption on membranes with adsorbed CD86 mAb antibodies or no antibodies. On reducing the temperature to 4 °C, the adhered cells were recovered together with antibodies from the PIPAAm-grafted PP membrane, because the PIPAAm grafted on the PP membrane became hydrophilic and tended to detach adhered cells. Using this method, CD80- or CD86-transfected cells were enriched to 72% or 66% from a 1 : 1 suspension. In a similar method, anti-human CD34 monoclonal antibodies adsorbed on a PIPAAm-grafted PP membrane enriched CD34-positive KG-1a cells to 85% from a 1 : 1 cell suspension of KG-1a cells and Jurkat cells.[110]

These methods enable simple separation of cells; however, they use antibodies, which are sometimes expensive and unstable, leading to high costs and low reproducibility. Additionally, the separated cells contain antibodies on the cell surfaces, which leads to problems in research or in transplants to the human body. To avoid these issues, temperature-modulated cell-separation methods without antibodies have been investigated.

A lymphocyte subpopulation, *i.e.*, B cells and T cells, was separated using PIPAAm-brush-modified glass beads packed in a column.[111] PIPAAm-brush-modified glass beads with various brush lengths were prepared, because previous reports indicated that the PIPAAm brush length determined the cell adhesion properties.[57,112] Lymphocyte retention at 37 °C decreased with increasing PIPAAm brush length, and the recovery rate at 5 °C increased, because the surface hydrophilicity increased with increasing PIPAAm brush length. The B cells showed temperature-dependent retention and elution properties, but the T cells were not eluted, even at low temperatures. The contents of lymphocyte subpopulations can therefore be changed using columns packed with PIPAAm-brush-modified beads simply by changing the temperature.

PIPAAm brushes have also been used to separate cells for cardiovascular tissue engineering and regenerative medicine (Fig. 9a).[113] Four types of cell, *i.e.*, human umbilical vein endothelial cells (HUVECs), normal human dermal fibroblasts (NHDFs), human aortic smooth muscle cells (SMCs), and human

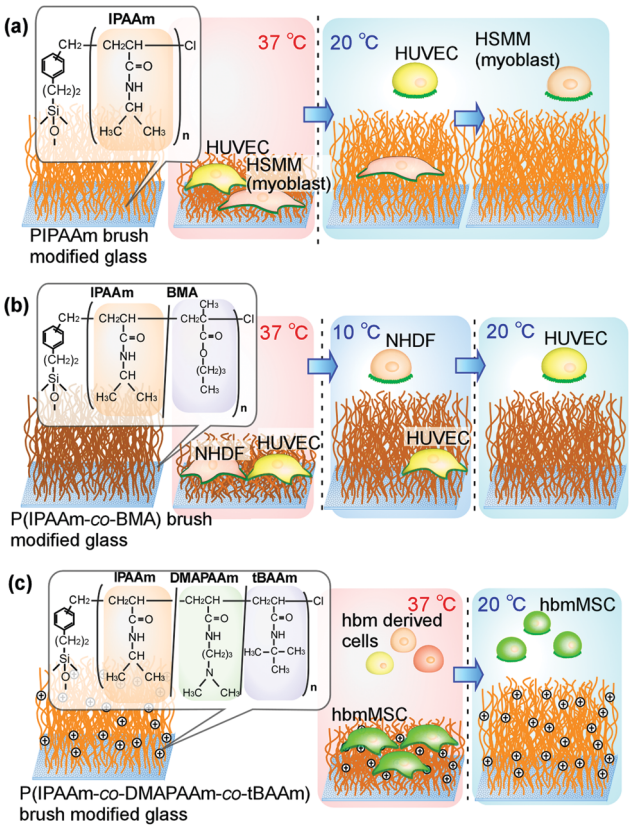

Fig. 9 Cell separations using thermoresponsive polymer brushes. (a) PIPAAm brush separations for cardiovascular tissue engineering. Adapted from ref. 113. Copyright 2012, Royal Society of Chemistry. (b) P(IPAAm-*co*-BMA) brushes for separation of endothelial and fibroblast cells. Adapted from ref. 114. Copyright 2013, American Chemical Society. (c) P(IPAAm-*co*-DMAPAAm-*co*-*t*BAAm) brushes for purification of human bone marrow mesenchymal stem cells. Adapted from ref. 116. Copyright 2015, American Chemical Society.

skeletal muscle myoblast cells (HSMMs), were adhered on PIPAAm brushes at 37 °C and detached from the brushes at 20 °C. The four types of cell showed similar cell adhesion rates at 37 °C, but at 20 °C, a range of cell detachment rates were observed, *e.g.*, the HUVECs were promptly detached from the PIPAAm brushes but the HSMMs were detached gradually. Based on the difference between their detachment rates from PIPAAm brushes, a mixture of HUVECs and HSMMs was separated simply by changing the temperature.[113] The temperature for effective detachment of each cell type can also be used for cell separation. Hydrophobized thermoresponsive polymer P(IPAAm-*co*-BMA) brushes were prepared on glass substrates (Fig. 9b).[114] HUVECs and NHDFs adhered to the brushes, and were detached at 20 and 10 °C, respectively.[114] This is because of the balance between hydrophilicity and the cell metabolic activities of these cells. When the temperature decreased, the hydrophilicity of the copolymer brushes increased, leading to increased cell detachment. However, the cell metabolic activities also decreased with decreasing temperature. A previous report indicated that cell detachment from a PIPAAm layer was caused not only by reduced interactions between the cells and the substrate surface, but also by morphological changes induced by cell metabolic activities.[115] The effective detachment temperature for a cell is therefore determined by its metabolic activity at lower temperatures. The difference between their cell detachment temperatures was used to separate a mixture of HUVECs and NHDFs. Both types of cell adhered to P(IPAAm-*co*-BMA) at 37 °C. The temperature was changed to 10 °C, and the NHDFs were detached. The temperature was then raised to 20 °C and the HUVECs were detached and recovered from the P(IPAAm-*co*-BMA) brushes. HUVECs and NHDFs were therefore separated using a multi-step temperature change.[114]

Thermoresponsive cationic copolymer P(IPAAm-*co*-DMAPAAm-*co*-*t*BAAm) brushes can be used to purify human bone marrow mesenchymal stem cells (hbmMSCs) (Fig. 9c).[116] P(IPAAm-*co*-DMAPAAm-*co*-*t*BAAm) brushes were prepared on glass substrates through surface-initiated ATRP. Adhesion and detachment of hbmMSCs on the thermoresponsive cationic copolymer brushes were more effective than those of other types of cell. The selective adhesion properties of hbmMSCs enabled purification of hbmMSC simply by changing the temperature. A mixture of cells derived from human bone marrow was seeded on thermoresponsive cationic copolymer brushes. At 37 °C, only hbmMSCs adhered to the copolymer brushes. The hbmMSCs were detached from the copolymer brushes and recovered by reducing the temperature to 20 °C.[116]

PIPAAm and its copolymer-brush-modified substrates can be used to separate cells by a simple procedure and without cell–surface modification, although the selectivity is lower than those achieved by separation methods using biological ligands such as antibodies. These methods are therefore suitable for rough cell separations such as preparation of cell compositions for tissue fabrication.

11. Conclusions

In this review article, bioanalytical and bioseparation methods using thermoresponsive polymers were summarized. Thermoresponsive chromatography using a stationary phase modified with PIPAAm or its copolymer has been developed for various bioanalytical and bioseparation applications. Various PIPAAm modification methods have been investigated for the development of effective stationary phases. Various separation modes, based on hydrophobic properties, ion-exchange, affinity modulation, and adsorption, have been used to separate target molecules and proteins. Cell separation without cell–surface modification can be achieved using thermoresponsive polymer brushes, based on the intrinsic adhesion properties, detachment rates, and detachment temperatures of the cells. The cell-separation methods described will be useful in regenerative medicine and tissue engineering, because cells can be separated without modification of the cell surfaces and with retention of cell activity.

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