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Aqueous chromatography utilizing hydrophobicity-modified anionic temperature-responsive hydrogel for stationary phases

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Abstract

A new pH-/temperature-responsive poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-*N*-*tert*-butylacrylamide) (poly(IPAAm-*co*-AAc-*co*-tBAAm)) hydrogel grafted on silica beads was evaluated as column matrix for a cation-exchange thermoresponsive chromatography. The stationary phase showed simultaneous changes in temperature-responsive surface charge density and hydrophobicity by incorporation of anionic AAc and hydrophobic tBAAm into IPAAm sequences. Thermoresponsive polymer property alterations were confirmed by temperature-responsive phase transition and shift in apparent pK_a values. Catecholamine derivatives were retained on poly(IPAAm-*co*-AAc-*co*-tBAAm)-modified column at pH 7.0. Analyte retention was primarily due to the electrostatic interaction. It was noted that the temperature-induced phase transition of poly(IPAAm-*co*-AAc-*co*-tBAAm) hydrogel layer on the stationary phases was evidenced by the apparent inflection point in van't Hoff plots around 36 °C. This suggests that solute interactions should be changed below and above the stationary phase transition temperature, reducing electrostatic interaction above the transition temperature.

Keywords: Stationary phases, LC; Hydrophobic interactions; Surface charge density; Electrostatic interaction; Poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-*N*-*tert*-butylacrylamide); Catecholamines

1. Introduction

We have recently proposed an anionic aqueous chromatography using poly(*N*-isopropylacrylamide-*co*-acrylic acid) (poly(IPAAm-*co*-AAc)) hydrogel-modified silica beads as stationary phases for separation of basic bioactive compounds, catecholamines [1]. The copolymer containing 3 mol% AAc exhibits soluble/insoluble changes across a lower critical solution temperature (LCST), which is affected by pH and ionic strength of the elution medium. Catecholamines were retained on poly(IPAAm-*co*-AAc) columns above pH 5.0 primarily due to electrostatic interaction while weak interaction with insufficient resolution of cationic catecholamines occurred on the nonionic PIPAAm-modified column. In this paper, we have introduced the possibility to control surface charge density by thermal stimulus.

Poly(*N*-isopropylacrylamide) (PIPAAm) exhibits a well-known temperature-responsive phase transition

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in aqueous solution at 32 °C; a lower critical solution temperature (LCST) [2]. We succeeded in obtaining reversible surface hydrophilic/hydrophobic property alterations for PIPAAm-grafted surfaces through changes in grafted polymer hydration state below and above PIPAAm's transition temperature [3–5]. In addition, we developed thermoresponsive PIPAAm-grafted cell culture dishes prepared by electron beam polymerization, and demonstrated noninvasive recovery of cultured cells [6] and cell sheets for tissue engineering applications [7,8] through temperature-induced surface hydration changes.

We have also been pioneering to demonstrate a new form of hydrophobic chromatography utilizing PIPAAm on the stationary phase for separation of steroids in a sole aqueous mobile phase [5,9–11]. Solute partitioning to PIPAAm-grafted stationary phases was modulated by external temperature changes. Several approaches by other groups have been directed toward the development of packing materials for HPLC to control the separation selectivity by changing external column temperature. In those cases, however, structural changes in stationary phase are not primary factors for separation [12–15], except one study that achieved successful separation by temperature-induced polarity changes of PIPAAm-grafted matrix surfaces [16]. By sharp contrast, successful separation of such hydrophobic bioactive compounds as steroids was achieved only through the modulation of hydrophilic/hydrophobic surface properties by external column temperature changes, with dramatic alterations of analyte retention times in response to temperature changes [5,9–11]. On the other hand, temperature-dependent changes in retention time of ionic analytes on poly(IPAAm-*co*-AAc) stationary phase were relatively small compared to that of hydrophobic steroids on the PIPAAm column. This was due to weakened thermoresponsive surface property alteration by incorporation of hydrophilic AAc within IPAAm polymer sequences. Carboxyl groups in hydrogels cause gel swelling primarily via Donnan ion exclusion [17], followed by swelling change through electrostatic repulsion. A repulsive force operating between the carboxylate anions of acrylic acid increases the hydration of the entire polymer chain, restricting the hydrophobic network aggregation [18].

To enhance resolution and selectivity in anionic temperature-responsive aqueous chromatography, it is desirable to improve poor thermosensitivity of the pH-/temperature-responsive polymer. Anionic thermoresponsive hydrogel-grafted surface would produce an alterable stationary phase with both thermally regulated hydrophobicity and charge density for separation of bioactive compounds. As temperature is increased, dehydration and collapse of the hydrogel layer on the gel surface might prevent charged groups within the hydrogel layer from accessing the hydrophilic mobile phase. This could lead to decreased surface charge density on the stationary phase. Urry et al. [19] reported that a carboxyl pK_a shift in synthetic polypeptides was induced by incorporation of hydrophobic amino acid residues. Thus, the dissociation degree of ionic groups in the polymer sequence is affected by hydrophobicity of the local environment of polymer chains. A modification of surface grafted polymer hydrogels with hydrophobic co-monomer would be a primary choice for improving thermal response of pH-/temperature-responsive polymer. In the present paper, hydrophobically modified poly(*N*-isopropylacrylamide-*co*-acrylic acid-*co*-*N*-*tert*-butylacrylamide) (poly(IPAAm-*co*-AAc-*co*-tBAAm)) hydrogel-grafted silica beads were prepared and examined as column matrices for temperature-dependent separation of bioactive compounds, with catecholamine derivatives as model cationic materials.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (IPAAm) was kindly provided from Kojin (Tokyo, Japan), and recrystallized from *n*-hexane. *N*-*tert*-butylacrylamide (tBAAm) was purchased from Polysciences (Warrington, PA) and recrystallized from acetone. Acrylic acid (AAc) was purchased from Wako Pure Chemical Industries (Osaka, Japan) and purified by distillation at 42 °C (7 mmHg). *N*,*N*′-Methylenebisacrylamide (MBAAm) was obtained from Eastman Kodak (Rochester, NY) and used as received. 2,2′-Azobisisobutyronitrile (AIBN; Wako) was purified by recrystallization from methanol. *N*,*N*-Dimethylformamide (DMF) and

tetrahydrofuran (THF), both from Kanto Chemical (Tokyo, Japan), were purified by distillation at 53 °C (9 mmHg) and at 67 °C (760 mmHg), respectively. 4,4′-Azobis(4-cyanovaleric acid) (ACV; Wako), 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (E-EDQ; Tokyo Kasei Kogyo, Tokyo, Japan), diethyl ether, and ethanol (Wako) were used without further purification.

Aminopropyl silica beads (average diameter, 5 μm; pore size, 120 Å; specific surface area, 310 m^2/g) were purchased from Nishio Industry (Tokyo, Japan). Sulfo-succinimidyl-4-*o*-(4,4′-dimethoxytrityl) butyrate (s-SDTB) was obtained from Pierce (Rockford, IL). All other chemicals were reagent-grade and used as received.

2.2. Synthesis of linear terpolymer

Poly(IPAAm-*co*-AAc-*co*-tBAAm)s were synthesized in THF (total monomer: 1.43 mol/l) with AIBN as an initiator (1.4 mmol AIBN per mol monomer). Monomer composition in feed was changed as: AAc 0, 3, 5, 10 mol%, while IPAAm/tBAAm mole ratio was fixed to 90/10. Copolymerization was performed with the protocol as previously described at 70 °C for 2 h [1]. Terpolymer is abbreviated as IAtB*X* where *X* represents the AAc content in terpolymer in mol%.

2.3. Characterizations of terpolymer

AAc content in the terpolymer was determined by acid–base titration in water at 4 °C [1]. Apparent dissociation constants (K'_a) of the linear polymers in 100 m*M* KCl solution were determined from titration using the following Henderson–Hasselbalch equation [20]:

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

where α is the degree of dissociation for carboxyl groups.

Experimental details of pK'_a measurement were as follows: terpolymer (100 mg) was dissolved in 20 ml of distilled water containing 100 m*M* KCl. A half of carboxyl groups in terpolymer were dissociated stoichiometrically by adding 0.05 *M* NaOH aq, resulting in α of 0.5. According to Eq. (1), the relationship between pH and pK'_a at $\alpha = 0.5$ represents:

$$pK'_a = pH \quad (2)$$

We measured the pH values of the terpolymer solution as pK'_a by pH-meter with vigorous stirring and with varying temperature. Transmittance changes during titration were also measured.

The content of tBAAm in linear terpolymer was measured with an NMR spectrometer (500 MHz, Lambda-500, JEOL, Tokyo, Japan) using chloroform-*d* containing 0.03% (v/v) tetramethylsilane as a solvent.

2.4. Determination of transition temperature for copolymers

Poly(IPAAm-*co*-AAc-*co*-tBAAm)s with different amounts of AAc were dissolved each at 1.0 w/v% in phosphate–citrate buffer adjusted to constant ionic strength ($I = 0.1$) by the addition of KCl. Optical transmittance change of each solution at a predetermined pH was monitored at 500 nm with a UV–Vis spectrometer (V-530, Japan Spectroscopic (JASCO), Tokyo, Japan). The sample cell was thermostated with a Peltier-effect cell holder (EHC-477, JASCO) with heating rate of 0.1 °C/min. Transition temperature was defined as the temperature at 90% transmittance of solution.

2.5. Terpolymer hydrogel polymerization on silica bead surfaces

Aminopropyl silica beads were used as base matrices for polymer gel modification. A polymerization initiator, ACV, was covalently immobilized on aminopropyl silica beads using the method as previously reported [1]. Cross-linked terpolymer hydrogel composed of IPAAm, AAc, tBAAm was prepared by radical copolymerization at the surfaces of initiator-immobilized silica beads. The typical preparation procedure was as follows: total monomer concentration was set at 8.84 mol/l with the following monomer composition in feed: IPAAm (16.2 g, 143 mmol), AAc (1.27 g, 17.6 mmol), tBAAm (2.02 g, 15.9 mmol) and MBAAm (0.272 g, 1.76 mmol) as a cross-linking agent (AAc 10 mol%, IPAAm/

tBAAm=90/10 mol/mol, 1.0 mol% MBAAm to monomer). To a monomer solution in ethanol (200 ml) was added ACV-immobilized silica beads (5.00 g). The reaction mixture was then degassed by subjecting to triplicate freeze–thaw cycles, and was sealed under reduced pressure. Polymerization was carried out at 70 °C for 15 h with vigorous stirring. Terpolymer hydrogel-modified silica beads were collected by filtration and washed three times with ethanol to remove unreacted monomers and un-immobilized polymers, followed by a drying process for 12 h under vacuum at 25 °C. Obtained terpolymer hydrogel-modified bead is abbreviated as IAtB10G, where 10 is the feed composition of AAc in mol% and G is the ''Gel''.

Elemental analyses of polymer hydrogel-modified surfaces were carried out by using electron spectroscopy for chemical analysis (ESCA; model ESCA750, Shimadzu, Kyoto, Japan) with take-off angle of 90°. The proportion of nitrogen to carbon atoms was calculated from core-level high resolution spectra areal integration of C1s and N1s peaks collected with sampling time of 200 ms and analyzed with ESPAC210 software (Shimadzu).

2.6. Temperature-responsive elution of basic compounds

Polymer hydrogel-modified silica beads were packed into a stainless steel column (150 mm×4.6 mm I.D.) from a slurry of beads in water–methanol (2:1 v/v) using a column packer at 350 kg/cm^2 for 1 h followed by equilibration with distilled water for 24 h. The obtained column was connected to a HPLC system (PU-980, AS-950, UV-970, JASCO) controlled by personal computer with Borwin analysis software version 1.21 (JASCO). Milli-Q water was used for preparation of elution buffer and sample solutions. Eluent was phosphate–citrate buffer adjusted to constant ionic strength ($I=0.1$) by the addition of KCl. Four substances were used as samples to produce chromatograms. These were D,L-DOPA, adrenaline, dopamine HCl, and tyramine. Concentration for each solute was 1.0 mg/ml. Na_2SO_3 (2.7 mg/ml) was added into the sample solution to prevent samples from oxidization. Thermoresponsive elution behavior for these sample molecules was monitored at 254 nm and recorded with a flow-rate of 0.50 ml/min at various temperatures. Column temperature was controlled using a water jacket connected to a thermostated Coolnics circulator (CTE42A, Yamato-KOMATSU, Tokyo, Japan) within a deviation of ±0.1 °C.

Resolution, R_s, for the two selected analytes at a given temperature was calculated using the following equation:

$$R_s = \frac{2(t_{R2} - t_{R1})}{w_{b1} - w_{b2}} \quad (3)$$

where t_{R1} and t_{R2} ($t_{R1} < t_{R2}$) are retention times, w_{b1} and w_{b2} are peak widths at the baseline for analytes 1 and 2, respectively.

3. Results and discussion

3.1. Syntheses and characterization of pH-/temperature-responsive terpolymers

In order to determine transition temperatures and temperature-dependent pK_a shifts for surface-grafted hydrogels, linear terpolymers, poly(IPAAm-*co*-AAc-*co*-tBAAm)s, were used as alternative models. The structural formula of terpolymer is shown in Fig. 1a. Table 1 summarizes the composition and pK'_a values of terpolymers at 4 °C. AAc and tBAAm contents were determined from acid–base titration and ^{1}H-NMR spectra, respectively. Mole fraction of tBAAm in the terpolymer was calculated by the peak areal ratio of the singlet at 1.3 ppm from methyl protons of *tert*-butyl side chain with the singlet at 4.0 ppm, attributed to the resonance of the methine proton of isopropyl groups in IPAAm units.

Transition temperature of terpolymers at pH 7.0, $I=0.1$ are also indicated in Table 1. The transition temperature of terpolymers was increased with increasing AAc content in terpolymer. Additionally, terpolymer with larger amounts of AAc showed transmittance change in broader temperature ranges, indicating poor sensitivities in phase transition behavior. This broadening arose from the weakened intra-/inter-polymer aggregation induced by increasing repulsive force among ionized carboxylates on polymer side-chains. Highly ionized carboxylates in poly(IPAAm-*co*-AAc) containing 10 mol% AAc in

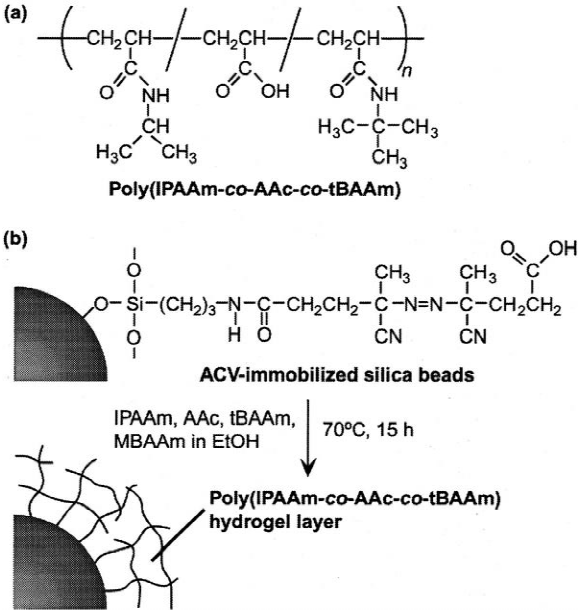

Fig. 1. (a) Structure of linear poly(IPAAm-*co*-AAc-*co*-tBAAm). (b) Preparation of cross-linked thermo-sensitive poly(IPAAm-*co*-AAc-*co*-tBAAm) hydrogel grafted onto silica bead surfaces.

feed prevented the polymer from dehydration and aggregation even at high temperature, resulting in a disappearance of the phase transition temperature (data not shown). However, IAtBs showed phase transition even in the case of IAtB8.9 probably because the temperature-induced polymer aggregation was enhanced by the presence of hydrophobic tBAAm co-monomers.

Fig. 2 shows the effect of pH on the optical transmittance changes of terpolymer IAtB8.9. Ionic strength was set at 0.1 regardless of solution pH. Higher transition temperature is observed with an

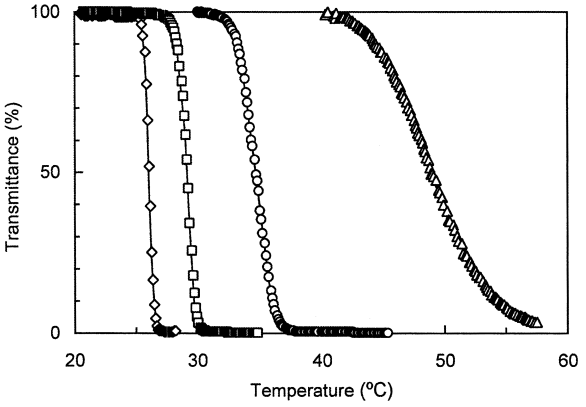

Fig. 2. Temperature-dependent optical transmittance changes of IAtB8.9 in phosphate–citrate buffer at pH 4.0 (diamond), pH 5.0 (square), pH 6.0 (circle), pH 7.0 (triangle) ($I=0.1$).

increase in buffer pH owing to the formation of larger amounts of dissociated carboxylate anions along the terpolymer molecule. Carboxyl groups in the terpolymers are protonated and thus are electrically neutral at low pH. These groups start to dissociate and possess negative charges with an increase in buffer pH. The more dissociated moieties induced the more ionic hydration, resulting in an increase in polymer hydrophilicity. The phase transition profile at lower pH was sensitive to temperature and occurred within a narrow temperature range. This is due to the small number of carboxylate anions; those should produce repulsive forces among polymer side-chains.

The apparent dissociation constant (K'_a) of terpolymer is estimated as a function of temperature. Fig. 3 shows temperature-dependent pK'_a shifts of IAtB8.9 in 100 m*M* KCl solution. The optical transmittance change of IAtB8.9 at 500 nm during

Table 1
Characterization of poly(IPAAm-*co*-AAc-*co*-tBAAm)s

Code	IPAAm/AAc/tBAAm (molar ratio)		pK'_a[b]	Transition temperature (°C)[c]
	In feed	In terpolymer[a]		
ItB	90.0/0/10.0	89.4/0/10.6	–	24.9
IAtB2.8	87.0/3.0/10.0	87.8/2.8/9.4	5.01 (0.05)	30.5
IAtB4.6	85.5/5.0/9.5	86.6/4.6/8.8	4.91 (0.03)	33.3
IAtB8.9	81.0/10.0/9.0	83.2/8.9/7.9	4.98 (0.03)	44.5

[a] Determined by acid–base titration ($n=3$) and ^{1}H-NMR measurement.
[b] Measured by acid–base titration at 4 °C with the standard deviation ($n=3$) in parentheses.
[c] Determined at 90% transmittance in phosphate–citrate buffer (pH 7.0, $I=0.1$).

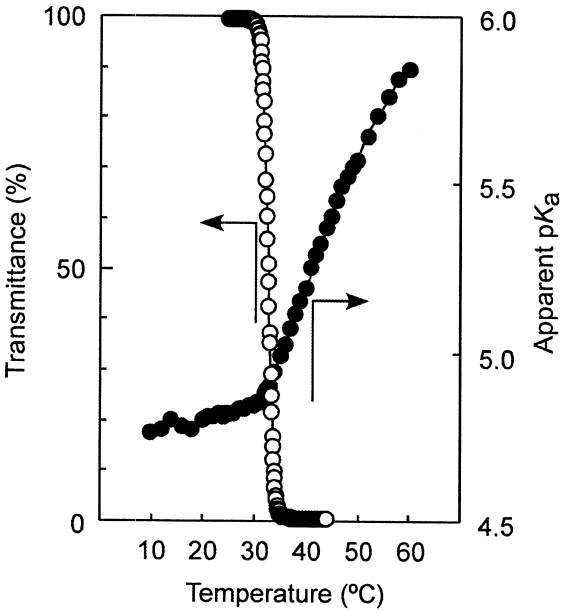

Fig. 3. Observed polymer carboxylate pK'_a shifts for IAtB8.9 in 100 m*M* KCl solution as a function of temperature.

titration is also indicated in Fig. 3. A drastic change in terpolymer pK'_a is observed above 32 °C. Terpolymer pK'_a value in a soluble state is slightly increased in 0.09 pH units from 10 to 32 °C. The pK'_a value is dramatically increased above 32 °C at which the terpolymer solution becomes turbid. The temperature with abrupt change in optical transmittance is almost the same with the temperature for pK'_a shift. This indicates that pK'_a shift is induced by solubility change of terpolymer. Above 32 °C, terpolymers are aggregated through hydrophobic interaction due to the dehydration of polymer side chains, and the solution thus becomes turbid. Intra- and inter-polymer aggregations cause increasing local hydrophobicity in the vicinity of carboxyl groups. Additionally, the interface between polymer molecules and solution environments was reduced by the aggregation of compact globules of dehydrated polymer chains. Incorporation of hydrophobic co-monomers into polyelectrolytes was reported to result in decreased acidity or basicity of weak acid or base moieties in polyelectrolytes [21,22]. Furthermore, the pK_a values for non-thermoresponsive acrylamide having weakly ionic moieties was almost constant regardless of temperature [23,24]. Thus, observed pK'_a shifts should be attributed to the temperature-induced reduction of dielectric constant around the polymer side-chain microenvironment in water above the polymer phase transition temperature [23,24].

We also investigated the ionic strength dependence of pK'_a values for IAtB8.9. To the extent of 10–100 m*M* KCl, pK'_a shift profiles of IAtB8.9 rarely depended on ionic strength, and the phase transition temperature during titration shifted from 34 to 32 °C with increasing ionic strength (data not shown). However, at higher ionic strength, 500 m*M* KCl, the phase transition temperature of IAtB8.9 during titration shifted to 27 °C. Lowering phase transition temperature is due to water structure-breaking effects by salt ions, inducing inter- and intramolecular hydrophobic interactions [25].

Terpolymer IAtB8.9 with the above-described characteristics was then introduced onto silica bead surfaces as will be discussed in the next section.

3.2. Preparation of terpolymer hydrogel-modified silica beads

To introduce thermoresponsive terpolymer on the silica bead surfaces, radical polymerization initiator with two carboxyl groups was covalently bound onto the aminated silica bead surfaces. The amount of immobilized initiator, ACV, was estimated by measuring the consumption of bead surface amino groups with s-SDTB [26]. ACV was introduced onto silica bead surfaces with 97% efficiency for an initial amount of amino groups of 250 μmol/g-beads [1]. Cross-linked poly(IPAAm-*co*-AAc-*co*-tBAAm), thin hydrogel layer having the analogous monomer composition with IAtB8.9 was introduced on the surface of silica beads (Fig. 1b). The molar ratio in feed was as follows: IPAAm/tBAAm = 90/10 mol/mol, AAc content of 10 mol%, and 1.0 mol% MBAAm per mol monomer. The obtained beads were abbreviated as IAtB10G.

Successful introduction of the hydrogel layer onto silica bead surfaces was confirmed by surface elemental analyses using ESCA. The detected surface composition from elements, C, N, O, and Si is summarized in Table 2. N/C ratio calculated from the ESCA data was not absolutely identical to theoretical values for polymer modified silica beads. However, slightly increased amounts of carbon and

Table 2
Elemental analyses of unmodified and polymer-modified silica beads by ESCA[a]

Code	Element (%)				N/C	N/C[b] (theor.)
	C	O	N	Si		
Amino silica	41.95 (2.09)	33.97 (1.73)	5.49 (1.93)	18.60 (2.45)	0.131 (0.043)	–
IAtB10G	47.41 (1.39)	33.13 (1.99)	5.81 (1.49)	13.66 (1.08)	0.123 (0.034)	0.140

[a] Data are calculated as mean of four samples with the standard deviation in parentheses.
[b] Calculated from theoretical molar ratios.

decreased amounts of silicon were observed on polymer-grafted silica beads than those on native amino silica beads. Polymerization reaction on the outer layer of silica bead surfaces was considered to proceed preferentially against inside pores of the matrix beads, since formation of gel layer on the outer surfaces may interfere with diffusion of monomers inside the pores. Considering that all surfaces were covered with polymers homogeneously, a polymer layer with 0.565 nm thickness on the silica beads was formed by calculating from elemental analyses data. Thus, it was concluded that surfaces of silica beads as base matrices were covered with very thin polymer hydrogel layer.

3.3. Separation of basic solutes with temperature-responsive HPLC

We previously studied an anionic aqueous chromatography using pH-/temperature-responsive copolymer; poly(IPAAm-*co*-AAc) hydrogel-grafted silica column [1]. Thermoresponsive property alteration was weakened by introduction of AAc into IPAAm sequences due to the creation of hydrophilic anionic sites in polymer chain and disruption of sequential similarity of the repeating IPAAm side group monomer structure. This resulted in the increased overall hydrophilicity of the polymer. More effective separations of catecholamine derivatives would be obtained by the modulation of the polymer hydrophobicity through copolymerization with hydrophobic comonomer, and/or to modulate carboxyl group dissociation [27]. Here, hydrophobically modified poly(IPAAm-*co*-AAc-*co*-tBAAm) hydrogel layer was applied as stationary phase to improve temperature-dependent elution of ionic analytes.

As the silica surfaces were covered with the thermoresponsive hydrogels, column void volume changes induced by hydrogel volume phase transition may occur during separation. The void volume change during the analytical procedure is undesired in chromatography. Therefore, the thermoresponsive void volume change on the hydrogel-grafted columns was estimated using deuterium oxide as a marker molecule. Retention times of D_2O were constant regardless of temperature and pH. The hydrogel volume phase transition on the stationary phase surfaces was thus negligible. This is a good indication of successful introduction of the thin hydrogel layer onto silica bead surfaces.

Fig. 4a and b shows chromatograms of several basic catecholamine derivatives separated on poly(IPAAm-*co*-AAc-*co*-tBAAm) hydrogel (IAtB10G)-grafted column with the mobile phase adjusted to ionic strength of 0.1. In Fig. 4a, an overlapped and unimodal peak is observed at pH 4.0 over the examined temperature ranges. Retention time of this unimodal peak is nearly equal to that of D_2O, indicating the negligible interaction with the surfaces. Dissociation of AAc carboxyl groups in stationary phases is suppressed under acidic pH 4.0 as judged from the pK_a' value of the analogous terpolymer IAtB8.9 as shown in Table 1 and Fig. 2. On the other hand, sample analytes are highly soluble due to the ionization of amino groups (DOPA shows an ampholyte property). Therefore, minimum interaction took place between hydrophilic cationic analytes and the non-charged thermoresponsive column surfaces, producing the void volume elution of analyte mixture. By sharp contrast, catecholamines except for the zwitterionic DOPA are all retained on the IAtB10G column at pH 7.0 as shown in Fig. 4b. Little change in retention time was observed for all catecholamines examined on a reference PIPAAm-hydrogel column regardless of temperature [1]. Catecholamine derivatives were considered to be inter-

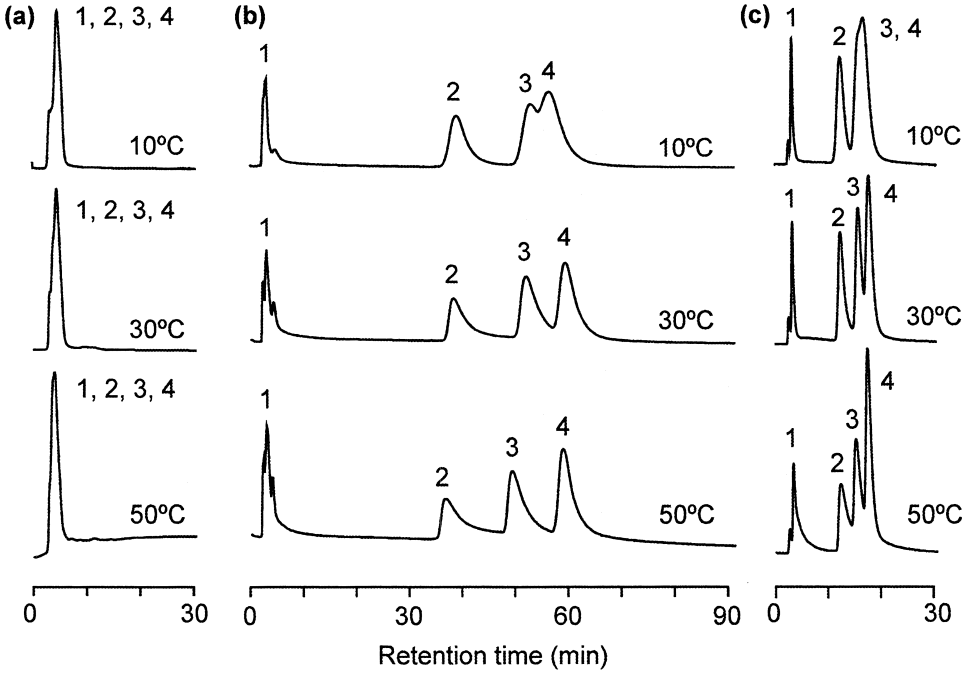

Fig. 4. Chromatograms of model basic analyte compounds on IAtB10G columns at 10, 30, and 50 °C. Mobile phase is phosphate–citrate buffer, (a) at pH 4.0, (b) at pH 7.0 ($I=0.1$), and (c) at pH 7.0 ($I=0.5$). Peaks: 1, DOPA; 2, adrenaline; 3, dopamine, and 4, tyramine.

acted with IAtB10G matrix surfaces at pH 7.0 primarily through electrostatic interaction. Elution times were increased in the order of DOPA< adrenaline<dopamine<tyramine. Analyte hydrophobicity (polarity), typically represented as the log *P* value, where *P* is the partition coefficient for a substance in an *n*-octanol–water system [28], is as follows: −2.74 for DOPA, −0.685 for adrenaline, 0.019 for dopamine, and 0.616 for tyramine, respectively. This order agreed with the observed order of elution times on IAtB10G columns. This indicated that the hydrophobic interaction has a substantial role in solutes–stationary phase interaction, and eventually enhanced the solute retention. Peaks of dopamine and tyramine were overlapped at 10 °C, and then divided into two peaks at higher temperature. Peak shapes and resolutions also are changed according to column temperature. This will be discussed in the following section.

The influence of mobile-phase ionic strength on elution behavior of analytes was then studied. Ionic strength was adjusted with KCl at constant pH of 7.0. Fig. 4c indicates that retention times of analytes are shortened with increasing ionic strength to 0.5 in the mobile phase. Electrostatic interaction between analytes and the stationary-phase surfaces is weakened at the increased ionic strength due to decreased Donnan effects. These results strongly support that the primary interaction force between analytes and the anionic IAtB10G stationary-phase surface is an electrostatic one.

Fig. 5 shows retention time changes for catecholamines as a function of temperature at pH 7.0 ($I=$ 0.1). The basic compound has an entirely protonated amino group, becoming highly hydrophilic at pH 7.0. Retention times of adrenaline and dopamine were decreased with increasing temperature. For tyramine, however, retention time was increased to reach a maximum at 40 °C, then was decreased.

As discussed in the previous section, the primary interaction force between catecholamine derivatives and the stationary-phase surface is an electrostatic one. With increasing temperature, surface charge density is considered to decrease, since surface

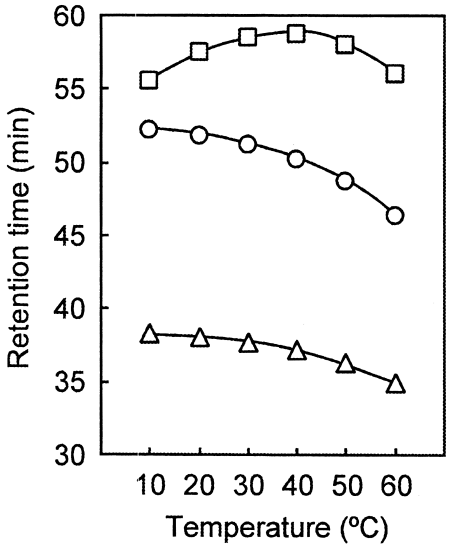

Fig. 5. Temperature-dependent retention time changes of model basic analytes on the anionic IAtB10G column at pH 7.0 ($I=0.1$). Adrenaline (triangle), dopamine (circle), and tyramine (square). Chromatographic condition is the same as Fig. 4b.

carboxyl group pK_a' is increased with temperature as shown in Fig. 3. This is supported by the decreased retention times of analytes except for tyramine at elevated temperature. Tyramine with higher hydrophobicity exhibited the longest retention time among the analytes examined. After neutralization of analyte through electrostatic interaction (salt formation) with the anionic IAtB10G column surfaces, analyte hydration should be disrupted, inducing a hydrophobic mode of retention. Below 40 °C, increasing retention times for tyramine against temperature were observed according to increasing hydrophobic partitioning into the stationary phases. Above 40 °C, the charge density on the stationary phases was significantly decreased, as observed in a drastic shift in pK_a' values for IAtB8.9 (Fig. 3). Through temperature-induced decreases in the surface charge density, interaction between tyramine and the stationary phase was decreased. This results in weaker interactions with the stationary phase surfaces. Therefore, different separation modes below and above 40 °C are considered to appear because of high hydrophobicity of analytes. Retention time of ionic analytes could be controlled by external column temperature. From these results, we consider the column temperature gradient to be an alternative to mobile-phase gradient in pH or ionic strength changes.

The van't Hoff plots for catecholamine derivatives on IAtB10G column are shown in Fig. 6. A linear relationship between ln k' values and reciprocal temperature ($1/T$) is commonly observed in the van't Hoff plots for commercially available reversed-phase columns under a normal chromatographic process. Interestingly, the van't Hoff plots of each analyte on IAtB10G column have an inflection point at 36 °C, respectively (Fig. 6). Above this temperature (or lower $1/T$ values), ln k' values largely decreased, indicating the weakened interaction of analyte molecules with hydrophobized surfaces. The inflection point in Fig. 6 should be represented in the phase transition of terpolymer-gel on the stationary phases. In the previous reports [5,9,10], we discussed the discontinuities in the van't Hoff plots observed in PIPAAm-grafted surfaces for steroid separations. These results suggest that interactions governing the solute retention should be changed below and above the stationary phase transition temperature, more reduced electrostatic interaction took place above the transition temperature.

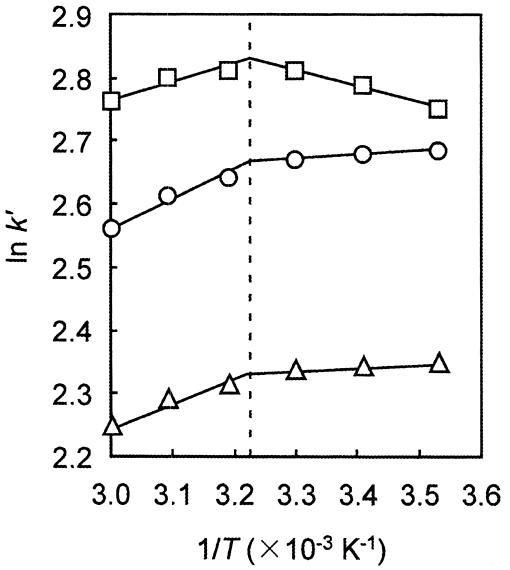

Fig. 6. The van't Hoff plots of catecholamine derivatives on IAtB10G column. Chromatographic condition is the same as Fig. 4b.

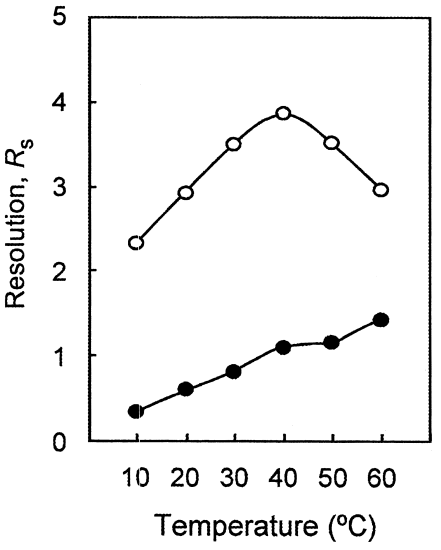

Fig. 7. Temperature-dependent changes in resolution between adrenaline and dopamine (open circle), dopamine and tyramine (closed circle), respectively. Chromatographic condition is the same as Fig. 4b.

Fig. 7 shows changes in the resolution of two neighboring solutes at various temperatures. With increasing column temperature, R_s value is monotonously increased, indicating more effective separation of dopamine and tyramine. This is probably due to the difference in the hydrophobicity of analytes. By contrast, the resolution of adrenaline and dopamine increased with increasing temperature up to 40 °C, and then decreased above this temperature. This might be due to the increasing peak widths of these analytes with temperature (see Fig. 4b). Two factors are considered to affect peak shapes: (1) analyte partitioning toward hydrogel layers, and (2) stronger hydrophobic interaction between analytes and hydrophobized matrix surfaces at elevated temperature [5]. Both of them have substantial influence on peak shapes. By tailoring the conformation of surface grafted thermoresponsive polymers from cross-linked hydrogels to linear polymer attached with one end, more pronounced resolution could be achieved.

4. Conclusions

We demonstrated the effect of hydrophobic comonomer incorporation of pH-/temperature-responsive stationary phases for utilization in cation-exchange chromatography of biological compounds. In the proposed chromatographic system, elution behavior of model basic analytes, catecholamine derivatives, is readily modulated with temperature-responsive hydrophobic and electrostatic property alterations of the stationary phases in the aqueous mobile phase. Since chromatographic separation is performed under a mild condition with aqueous mobile phase, this system would be advantageous for preserving solute bioactivity and for low environmental impact (eliminated organic solvent use/disposal). Moreover, there is no need to operate complicated desalting after separation because of the use of mobile phase with relatively low ionic strength. We believe that the packing materials might provide another choice for the analysis of basic compounds that are often difficult to separate by conventional chromatographic techniques. More practical examples, for instance, separation of bioactive compounds such as peptides will be reported in forthcoming papers.

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