Thermoresponsive Polymer Brush Surfaces with Hydrophobic Groups for All-Aqueous Chromatography

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ABSTRACT For developing thermoresponsive chromatographic matrices with a strong hydrophobicity, poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate) (poly(IPAAm-*co*-BMA)) brush grafted silica beads were prepared through a surface-initiated atom transfer radical polymerization (ATRP) with a $CuCl/CuCl_2/Me_6TREN$ catalytic system in 2-propanol at 25 °C for 16 h. The prepared beads were characterized by chromatographic analysis. Chromatograms of the benzoic-acid family and phenol as model analytes were obtained with high-resolution peaks because of their strong hydrophobic interactions to the densely grafted hydrophobized copolymers on the beads. Retention times of the analytes increased with the increase in BMA composition ratio. Dehydration of grafted copolymer with large BMA composition was performed at low temperature. These results indicated that the copolymer-brush-grafted surface prepared by ATRP was an effective tool for separating hydrophilic analytes at low temperature through modulating the strong hydrophobic interaction.

KEYWORDS: thermoresponsive surface • hydrophobic interaction • polymer brush • chromatography • separation

INTRODUCTION

Recently, intelligent interfaces that respond to external stimuli have been developed and utilized in biomedical fields (1−5). One of the most attractive intelligent interfaces is thermoresponsive surface prepared by grafting poly(*N*-isopropylacrylamide) (PIPAAm) to substrates. PIPAAm exhibits reversible temperature-dependent phase transition in aqueous solutions at its lower critical solution temperature (LCST) of 32 °C (6), and this intrinsic thermoresponsive property is widely used in biomedical applications, such as controlled drug and gene delivery systems (7, 8), enzyme bioconjugates (9, 10), microfluidics (11), cell culture substrates (12), and tissue engineering for regenerative medicine (13−16). Furthermore, temperature-responsive chromatography utilizing PIPAAm as a stationary phase has been developed for the thermally induced separation of bioactive compounds in aqueous mobile phase without organic phase (17, 18). This chromatography system is highly useful for controlling the properties of stationary phase of high performance liquid chromatography (HPLC) by changing only column temperature. Additionally, requiring no organic solvents as a mobile phase for separation, this system preserves the biological activity of analytes with minimizing the environmental loads. To improve the performance of PIPAAm grafted silica beads, researchers investigated the grafting method of PIPAAm on silica bead surfaces and the elution behavior of analytes from them (17−21). As the results of investigation, chromatographic matrices prepared by a surface-initiated atom transfer radical polymerization (ATRP) exhibit a strong interaction with analytes, since the polymerization procedure give a densely packed polymer, called a polymer brush, on the surfaces (21). ATRP is an attractive polymer grafting method allowing to prepare surface with well-defined polymer brushes by surface-immobilized ATRP initiators (22−27). The methodology can control graft chain length by varying the duration of polymerization (21) and graft density by varying the concentration of ATRP initiators (28). Thus, surface-initiated ATRP permits the preparation of effective stationary phase for temperature-responsive chromatography.

On the contrary, previous reports demonstrated that the copolymerization of functional monomer into PIPAAm copolymer gives other properties to thermo-responsive polymer (29−31). Incorporation of anionic or cationic monomer into PIPAAm copolymer exhibits thermoresponsive anionic or cationic properties (29, 30). These characteristics were utilized as thermoresponsive ion-exchange chromatography that modulates electrostatic interaction with analytes by

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changing temperature (32, 33). Additionally, *n*-butyl methacrylate (BMA), as an incorporated hydrophobic monomer, in PIPAAm copolymer provides a strong hydrophobicity to the copolymer (31). In a previous report, P(IPAAm-*co*-BMA) was sparsely grafted to silica bead surfaces via a coupling reaction, and the copolymer grafted silica bead surfaces exhibit a strong hydrophobic interaction with analytes compared to that using PIPAAm grafted beads (31). From these results, if P(IPAAm-*co*-BMA) can be densely grafted on silica beads surface by surface-initiated ATRP, the hydrophobized thermo-responsive chromatography stationary phase could be prepared for separating hydrophilic analytes that may be difficult to be separate by previously reported thermoresponsive chromatography.

In this study, we describe the preparation of high-density thermoresponsive copolymer brush comprising P(IPAAm-*co*-BMA) on silica bead surfaces using a surface-initiated ATRP. Characterization of the dense hydrophobized copolymer brush surfaces on silica beads was also investigated by chromatographic analysis using benzoic-acid family and phenol as model analytes.

EXPERIMENTAL SECTION

Materials. *N*-Isopropylacrylamide (IPAAm) was kindly provided by Kohjin (Tokyo, Japan) and recrystallized from *n*-hexane. *n*-Butyl methacrylate (BMA), obtained from Wako Pure Chemicals Industries (Osaka), was purified by distillation at 35 °C (3 mmHg). CuCl and $CuCl_2$ were purchased from Wako Pure Chemicals. Tris(2-aminoethyl)amine (TREN) was purchased from Acros Organics (Pittsburg, PA, USA). Formaldehyde, formic acid, and sodium hydroxide were purchased from Wako Pure Chemicals. Tris(2-(*N*,*N*-dimethylamino)ethyl)amine (Me_6-TREN) was synthesized from TREN, according to a previous reports (34). Silica beads (the average diameter: 5 μm, the pore size: 300 Å, the specific surface area: 100 m^2/g) were purchased from Chemco Scientific (Osaka). Hydrochloric acid, hydrofluoric acid, and ethylenediamine-*N*, *N*, *N*′,*N*′-tetraacetic acid disodium salt dehydrate (EDTA · 2Na) were purchased from Wako Pure Chemicals. 2-(*m*/*p*-Chloromethylphenyl)ethyltrichlorosilane was obtained from ShinEtsu Chemical Industry (Tokyo). 2-Propanol (HPLC grade), dichloromethane, and toluene (dehydrate) were purchased from Wako Pure Chemicals. Benzoic acids and phenol utilized as chromatographic analyte were purchased from Wako Pure Chemicals. Water used in this study was Milli-Q water prepared by an ultrapure water purification system (Synthesis A10) (Millipore, Billerica, MA) unless otherwise mentioned.

Preparation of ATRP Initiator Immobilized Silica Beads. 2-(*m*/*p*-Chloromethylphenyl) ethyltrichlorosilane as an ATRP-initiator modified silica were prepared as shown in the first step in Figure 1, according to the previous reports (22, 28). First, silica beads were washed with concentrated hydrochloric acid for 3 h at 90 °C, then rinsed with a large amount of distilled water repeatedly until the washing water pH became neutral, followed by thorough drying in a vacuum oven at 110 °C for 18 h. Formation of silane layers comprising the ATRP initiator on silica surfaces was performed as follows: silica beads (15.1 g) were placed into a round-bottom flask and humidified at 60% relative humidity for 4.0 h, followed by the addition of 3.53 mL of 2-(*m*/*p*-chloromethylphenyl)ethyltrichlorosilane in 302 mL of dried toluene. Nitrogen gas was flowed over the reaction mixture for first 5 min as HCl gas evolved, and then the flask was sealed. The reaction proceeded at room temperature for overnight with continuous stirring. ATRP initiator-immobilized silica beads were collected by filtration and extensively rinsed with toluene, methanol, dichloromethane, and acetone, and dried in a vacuum oven at 110 °C.

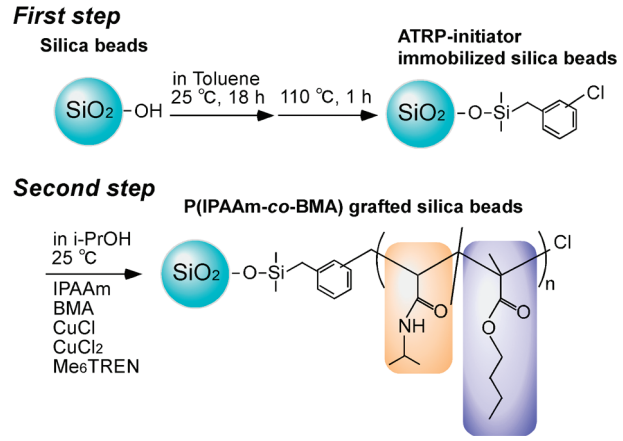

FIGURE 1. Scheme of the preparation of poly(*N*-isopropylacrylamide-*co*-butyl methacrylate) grafted silica bead surfaces using a surface-initiated atom transfer radical polymerization (ATRP).

Table 1. Characterization of P(IPAAm-*co*-BMA) Copolymer

	IPAAm/tBA/tBAAm (molar ratio)				
code[a]	in feed	in the copolymer[b]	Mn[c]	Mw/Mn[c]	LCST [d]
IP	100/0		6300	1.14	34.0
IPB-2.5	99.0/1.0	97.5/2.45	6400	1.17	26.6
IPB-5.2	97.0/3.0	94.8/5.18	5700	1.22	19.6
IPB-8.8	95.0/5.0	91.2/8.79	6400	1.22	13.7

[a] Abbreviated as IPB-*x*, where *x* represents the mole fraction of BMA in the copolymer. [b] Determined by ^{1}H-NMR measurement. [c] Measured by GPC using DMF containing 50 mmol/L LiCl with PEG standards. [d] Defined as the temperature at 90% transmittance in Milli-Q water.

Surface Modification of Silica Beads with Thermoresponsive Copolymer by ATRP. Thermoresponsive copolymer brushes composed of IPAAm and BMA were prepared on the ATRP-initiator immobilized silica beads by ATRP as shown in the second step in Figure 1. Typical preparation procedure was as follows: the total monomer concentration was set to be 1 mol/L with the following monomer composition in feed: IPAAm (4.81 g, 42.5 mmol) and BMA (61 mg, 0.43 mmol) (the monomer composition: BMA 1mol%) were dissolved in 42.8 mL of 2-propanol, and the solution was deoxygenated by nitrogen gas bubbling for 30 min. The feed composition of BMA was varied to be 0, 1, 3, or 5 mol % (Table 1). CuCl (84.7 mg, 0.86 mmol), $CuCl_2$ (11.5 mg, 0.086 mmol), and Me_6TREN (0.22 g, 0.959 mmol) were added under a nitrogen atmosphere, and the solution was stirred for 20 min to obtain a CuCl/$CuCl_2$/Me_6TREN catalyst system. ATRP initiator-immobilized silica beads (1.0 g) were placed into a clean dry 50 mL glass vessel. Both monomer solution and the silica beads were placed into a glove bag purged with dry nitrogen gas by repeated vacuum and nitrogen flush (three times). The monomer solution was then poured into the glass vessel containing the silica beads, and the vessel was sealed under nitrogen. The ATRP reaction proceeded for 16 h at 25 °C under continuous shaking on a desk-top shaker (SN-M40S) (NISSIN, Tokyo). Copolymer-grafted silica beads were washed by ultrasonication with acetone for 30 min followed by centrifugation to remove unreacted monomers and ungrafted copolymers. This washing process by ultrasonication was repeated twice. Copolymer-grafted silica beads were further

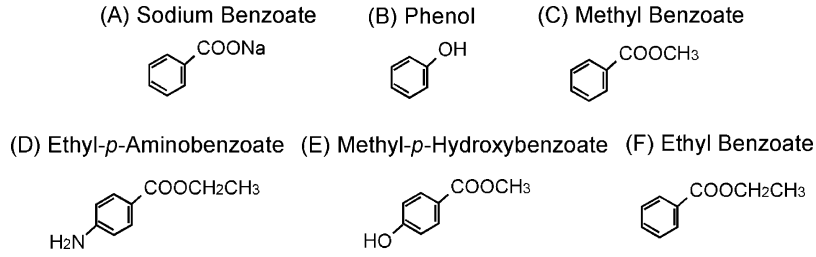

FIGURE 2. Chemical structures of benzoic-acid family and phenol used as analytes.

washed by sequential centrifugation and resuspension in methanol, 50 mM EDTA solution, and finally with Milli-Q water. Modified silica beads were filtered and rinsed with Milli-Q water and acetone, and dried in a high vacuum oven at 50 °C for 5 h.

Characterization of Initiator Immobilized Silica and Grafted PIPAAm. In order to determine the amount of ATRP-initiator and grafted copolymer on the silica beads, the silica beads were subject to elemental analysis using a CHN elemental analyzer VarioEL (Elementar, Hanau, Germany). ATRP-initiator and copolymer (milligrams per square meter) on silica beads was calculated by the following equations

$$\text{ATRP-initiator} = \frac{\%C_I}{\%C_I(\text{calcd})(1 - \%C_I/\%C_I(\text{calcd}))S} \quad (1)$$

$$\text{grafted copolymer} = \frac{\%C_C}{\%C_C(\text{calcd}) \times (1 - \%C_C/\%C_C(\text{calcd}) - \%C_I/\%C_I(\text{calcd}))S} \quad (2)$$

where $\%C$ is percent carbon increase as determined by elemental analysis, $\%C$(calcd) is the calculated weight percent of carbon in initiator or copolymers, S is the specific surface area of silica beads in square meters per gram (the manufacture's data: 100 m^2/g), and the subscripts I and C denote initiator and copolymer, respectively.

Grafted copolymer on silica bead surfaces was also retrieved and analyzed by gel permeation chromatography (GPC) for obtaining both the molecular weight and polydispersity index (PDI). Detailed procedure for retrieving and analyzing grafted polymer is described in the Supporting Information. The graft density of coplymer on the silica beads surfaces was estimated using the follow equation:

$$\text{graft density} = \frac{m_C N_A}{M_n} \quad (3)$$

where m_c is the amount of grafted copolymer on the silica bead surfaces per square meter (g/m^2), N_A is Avogadro's number, and M_n is the number average molecular weight of grafted copolymer.

Synthesis of Hydrophobized Thermoresponsive Copolymer by ATRP. To characterize the thermoresponsive copolymer including hydrophobic group, P(IPAAm-*co*-BMA) products with various feed ratios (the monomer composition in feed: BMA 0, 1, 3, or 5mol%) were synthesized by solution-phase ATRP in the similar conditions as the copolymer grafting onto silica bead surfaces. Copolymerization was performed by the same protocol as the grafting copolymer onto silica except that α-chloro-*p*-xylene (53.4 mg, 380 μmol) was added as an initiator in the reaction solution instead of silica beads. After the copolymerization, the solution was dialyzed against EDTA solution using the dialysis membrane for 3 days with changing EDTA solution every day, followed by dialysis against Milli-Q water for 2 days, and the copolymer was obtained by lyophilization.

Table 2. Properties of Benzoic Acids and Phenol Utilized As Analytes

analyte	mol wt	log P^a
sodium benzoate	144.1	−2.27
phenol	94.11	1.46
methyl benzoate	136.15	2.12
ethyl *p*-aminobenzoate	165.19	1.86
methyl *p*-hydroxybenzoate	152.15	1.96
ethyl benzoate	150.17	2.64

[a] Partition coefficient in *n*-octanol/water system.

Characterization of Hydrophobized Thermoresponsive Copolymer. Prepared copolymer, P(IPAAm-*co*-BMA), was analyzed by the GPC system to determine both the molecular weight and PDI. Phase transitions of the copolymer solutions in water were investigated by optical transmittance changes. Solutions of P(IPAAm-*co*-BMA) containing various amounts of BMA were prepared using Milli-Q water (10 mg/mL). Optical transmittance changes of the copolymer solutions were monitored at 600 nm by a UV/visible spectrometer (V-530) (JASCO, Tokyo). The sample cuvette was thermostated with a Peltier-effect cell holder (EHC-477) (JASCO) with a heating rate of 0.10 °C/min. The LCST was defined as the temperature at 90% transmittance of solution. BMA content in the copolymers was determined by ^{1}H-NMR (^{UNITY}INOVA 400MHz spectrometer) (Varian, Palo Alto, CA) using chloroform-d containing 0.03 v/v% tetramethylsilane as a solvent.

Temperature Modulated Elution of Analytes. P(IPAAm-*co*-BMA) grafted silica beads were packed into a stainless steel column (4.6 mm i.d. × 50 mm). A slurry of copolymer-grafted silica beads in water/methanol mixed solvents (1:1) was poured into a slurry reservoir (TOSOH, Tokyo) connected to a stainless steel column. Water/methanol mixed solvent (1:1) was flowed through the slurry reservoir using an HPLC pump (PU-980) (JASCO) at 350 kg/cm^2 for 1 h, followed by equilibration with Milli-Q water for at least 12 h. Copolymer-grafted bead-packed columns were connected to an HPLC system (PU-980 and UV-970) (JASCO) controlled by a personal computer with Borwin analysis software version 1.21 (JASCO). Six model analytes, benzoic acid family (sodium benzoate, methyl benzoate, ethyl *p*-aminobenzoate, methyl *p*-hydroxybenzoate, and ethyl benzoate) and phenol, were used for obtaining chromatograms at a concentrations of 0.2 mg/mL with Milli-Q water. Chemical structures and hydrophobic properties of these analytes are shown in Figure 2 and Table 2, respectively. Milli-Q water was used as a mobile phase. Thermoresponsive elution behaviors of benzoic acids and phenol were monitored at 254 nm with a flow rate of 1.0 mL/min. The column temperature was controlled with a deviation of ±0.1 °C using a thermostated water bath (RE206) (Lauda, Lauda-Königshofen). After the observation

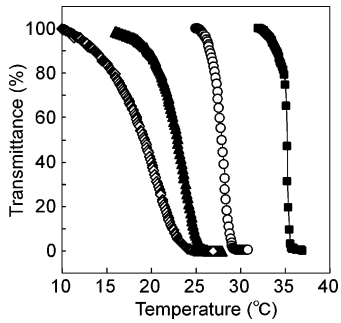

FIGURE 3. Phase transition profiles of poly(*N*-isopropylacrylamide-*co*-butyl methacrylate) in Milli-Q water. Closed squares, PIPAAm homopolymer; open circles, IPB-2.5; closed triangles, IPB-5.2; open diamonds, IPB-8.8.

of elution profiles of analytes using the column, the column was cleaned by flowing water with a flow rate of 1.0 mL/min for 2 h at 10 °C.

To observe analytes retention behavior on the prepared columns, van't Hoff plots for these analytes were obtained. The retention factor k' value was defined using the follow equation

$$\text{retention factor} = \frac{t_R - t_0}{t_0} \quad (4)$$

where t_R is the retention time for known sample at a specific temperatures, and t_0 is the retention time for uracil as the initial standard.

RESULTS AND DISCUSSION

Characterization of Thermoresponsive Copolymer Including Hydrophobic Group. Characteristics of thermoresponsive copolymers of P(IPAAm-*co*-BMA) are summarized in Table 1. Prepared copolymers are abbreviated as IPB-x, where x represents the mole fraction of BMA in the copolymer. Mole fraction of BMA in the copolymer was larger than that in the feed composition, probably due to the higher reactivity ratio of BMA, compared to that of IPAAm in ATRP procedure using a CuCl/$CuCl_2$/Me_6TREN catalyst system with 2-propanol as a solvent. Polydispersity of prepared copolymers were approximately 1.2, indicating that the polymerization was well-controlled compared to a conventional radical polymerization (8). Furthermore, the polydispersity of the copolymers including BMA were relatively large compared to that of PIPAAm homopolymer, because the ATRP catalytic system CuCl/$CuCl_2$/Me_6TREN is ordinarily used for acrylamide derivatives including IPAAm, and it may be unsuitable for BMA (35, 36).

LCST values for the copolymers in water decreased with the increase of BMA content in the copolymer (Figure 3). Incorporation of hydrophobic co-monomer decreases copolymer's LCST, because the incorporation of BMA increase the hydrophobicity of the random copolymer (31), and the increased hydrophobicity enhances the aggregation of copolymers. Thus, the incorporation of BMA monomer in the copolymer decreased the copolymers LCST. Additionally, the temperature range of the transmittance changes became wider with the increase in BMA contents, because BMA molecules interrupt the IPAAm sequences, disrupting any segmental cooperative effects in temperature-dependent dehydration upon temperature increase (31). However, the observed phase transition temperatures of the copolymers including BMA monomer were suitable for temperature-responsive chromatography, because the controlling column temperature for modulating interactions with analytes could be performed at a specific temperature region for avoiding possible deactivations of analytes.

Characterization of Initiator Surfaces and Copolymer Grafted Silica Beads. Initiator immobilized silica beads and copolymer-grafted silica beads were characterized by elemental analysis. Detected elements (C, H, and N) and the amounts of immobilized initiator are summarized in Table 3. These surfaces are abbreviated as IPB-xB where x represents the feed composition of BMA. B in xB denotes the "brush". Amounts of the grafted copolymer were greater than that of polymer hydrogel-modified silica beads prepared by the conventional radical polymerization we reported previously (32). This was due to the graft configuration of polymer brush prepared by the surface initiated ATRP. Polymer brushes prepared by the surface-initiated ATRP formed densely packed configurations compared to that by other radical polymerizations, since the initiation efficiency of ATRP was quite high. Thus, P(IPAAm-*co*-BMA) was densely grafted on silica bead surfaces, leading to the significantly large amount of grafted copolymer on these surfaces.

To characterize the chain lengths and graft densities of copolymers on the silica surfaces, the molecular weight of grafted copolymer was determined by GPC after the chains was cleaved from silica beads with hydrofluoric acid. These data are also summarized in Table 3 (GPC charts of cleaved polymer are shown in Figure S1 of the Supporting Information). The polydispersity index of the cleaved copolymer was larger than that of copolymers prepared in solution. The larger polydispersity was suggested to be attributed to the porous geometry of silica beads (28, 37). Polymerization reaction inside the pores of beads was limited by the insufficient of monomer supply compared to outer exposed surfaces. In addition, the propagation of the polymer chains from the initiator inside the pores was also restricted to the pore diameter. These factors gave the large polydispersity of grafted copolymers on porous silica bead surfaces.

Elution Behavior of Benzoic Acids and Phenol from P(IPAAm-co-BMA) Brush Surfaces. To investigate the interfacial hydrophobic properties of the P(IPAAm-*co*-BMA) brush surfaces, the elution behavior of the benzoic acids and phenol from the copolymer grafted silica beads used as chromatographic stationary phases was investigated. In previous reports, hydrophobic steroids were utilized as model analytes for investigating hydrophobic interaction between stationary phase and analytes. However, no steroids elution was observed from the prepared P(IPAAm-*co*-BMA) copolymer brush surfaces, probably due to the adsorption of steroids onto the hydrophobized surfaces (chromatograms are shown in Figure S2 in the Supporting Information). Thus, we used several different kinds of ben-

Table 3. Characterization of P(IPAAm-*co*-BMA) Grafted Silica Beads

code[a]	elemental composition (%)[b] C	H	N	immobilized initiator (μmol/m^2)	grafted polymer (mg/m^2)	M_n[c]	M_w/M_n[c]	graft density (chains/nm^2)
initiator-immobilized silica	4.7	1.1	0.3	4.56				
IPB-0B	18.8	3.1	2.9		3.16	16900	3.00	0.085
IPB-1B	19.5	3.1	2.9		3.37	18200	3.97	0.111
IPB-3B	19.9	3.2	2.8		3.48	20300	3.20	0.103
IPB-5B	20.5	3.3	2.6		3.66	22700	2.89	0.097

[a] Abbreviated as IPB-*x*B where*x* represents the feed composition of BMA and the B in xB denotes the "brush". [b] Determined by elemental analysis ($n = 2$). [c] Determined by GPC using DMF containing 50 mmol/L LiCl.

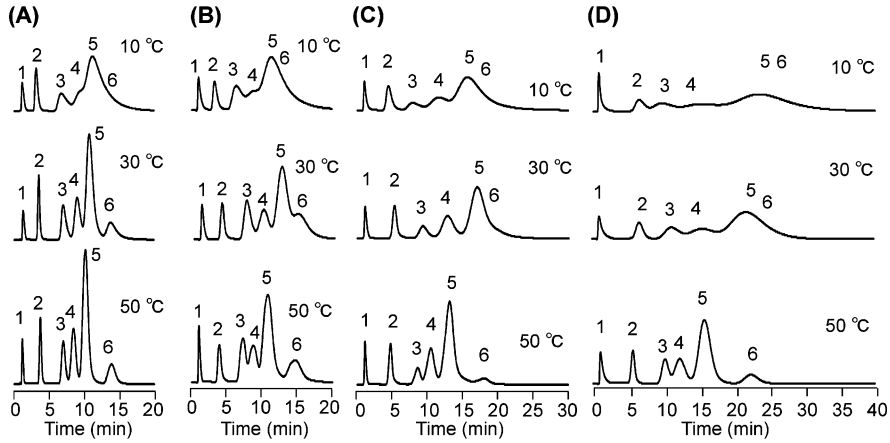

FIGURE 4. Chromatograms of benzoic acids and phenol separated on HPLC of which packing material was poly(*N*-isopropylacrylamide-*co*-butyl methacrylate) grafted silica beads at various temperatures: (A) IPB-0B (PIPAAm), (B) IPB-1B, (C) IPB-3B, and (D) IPB-5B columns (Table 3). The mobile phase is Milli-Q water. Peak 1 represents sodium benzoate; 2, phenol; 3, methyl benzoate; 4, ethyl *p*-aminobenzoate; 5, methyl *p*-hydroxybenzoate; 6, ethyl benzoate.

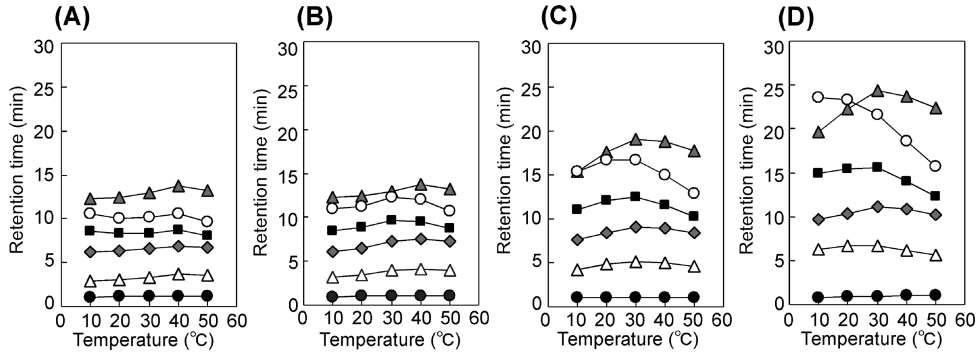

FIGURE 5. Temperature-dependent retention time changes of benzoic acids and phenol on (A) IPB-0B (PIPAAm), (B) IPB-1B, (C) IPB-3B, and (D) IPB-5B columns (Table 3). Closed circles, sodium benzoate; open triangles, phenol; closed diamonds, methyl benzoate; closed squares, ethyl *p*-aminobenzoate; open circles, methyl *p*-hydroxybenzoate; closed triangles, ethyl benzoate.

zoic acids and phenol, which are used as fragrance materials or preservatives in foods and cosmetics, as model analytes. Separation of these analytes would be applicable for the contents analysis of foods and cosmetics. Figure 4A–D shows the chromatograms of benzoic acids and phenol at various temperatures on IPB-0B, IPB-1B, IPB-3B, and IPB-5B bead-packed columns using Milli-Q water as a mobile phase, respectively. Figure 5A–D show changes in the retention times with various temperatures on these columns. Chromatograms with high resolution were obtained at the elevated column temperature on all columns. This is attributed to the increased hydrophobicity of grafted copolymer on silica beads with increased temperature. Grafted copolymers on silica beads dehydrated and increased their hydrophobicity with increasing column temperature, leading to the increase in hydrophobic interaction between the surface and analytes. Thus, high-resolution peaks were obtained in a higher-temperature region.

Benzoic acids and phenol were all retained on the prepared copolymer modified columns, and the retention times of these anlaytes increased with the increase of the feed composition of BMA. These results indicated that the hydrophobicity of copolymer brush modified surface increased with increasing the content of BMA in grafted copolymer. Additionally, the retention times increased in the following order; sodium benzoate < phenol < methyl benzoate < ethyl *p*-aminobenzoate < methyl *p*-hydroxybenzoate < ethyl benzoate, which is almost in agreement with the hydrophobicity

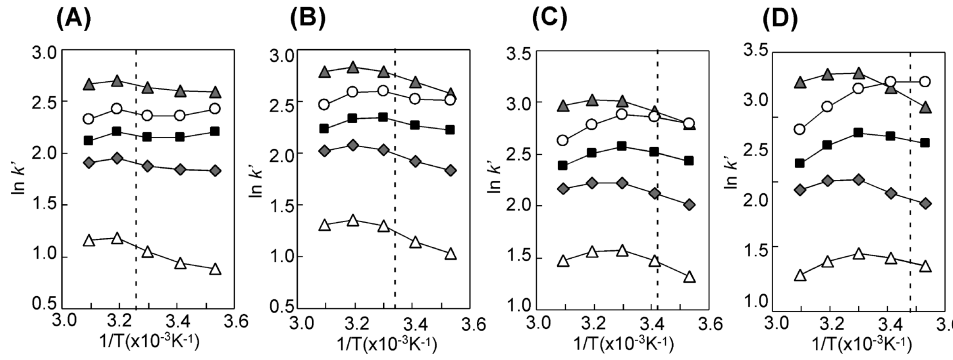

FIGURE 6. Van't Hoff plots of benzoic acids and phenol on (A) IPB-0B (PIPAAm), (B) IPB-1B, (C) IPB-3B, and (D) IPB-5B columns (Table 3). Closed circles, sodium benzoate; open triangles, phenol; closed diamonds, methyl benzoate; closed squares, ethyl *p*-aminobenzoate; open circles, methyl *p*-hydroxybenzoate; closed triangles, ethyl benzoate.

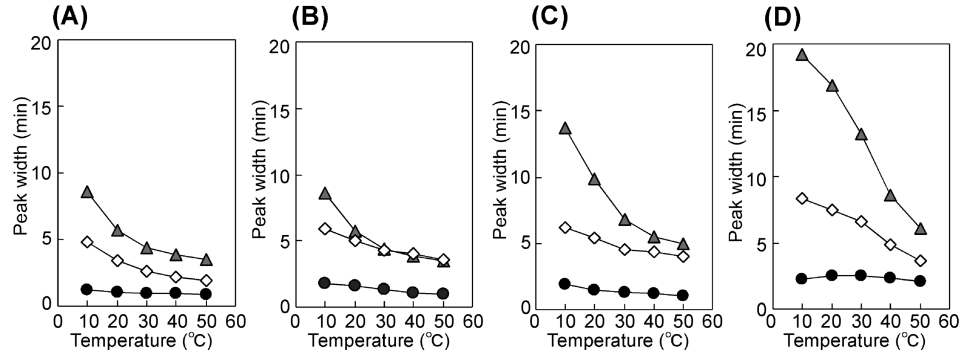

FIGURE 7. Temperature-dependent peak width changes of benzoic acids and phenol on (A) IPB-0B (PIPAAm), (B) IPB-1B, (C) IPB-3B, and (D) IPB-5B columns (Table 3). The closed circles represent sodium benzoate; open diamonds, methyl benzoate; closed triangles, ethyl benzoate.

(polarity) of analytes. This result indicated that a hydrophobic interaction between the stationary phase and analytes can predominantly contribute the holding capacity for analytes. Two analytes having functional goups, ethyl *p*-aminobenzoate and methyl *p*-hydroxybenzoate, exhibited longer retention times, although their hydrophobicities are relatively low. These unpredicted observations are probably due to additional interactions between the copolymer brush grafted silica beads and these analytes. Amino group of ethyl *p*-aminobenzoate is speculated to electrostatically interact with silanol groups appeared on silica surface. Also, the hydroxyl group of methyl *p*-hydroxybenzoate would interact with IPAAm units through hydrogen bonding. These possible interactions would contribute to the longer retention time of these analytes. Actually, using a pH 2.5 mobile phase, retention time of ethyl *p*-aminobenzoate was decreased compared to that using a neutral mobile phase, probably due to suppression of silanol activity at lower pH (see the Supporting Information, S3). To confirm the interactions, further study will be necessary.

To investigate the detailed retention mechanism of analytes, the van't Hoff plots of these analytes were obtained. Figure 6 shows the van't Hoff plots of these anlytes, which exhibit a relationship between the analyte retention and the column temperature. A linear relationship between ln k' values and reciprocal temperature (1/T) is commonly observed in the van't Hoff plots of commercially available reversed phase columns in a normal chromatographic process. However, as shown in Figure 6, the inflection points were observed near the LCSTs of copolymers, indicating that the hydrophobic interaction with analytes increased near the LCSTs. Our research group has previously reported that hydrophobic steroids retention through hydrophobic interaction was dramatically increased above the LCSTs of grafted PIPAAm (19). Similar retention behavior was observed on the surfaces with relatively lower BMA content copolymers, IPB-0B and IPB-1B. On the contrary, on IPB-3B and IPB-5B columns, the retention k' decreased at higher temperature region. This is attributed to the low LCST of grafted copolymers. Copolymer with high BMA composition exhibited low LCSTs, indicating that the dehydration of copolymer have terminated at low temperature. Thus, at higher temperature region, the IPB-5B surface no longer increased its hydrophobicity with increasing column temperature. On the contrary, the solubility of analytes into the mobile phase increased with the increase of column temperature. Thus, at high temperature on IPB-3B and IPB-5B columns, the retention of analytes decreased with increasing temperature.

To observe the separation efficiency of the prepared columns, we obtained temperature-dependent peak width change of three analytes, sodium benzoate, methyl benzoate, and ethyl benzoate. Figure 7 show the temperature-dependent peak width changes. Peak widths of analytes decreased with the increase in column temperature. The large peak width change was predominantly attributed to the shrinking of the grafted copolymer with increasing temperature. At low temperature, the grafted copolymer is highly extended on the silica bead surfaces, and the analytes

molecules tend to penetrate into the copolymer brush layer and interact with the inner brush layer (21). Additionally, at low temperature, a larger peak width was observed at higher BMA composition columns. Incorporation of BMA into copolymer increased the copolymer's hydrophobicity, which enhanced a hydrophobic interaction with analytes. Thus, analyte retention at the inner brush layer was promoted with the increase in BMA composition, which gave a wide peak at low temperature.

Incorporation of BMA into a PIPAAm copolymer brush provided a strong hydrophobicity to thermo-responsive chromatographic stationary phase. Also, the dehydration of grafted copolymer was promoted at lower temperature, because the copolymer's LCST was decreased with the hydrophobicity of incorporated BMA. Thus, these prepared hydrophobized thermoresponsive copolymer brush surfaces would be a useful thermoresponsive stationary phase that separates relatively hydrophilic compounds at lower temperature region.

CONCLUSIONS

Thermoresponive copolymer including hydrophobic group, P(IPAAm-*co*-BMA), is successfully grafted onto silica bead surfaces using surface initiated ATRP. The grafted amount of P(IPAAm-*co*-BMA) was relatively large, compared to the previously reported hydrogel grafted silica beads. LCST value was modulated by changing the feed composition of BMA in ATRP procedure. A mixture of benzoic acids and phenol as model analytes were separated on the copolymer grafted column. Chromatograms of these analytes with a high resolution were obtained at high temperature, because a hydrophobic interaction between the stationary phase and the analytes contribute the high resolution separation. Retention times for the analytes prolonged with the BMA composition, because the hydrophobicity of grafted copolymer increased. The analysis of retention mechanism using van't Hoff plot indicated that the dehydration of grafted copolymer brush with high BMA composition was obtained at low-temperature regions. The prepared beads were speculated to provide an opportunity to control column temperature at a specific temperature region for avoiding possible deactivations of analytes. These results suggested that the prepared copolymer brush surfaces interacted with analytes through the strongest hydrophobic interaction among previously reported thermo-responsive chromatography, and the modulation of column temperature allowed the surfaces to be an attractive separation tool for hydrophilic analytes at low temperature.

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Supporting Information Available: GPC molecular weight chart for cleaved copolymers from silica bead surfaces and chromatograms of hydrophobic steroids on a prepared bead-packed column (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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