High Stability of Thermoresponsive Polymer-Brush-Grafted Silica Beads as Chromatography Matrices

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Ⓢ *Supporting Information*

ABSTRACT: Thermo-responsive chromatography matrices with three types of graft architecture were prepared, and their separation performance and stability for continuous use were investigated. Poly(N-isopropylacrylamide)(PIPAAm) hydrogel-modified silica beads were prepared by a radical polymerization through modified 4,4'-azobis(4-cyanovaleric acid) and N,N'-methylenebisacrylamide. Dense PIPAAm brush-grafted silica beads and dense poly(N-tert-Butylacrylamide (tBAAm)-b-IPAAm) brush-grafted silica beads were prepared through a surface-initiated atom transfer radical polymerization (ATRP) using CuCl/CuCl$_2$/ Tris(2-(N,N-dimethylamino)ethyl)amine (Me$_6$TREN) as an ATRP cata-

lytic system and 2-propanol as a reaction solvent. Dense PIPAAm brush-grafted silica beads exhibited the highest separation performance because of their strong hydrophobic interaction between the densely grafted well-defined PIPAAm brush on silica-bead surfaces and analytes. Using an alkaline mobile phase, dense themoresponsive polymer brushes, especially having a hydrophobic basal layer, exhibited a high stability for continuous use, because polymer brush on the silica bead surfaces prevented the access of water to silica surface, leading to the hydrolysis of silica and cleavage of grafted polymers. Thus, the precisely modulating graft configuration of thermoresponsive polymers provided chromatography matrices with a high separation efficiency and stability for continuous use, resulting in elongating the longevity of chromatographic column.

KEYWORDS: *intelligent materials, temperature-responsive polymer, poly(N-isopropylacrylamide), polymer brush, chromatography, bioactive compounds*
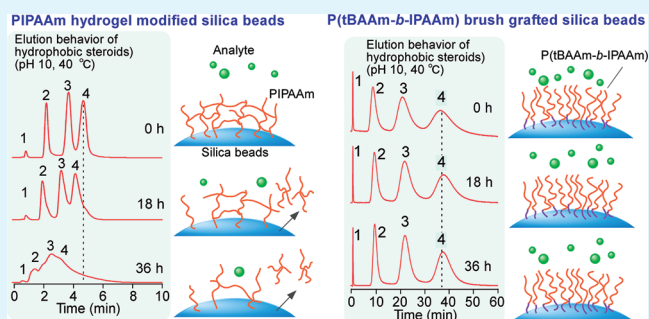
■ INTRODUCTION

Functionalized interfaces, prepared by modifying functional groups or functional polymers, have been investigated for various applications including sensor, membrane, or implantable device.[1−5] Especially, thermoresponsive interfaces prepared with poly(N-isopropylacrylamide) (PIPAAm) have been developed for various biomedical applications,[6−8] because PIPAAm exhibits a reversible temperature-dependent phase transition[9] in aqueous solutions at its lower critical solution temperature (LCST) of 32 °C, near the body temperature. The thermoresponsive characteristics are utilized for drug and gene deliveries,[10,11] sensors and actuators,[12,13] and bioconjugate materials.[14,15] Additionally, our research institute has investigated the intrinsic characteristics of thermo-responsive hydrophobic/hydrophilic alteration of PIPAAm-modified surface, and applied them to biomedical research fields for developing remarkable medical devices. Especially, the interaction between grafted PIPAAm on surface and cells can be modulated by external temperature change,[16,17] and

confluently cultured cells can be successfully harvested as a contiguous "cell sheet" having a tissuelike cellular architecture with simply reducing temperature. Fabricated cell sheets have been utilized for the various types of tissue engineering and regenerative medicines.[18−21]

Furthermore, thermoresponsive chromatography using a PIPAAm-modified stationary phase has been investigated as a new type of analytical tool.[22−25] Modified PIPAAm on stationary phase alters its hydrophobicity by changing temperature, leading to the modulation of the hydrophobic interaction between PIPAAm and analytes. Additionally, this system requires no organic solvents as a mobile phase for separation, leading to preserving the biological activity of analytes with minimizing the environmental loads. For improving the thermoresponsive chromatography system, various types of
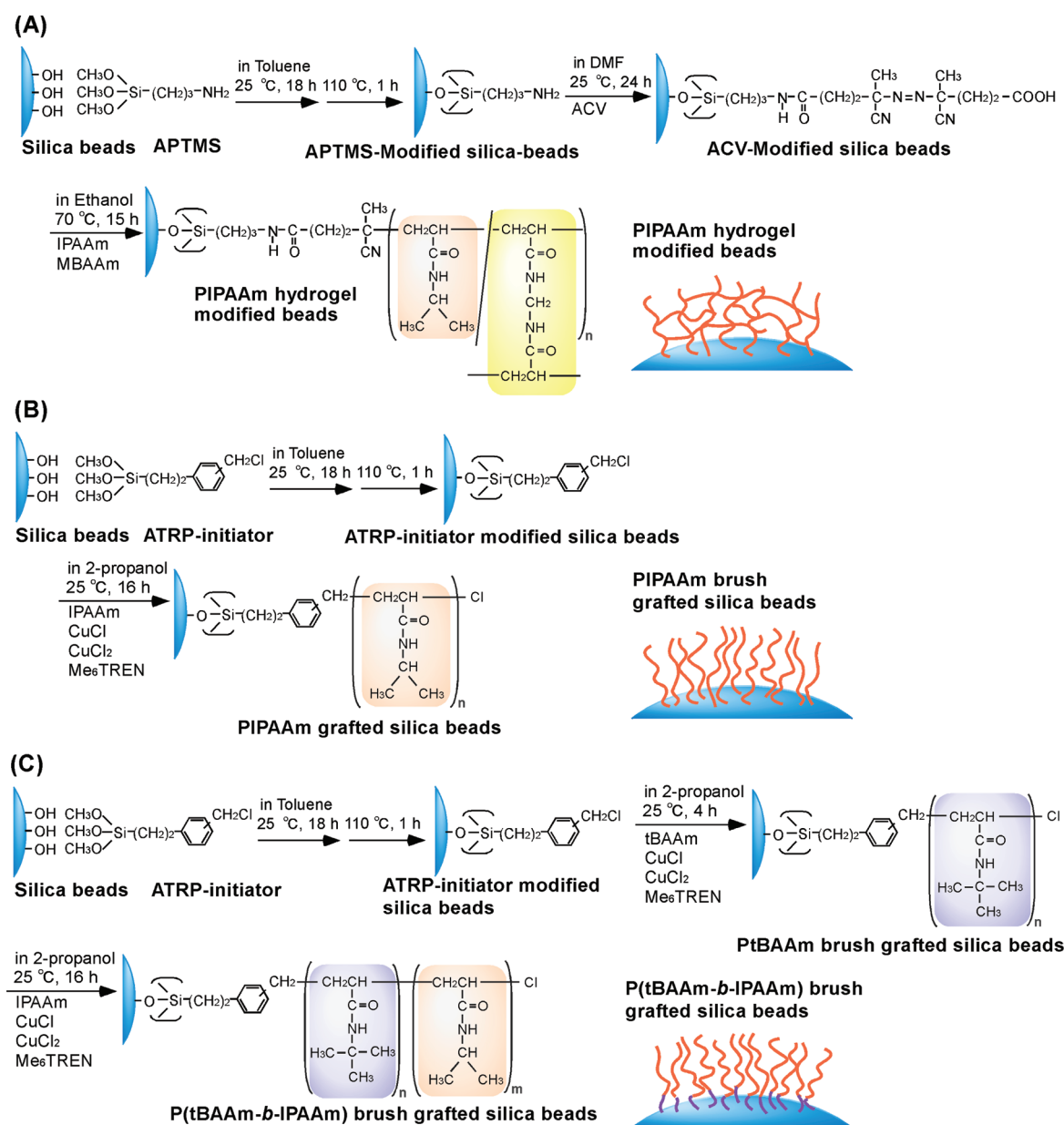
Figure 1. Scheme of the preparation of thermoresponsive polymer-grafted silica-beads using a surface-initiated atom transfer radical polymerization (ATRP); (A) poly(N-isopropylacrylamide) (PIPAAm) hydrogel-modified beads and (B) PIPAAm brush-grafted surface, and (C) poly(tert-butylacrylamide (tBAAm)-b-IPAAm).

PIPAAm-modified chromatography matrices have been developed by attempting to use various modification methods and base materials.

Various types of PIPAAm grafting methods have been utilized for preparing temperature responsive chromatography. First, using a "grafting to" method, PIPAAm-grafted silica beads are prepared by a standard ester-amine coupling reaction.[22] A second "grafting from" method for PIPAAm uses a surface-immobilized azo-initiator and cross-linker to prepare polymer layers with a conventional radical polymerization.[26] Recently, well-defined PIPAAm-grafted surface on silica beads by a surface initiated atom transfer radical polymerization (ATRP).[27] Allowing surfaces to be modified with well-defined dense polymer brush using surface-immobilized ATRP initiators,[28−33] ATRP is an attractive polymer grafting method.

Regarding the base matrices of stationary phase, silica beads,[34] polystyrene beads,[35] poly(hydroxyl methacrylate)

(PHMA) beads,[36] and monolithic silica rods[37] are used as a base material. In terms of the separation efficiency of analytes, silica beads and silica monoliths (silica rods) exhibit a higher separation efficiency compared to those of other polymer based beads, because silica based stationary phases have a relatively high mechanical strength and can be designed easily to their particle diameter, pore-size, and surface area for various separation purposes. Thus, PIPAAm-grafted silica beads or silica monoliths are mainly utilized.

However, previous reports indicate that all silica stationary phases used in reversed-phase chromatography become instable under an alkaline condition and stable in a neutral pH mobile phase.[38] This is attributed to the hydrolysis of silica surfaces under an alkaline condition, and the modified functional groups are cleaved. Empirically, a separation reproducibility decreases with the repeated use of silica-based stationary phases even at a neutral condition. Thus, stationary phases providing a high

stability at neutral-high pH are required for improving an aqueous separation reproducibility. To improve the stability of silica beads as chromatography matrices, several investigators applied various coating methods for silica beads for preventing hydrolysis.[39,40] These coated materials can prevent the access of water molecule to silica-bead surfaces, leading to the prevention of hydrolysis. In the same way, dense polymer brush on silica-bead surfaces would prevent the hydrolysis, because a densely packed polymer-brush structure on silica-bead surfaces would prevent the access of water to silica-bead surfaces.

This study described the preparations of three kinds of thermoresponsive polymer-grafted silica beads. Separation efficiency and stability were investigated by observing the time course of separation efficiency of hydrophobic steroids. To the best of our knowledge, this is the first investigation for improving the stability of temperature-responsive chromatographic stationary phase.

■ EXPERIMENTAL SECTION

Materials. N-isopropylacrylamide (IPAAm) was kindly provided by Kohjin (Tokyo, Japan) and recrystallized from n-hexane. N-tert-Butylacrylamide (tBAAm) was obtained by Wako Pure Chemicals (Osaka) and recrystallized from acetone. N,N'-Methylenebisacrylamide (MBAAm) was obtained from Wako. CuCl and CuCl$_2$ were purchased from Wako. Tris(2-aminoethyl)amine (TREN) was purchased from Acros Organics (Pittsburgh, PA, USA). Formaldehyde, formic acid, and sodium hydroxide were purchased from Wako. Tris(2-(N,N-dimethylamino)ethyl)amine (Me$_6$TREN) was synthesized from TREN, according to the previous reports.[41] 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). Stainless steel column (50 × 4.6 mm i.d.) was obtained from GL Science (Tokyo). Hydrochloric acid, and ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt dehydrate (EDTA·2Na) were purchased from Wako. ((Chloromethyl)phenylethyl)trimethoxysilane (mixed m, p isomers) as an ATRP initiator and 3-aminopropyltrimethoxysilane (APTMS) were obtained from Gelest (Morrisville, PA). 4,4′-Azobis(4-cyanovaleric acid)(ACV) was purchased from Wako. 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was obtained from Tokyo Chemical Industries (Tokyo). 2-Propanol (HPLC grade), ethanol, N,N-dimethylformamide, and toluene (dehydrate) were purchased from Wako. Steroids and uracil were also purchased from Wako. 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 PIPAAm Hydrogel-Modified Silica Beads. PIPAAm hydrogel-modified silica beads were prepared according to the previous reports[26,42] except that 3-aminopropyltrimethoxysilane (APTMS) was modified on silica beads having 300 Å pore diameter. APTMS-modified silica beads were prepared as shown in the first step in Figure 1A. 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 through drying in a vacuum oven at 110 °C for 18 h. Formation of silane layers comprising APTMS on silica surface was performed as follows. Silica beads (16.2 g) were placed into a round-bottom flask and humidified at 60% relative humidity for 4.0 h, followed by the addition of 3.03 mL of APTMS in 324 mL of dried toluene. The reaction proceeded at room temperature for overnight with continuous stirring. APTMS-immobilized silica beads were collected by filtration, extensively rinsed with toluene and acetone, and dried in a vacuum oven at 110 °C. Preparation of initiator immobilized silica bead surface was performed as shown in the second step in Figure 1A. APTMS-modified beads (16.5 g) were placed into a round-bottom flask and dissolved in 165 mL of DMF, followed by the addition of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (12.4 g, 50.1 mmol), a condensing agent. Then, 4,4′-azobis(4-cyanovaleric acid)(ACV) (6.93 g, 24.7 mmol), an initiator, was

dissolved in 165 mL of DMF, and the solution was poured into the flask. The reaction proceeded at 25 °C for 12 h with continuous stirring. ACV immobilized silica beads were collected by filtration, extensively rinsed with DMF and ethanol, and dried in a vacuum oven at 25 °C for 12 h. PIPAAm hydrogel-modified surfaces were prepared as shown in the third step in Figure.1 (A). The ACV immobilized silica beads were placed in the flask. IPAAm (36.0 g, 318 mmol) and MBAAm (0.492 g, 3.19 mmol) were dissolved in 240 mL of ethanol, and the solution was poured into the beads placed flask. The reaction solution containing the beads was degassed by subjecting to it three freeze−thaw cycles, and the flask was sealed under reduced pressure. The polymerization reaction proceeded at 70 °C for 15 h. The PIPAAm hydrogel-modified silica beads were collected by filtration and extensively rinsed with ethanol and acetone in a vacuum oven at 50 °C for 5 h.

Preparation of PIPAAm Brush-Grafted Silica Beads. ((Chloromethyl)phenylethyl)trimethoxysilane (CPTMS), an ATRP-initiator, modified silica beads were prepared as shown in the first step in Figure 1B, according to the previous reports.[28,43] First, silica beads were washed as the same procedure described above. Formation of silane layers comprising the ATRP initiator on silica surface was performed as follows; silica beads (20.5 g) were placed into a round-bottom flask and humidified at 60% relative humidity for 4.0 h, followed by the addition of 5.53 mL of CPTMS in 410 mL of dried toluene. The reaction proceeded at room temperature for overnight with continuous stirring. ATRP initiator-immobilized silica beads were collected by filtration, extensively rinsed with toluene, methanol, dichloromethane, and acetone, and dried in a vacuum oven at 110 °C. PIPAAm brush-grafted silica beads were prepared as shown in the second step in Figure 1B, according to the previous report.[43] IPAAm (48.6 g, 429 mmol) were dissolved in 428 mL of 2-propanol, and the solution was deoxygenated by argon gas bubbling for 2 h. CuCl (847 mg, 8.6 mmol), CuCl$_2$ (115 mg, 0.86 mmol), and Me$_6$TREN (2.22 g, 9.59 mmol) were added under an argon atmosphere, and the solution was stirred for 20 min for obtaining a CuCl/CuCl$_2$/Me$_6$TREN catalyst system. ATRP initiator-immobilized silica beads (10 g) were placed into a 500-mL of round-bottom flask. Both monomer solution and the silica beads in the flask were placed into a glovebox purged with dry argon gas by repeated vacuum and argon flush (three times). The monomer solution was poured on the silica beads in the flask, which was sealed under argon. The ATRP reaction proceeded for 16 h at 25 °C under continuous stirring on a magnetic stirrer (AMGS, ASH, Chiba). PIPAAm-grafted silica beads were washed by ultrasonication with acetone for 30 min followed by centrifugation for removing unreacted monomers and ungrafted polymers. This washing process with ultrasonication was repeated twice. Polymer-grafted silica beads were further washed by sequential centrifugation and resuspension in methanol, 50 mmol/L EDTA solution, and finally with Milli-Q water. Modified silica beads were filtered, rinsed with Milli-Q water and acetone, and dried in a high vacuum oven at 50 °C for 5 h.

Preparation of P(tBAAm-b-IPAAm) Brush-Grafted Silica Beads. Preparation of thermoresponsive block copolymer brush having hydrophobic basal layer was performed as shown in the Figure 1C. First, hydrophobic tert-butylacrylamdie (tBAAm) brush was grafted as shown in the second step in Figure 1C. tBAAm (5.47 g, 42.9 mmol) were dissolved in 428 mL of 2-propanol, and the solution was deoxygenated by argon gas bubbling for 2 h. CuCl (847 mg, 8.6 mmol), CuCl$_2$ (115 mg, 0.86 mmol), and Me$_6$TREN (2.22 g, 9.59 mmol) were added under an argon atmosphere, and the solution was stirred for 20 min for obtaining a CuCl/CuCl$_2$/Me$_6$TREN catalyst system. The ATRP initiator-immobilized silica beads (10 g) that were prepared and described in the previous section were placed into a 500-mL of round-bottom flask. Both monomer solution and the silica beads in the flask were placed into a glovebox purged with dry argon gas by repeated vacuum and argon flush (three times). The monomer solution was then poured on the silica beads in the flask, which was sealed under argon. The ATRP reaction proceeded for 4 h at 25 °C under continuous stirring on the magnetic stirrer. PtBAAm brush-grafted silica beads were collected by filtration, extensively rinsed with acetone and methanol, and dried in a vacuum oven at 50 °C. The

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second thermoresponsive PIPAAm brush layer was prepared on the first hydrophobic tBAAm brush layer as shown in the third step in Figure 1C. IPAAm (43.7 g, 387 mmol) were dissolved in 428 mL of 2-propanol, and the solution was deoxygenated by argon gas bubbling for 2 h. CuCl (847 mg, 8.6 mmol), CuCl$_2$ (115 mg, 0.86 mmol), and Me$_6$TREN (2.22 g, 9.59 mmol) were added under an argon atmosphere, and the solution was stirred for 20 min for obtaining a CuCl/CuCl$_2$/Me$_6$TREN catalyst system. The tBAAm brush-grafted silica beads (9.0 g) were placed into a 500 mL round-bottom flask. Both monomer solution and the silica beads in the flask were placed into a glovebox purged with dry argon gas by repeated vacuum and argon flush (three times). The monomer solution was then poured on the silica beads in the flask, which was sealed under argon. The ATRP reaction proceeded for 16 h at 25 °C under continuous stirring on the magnetic stirrer. This washing process by ultrasonication was repeated twice. Polymer-grafted silica beads were further washed by sequential centrifugation and resuspension in methanol, 50 mM EDTA solution, and finally with Milli-Q water. Modified silica beads were filtered, 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. For determining the amount of silane layer, initiator, and grafted polymer, the prepared silica beads were subject to elemental analysis using a CHN elemental analyzer a PE 2400 series II CHNS/O analyzer (PerkinElmer, Waltham, MA). The amount of modified silane, initiator, and polymer (milligrams per square meter) on silica beads were calculated by the following equations

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

grafted polymer

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

where %C is percent carbon increase as determined by elemental analysis, %C(calcd) is the calculated weight percent of carbon in initiator or polymers, 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 P denote initiator and polymer, respectively.

Grafted polymer on silica bead surface was also retrieved and analyzed by GPC for determining both the molecular weight and polydispersity (PDI). Polymer-grafted silica beads were treated with concentrated sodium hydroxide solution for overnight, and the solution was neutralized by the addition of hydrochloric acid.[44] The solution was filtered and dialyzed against Milli-Q water using dialysis membrane [Spectra/Por standard regenerated cellulose dialysis membrane, Molecular Weight Cut Off (MWCO): 1000] (Spectrum Laboratories, Rancho Dominguez, CA) for 1 week with water changed daily, and the polymer was recovered by freeze-drying. Number-average molecular weights and PDI values of the polymer were determined using the GPC system. A calibration curve was obtained using poly(N-isopropylacrylamide) standards. The flow rate was 1.0 mL/min. The mobile phase was DMF containing 50 mmol/L LiCl, and the column temperature was controlled at 45 °C using an equipped column oven, and the elution profiles were monitored by an equipped refractometer. Graft density of PIPAAm on silica bead surfaces was estimated using the follow equation

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

where m_c the amount of grafted PIPAAm 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 the grafted PIPAAm.

For obtaining surface elemental composition, X-ray photoelectron spectroscopy (XPS) measurement was performed for ATRP initiator-modified and polymer-grafted silica-bead surfaces by an XPS (K-Alpha, Thermo Fisher Scientific, Waltham, MA). Excitation X-rays were produced from a monochromatic Al K$\alpha_{1,2}$ source with a takeoff angle of 90°. Wide scans were recorded to analyze all existing elements on the surfaces, and a high-resolution narrow scan analysis was performed for the peak deconvolution of carbon C1s signals. All binding energies were referenced to a C1s hydrocarbon peak at 285.0 eV.

Surface morphology of osmium tetroxide (OsO4)-stained the prepared beads were observed using a scanning electron microscopy (SEM) S-4300 (Hitachi, Tokyo).

Nitrogen adsorption analyses were performed prepared silica beads at −196 °C with a nitrogen-adsorption measuring apparatus (BELSORP18PLUS-HT) (BEL Japan, Osaka). The beads were degassed at 50 °C under vacuum for 5 h before adsorption measurements.[45]

Temperature Modulated Elution of Bioactive Compounds. Thermoresponsive polymer-modified silica beads were packed into a stainless steel column (4.6 mm i.d. × 50 mm). A slurry of polymer-grafted silica beads in water/methanol mixed solvent (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, Tokyo) at 350 kg/cm^2 for 1 h, followed by equilibration with Milli-Q water for at least 12 h. Polymer-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). Hydrophobic steroids and adenosine were used for obtaining chromatograms at a concentration of 0.5 mg/mL ethanol

Table 1. Properties of Hydrophobic Steroids

analyte	mol wt	log P^a
adenosine	267.24	−1.05
hydrocortisone	362.46	1.61
dexamethasone	392.46	1.83
hydrocortisone acetate	404.50	2.19

^{a}Partition coefficient in n-octanol/water system.[46]

solution. The properties of analytes were summarized in Table 1.[46] Buffer solutions with various pHs (pH 7.0, 8.0, 9.0, and 10.0) were used as a mobile phase. The compositions of these buffer solutions were shown in Supporting Information. Thermoresponsive elution behaviors of steroids were monitored at 254 nm with a flow rate of 1.0 mL/min. Column temperature was controlled with a deviation of ±0.1 °C using a constant temperature water circulator (CTA400) (Yamato, Tokyo). For observing the time courses of steroids retention on the column, the elution behavior of steroids at 40 °C was observed every 6 h with the continuous flowing of various pH-mobile phases.

For observing elution profiles using step-temperature gradient,[47] two thermostatted water baths (RE206, Lauda, Lauda-Konigshofen), set at 10 and 50 °C individually were used. First, relatively hydrophilic steroids were eluted at 50 °C. Then, column temperature was reduced by immersing the column into another thermostatted water bath set at 10 °C, and elution behavior at 10 °C was observed. Because the outer casing of column was a stainless steel with small size diameter, the temperature of column was speculated to be promptly equilibrate with those of water baths.

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:

$$\text{retention factor} = \frac{t_R - t_0}{t_0} \quad (5)$$

where t_R is the retention time of known sample at a specific temperatures, and t_0 is the retention time of uracil as an initial standard,[48] because there is no interaction between uracil and PIPAAm, confirmed by observing the same retention time of

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Table 2. Elemental Analyses of Initiators and Polymer-Grafted Silica Beads by XPS with a Take-off Angle of 90°

code[a]	atom (%)[d]					N/C ratio
	C	N	O	Si	Cl	
APTMS modified	14.98 ± 3.49	2.28 ± 0.24	53.52 ± 2.60	28.54 ± 1.52	0.68 ± 0.45	0.152
ACV modified	20.48 ± 0.96	4.00 ± 0.25	48.65 ± 0.29	26.65 ± 1.18	0.22 ± 0.38	0.195
IP-G	34.03 ± 1.78	5.06 ± 0.31	41.29 ± 1.36	19.62 ± 2.48	n.d.	0.149
CPTMS modified	18.91 ± 0.27	0.19 ± 0.33	51.08 ± 0.57	28.88 ± 0.57	0.94 ± 0.25	0.010
IP-B	66.2 ± 1.38	9.72 ± 0.23	18.18 ± 0 0.61	5.77 ± 1.04	0.14 ± 0.24	0.147
tB-B	27.62 ± 0.77	2.00 ± 0.13	44.70 ± 0.59	24.52 ± 0.93	1.16 ± 0.27	0.072
tBIP-B	53.19 ± 0.69	7.05 ± 0.76	28.09 ± 2.33	10.93 ± 1.29	0.73 ± 0.39	0.133
calcd IPAAm[b]	75.0	12.5	12.5			0.167
calcd tBAAm[c]	77.8	11.1	11.1			0.143

[a]Thermoresponsive polymer-modified silica beads are named using polymer component and its structure. IP and tB represent "IPAAm" and "tBAAm", respectively. G and B denote "hydrogel" and "brush", respectively. [b]Theoretical atomic composition of (N-isopropylacrylamide) monomer. [c]Theoretical atomic composition of (N-tert-butylacrylamide) monomer. [d]Data from three separate experiments are shown as mean ± SD.

Table 3. Characterization of Thermoresponsive Polymer-Modified Silica Beads

code[a]	elemental composition (%)			immobilized initiator[c] (μmol/m²)	immobilized silane[d] (μmol/m²)	grafted polymer (mg/m²)	M_n[e]	M_n/M_w[e]	graft density (chains/nm²)	surface area[g] (m²/g)
	C[b]	H[b]	N[b]							
APTMS modified	2.58 ± 0.02	0.45 ± 0.03	1.00 ± 0.05		9.18					
ACV modified	5.84 ± 0.02	0.72 ± 0.01	1.74 ± 0.04	6.53						
IP-G	10.19 ± 0.01	1.41 ± 0.02	2.33 ± 0.03			0.328	9100	2.68	0.021[f]	84
CPTMS modified	4.82 ± 0.04	0.44 ± 0.06	0.16 ± 0.02	5.06						
IP-B	17.70 ± 0.04	2.49 ± 0.02	2.78 ± 0.08			2.83	16100	2.94	0.106	55
tB-B	8.33 ± 0.04	0.91 ± 0.03	0.85 ± 0.04			0.62	4700	1.77	0.080	
tBIP-B	15.7 ± 0.04	2.21 ± 0.03	2.32 ± 0.07			2.07	18600	1.89	0.067	69

[a]Thermoresponsive polymer-modified silica beads are named using polymer component and its polymer structure. IP and tB represent "IPAAm" and "tBAAm", respectively. G and B denote "hydrogel" and "brush", respectively. [b]Determined by elemental analysis (n = 3). [c]Estimated from carbon composition. [d]Estimated from carbon composition. [e]Determined by GPC using DMF containing 50 mmol/L LiCl. [f]Graft density for IP-G denotes hydrogel-modified point onto silica bead surfaces. [g]Measured by N² adsorption method.

deuterium oxide. Resolution between two hydrophobic steroids was calculated according to the following equation[49]

$$resolution = 2.0 \times \frac{t_{R2} - t_{R1}}{W_1 + W_2} \qquad (6)$$

where t_{R1} and t_{R2} ($t_{R1} < t_{R2}$) are the retention times of analytes, W_1 and W_2 are the peak widths of analytes at the baseline.

■ RESULTS AND DISCUSSION

Characterization of Initiator and Thermoresponsive Polymer-Modifed Silica Beads. For obtaining the information of elemental composition of the prepared silica-bead surfaces, XPS measurement was performed (Table 2). Thermoresponsive polymer-modified silica beads are named using polymer component, and the polymer structure. IP and tB represent the "IPAAm" and "tBAAm", respectively. G and B denote "hydrogel" and "brush", respectively.

With modifying ACV, carbon and nitrogen contents increased, and silicon and oxygen content decreased compared to APTMS-modified beads. Similarly, PIPAAm hydrogel-modified bead (IP-G) exhibited higher carbon and nitrogen contents compared to that of APTMS-modified bead. These results indicated that ACV and PIPAAm hydrogel were modified through the reaction and polymerization, because these modifiers contain carbon and nitrogens and prevented the detection of chemical components of the base surface. Additionally, in the case of IP-B, tB-B, and tBIP, carbon and
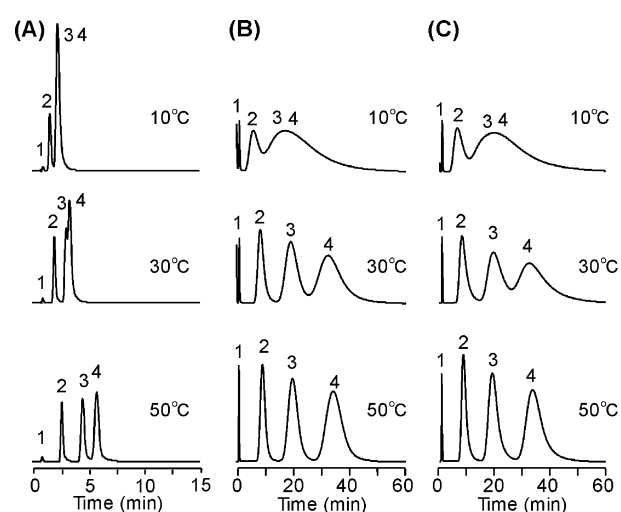

Figure 2. Chromatograms of steroids separated on HPLC of which packing materials were poly(N-isopropylacrylamide) (PIPAAm)-grafted silica beads at various temperatures: (A) PIPAAm hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(tert-butylacrylamide(tBAAm)-b-IPAAm) brush-grafted silica-bead column (tBIP-B). Mobile phase was Milli-Q water. The peak 1 represents adenosine; peak 2 , hydrocortisone; peak 3, dexamethasone; peak 4, hydrocortisone acetate.
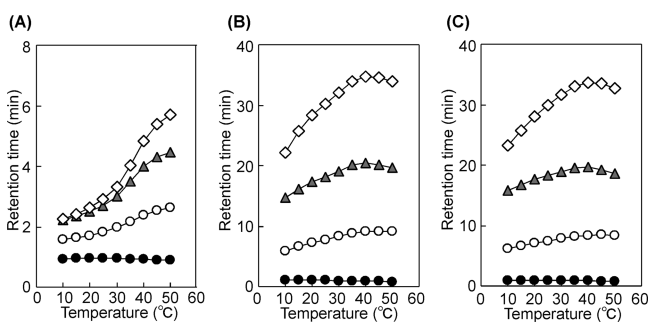
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Figure 3. Temperature-dependent retention time changes of steroids on (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(*tert*-butylacrylamide-(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydro-cortisone acetate.

nitrogen contents increased, and silicone and oxygen contents decreased after ATRP reaction. These results also indicated that ATRP procedures successfully modified PIPAAm or PtBAAm on silica bead surfaces.

For measuring the amount of initiator-immobilized and grafted polymers on the silica-bead surface, we performed CHN elemental analyses. Elemental composition of carbon, hydrogen, and nitrogen were summarized in Table 3. IP-B exhibited a larger amount of grafted PIPAAm compared to that on IP-G, although the almost same initiator density on both beads surfaces. This was attributed to the difference in the graft configuration of PIPAAm on both silica bead surfaces. Modified PIPAAm on IP-B bead surface forms a densely packed polymer-brush structure, which was suggested by the higher graft density above 0.1 chains/nm^2, because ATRP provided a remarkably high initiation efficiency compared to a conventional radical polymerization using azo-initiators.[50] On the contrary, the graft density of modified PIPAAm would be small compared to that on IP-B bead surfaces, because the initiation efficiency of this polymerization procedure was lower than that on ATRP. Actually, the estimated PIPAAm graft density on IP-G surfaces was smaller than that of IP-B surface, although the precise molecular weight and graft density of IP-G surface was unable to be observed because of the cross-linking structure of PIPAAm hydrogel. These polymerization properties led to a
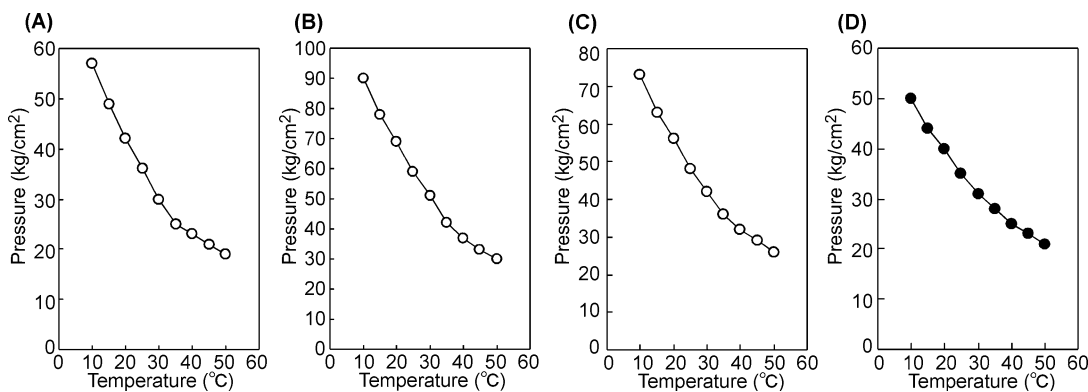

Figure 5. The van't Hoff plots of steroids on (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(*tert*-butylacrylamide(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydrocortisone acetate.

Figure 6. Temperature-dependent peak width changes of steroids on (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(*tert*-butylacrylamide-(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydro-cortisone acetate.

large difference in the amount of grafted PIPAAm on each surface.

In comparison between IP-B and tB-B, the grafted amount of PtBAAm was smaller than that of IPAAm on IP-B, because of the lower monomer concentration and a short polymerization period in tB-B preparation. The PtBAAm graft density of tB-B

Figure 4. Temperature-dependent back pressure changes on (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), (C) poly(*tert*-butylacrylamide(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B), and (D) unmodified bead packed column.
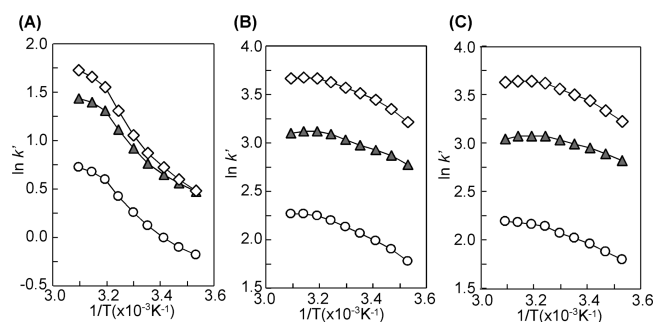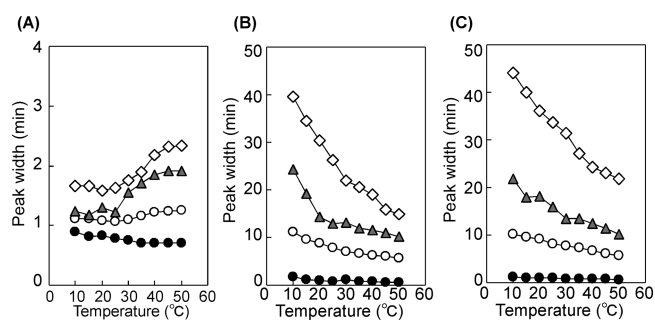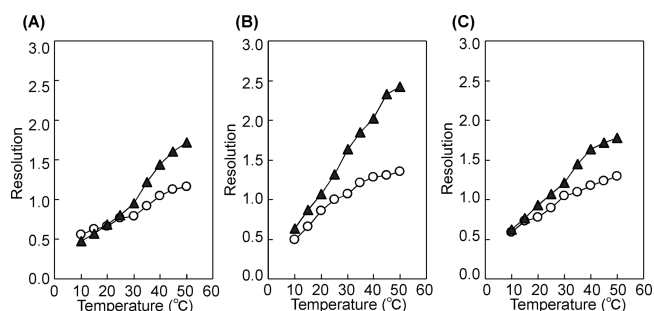
Figure 7. Temperature-dependent resolution changes of steroids on (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(*tert*-butylacrylamide-(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B). The open circles represent resolution between hydrocortisone and dexamethasone; the closed triangles, between hydrocortisone and hydrocortisone acetate.

was relatively smaller compared to that of PIPAAm on IP-B. In considering that the initiation efficiency of immobilized initiator of two surfaces, tB-B and IP-B, was almost the same, because the same initiator immobilized surface was used. However, a monomer having a larger molecular weight (size) tends to occupied larger initiator-immobilized surface area, leading to decreased graft density of the surface (chains/nm^2). In this situation, tBAAm, a larger molecular weight monomer, tended to occupied larger surface area compared to IPAAm, a relatively smaller molecular weight monomer. Thus, the graft density of PtBAAm was found to be smaller than that of PIPAAm.

Regarding the molecular weight of polymers on the prepared beads, the molecular weight of cleaved polymer from IP-G was speculated to be unable to be measured precisely, because PIPAAm hydrogel was cross-linked and became enormously large molecules. However, GPC measurement of cleaved IP-G hydrogel exhibited a relatively small molecular weight (Mn: 9100), indicating that one or two cross-linking point in the grafted PIPAAm chain on silica beads. In addition, the PDIs of
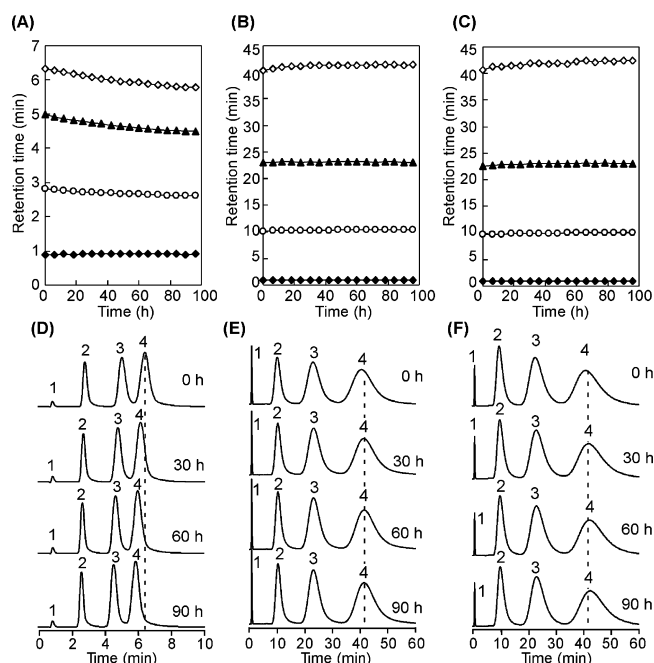

Figure 9. Temperature-dependent retention time changes of steroids using pH 7.0 phosphate buffer as a mobile phase at 40 °C on (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(*tert*-butylacrylamide-(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydro-cortisone acetate. Time course of chromatograms of steroids separated on (D) IP-G, (E) IP-B, and (F) tBIP-B columns. Peak 1 represents adenosine; peak 2, hydrocortisone; peak 3, dexamethasone; peak 4, hydrocortisone acetate.

cleaved polymers from IP-B, tB-B, and tBIP-B were relatively larger than those reported in previous studies,[34,45] although these polymers were grafted through the surface-initiated
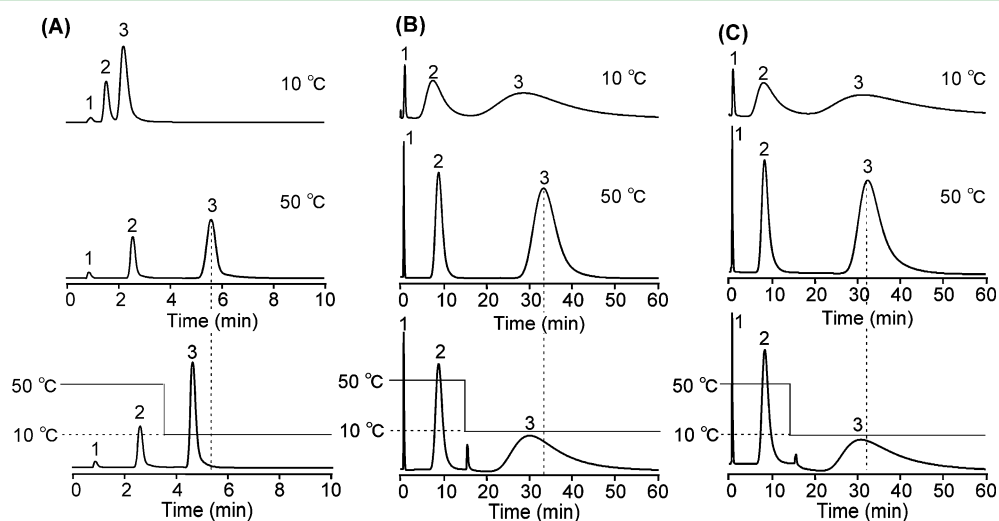

Figure 8. Step-temperature gradient on steroids elution from (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(*tert*-butylacrylamide(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B). Mobile phase is Milli-Q water. Peak 1 represents adenosine; peak 2, hydrocortisone; peak 3, hydrocortisone acetate. Chromatograms of steroids at 10 and 50 °C on upper and middle rows are shown for comparing their retention times on constant temperature and step-temperature gradient.
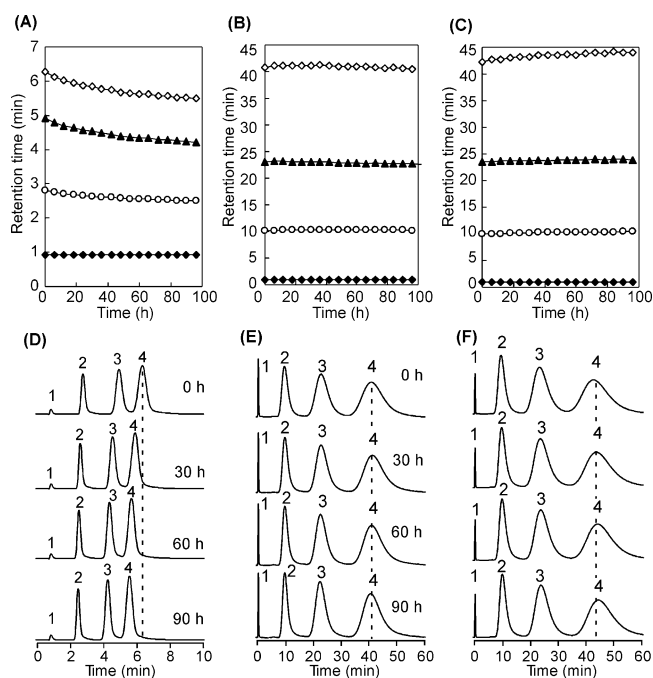
Figure 10. Temperature-dependent retention time changes of steroids using pH 8.0 phosphate buffer as a mobile phase at 40 °C on (A) poly(N-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(tert-butylacrylamide-(tBAAm)-b-IPAAm) brush-grafted silica-bead column (tBIP-B). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydrocortisone acetate. Time course of chromatograms of steroids separated on (D) IP-G, (E) IP-B, and (F) tBIP-B columns. Peak 1 represents adenosine; peak 2, hydrocortisone; peak 3, dexamethasone; peak 4, hydrocortisone acetate.

Figure 11. Temperature-dependent retention time changes of steroids using pH 9.0 glycine−sodium hydroxide buffer as a mobile phase at 40 °C on (A) poly(N-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(tert-butylacrylamide-(tBAAm)-b-IPAAm) brush-grafted silica-bead column (tBIP-B). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydrocortisone acetate. Time course of chromatograms of steroids separated on (D) IP-G, (E) IP-B, and (F) tBIP-B columns. Peak 1 represents adenosine; peak 2, hydrocortisone; peak 3, dexamethasone; peak 4, hydrocortisone acetate.

ATRP. The larger polydispersity was speculated to be attributed to the porous geometry of silica beads.[27,34] Polymerization reaction inside the pores 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. Especially, the GPC chromatograms of IP-B exhibited two overlapped peaks, which were attributed to the grafted polymers inside and outside pores, because the propagation of grafted PIPAAm inside the pore was restricted to the pore diameter. (GPC charts of cleaved polymer are shown in Figure S1 in the Supporting Information).

Surface area of the prepared beads was measured by nitrogen adsorption methods (Table 3), because the surface area affects the separation efficiency of chromatographic matrices. The surface areas were decreased compared to unmodified silica beads (100 m²/g), which was attributed to the grafted polymer infilled pores to some extent.[45]

Separation Efficiency of Prepared Thermoresponsive Stationary Phase. The elution behaviors of hydrophobic steroids from three types of thermoresponsive polymer-grafted silica beads used as chromatographic stationary phases were investigated. Figure 2A−C) shows the chromatograms of steroids at various temperatures on IP-G, IP-B, and tBIP-B columns using Milli-Q water as a mobile phase, respectively. Figure 3A−C show changes in the retention times of analytes with various temperatures on these columns. Retention times of
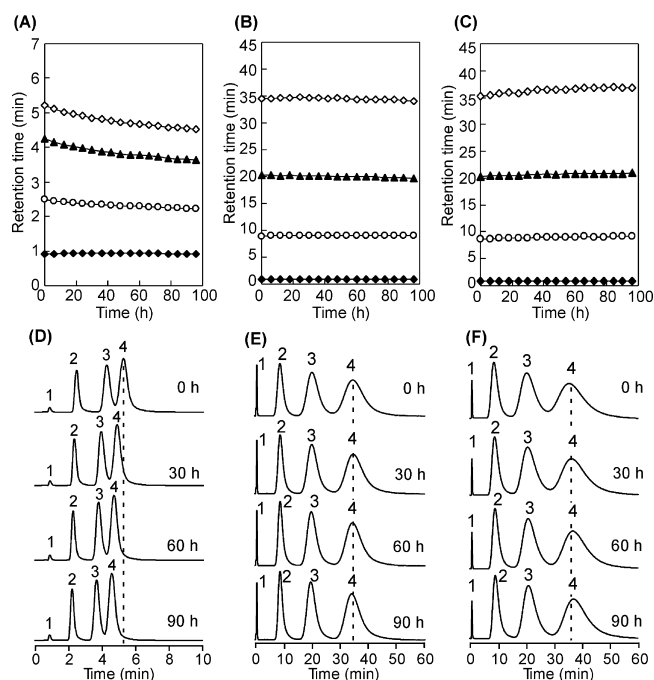
steroids on all columns were increased, and a mixture of steroids was separated with increasing column temperature, as explained by the hydrophobic drive partitioning interactions between dehydrated PIPAAm chains or PIPAAm segment of P(tBAAm-b-IPAAm) and steroids. The longer retention times was observed on IP-B and tBIP-B columns compared to those on IP-G columns, due to a larger amount of grafted polymers on silica bead surfaces. Densely packed polymers were grafted on IP-B and tBIP-B surfaces, leading to a significant strong hydrophobic interaction between grafted polymer and analyes. Additionally, IP-B and tBIP-B separated a mixture of steroids at 30 °C, whereas dexamethasone and hydrocortisone acetate were eluted as one peak on IP-G column. This was probably attributed to the intrinsic hydration property of dense PIPAAm brush. PIPAAm on IP-B and tBIP-B would tend to be dehydrated state and became hydrophobic even though at a relatively lower temperature, due to the densely packed polymer configuration. On the contrary, PIPAAm was sparsely grafted on IP-G and tend to hydrate at lower temperatures. Thus, IP-B and tBIP-B columns separated analytes, even though the column temperature was below PIPAAm's LCST. Additionally, on the retention times of IP-B and tBIP-B, peaks were observed at 40 °C (Figure 3B, C), whereas no peak was observed on IP-G (Figure 3A). This was also attributed to the different dehydration properties between dense PIPAAm brush and sparsely grafted PIPAAm hydrogel. Dense PIPAAm brush was dehydrated by increasing the temperature up to 40 °C,
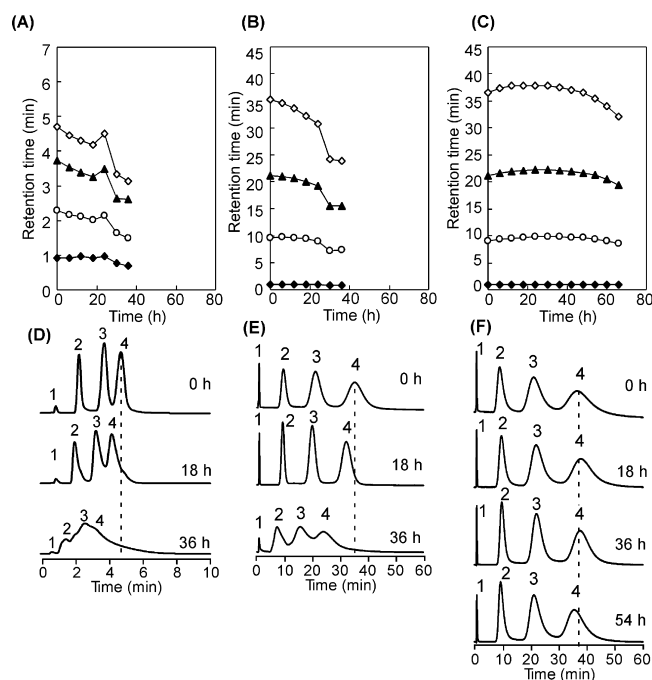
Figure 12. Temperature-dependent retention time changes of steroids using pH 10.0 sodium carbonate–sodium hydrogen carbonate buffer as a mobile phase at 40 °C on (A) poly(*N*-isopropylacrylamide) (PIPAAm) hydrogel-modified silica-bead-packed column (IP-G in Table 2), (B) PIPAAm-brush-grafted silica-bead column (IP-B), and (C) poly(*tert*-butylacrylamide(tBAAm)-*b*-IPAAm) brush-grafted silica-bead column (tBIP-B). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydrocortisone acetate. Time course of chromatograms of steroids separated on (D) IP-G, (E) IP-B, and (F) tBIP-B columns. Peak 1 represents adenosine; peak 2, hydrocortisone; peak 3, dexamethasone; peak 4, hydrocortisone acetate.

leading to an increase in the hydrophobic interactions between PIPAAm and steroids and increased retention times of steroids. The retention time decreased as column temperature increased beyond this point,[27] because PIPAAm dehydration completed at this temperature, and no hydrophobic interaction increased further with increasing temperature. By contrast, steroid solubility in the mobile phase increases with increasing temperature. Thus, the retention times of steroids decreased above 40 °C. On the contrary, the retention times on sparsely grafted PIPAAm hydrogel increased with increasing temperature beyond the phase transition temperature of PIPAAm (32 °C), because its dehydration failed to complete at this point, and the hydrophobicity of PIPAAm increased beyond the temperature.[27,37]

Figure 4 shows the back pressure of these columns at various temperatures by flowing Milli-Q water at a flow rate of 1.0 mL/min. The back pressure of the prepared beads packed columns at various temperatures provides information regarding the grafted PIPAAm's swelling and shrinking behavior. Larger decrease in the back pressure with increasing column temperature was observed on the prepared beads packed column than that of the control unmodified beads packed column, because the grafted PIPAAm shrunk with the dehydration of PIPAAm, leading to increasing flow path inside the column. Particularly, an inflection point was observed in the back pressure of IP-G column near polymer's LCST, whereas there was no inflection point was observed compared to IP-B or

tBIP-B columns. The different back-pressure-changing behavior was speculated to be attributed to a difference in the graft configuration between sparsely grafted PIPAAm hydrogel and densely packed PIPAAm brush. Previous reports indicate that densely packed PIPAAm brush, prepared by surface-initiated ATRP, dehydrates and decreases its thickness broad temperature range,[29,30] whereas sparsely grafted PIPAAm exhibited a remarkable phase-transition around its LCST. Similarly, in the case of PIPAAm grafted silica bead surfaces, relative sparsely grafted PIPAAm hydrogel was speculated to be shrunk at near the LCST, whereas PIPAAm brush gradually shrank at a broad range of temperature.

To investigate the detailed elution profiles of analytes from these bead-packed columns, we observed the van't Hoff plots (Figure 5). Change in the retention factor of IP-G column was relatively large compared to those of IP-B and tBIP-B columns. This was due to the hydration properties of the sparsely grafted PIPAAm of IP-G column. Also, change in the retention time of IP-B was relatively large than that of tBIP-B, because longer PIPAAm was grafted on IP-B surface compared to PIPAAm segment on tBIP-B surface, leading to a large change in hydrophobicity by changing temperature.

Peak width of the prepared bead column was observed (Figure 6). IP-B and tBIP-B exhibited relatively large peak widths compared to that of IP-G column because of the penetrations of analytes into the polymer brush layer and the interactions with relatively dehydrated polymers. Resolution of analyte on the bead-packed column was calculated from these obtained data. Figure 7 shows the temperature-dependent resolution change of steroids. IP-B column exhibited the highest resolution among these columns, attributed to the strong hydrophobic interaction between the densely grafted PIPAAm and analytes. Resolution of tBIP-B columns was relatively small compared to that on IP-B columns, which was attributed to the tailings of peaks on chromatogram (shown in Figure 2C). The graft density of PIPAAm segment in P(tBAAm-*b*-IPAAm) brush on tBIP-B was speculated to be decreased compared to dense PIPAAm brush, because the trace amount of halogen group was deactivated, and the block copolymerization of PIPAAm was hardly initiated on the site, which was suggested in the decrease in the graft density after the second polymerization in Table 3. This slightly decreased density of PIPAAm segment in tBIP-B led to the uneven elutions and peak tailing of steroids in chromatogram.

In addition, the effect of step-temperature gradient on these prepared beads packed columns was investigated (Figure 8). First, adenosine and hydrocortisone, relatively hydrophilic steroids, were separated with relatively strong hydrophobic interactions at 50 °C. After the column temperature was reduced to 10 °C, the retention time of hydrocortisone acetate was found to be shortened. On all columns with various PIPAAm graft configuration, the retention time of hydrocortisone acetate was promptly shorten by decreasing column temperature, suggesting that all PIPAAm graft configurations were promptly hydrated and become hydrophilic after the column temperature was decreased from 50 to 10 °C.

Stability of Thermoresponsive Stationary Phase Under Vaious pHs Mobile Phase. Stability of the prepared thermoresponsive chromatography matrices was investigated by observing the time course of retention times of analytes using various pH-mobile phases. Figure 9 shows the time course of retention time of analytes on the prepared beads packed column using pH 7.0 phosphate buffer. Retention times of

analytes on IP-G column slightly decreased with increasing the time, while IP-B and tBIP-B columns maintained the same retention times. This was attributed to the cleavage of grafted polymer on silica bead surface by the hydrolysis of silica bead surface. As shown in Table 3, PIPAAm graft density of IP-G was small compared to those of IP-B and tBIP-B, and XPS measurement indicated that IP-G surface was exposed from its base surface compared to IP-B and tBIP-B surfaces (Table 2). Thus, on IP-G surfaces, the accessibility of mobile phase to the silica bead surface was relatively high, leading to the hydrolysis of base silica and cleaved grafted PIPAAm with continuous use. On the contrary, IP-B and tBIP-B surfaces were covered with dense polymer brushs, preventing the access of mobile phase to base silica materials. Thus, a dense polymer brush structure prevented the hydrolysis of base silica and the cleavage of grafted polymers, leading to the maintenance of the retention of analyte.

Figures 10 and 11 show the time course of retention of analyte on the prepared bead surfaces with continuous flowing pH 8 and pH 9 mobile phases, respectively. Also, IP-G exhibited a slightly decrease in the retention time of analytes with continuous flowing pH 8 and pH 9 mobile phases. On the contrary, IP-B and tBIP-B maintained the retention time of analytes. This was also attributed to the hydrolysis and the cleavage of grafted PIPAAm on IP-G and the prevention of hydrolysis by dense polymer brush on IP-B and tBIP-B.

Figure 12 shows the time course of retention time on the prepared beads packed column using pH 10.0 mobile phase, where (1) the retention times of analytes on IP-G and IP-B decreased and (2) the peak shapes on the chromatograms were lost with the continuous flow of mobile phase even for 36 h. Finally, the mobile phase was unable to be flow into the column at approximately 40 h, because the possible dissolved silica beads gave the clogging of the packed column. SEM observation also indicated that the porosity of silica beads slightly increased after the flowing of an alkaline mobile phase (see the Supporting Information, Figure S2). Additionally, XPS measurement revealed that carbon and nitrogen contents decreased after the flowing of the mobile phase, attributed by the cleavage of grafted polymer due to the hydrolysis of silica surface. However, on the tBIP-B columns, the retention time of analytes and the peak shape of chromatogram were relatively maintained even after the mobile phase was flowed into the column for 54 h. This was attributed to the hydrophobic basal tBAAm segment of copolymer brush layer on tBIP-B column. Hydrophobic tBAAm segment provided a hydrophobicity to the graft interface of silica bead surfaces, leading to the prevention of hydrolysis of silica base materials under a relatively strong alkaline condition. Thus, tBIP-B exhibited a high stability for its continuous use and an alkaline mobile phase.

These results demonstrated that (1) a thermoresponsive polymer graft architecture on silica bead surfaces, was able to be modulated by a polymerization procedure, and the architecture influenced the separation efficiency of analytes, (2) the high graft density of thermoresponsive polymer and the hydrophobic segment provided a high stability against the hydrolysis and cleavage of polymer, leading to the maintenance of the separation performance. Thus, the precisely modulating graft configuration of thermoresponsive polymers was essential for preparing thermoresponsive chromatography matrices with a high separation efficiency and stability.

CONCLUSIONS

Thermoresponsive chromatography matrices with three kinds of thermoresponsive-polymer graft architectures were prepared by radical polymerization and surface-initiated ATRP. Grafted polymer amount and polymer graft density on silica surface prepared by a surface-initiated ATRP were significantly higher than those of silica surfaces prepared by a conventional radical polymerization. Dense PIPAAm homopolymer brush on silica bead surfaces exhibited the highest separation efficiency in the separation of hydrophobic steroids, because of the strong hydrophobic interaction between the well-defined polymer brush and analyets. Stabilities of prepared chromatography matrices upon continuous use were also investigated by using various pH-mobile phases. As the result, prepared beads with a dense polymer brush structure maintained a stable separation efficiency, while beads with sparsely grafted polymers gradually decreased their separation capacity. Especially, using pH 10 mobile phase, dense copolymer brush with a hydrophobic basal layer exhibited a high stability against a relatively high alkaline condition, probably attributed to the provided strong hydrophobicity to graft interfaces of silica beads. Thus, the precisely modulating graft configuration of thermoresponsive polymers on interfaces provided chromatography matrices with a high separation efficiency and stability for continuous use, resulting in elongating the longevity of chromatographic column.

ASSOCIATED CONTENT

S Supporting Information

Protocols for preparation of various pH-mobile phases, GPC chart of the grafted polymer on silica surfaces for obtaining the molecular weight, SEM photograph, and XPS measurement of used beads from the prepared bead-packed columns. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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