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Thermally Modulated Cationic Copolymer Brush on Monolithic Silica Rods for High-Speed Separation of Acidic Biomolecules

Kenichi Nagase,[†] Jun Kobayashi,[†] Akihiko Kikuchi,[‡] Yoshikatsu Akiyama,[†] Hideko Kanazawa,[§] and Teruo Okano*[,†]

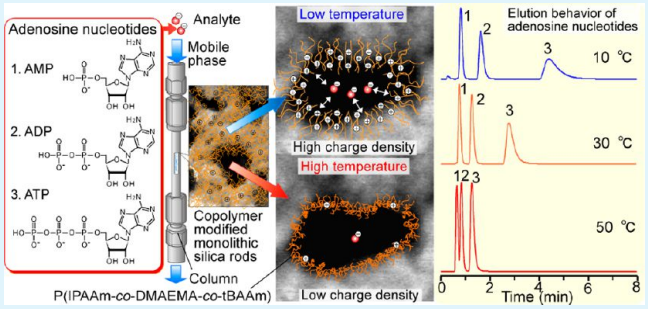
[†]Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, TWIns, 8-1 Kawadacho, Shinjuku, Tokyo 162-8666, Japan

[‡]Department of Materials Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan

[§]Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato, Tokyo 105-8512, Japan

Supporting Information

ABSTRACT: Poly(*N*-isopropylacrylamide (IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide (tBAAm)), a thermoresponsive-cationic-copolymer, brush-grafted monolithic-silica column was prepared through surface-initiated atom transfer radical polymerization (ATRP) for effective thermoresponsive anion-exchange chromatography matrices. ATRP-initiator was grafted on monolithic silica-rod surfaces by flowing a toluene solution containing ATRP initiator into monolithic silica-rod columns. IPAAm, DMAEMA, and tBAAm monomers and CuCl/$CuCl_2$/Me_6TREN, an ATRP catalytic system, were dissolved in 2-propanol, and the reaction solution was pumped into the preprepared initiator modified columns at 25 °C for 16 h. The constructed copolymer-brush structure on monolithic silica-rod surface was confirmed by X-ray photoelectron spectroscopy (XPS), elemental analysis, scanning electron microscopy (SEM) observation, and gel permeation chromatography (GPC) measurement of grafted copolymer. The prepared monolithic silica-rod columns were also characterized by chromatographic analysis. The cationic copolymer brush modified monolithic silica-rod columns were able to separate adenosine nucleotides with a shorter analysis time (4 min) than thermoresponsive copolymer brush-modified silica-bead-packed columns, because of the reduced diffusion path length of monolithic supporting materials. These results indicated that thermoresponsive cationic copolymer brush grafted monolithic silica-rod column prepared by ATRP was a promising tool for analyzing acidic-bioactive compounds with a remarkably short analysis time.



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

INTRODUCTION

Intelligent biointerfaces have been increasing their importance because progress in biomedical technology strongly demands these biointerfaces. Several types of biointerfaces have been developed by modifying functional groups or polymers to substrates for applying these biointerfaces to biosensors, membrane devices, or implantable devices.[1−7] These biointerfaces have been used for avoiding a specific biological reaction such as proteins and cells adhesion to the surfaces. On the contrary, poly(*N*-isopropylacrylamide)(PIPAAm) modified surface has been used as thermoresponsive biointerfaces that can control the interactions among proteins, cells, and the surfaces. PIPAAm exhibits a reversible temperature-dependent phase transition in aqueous solutions at its lower critical solution temperature (LCST) of 32 °C, near the body temperature.[8] Thus, PIPAAm modified materials exhibits a temperature dependent hydrophilic/hydrophobic alteration,[9] and its properties were used in various biomedical applications.[5,10−13] Especially, one of the most successful applications of the thermoresponsive interfaces is thermoresponsive cell culture dishes.[14,15] The interaction between grafted PIPAAm on the culture dishes and cells can be modulated by external temperature change, and confluently cultured cells can be successfully harvested as a contiguous "cell sheet" having a tissue-like cellular architecture with simply reducing temperature. Fabricated cell sheets have been used for the various types of tissue engineering and regenerative medicines.[16−18] Especially, in several types of tissues, clinical trials using cell sheets have already started.[19−21]

Furthermore, our research group has investigated thermoresponsive chromatography using a PIPAAm modified stationary phase as a new type of analytical tool. Modified

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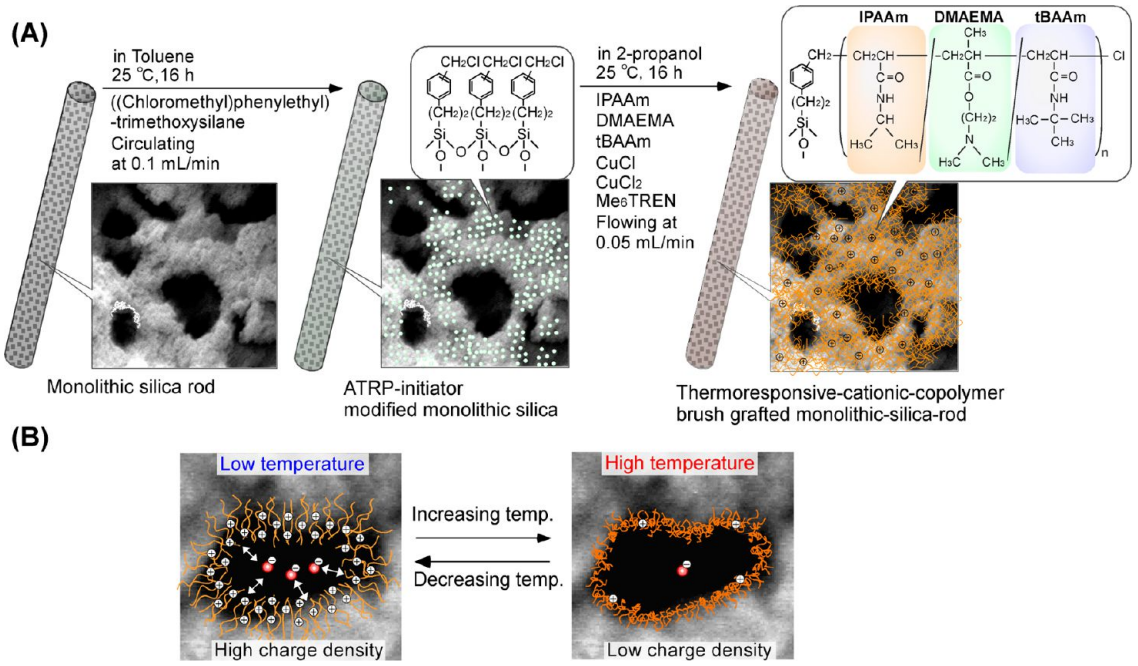

Figure 1. (A) Scheme of the preparation of poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate (DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) brush-grafted monolithic silica-rod surfaces using surface-initiated atom transfer radical polymerization (ATRP). (B) Schematic illustration of the thermally modulated interaction between cationic copolymer and acidic biomolecules.

PIPAAm on stationary phase alters its hydrophobicity by changing temperature, leading to the modulation of the hydrophobic interaction between PIPAAm and analytes.[22] 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 performance of PIPAAm modified beads, several types of PIPAAm modifying methods have been investigated.[22,23] As a result, dense PIPAAm brush grafted matrices prepared by surface initiated atom transfer radical polymerization (ATRP) exhibited a high separation efficiency due to a strong interaction with analytes,[24] since the polymerization procedure forms a densely packed polymer, called a polymer brush, on the surfaces.[24−27] Additionally, thermoresponsive ion-exchange chromatography has been developed for separating ionic biomolecules. This chromatography system can modulate the electrostatic interaction between the grafted ionic-copolymer and analytes with changing temperature, because the ionic properties (acidity or basicity) of grafted polymers change by changing temperature.[28,29] In this chromatography system, thermoresponsive ionic polymer brush grafted matrices, prepared by surface-initiated ATRP, also exhibited a high separation efficiency because of the strong electrostatic interaction between dense ionic polymer brush and analytes.[30,31] Using these dense thermoresponsive ionic polymer brush grafted beads, several pharmaceutical proteins have been successfully purified only by changing column temperatures.[32,33] However, for separating low-molecular-weight compounds with a strong acidity, such as adenosine nucleotides, relatively longer retention time is required because of the excessive electrostatic interaction between grafted copolymer and analytes.[30]

On the other hand, in the fields of analytical science, monolithic silica-rod columns have attracted attention as a new chromatographic support, an alternative to silica-bead-packed column.[34−39] Monolithic silica-rod column is a single piece of silica rod having a three-dimensionally interconnected skeleton structure, which provides through-pores to the column. The three-dimensional (3D) skeleton structure and large through-pores reduce the diffusion path length and flow resistance, leading to a higher column efficiency and column permeability than those of conventional particle-packed columns.[34] Actually, the present authors' group has succeeded in preparing PIPAAm grafted monolithic-silica-rod column that can separate hydrophobic steroids with a high resolution and a remarkably short analysis time.[40] The result suggests that monolithic-silica-rod would be an alternative to silica beads as base materials of temperature responsive chromatography matrices. Thus, a thermoresponsive ionic copolymer brush modified monolithic silica-rod column is thought to be a good candidate as an effective thermoresponsive ion-exchange chromatography stationary phase that can separate ionic low molecular bioactive compounds with short analysis time.

In this study, we described the preparation of thermoresponsive cationic copolymer brush comprising poly(IPAAm-*co*-2-(dimethylamino)ethylmethacrylate-*co*-*N*-*tert*-butylacrylamide), on monolithic silica-rod surface using surface-initiated ATRP. 2-(dimethylamino)ethylmethacrylate (DMAEMA) was used as a cationic-comonomer, because the pK_a of poly-DMAEMA is 7.4,[41] and the basicity of the copolymer would tend to decrease by temperature alteration at neutral pH.[30] Also, for modulating the hydrophobicity of copolymer, *N*-*tert*-butylacrylamide (tBAAm) was used as hydrophobic comonomer, which is known to suppress the possible uncontrollable progress of polymerization[31,33] compared with other hydrophobic comonomer, such as *n*-butyl methacrylate (BMA).[32,42] Characterizations of the thermoresponsive cationic copolymer and the copolymer grafted monolithic silica rod were performed. Separation efficiency of the prepared column was investigated by observing the temperature-responsive elution profiles of adenosine nucleotides.

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EXPERIMENTAL SECTION

Materials. *N*-isopropylacrylamide (IPAAm) was kindly provided by Kohjin (Tokyo, Japan) and recrystallized from *n*-hexane. *N*,*N*-dimethylaminoethylmethacrylate (DMAEMA), purchased from Wako Pure Chemicals (Osaka), was purified by distillation at 46 °C and 3 mmHg. *N*-*tert*-Butylacrylamide (tBAAm) was obtained from Wako Pure Chemicals and recrystallized from acetone. CuCl and $CuCl_2$ were purchased from Wako Pure Chemicals. Tris(2-aminoethyl)amine (TREN) was purchased from Acros Organics (Pittsburgh, PA, USA). Formaldehyde, formic acid, and sodium hydroxide were purchased from Wako Pure Chemicals. Tris(2-(*N*,*N*-dimethylamino)-ethyl)amine (Me_6TREN) was synthesized from TREN, according to the previous reports.[43] Monolithic silica column (MonoBis column, 50 × 3.2 mm i.d., the meso pore diameter: 30 nm, surface are: 71.8 m^2/g) was purchased from Kyoto Monotech (Kyoto). 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 Pure Chemicals. ((Chloromethyl)phenylethyl)-trimethoxysilane (mixed *m*, *p* isomers) as an ATRP initiator was obtained from Gelest (Morrisville, PA). 2-Propanol (HPLC grade), dichloromethane, and toluene (dehydrate) were purchased from Wako Pure Chemicals. Adenosine nucleotides and steroids 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.

Surface Modification of Monolithic Silica Column with Silane Agents. Formation of silane layer of ATRP initiator on monolithic silica-rod surfaces was performed as shown in the first step in Figure 1A. Monolithic silica-rod columns were placed into a humidified container for 18 h at approximately 75% relative humidity. ATRP initiator solution was prepared by adding 6.0 mL of ((chloromethyl)phenylethyl)trimethoxysilane in 14 mL of dried toluene, followed by circulating the solution into the column using an HPLC pump (PU-980) (JASCO) with a flow rate of 0.1 mL/min for 16 h. The reaction proceeded at room temperature for overnight with continuously circulating the solution. The ATRP initiator modified column was extensively rinsed with flowing toluene and acetone, and dried in a vacuum oven at 110 °C. ATRP initiator modified silica beads was also prepared according to the previous report.[44,45] Briefly, silica beads (21.0 g) was humidified at 60% relative humidity for 4 h, followed by adding toluene solution of ATRP initiator (53.4 mmol/L) into silica beads in a round-bottom flask and stirring for 16 h. The ATRP initiator immobilized silica beads was rinsed with toluene and acetone, and dried in a vacuum oven at 110 °C.

Copolymer Modification of Monolithic Silica by ATRP. P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brushes were prepared on the ATRP-initiator immobilized monolithic silica-rod column by ATRP as shown in the second step in Figure 1A. First, IPAAm (11.7 g, 103 mmol), DMAEMA (2.03 g, 12.9 mmol) and tBAAm (1.64 g, 12.9 mmol) was dissolved in 85.6 mL of 2-propanol, and the solution was deoxygenated by argon gas bubbling for 60 min. CuCl (168 mg, 1.70 mmol), $CuCl_2$ (23.0 mg, 0.171 mmol), and Me_6TREN (0.44 g, 1.91 mmol) were added into the solution under argon atmosphere, and the solution was stirred for 20 min for allowing a CuCl/$CuCl_2$/Me_6TREN catalyst system to appear. Both monomer solution and the initiator modified monolithic silica-rod column were placed into a glovebox purged with argon gas by repeated evacuation and argon flushes three times. The reaction solution was pumped into the column by the HPLC pump with a flow rate of 0.05 mL/min for 16 h. The PIPAAm grafted column was extensively rinsed with flowing acetone, methanol, EDTA aqueous solution, and water, and dried in a vacuum oven at 50 °C. The copolymer-brush grafted silica beads were also prepared, according to the previous reports.[30,31] Briefly, IPAAm, DMAEMA and tBAAm (feed composition: 8:1:1) were dissolved in 2-propanol (total monomer concentration: 1.0 mol/L). The monomer solution was reacted with the silica beads using CuCl/$CuCl_2$/Me_6TREN as catalyst was reacted with continuous shaking on a desktop shaker (SN-M40S) (NISSIN, Tokyo) for 4 or 16 h at 25 °C. Then, the copolymer brush-grafted silica beads were rinsed with acetone, methanol, EDTA aqueous solution, and water, and dried a vacuum oven at 50 °C.

Characterization of Initiator Immobilized Silica and Grafted Copolymer. To determine the amounts of initiator and copolymer, monolithic silica-rod and silica beads were analyzed using a PE 2400 series II CHNS/O analyzer (PerkinElmer, Waltham, MA). Amount of modified ATRP initiator on silica supports (g/m^2) was calculated using the following equation:

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

where $\%C_I$ is the percent carbon as determined by elemental analysis, $\%C_I$(calcd) is the calculated weight percent of carbon in initiator, and S is the specific surface area of the monolithic silica-rod (the data measured by nitrogen adsorption: 71.8 m^2/g) and silica beads in square meters per gram (the manufacture's data: 100 m^2/g). Amount of grafted copolymer on silica bead surfaces (g/m^2) was calculated using the following equation

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

where $\%C_p$ is the percent carbon increase from that of the modified initiator layers as determined by elemental analysis, and $\%C_p$(calcd) is the calculated weight percent of carbon in the copolymer monomer.

Grafted copolymer on the monolithic silica-rod surfaces was also retrieved and analyzed by gel permeation chromatography (GPC) for determining both the molecular weight and polydispersity index (PDI). Copolymer grafted monolithic silica-rod surfaces were treated with concentrated sodium hydroxide solution for overnight, and the solution was neutralized by the addition of hydrochloric acid.[46] The solution was filtered and dialyzed against Milli-Q water using a dialysis membrane [Spectra/Por standard regenerated cellulose dialysis membrane, molecular weight cut off (MWCO): 1000] (Spectrum Laboratories, Rancho Dominguez, CA) for 1 week with daily water changed, and copolymer was recovered by freeze-drying. Number-average molecular weights and PDI values of the polymer were determined using a GPC system (the columns: TSKgel Super-AW2500, TSKgel SuperAW3000, and TSKgel SuperAW4000) (Tosoh, Tokyo) controlled with GPC-8020 model II ver. 5.0 (Tosoh). A calibration curve was obtained using PEG standards. The flow rate was 1.0 mL/min. The mobile phase was *N*,*N*-dimethylformamide (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 a refractometer. Graft density of copolymer on the monolithic silica and silica bead 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 monolithic silica-rod and 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 copolymer.

For obtaining surface elemental composition, X-ray photoelectron spectroscopy (XPS) measurement was performed for ATRP-initiator modified and copolymer-brush grafted silica-rod and 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 and a takeoff angle of 90°. Wide scans were recorded to analyze all existing elements on the surfaces, and high-resolution narrow scan analysis was performed for the peak deconvolution of carbon C1s

Table 1. Characterization of P(IPAAm-*co*-DMAEMA-*co*-tBAAm) Cationic Copolymer

code[a]	IPAAm/DMAEMA/tBAAm (molar ratio) in feed	IPAAm/DMAEMA/tBAAm (molar ratio) in copolymer[b]	Mn	M_w/M_n[c]	LCST[d] in 66.7 mmol/L PB	pK_a[e]
IDtB-21.6	80.0/10.0/10.0	66.5/21.6/12.0	7100	1.31	41.4	7.94
ID-21.6	90.0/10.0/0	78.4/21.6/0	9000	1.25	55.2	8.40
ItB	90.0/0/10.0	88.0/0/12.0	7700	1.21	25.2	
IP	100/0/0		7700	1.21	31.9	

[a]Abbreviated as IDB-x where x represents the mole fraction of DMAEMA in the copolymer. "I", "D", and "tB" represents IPAAm, DMAEMA, and tBAAm, respectively. IP represents PIPAAm homopolymer. [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 phosphate buffer solution (pH 7.0). [e]Measured by acid−base titration at 4 °C.

signals. All binding energies were referenced to a C1s hydrocarbon peak at 285.0 eV.

Surface morphology of osmium tetroxide (OsO_4)-stained ATRP initiator-immobilized and copolymer-brush-grafted monolithic silica-rod and silica beads were observed using a scanning electron microscopy (SEM) S-4300 (Hitachi, Tokyo).

Synthesis of Cationic Copolymer by ATRP. For characterizing the thermoresponsive cationic copolymer, P(IPAAm-*co*-DMAEMA-*co*-tBAAm) were synthesized by solution-phase ATRP in the similar conditions as the copolymer grafting onto monolithic-silica-rod and silica beads surfaces,[30] because the amount of retrieved copolymer from silica surface with concentrated sodium hydroxide was too small (below 10 mg) to be used performing all characterization. Copolymerization was performed by the same protocol as grafting copolymer onto silica beads except that α-chloro-*p*-xylene (53.4 mg, 380 μmol) was added as an initiator in the reaction solution instead of silica beads.[30] 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.

Characterization of Cationic Copolymer. Prepared copolymer, P(IPAAm-*co*-DMAEMA-*co*-tBAAm), was analyzed by the GPC system to determine both the molecular weight and PDI. Phase transition of the thermoresponsive cationic copolymer solutions in phosphate buffer solution was observed by optical transmittance changes. Solutions of P(IPAAm-*co*-DMAEMA-*co*-tBAAm) were prepared using 66.7 mmol/L phosphate buffer solution at pH 7.0 (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 thermostatted with a Peltier-effect cell holder (EHC-477) (JASCO) with a heating rate of 0.20 °C/min. LCST was defined as the temperature at 90% transmittance of solution. DMAEMA and tBAAm content in the copolymers was determined by ^{1}H NMR (^{UNITY}INOVA 400 MHz spectrometer) (Varian, CA) using CD_3OD containing 0.05v/v% tetramethylsilane as a solvent. Mole fraction of DMAEMA was calculated from the peak areal ratio of the singlet methylene protons adjacent to oxygen in DMAEMA at 4.4 ppm to the singlet of the resonance of the methine proton of isopropyl groups in IPAAm units at 4.0 ppm. Mole fraction of tBAAm in the copolymer was also calculated from the peak areal ratio of the methyl protons of *tert*-butyl side chains at 1.3 ppm to the same peak of isopropyl groups in IPAAm units at 4.0 ppm. Apparent dissociation constants pK'_a of the copolymers in a 66.7 mmol/L KCl solution were determined by titration using the following Henderson−Hasselbalch equation.[47]

$$pK'_a = pH - \log \frac{\alpha}{1-\alpha} \quad (4)$$

where α is the degree of protonation of dimethyl amino group. Experimental detail of pK'_a measurement procedure was as the following procedures.[28,29] Copolymer (100 mg) was dissolved in 20 mL of distilled water containing 66.7 mmol/L KCl. A half of amino groups in copolymer were protonated stoichiometrically by adding NaOH aq or HCl aq, and α became 0.5. According to eq 4, the relationship between pH and pK'_a at an α-value of 0.5 represents

$$pK'_a = pH \quad (5)$$

Therefore, pH values of the copolymer solution showing pK'_a value was measured by a pH-meter with vigorous stirring and at various temperatures.

Temperature-Modulated Elution of Adenosine Nucleotides. P(IPAAm-*co*-DMAEMA-*co*-tBAAm) grafted silica beads were packed into a stainless steel column (4.6 mm i.d. × 50 mm), according to the previous report.[24] The surface area of silica-bead-packed column is speculated to be relatively larger than that of monolithic silica rod column. Although two different kinds of columns having the same surface area are suitable for comparison, unfortunately these columns are unable to be obtained. Therefore, column performances were evaluated using two columns having the same column length and similar diameter. P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted monolithic silica-rod column or bead-packed column was connected to an HPLC system (PU-980 and UV-970) (JASCO) controlled by a personal computer with Borwin analysis software version 1.21 (JASCO). AMP, ADP, and ATP, which are adenosine nucleotides, were used as a marker for obtaining chromatograms at concentrations of 0.201 mg/mL, 0.283 mg/mL, and 0.588 mg/mL, respectively. Also, hydrocortisone and dexamethasone, which are hydrophobic steroids, were dissolved in ethanol and used for investigating the hydrophobic properties of the grafted cationic copolymer brush at concentrations of 0.385 mg/mL and 0.467 mg/mL, respectively. Properties of these analytes were shown in Table S1 in the Supporting Information.[48] Phosphate buffer solution (pH 7.0, 66.7 mmol/L) was used as a mobile phase, because the same mobile phase was used in the previous reports, and the elution behavior was able to be compared to those in the reports.[30,33] Thermoresponsive elution behaviors of adenosine nucleotides and 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 elution profiles using a step-temperature gradient, a thermostatted water bath (RE206, Lauda, Lauda-Konigshofen) and a constant temperature water circulator (CTA400) were used, and their temperatures were set at 50 and 10 °C, respectively. First, AMP and ADP, relatively weak acidic compounds, were eluted at 10 °C. Then, column temperature was raised by immersing the columns into 50 °C-thermostatted water bath, and the elution behavior of ATP at 50 °C was observed. Since the outer casing of column was a stainless steel with small size diameter, the temperature of column was speculated to be promptly equilibrate with that of water bath.

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

$$\text{retention factor} = \frac{t_R - t_0}{t_0} \quad (6)$$

where t_R is the retention time of known sample at a specific temperatures, and t_0 is the retention time of deuterium oxide as an initial standard,[49] because there is no interaction between deuterium oxide and grafted copolymer.

RESULTS AND DISCUSSION

Characterization of the Thermoresponsive Cationic Copolymer. For investigating the temperature-dependent ionic and hydrophobic properties of P(IPAAm-*co*-DMAEMA-*co*-tBAAm), the characterization of thermoresponsive cationic copolymer was performed and summarized in Table 1. Prepared copolymers are abbreviated as IDtB-x or ID-x where "I", "D", and "tB" represents IPAAm, DMAEMA, and tBAAm, respectively, and "x" in IDtB-x or ID-x represents the mole fraction of DMAEMA in the copolymer. Additionally, "IP" represents PIPAAm homopolymer. Mole fraction of DMAEMA were approximately two times higher than those of their feed compositions because of the higher reactivity ratio of methacrylate monomer than that of acrylamide monomer in ATRP procedure using the $CuCl/CuCl_2/Me_6TREN$ catalyst system with 2-propanol as the solvent.[30,42] Polydispersity of prepared copolymers ranged from 1.2 to 1.3, indicating that the polymerization was relatively controlled compared to a conventional radical polymerization.[50] Furthermore, the polydispersity of IDtB-21.6 and ID-21.6, cationic copolymers including DMAEMA, were relatively larger than that of IP, PIPAAm homopolymer, and ItB, P(IPAAm-*co*-tBAAm) copolymer, because the ATRP catalytic system $CuCl/CuCl_2/Me_6TREN$ was ordinary used for acrylamide derivatives, such as IPAAm, and tBAAm, and except for DMAEMA.

LCST values for the cationic copolymers in phosphate buffer (PB; pH 7.0, 66.7 mmol/L) exhibited higher values than those of IP and ItB (Figure 2), because the incorporation of ionic

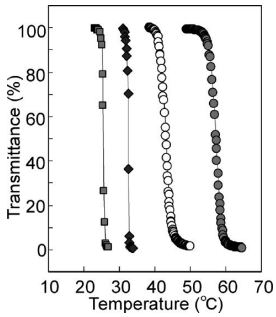

Figure 2. Phase transition profiles of poly(*N*-isopropylacrylamide-(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate (DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) in phosphate buffer (PB) (pH 7.0). The closed diamond represents PIPAAm homopolymer (IP in Table 1); the closed square, P(IPAAm-*co*-tBAAm) (ItB); the open circles, P(IPAAm-*co*-DMAEMA-*co*-tBAAm) (IDtB-21.6); the closed circle, P(IPAAm-*co*-DMAEMA) (ID-21.6) in 66.7 mmol/L PB.

monomer increased LCST with increasing the hydrophilicity of the random copolymer.[51] Additionally, the temperature range of the transmittance changes became slightly wider with the incorporation of DMAEMA because of the disruption of the hydrophobic aggregation of PIPAAm sequences by the incorporation of ionic DMAEMA units. However, the observed phase transition temperature of the thermoresponsive cationic copolymer, P(IPAAm-*co*-DMAEMA-*co*-tBAAm), was found to be approximately 40 °C and suitable for temperature-responsive chromatography, because adenosine nucleotides tend to hydrolysis at higher temperatures. Also, pharmacological peptides and proteins, candidate analytes of temperature responsive chromatography, are presumed to tend to lose their activity at higher temperature region. On the contrary, P(IPAAm-*co*-DMAEMA) was unsuitable for temperature-responsive chromatography because of its excessively high LCST.

Temperature-dependent apparent dissociation constant (p*K*′a) of the thermoresponsive copolymer was observed (Figure 3). Although p*K*′a values slightly decreased below the

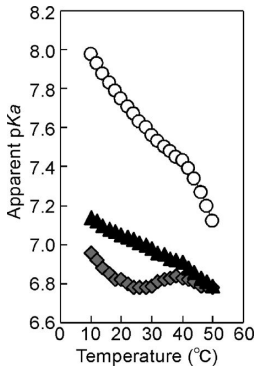

Figure 3. Temperature-induced pK_a shifts for amino side groups in poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)-ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide (tBAAm)) in 66.7 mmol/L KCl solution. The open circle represents P(IPAAm-*co*-DMAEMA-*co*-tBAAm) (IDtB-21.6) (IDtB-21.6 in Table 1); the closed triangle, PIPAAm (IP); the closed diamond, P(IPAAm-co-tBAAm) (ItB).

LCSTs, a drastic change in the copolymer p*K*′a was observed above LCST because of the increase of hydrophobicity of the copolymer with increasing temperatures. Incorporation of hydrophobic comonomers into polyelectrolytes is reported to result in the decreased acidity or the basicity of weak acidic and basic moieties in the polyelectrolytes.[52,53] Above the LCST of copolymers, their hydrophobicity dramatically increased due to their dehydration, leading to increasing the local hydrophobicity in the vicinity of dimethylamino groups in DMAEMA. Additionally, the intra- and interaggregations of compact globules of dehydrated copolymer chains reduced the interface between the copolymer molecules and the solution environment. Thus, the protonation of dimethylamino group in the copolymer was modulated by changing temperature.

Characterization of Initiator and Copolymer Brush Grafted Monolithic Silica Surfaces. For obtaining the information of elemental composition of the prepared monolithic silica-rod and silica bead surfaces, XPS measurement was performed (Table 2). All samples are named using silica structure and component of grafted copolymer. IM and IB denote the initiator modified "monolith" and "beads", respectively. "I" and "D" and "tB" represents IPAAm, DMAEMA, and tBAAm, respectively.

The peak deconvolution was performed according to the previous reports.[54,55] The peak deconvolutions of XPS carbon C1s peaks for the surfaces were shown in Figure S1 in the Supporting Information. The different peaks were observed between ATRP-initiator modified surfaces (IM and IB) and copolymer grafted monolithic silica-rod surfaces (IM-IDtB, IB-IDtB-S, and IB-IDtB-L). In the spectrum of cationic copolymer grafted monolithic silica-rod surfaces, an additional peak was observed at 288 eV (see Figure S1B, D, and E in the Supporting Information), corresponding to the C═O bond of copolymer, whereas there were no peaks in the spectrum of ATRP-initiator modified monolithic silica-rod surfaces (see Figure S1A, C in the Supporting Information). Additionally, nitrogen contents

Table 2. Elemental Analyses of P(IPAAm-*co*-DMAEMA-*co*-tBAAm) Brush-Grafted Silica Rod and Silica Beads by an XPS Take-off Angle of 90°

	at %[c]					
code[a]	C	N	O	Si	Cl	N/C ratio
IM	4.16 ± 1.66	0.43 ± 0.37	62.3 ± 1.15	32.1 ± 0.59	1.08 ± 0.29	
IM-IDtB	17.0 ± 5.61	2.21 ± 0.46	53.8 ± 2.77	26.4 ± 2.65	0.67 ± 0.51	0.130
IB	20.8 ± 2.79	0.36 ± 0.34	50.4 ± 0.90	26.3 ± 2.41	2.09 ± 1.24	
IB-IDtB-S	56.4 ± 1.48	6.17 ± 2.39	28.1 ± 3.22	8.61 ± 0.78	0.80 ± 0.73	0.109
IB-IDtB-L	61.4 ± 3.00	7.36 ± 1.32	26.4 ± 5.65	4.33 ± 1.66	0.56 ± 0.50	0.112
calcd[b]	74.9	11.6	13.6	-	-	0.155

[a]All samples were named using silica structure, and the component of grafted copolymer. IM and IB denote "initiator modified monolith" and "initiator modified beads", respectively. "I","D", and "tB" represents IPAAm, DMAEMA, and tBAAm, respectively. "S" and "L" represents short and long brush, respectively. [b]Estimated atomic composition of P(IPAAm-*co*-DMAEMA-*co*-tBAAm). [c]Data from three separate experiments are shown as mean ± SD.

Table 3. Characterization of P(IPAAm-*co*-DMAEMA-*co*-tBAAm) Brush-Grafted Monolithic Silica Rod and Silica Beads

	elemental composition (%)							
code[a]	C[b]	H[b]	N[b]	immobilized initiator[c] (μmol/m^2)	grafted copolymer (mg/m^2)	M_n[d]	M_w/M_n[d]	graft density (chains/nm^2)
IM	2.03 ± 0.37	n.d.	0.32 ± 0.02	2.67				
IM-IDtB	8.17 ± 0.51	1.05 ± 0.20	1.43 ± 0.14		1.55	12900	2.53	0.072
IB	4.73 ± 0.02	0.28 ± 0.08	0.23 ± 0.02	4.60				
IB-IDtB-S	17.3 ± 0.11	2.09 ± 0.05	2.65 ± 0.04		2.74	14300	2.23	0.115
IB-IDtB-L	18.8 ± 0.02	2.32 ± 0.01	2.91 ± 0.13		3.18	17400	3.13	0.110

[a]All samples were named using silica structure, and the component of grafted copolymer. IM and IB denote "initiator modified monolith" and "initiator modified beads", respectively. "I" and "D" and "tB" represents IPAAm, DMAEMA, and tBAAm, respectively. "S" and "L" represents short and long brush, respectively. [b]Determined by elemental analysis (n = 3). [c]Estimated from carbon composition. [d]Determined by GPC using DMF containing 50 mmol/L LiCl.

increased, and silicon contents decreased after grafting cationic copolymer on the surfaces, because cationic copolymer brush layer covered onto monolithic silica-rod and silica beads surfaces. These results indicated that cationic copolymer was successfully grafted on monolithic silica-rod surfaces through surface-initiated ATRP.

For measuring the amount of initiator immobilized and grafted copolymer on monolithic silica-rod and silica beads surfaces, CHN elemental analyses were performed. Elemental composition of carbon, hydrogen, and nitrogen were summarized in Table 3. The amount of immobilized initiator on monolithic silica-rod estimated from carbon composition was slightly smaller than that on silica beads surface, although the higher concentration of initiator solution was reacted with monolithic-silica-rod surface than that with silica beads. This unexpected result was caused by the different reactivity between the monolithic silica surface and silica beads surface, probably attributed to difference in the washing reaction before silanization.

Copolymer-brush was grafted from these initiator-modified monolithic silica -rod and silica beads via surface-initiated ATRP. These copolymer-brush modified silica rods and silica beads were named as IM-IDtB, IB-IDtB-S, and IB-IDtB-L. Amounts of grafted copolymer on these surfaces including silica-rod and -bead were larger than that of polymer hydrogel-modified silica beads prepared by conventional radical polymerization as reported previously.[56,57] This was due to the graft configuration of copolymer-brush prepared by surface initiated ATRP. Polymer brushes prepared by surface-initiated ATRP formed densely packed configurations, compared to that by other radical polymerizations, because the initiation efficiency of ATRP is quite high.[25] Thus, copolymer was densely grafted on monolithic silica-rod surfaces, leading to the significantly large amount of grafted copolymer on the surfaces. In addition, the grafted amount of copolymer on IM-IDtB was smaller than those on IB-IDtB-S and IB-IDtB-L, attributed to the different ATRP-initiator-density of silica surface and different reaction condition of ATRP.

To characterize the chain lengths and graft densities of copolymers on the silica surfaces, we cleaved the molecular weight of grafted copolymer was determined by GPC after copolymer chains from silica-rod and silica beads with concentrated sodium hydroxide solution. These data were also summarized in Table 3 (GPC charts of cleaved polymer are shown in Figure S2 in the Supporting Information). The polydispersity index of the cleaved copolymer was larger than that of copolymer prepared in solution, and two narrow peaks overlapped in GPC chromatograms. The larger polydispersity was speculated to be attributed to the porous geometry of monolithic silica-rod and silica beads.[24,58] Polymerization reaction inside the pores of both rods and beads was limited by the insufficient of monomer supply compared to outer exposed surfaces. In addition, the propagation of the copolymer chains from the initiator inside the pores was also restricted to the pore diameter. These factors led to the large polydispersity of grafted copolymer on porous silica substrates. The copolymer graft densities on monolithic silica rod and silica beads exhibited larger values, indicating that the dense copolymer brush layer formed on these surfaces.

SEM observation of before and after copolymer grafting onto monolith silica-rod and silica beads was performed (Figure 4). SEM photograph of initiator modified monolithic silica-rod and beads were also shown in the Supporting Information Figure S3. The macropore structure in monolithic silica rod remained,

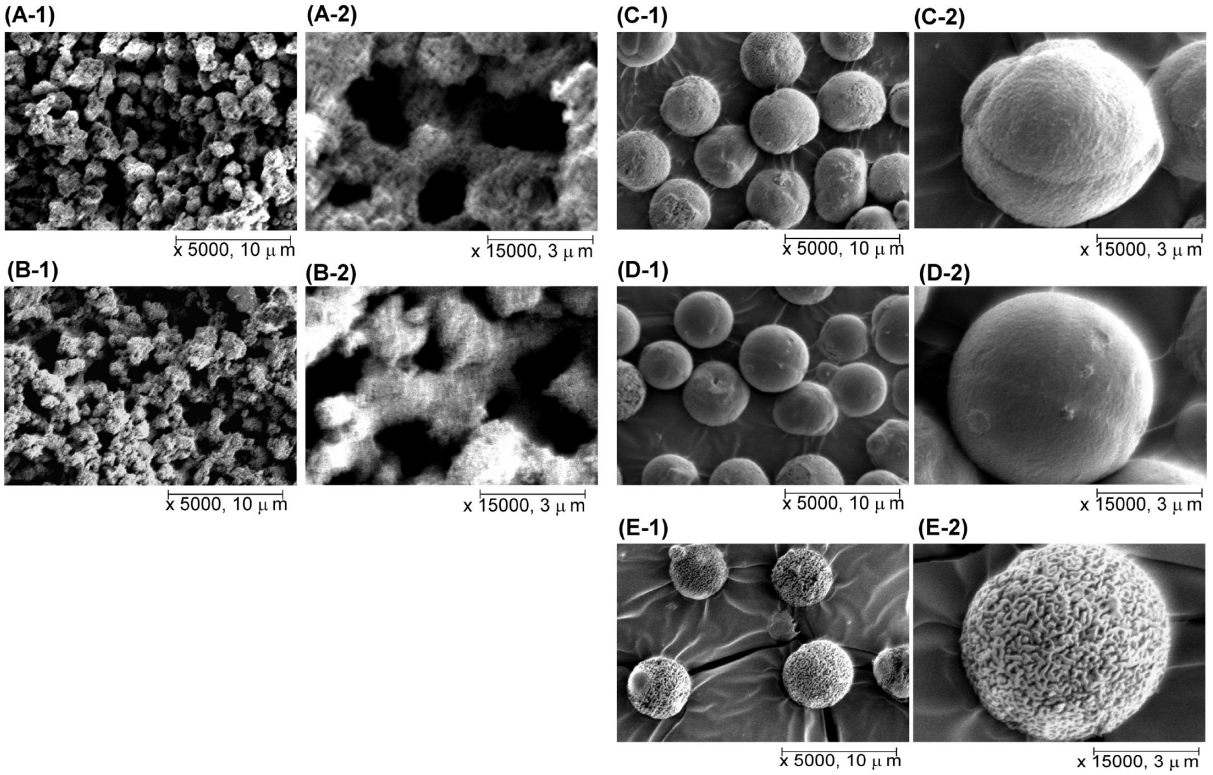

Figure 4. SEM photograph of poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate (DMAEMA)-*co*-*tert*-butylacrylamide-(tBAAm)) brush-modified monolithic silica rods and silica beads. (A) SEM of P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted monolithic silica-rod (IM-IDtB in Table 2); (B) unmodified monolithic silica rod; (C) short P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica beads (IB-IDtB-S); (D) long P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica beads (IB-IDtB-L); (E) unmodified silica beads. The back ground patterns in C−E are double-stick tape for fixing the sample. The photographs (A-2−E-2) (15 000 fold magnification) are the high-resolution photographs of (A-1−E-1) (5000 fold magnification).

although the sufficient amount of copolymer was modified on the silica surface, which would lead to large flow paths and a low back pressure. Figure 5 shows the back pressure of these columns at various temperatures by flowing phosphate buffer solution at a flow rate of 1.0 mL/min. The back pressure of the copolymer modified monolithic silica rod columns was found to keep a low back pressure,[59] indicating that the copolymer grafting method in this study gave no clogging character to the monolithic silica-rod. Additionally, the prepared copolymer brush modified monolithic silica columns would be used various conditions, such as lower temperature and higher flow rate of mobile phase, because of its relatively low back pressure.

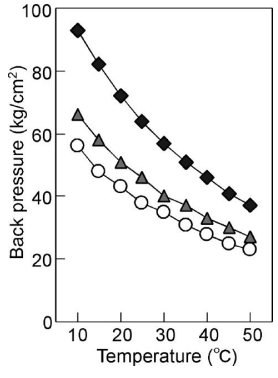

Figure 5. Temperature-dependent back pressure changes on prepared poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)-ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) brush grafted monolithic silica-rods and P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush grafted silica beads at various temperatures The open circles represent back pressure observed on P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted monolithic silica-rod column (IM-IDtB in Table 2); the closed triangles, IB-IDtB-S bead-packed column; the closed diamonds, IB-IDtB-L bead-packed column.

Elution Behavior of Acidic Analytes from Cationic Copolymer Brush Surfaces. The elution behavior of adenosine nucleotides from copolymer grafted monolithic silica-rod column and silica beads used as chromatographic stationary phases was investigated. Figure 6A−C show the chromatograms of adenosine nucleotides at various temperatures on IM-IDtB, IB-IDtB-S, and IB-IDtB-L beads packed columns using 66.7 mmol/L phosphate buffer (pH7.0) as a mobile phase, respectively. Figure 7A−C show changes in the retention times of analytes with various temperatures on these columns. Retention times of AMP, ADP, and ATP increased with increasing the number of phosphate units, attributed to the electrostatic interaction between analyte and grafted copolymer on monolithic silica rod and silica beads surfaces. Also, the retention times of adenosine nucleotides on all columns decreased with increasing column temperature, explained by the decrease in basicity in the grafted copolymer with increasing temperature. As shown in Figure 3, the basic property of P(IPAAm-*co*-DMAEMA-*co*-tBAAm) decreased with increasing temperature. Thus, the electrostatic interaction between grafted copolymer and adenosine nucleotides was weakened by increasing column temperature. On IM-IDtB column, adenosine nucleotides were successfully separated with

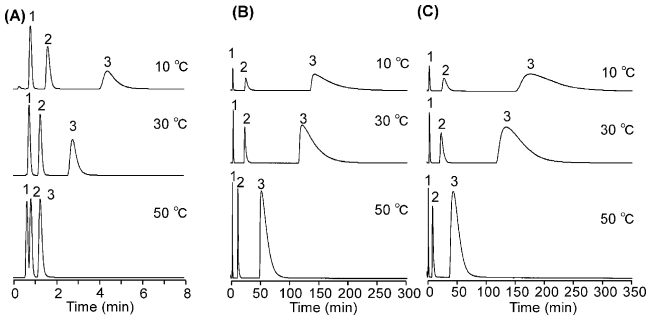

Figure 6. Chromatograms of adenosine nucleotides separated on HPLC of which packing materials were poly(*N*-isopropylacrylamide-(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) brush grafted monolithic silica-rods and P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush grafted silica beads at various temperatures (A) P(IPAAm-*co*-DMAEMA-*co*-tBAAm)-brush grafted monolithic silica-rod column (IM-IDtB in Table 2), (B) short P(IPAAm-*co*-DMAEMA-*co*-tBAAm)-brush grafted silica-beads packed column (IB-IDtB-S), and (C) long P(IPAAm-*co*-DMAEMA-*co*-tBAAm)-brush grafted silica-beads packed column (IB-IDtB-L). Mobile phase is 66.7 mM phosphate buffer (PB) (pH 7.0). The peak No.1 represents AMP; No.2, ADP; No.3, ATP. Because of their high-speed separation abilities, the time scale of monolithic column (IM-IDtB) is expanded than those of silica beads columns (IB-IDtB-S and IB-IDtB-L).

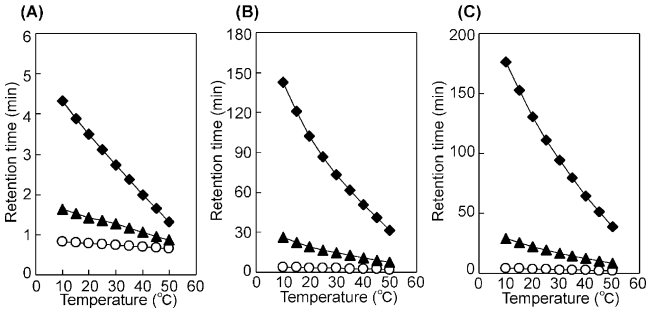

Figure 7. Temperature-dependent retention time changes of adenosine nucleotides on (A) poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) brush grafted monolithic silica-rod column (IM-IDtB in Table 2), (B) short P(IPAAm-*co*-DMAEMA-*co*-tBAAm)-brush grafted silica-beads packed column (IB-IDtB-S), and (C) long P(IPAAm-*co*-DMAEMA-*co*-tBAAm)-brush grafted silica-beads packed column (IB-IDtB-L). The open circles represent AMP; the closed triangles, ADP; the closed diamonds, ATP.

a remarkably short time (6 min) compared to those on IB-IDtB-S and IB-IDtB-L, although the same cationic copolymer was grafted on both silica surfaces in the same column length (50 mm). This high resolution separation with a remarkably short retention time was attributed to the different structure between monolithic silica-rod and silica-bead-packed columns. The liner velocity of mobile phase in monolithic silica-rod is relatively higher than that in beads packed column although flow rate of mobile phase is same.[40] This is attributed to low flow resistance character of monolithic silica rod columns compared to beads packed column. Monolithic supporting materials have a 3D fine-dendritic skeleton structure with large through-pores, reducing the diffusion path length and fluid flow resistance, compared to those of the bead-packed column.[34,35,37] In addition, as shown in the SEM photograph in Figure 4, the narrower skeleton structure of monolithic silica rod than the diameter of silica beads more contributed to the effective utilization of surface area with which the analyte has interaction. Thus, the 3D structure of thermoresponsive cationic copolymer brush-grafted monolithic silica rod was able to rapidly separate a mixture of adenosine nucleotides. In our previous report, PIPAAm brush modified monolithic silica columns rod column is able to separate a mixture of steroids with short analysis time (10 min).[40] Similarly, the prepared thermoresponsive cationic copolymer brush-grafted column is also able to separate acidic biomolecules with in remarkably short retention time (6 min).

For investigating the detailed retention profile of analytes, analysis using the van't Hoff plots were performed (Figure 8).

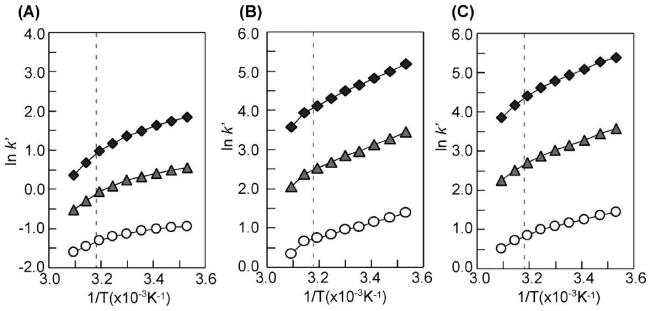

Figure 8. van't Hoff plots of adenosine nucleotides on (A) poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate-(DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) brush grafted monolithic silica-rod column (IM-IDtB in Table 2), (B) short P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica-bead-packed column (IB-IDtB-S), and (C) long P(IPAAm-*co*-DMAEMA-*co*-tBAAm)-brush-grafted silica-bead-packed column (IB-IDtB-L). The open circles represent AMP; the closed triangles, ADP; the closed diamonds, ATP. The dashed line indicate LCST of P(IPAAm-*co*-DMAEMA-*co*-tBAAm).

The retention factor k' value was defined as $k' = R_t/(R_t - R_0)$; where R_t is the retention time of a known sample at predetermined temperature, and R_0 is the retention time of deuterium oxide as an initial standard. The slope of the retention factor increased above the LCSTs of copolymers. This result also indicated that the retention of adenosine nucleotides was related to the phase transition of grafted copolymer, because the copolymer's basicity dramatically changed around LCST.

For investigating the surface hydrophobicity of the prepared monolithic rod and beads surfaces, the temperature-dependent elution profiles of hydrophobic steroids were observed. Figure 9A–C show the chromatograms of hydrophobic steroids at various temperatures on IM-IDtB, IB-IDtB-S and IB-IDtB-L beads packed columns using 66.7 mmol/L phosphate buffer (pH 7.0) as a mobile phase, respectively. Figure 10A–C show changes in the retention times of analytes with various temperatures on these columns. Two hydrophobic steroids were separated on IB-IDtB-S and IB-IDtB-L columns, whereas these were scarcely separated on IM-IDtB column. These results indicated that hydrophobicities of IB-IDtB-S and IB-IDtB-L were higher than that of IM-IDtB. Additionally, relatively longer copolymer was grafted on IB-IDtB-S and IB-IDtB-L compared to that on IM-IDtB. Steroids molecules tend to penetrate into longer copolymer brush layer, leading to a strong interaction with grafted copolymer. Thus, hydrophobic steroids were separated on IB-IDtB-S and IB-IDtB-L. Retention time of steroids on IM-IDtB increased with increasing

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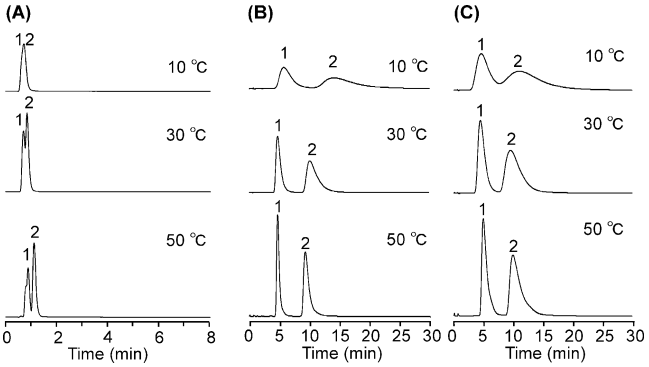

Figure 9. Chromatograms of steroids separated on HPLC of which packing materials were poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) brush-grafted monolithic silica-rods and P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica beads at various temperatures (A) P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted monolithic silica-rod column (IM-IDtB in Table 2), (B) short P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica-bead-packed column (IB-IDtB-S), and (C) long P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica-bead-packed column (IB-IDtB-L). Mobile phase is 66.7 mM phosphate buffer (PB) (pH 7.0). Peak no. 1 represents hydrocortisone; no. 2, dexamethasone. Because their high-speed separation abilities, the time scale of monolithic column (IM-IDtB) is expanded compared to those of silica beads columns (IB-IDtB-S and IB-IDtB-L).

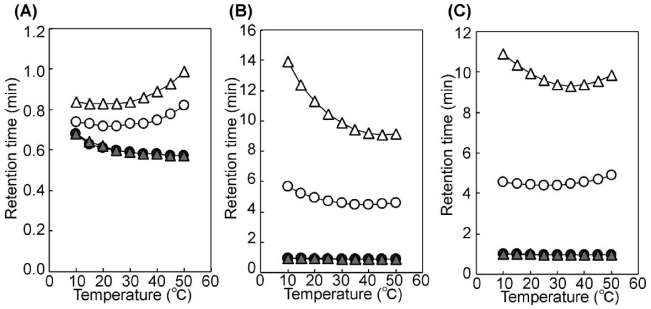

Figure 10. Temperature-dependent retention time changes of steroids on (A) poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)-ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) brush-grafted monolithic silica-rod column (IM-IDtB in Table 2), (B) short P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica-bead-packed column (IB-IDtB-S), and (C) long P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica-bead-packed column (IB-IDtB-L). The open circles represent hydrocortisone; the open triangles, dexamethasone. The closed circle and closed triangles represents retention of hydrocortisone and dexamethasone on unmodified monolithic silica rod and silica beads, respectively.

temperature, while that on unmodified monolithic silica rod decreased (Figure 10A). This indicated that grafted copolymer on IM-IDtB dehydrated and became hydrophobic with increasing temperature, leading to increase in the hydrophobic interaction between copolymer and steroids. On IB-IDtB-S and IB-IDtB-L columns, the retention time of steroids decreased with increasing temperature. Hydrophobic steroids tend to penetrate into swollen copolymer brush layer and interact with copolymer brush at lower temperature, while the steroids had difficulty in penetrating into shrunken copolymer brush at higher temperature. Thus, steroids retention scarcely increased with increasing temperature on IB-IDtB-S and IB-IDtB-L columns.

Grafted thermoresponsive cationic copolymer brush would rapidly change their basic properties with changing temperature. Thus, step-temperature gradients shorten total analysis time with high resolution in temperature-responsive chromatography.[29,56] Figure 11 shows the effects of a step-temperature

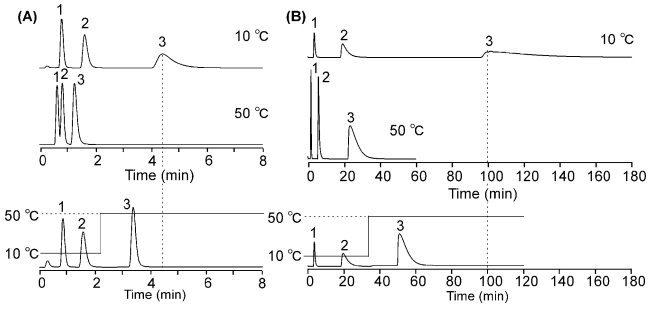

Figure 11. step-temperature gradient on adenosine nucleotides elution from (A) poly(*N*-isopropylacrylamide(IPAAm)-*co*-2-(dimethylamino)ethylmethacrylate(DMAEMA)-*co*-*tert*-butylacrylamide(tBAAm)) grafted monolithic silica-rod column (IM-IDtB in Table 2), and (B) short P(IPAAm-*co*-DMAEMA-*co*-tBAAm) brush-grafted silica-bead-packed column (IB-IDtB-S). Mobile phase is 66.7 mmol/L phosphate buffer (PB) (pH 7.0). Peak no.1 represents AMP; no. 2, ADP; no. 3, ATP. Chromatograms of adenosine nucleotides at 10 and 50 °C are represented for comparing retention times. Because of their high-speed separation abilities, the time scale of monolithic column (IM-IDtB) is expanded compared to those of silica beads columns (IB-IDtB-S).

gradient on steroid elution from IM-IDtB and IM-IDtB-S columns. First, AMP and ADP, relatively weak acidic adenosine nucleotides, were separated by relatively strong electrostatic interactions at 10 °C. After the column temperature was increased to 50 °C, the retention time of ATP were found to be shortened. Using the copolymer brush grafted beads packed column, the step-temperature gradient shortened the retention time of adenosine nucleotides in relatively longer analysis time, approximately 60 min. On the contrary, copolymer brush-grafted monolithic silica-rod column was able to shorten the retention time (total analytical time < 4 min), speculated to be attributed to the suppressed diffusion path length and thermoresponsive properties of copolymer-brush structure.

These results demonstrated that (1) thermoresponsive-cationic-copolymer-brushes was successfully formed on monolithic silica-rod surfaces through surface initiated ATRP with flowing the reaction solution into the column, and (2) thermoresponsive-cationic-copolymer-brushes-grafted monolithic-silica-rod column allows us to perform the high-speed separation of acidic bioactive compounds compared to that using the copolymer brush-grafted bead-packed-column. Thus, thermoresponsive cationic copolymer brush-grafted monolithic silica-rod column would be alternative to thermoresponsive cationic copolymer brush-grafted silica beads packed column.

CONCLUSIONS

Thermoresponsive cationic copolymer brush was successfully grafted onto monolithic silica-rod surfaces through surface-initiated ATRP, and the elution behavior of adenosine nucleotides were observed for investigating the separation efficiency of the prepared columns. The prepared copolymer brush-grafted monolithic silica-rod column separated adenosine nucleotides with a remarkable short time, compared to that using the copolymer brush-grafted bead-packed-columns,

because of the suppressed diffusion path length of thermoresponsive cationic copolymer brush grafted monolithic silica rod column. Furthermore, a step-temperature gradient using the monolithic silica rod column further shortened the retention time (total analytical time < 4 min). Thus, thermoresponsive cationic copolymer brush-grafted monolithic silica-rod column would be an effective tool for analyzing acidic bioactive compounds with a short analysis time and a high resolution.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Properties of analytes, XPS Peak deconvolution of XPS C1s peaks, GPC chart of the grafted copolymer on silica surfaces for obtaining the molecular weight, SEM photograph of monolithic silica rod and silica beads. This material is available free of charge via the Internet at http://pubs.acs.org/.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +81-3-5367-9945 ext. 6201. Fax: +81-3-3359-6046. E-mail: tokano@abmes.twmu.ac.jp.

Notes

The authors declare no competing financial interest.

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