For *Journal of Chromatography A*

Effect of Reaction Solvent on the Preparation of Thermoresponsive Stationary Phase through a Surface Initiated Atom Transfer Radical Polymerization

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Keywords: Thermo-responsive polymer; Poly(*N*-isopropylacrylamide); Atom transfer radical polymerization; Polymer brush; Reaction solvent

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ABSTRACT

Poly(*N*-isopropylacrylamide) (PIPAAm) brush grafted silica beads, a thermo-responsive chromatographic stationary phase, were prepared through a surface-initiated atom transfer radical polymerization (ATRP) using 2-propanol, *N*,*N*-dimethylformamide (DMF), and water as reaction solvents. The rate of grafting PIPAAm on silica bead surfaces was different and found to be dependent on the reactivity of reaction solvent. Temperature-dependent elution profiles of hydrophobic steroids from the prepared-beads-packed columns were found to be different, although the graft amounts of PIPAAm were similar on silica bead surfaces. Especially, prepared beads using 2-propanol exhibited a higher resolution than those using DMF. Calibration curves using glucose and pullulan suggested that beads prepared using DMF prohibited analytes to diffuse into the pores. On the contrary, beads prepared using 2-propanol allowed anlaytes to diffuse into the pores. The pore diameter of the prepared beads, measured by N_2 adsorption–desorption measurement, suggested that beads using 2-propanol has relatively larger pore diameter than those using DMF. Thus, the reaction solvent in surfaces-initiated ATRP affected the grafting configuration of PIPAAm on porous silica-bead surfaces, leading to the different separation efficiency of stationary phase for bioactive compounds.

1. INTRODUCTION

Intelligent polymers, which can respond to external stimuli, are widely used in biomedical fields. Especially, one of the most attractive intelligent polymers is poly(*N*-isopropylacrylamide)(PIPAAm). PIPAAm exhibits a reversible temperature-dependent phase transition in aqueous solutions at its lower critical solution temperature (LCST) of 32 °C [1], leading to its hydrophilic/hydrophobic alteration and extending and shrinking conformational change with temperature. This intrinsic thermo-responsive property is widely used in biomedical appliccatalystations, such as controlled drug and gene delivery systems [2,3], enzyme bioconjugates [4,5], microfluidics [6], cell culture substrates [7], and tissue engineering for regenerative medicine [8-11]. Furthermore, temperature-responsive chromatography utilizing PIPAAm as a stationary phase has been developed for the thermally induced separation of bioactive compounds in aqueous mobile phase without organic phase [12-16]. This chromatography system is highly useful to control the properties of stationary phase for high performance liquid chromatography (HPLC) by only changing the column temperature. Modified PIPAAm 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, preserves the biological activity of analytes, and minimizes the environmental loads. In order to improve the performance of PIPAAm grafted silica-beads, the grafting method of PIPAAm on silica bead surfaces and the elution behavior of analytes from them were intensively investigated [17-20]. As the results, chromatographic matrices prepared by a surface-initiated atom transfer radical polymerization (ATRP) exhibit a strong interaction with analytes, since the polymerization procedure forms a densely packed polymer, called a polymer brush on the surfaces [19,21]. ATRP is an attractive polymer grafting method allowing surface to obtain well-defined polymer brushes by surface-immobilized ATRP initiators [22-28]. The methodology can control the graft chain length by varying the duration of polymerization or initial

monomer concentration [19] and the graft density by varying the concentration of modified ATRP initiator on surface[29].

Additionally, the effect of reaction solvent on ATRP has been investigated in previous studies, indicating that appropriate solvents promote control of the polymerization of PIPAAm and other polymers [30-32]. These reports indicated that reaction solvents affected the activity of ATRP catalyst, monomer, and polymer solubility, and consequently the resulted molecular weight and graft density. Thus, only changing solvent in ATRP is expected to be a key-factor for preparing an efficient thermoresponsive stationary phase. However, to our best knowledge, no report on ideal effective reaction solvents for PIPAAm brush grafting on porous silica bead surfaces was founded.

This study described the preparation of PIPAAm brush grafted silica beads using surface-initiated ATRP with various reaction solvents. The prepared silica beads were characterized by analyzing the graft configuration on silica bead surfaces. Separation efficiencies of the prepared columns were also investigated by observing the temperature-responsive elution profiles of hydrophobic steroids. These results gave an ideal effective reaction solvent for grafting PIPAAm on porous silica bead surfaces.

2. EXPERIMENTAL SECTION

2.1 Materials. *N*-isopropylacrylamide (IPAAm) was kindly provided by Kohjin (Tokyo, Japan) and recrystallized from *n*-hexane. CuCl and $CuCl_2$ were purchased from Wako Pure Chemicals (Osaka). Tris(2-aminoethyl)amine (TREN) was purchased from Acros Organics (Pittsburg, PA, USA). Formaldehyde, formic acid, and sodium hydroxide were purchased from Wako Pure Chemicals. Tris(2-(*N*,*N*-dimethylamino)ethyl)amine (Me_6TREN) was synthesized from TREN, according to a previous report [33]. Silica beads (the average diameter: 5 μm, the pore size: 300 Å, the specific surface area: 100 m^2/g) were purchased from Chemco Scientific (Osaka). Hydrochloric acid, hydrofluoric acid, and ethylenediamine-*N*, *N*, *N'*, *N'*-tetraacetic acid disodium salt dehydrate (EDTA·2Na) were purchased from Wako Pure Chemicals. 2-(*m/p*-Chloromethylphenyl)ethyltrichlorosilane was obtained from ShinEtsu Chemical Industry (Tokyo). 2-Propanol (HPLC grade), *N,N*-dimethylformamide (DMF) (dehydrate), dichloromethane, and toluene (dehydrate) were also purchased from Wako Pure Chemicals. Steroids and uracil 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.

2.2 Preparation of ATRP Initiator Immobilized Silica Beads. 2-(*m/p*-Chloromethylphenyl) ethyltrichlorosilane as an ATRP-initiator modified silica were prepared as shown in the first step in Fig. 1, according to the previous reports [24,29]. First, silica beads were washed with concentrated hydrochloric acid for 3 h at 90 °C, then rinsed with a large amount of distilled water repeatedly until the washing water pH became neutral, followed by thorough drying in a vacuum oven at 110 °C for 18 h. Formation of silane layers comprising the ATRP initiator on silica surfaces was performed as follows; silica beads (15.1 g) were placed into a round-bottom flask and humidified at 60% relative humidity for 4.0 h, followed by the addition of 3.53 mL of 2-(*m/p*-chloromethylphenyl)ethyltrichlorosilane in 302 mL of dried toluene. Nitrogen gas was flowed over the

reaction mixture for first 5 min as HCl gas evolved, and then the flask was sealed. The reaction proceeded at room temperature for overnight with continuous stirring. ATRP initiator-immobilized silica beads were collected by filtration and extensively rinsed with toluene, methanol, dichloromethane, and acetone, and dried in a vacuum oven at 110 °C.

2.3 Modification of thermo-responsive polymer brush by ATRP using various solvents. Dense PIPAAm brush was grafted on ATRP-initiator silica beads through surface-initiated ATRP as shown in the second step in Fig.1. DMF, 2-propanol, and water were used as reaction solvents for surface-initiated ATRP, because previous reports showed that the controlled polymerization of PIPAAm was performed using these solvents as reaction solvents [30,31]. The properties of reaction solvent were summarized in Table S1 in Supplementary Materials. Typical preparation procedure was as follows; the total monomer concentration was set to be 1 mol/L with the following monomer composition in feed; IPAAm (4.86 g, 42.9 mmol) were dissolved in 42.8 mL of 2-propanol, and the solution was deoxygenated by nitrogen gas bubbling for 30 min. CuCl (84.7 mg, 0.86 mmol), $CuCl_2$ (11.5 mg, 0.086 mmol), and Me_6TREN (0.22 g, 0.959 mmol) were added under a nitrogen atmosphere, and the solution was stirred for 20 min to obtain a CuCl/$CuCl_2$/Me_6TREN catalyst system. ATRP initiator-immobilized silica beads (1.0 g) were placed into a clean dry 50 mL glass vessel. Both monomer solution and the silica beads were placed into a glove bag purged with dry nitrogen gas by repeated vacuum and nitrogen flush (three times). The monomer solution was then poured into the glass vessel containing the silica beads, and the vessel was sealed under nitrogen. The ATRP reaction proceeded for 5 h, 10 h, and 15 h at 25 °C under continuous shaking on a desk-top shaker (SN-M40S) (NISSIN, Tokyo). Polymer-grafted silica beads were washed by ultrasonication with acetone for 30 min followed by centrifugation to remove unreacted monomers and ungrafted copolymers. This washing process by ultrasonication was repeated twice. 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 and rinsed with Milli-Q water and acetone, and dried in a high vacuum oven at 50 °C for 5 h. For grafting PIPAAm using DMF, polymerization was performed by the same protocol using DMF as the reaction solvent instead of 2-propanol, and the ATRP reactions were performed for 1.5 h, 3.0 h, and 4.5 h. In the ATRP using water as the reaction solvent, initial monomer concentration was reduced to 0.2 mmol/L, because previous reports indicated that water provides relatively higher conversion [30]. The ATRP reactions using water were performed for 0.5 h, 1.0 h, 1.5 h, and 2.0 h.

2.4 Synthesis of Thermo-responsive Polymer by ATRP using various solvents. For investigating the effects of reaction solvent on the polymerization, unbound PIPAAm were synthesized by solution-phase ATRP in a reaction conditions similar to that of silica-bead surfaces. Polymerization was performed by the same protocol as grafting PIPAAm onto silica using α-chloro-*p*-xylene (26.7 mg, 190 μmol) as an initiator in the reaction solution instead of initiator modified silica-beads, and the half amounts of monomer, catalyst, solvents were used. After the polymerization, the solution was dialyzed against EDTA solution using a dialysis membrane [Spectra/Por standard regenerated cellulose dialysis membrane, Molecular Weight Cut Off (MWCO): 1000] (Spectrum Laboratories, Rancho Dominguez, CA) for 3 days with changing EDTA solution every day, followed by dialysis against Milli-Q water for 2 days, and PIPAAm was obtained by lyophilization. Number-average molecular weights and polydispersity (PDI) values of the polymer were determined using a GPC system (the columns: TSKgel SuperAW2500, TSKgel SuperAW3000, and TSKgel SuperAW4000) (Tosoh, Tokyo) controlled with GPC-8020 model II ver. 5.0 (Tosoh). A calibration curve was obtained using poly(ethylene glycol) 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 a equipped column oven, and the elution profiles were monitored by a equipped refractometer.

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

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

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

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 *C* denote initiator and polymer, respectively.

Grafted PIPAAm on the silica bead surfaces was also retrieved and analyzed by GPC for determining both the molecular weight and PDI. PIPAAm grafted silica beads were treated with concentrated sodium hydroxide solution for overnight, and the solution was neutralized by the addition of hydrochloric acid [34]. The solution was filtered and dialyzed against Milli-Q water using the dialysis membrane described above for 1 week with daily water changed, and PIPAAm 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 a equipped column oven, and the elution profiles were monitored by the refractometer. Graft density of PIPAAm on silica bead surfaces was estimated using the follow equation:

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

where m_c is 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.

Adsorption and desorption isotherm analyses of PIPAAm brush-grafted silica beads were measured at -196 °C with a nitrogen-adsorption measuring apparatus (BELSORP18PLUS-HT) (BEL Japan, Osaka) using N_2 gas. The beads were degassed at 50 °C under vacuum for 5 h before adsorption measurements.

2.6 Temperature Modulated Elution of Analytes. PIPAAm brush grafted silica beads prepared through ATRP using various solvents were packed into a stainless steel column (4.6 mm i.d. x 50 mm). A slurry of polymer-grafted silica beads in water/methanol mixed solvents (1:1) was poured into a slurry reservoir (TOSOH, Tokyo) connected to a stainless steel column. Water/methanol mixed solvent (1:1) was flowed through the slurry reservoir using an HPLC pump (PU-980) (JASCO) at 350 kg/cm^2 for 1 h, followed by equilibration with Milli-Q water for at least 12 h. 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 solution. The properties of analytes were summarized in Table 1 [35]. Milli-Q water was used as a mobile phase. 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 elution profiles using step-temperature gradient[36], two thermostated water baths (RE206, Lauda, Lauda-Konigshofen), set at 10 and 50 °C respectively, were used. First, relatively hydrophilic steroids were eluted at 50 °C. Then, column temperature was reduced by immersing the columns into 10-°C thermostated water bath, and elution behavior at 10 °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 analytes retention behavior on prepared columns, van't Hoff plots for these analytes were obtained. The retention factor *k'* value was defined using the follow equation:

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

where t_R is the retention time of known sample at a specific temperatures, and t_0 is the retention time of uracil as an initial standard [37], because there is no interaction between uracil and PIPAAm, after being confirmed by observing the almost same retention time as deuterium oxide. Resolution between two hydrophobic steroids was calculated according to the following equation:

$$\text{Resolution} = 2.0 \times \frac{t_{R2} - t_{R1}}{W_1 + W_2} \quad (5)$$

where tR_1 and tR_2 ($tR_1 < tR_2$) are the retention times of analytes, W_1 and W_2 are the peak widths of analytes at the baseline.

To investigate the diffusion of analyte into the pores, the prepared beads packed columns were calibrated with glucose and pullulan standards at 10-50 °C. Glucose and pullulan standards were dissolved in Milli-Q water at a concentration of 0.5 mg/mL. Molecular weight (MW) and radius of gyration (Rg) of glucose and pullulan standards are shown in Table 2. Rg was calculated according to the following equation [38]:

$$\text{Rg} = 1.47 \times 10^{-2} Mw^{0.58} \quad (6)$$

Thermo-responsive elution behaviors for glucose and pullulan standards were monitored by an RI detector (830-RI) (JASCO) with a flow rate of 1.0 mL/min. Column temperature was controlled with a deviation of ± 0.1 °C using the same constant temperature water circulator described above.

3. RESULTS AND DISCUSSION

3.1 Characterization of Thermo-responsive Polymer Brush Grafted Silica Beads.

Initiator immobilized silica beads and polymer-grafted silica beads were characterized by elemental analysis. Detected elements (C, H, and N) and the amounts of immobilized initiator are summarized in Table 3. These silica beads are abbreviated as IP-x-y where x denotes initial of reaction solvent, and y represents the amount of PIPAAm on silica bead surfaces. Fig.2(A) shows the time-course changes in the amount of grafted PIPAAm on silica beads. Amount of the grafted PIPAAm on silica bead surfaces increased with proceeding ATRP reaction. However, different increase rates were observed using each solvent, indicating that the rate of polymerization reaction was affected by the properties of reaction solvent. Polymerization rate decreased in the order of water> DMF >2-propanol. In addition, the polydispersity index of the cleaved PIPAAm was larger than that of PIPAAm prepared in solution, and two narrow peaks overlapped in GPC chromatograms (Supplementary Information; Fig. S1). The larger polydispersity was speculated to be attributed to the porous geometry of silica beads. 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. These factors gave the large polydispersity of grafted polymer on porous silica substrate [19,29,39].

For further investigating the polymerization rate in these solvent, a solution-phase ATRP was performed, and molecular weight and polydispersity were observed. Fig.2(B) shows the reaction time-course changes in the molecular weight of PIPAAm synthesized by solution-phase ATRP, and Table 4 summarized the molecular weight and polydispersity of the synthesized polymers. In solution phase ATRP, polymerization rate decreased in the same order (water > DMF > 2-propanol).

Previous reports indicated that solvent properties influence the ATRP reaction, especially, water strongly increases the rate of ATRP [30]. Thus, ATRP using water exhibited the higher amount of grafted PIPAAm and molecular weight in solution phase ATRP, although the initial monomer concentration was set at one-fifth of other solvents. Polymerization rate of PIPAAm using DMF were higher than that using 2-propanol. This was also probably due to the different solubility of ATRP

catalyst and monomer solubility in solvents. Empirically, the solubility of the catalyst in DMF was slightly higher than that in 2-propanol, leading to the higher polymerization rate of PIPAAm in DMF. Additionally, previous report suggest that DMF affects the ATRP not only as a solvent but also as a catalyst for the polymerization to some extent [40]. The catalytic character of the solvent was also speculated to accelerate the polymerization rate of PIPAAm. However, the polydispersity of synthesized polymers using 2-propanol was smaller than that of other solvents, indicating that more controlled polymerization was performed in 2-propanol compared to other solvents.

3.2 Temperature-modulated elution of steroids. Temperature-dependent elution profiles of hydrophobic steroids on the prepared PIPAAm-brush beads packed columns were observed. Adenosine was also used as a model hydrophilic analytes, which scarcely interact with the stationary phase. Fig. 3 shows the chromatograms of hydrophobic steroids and adenosine at various temperatures on the prepared beads-packed columns using Milli-Q water as a mobile phase, and Fig. 4 shows the temperature-dependent retention time changes of hydrophobic steroids on the columns. On all columns, the retention of hydrophobic steroids were enhanced with increasing column temperature, explained by the increase of hydrophobic interaction between dehydrated PIPAAm on silica beads and hydrophobic analytes. Additionally, the retention time of steroids increased with increasing the grafted amount of PIPAAm, because the hydrophobic interaction between PIPAAm and analytes increased with increasing the grafted amount of PIPAAm. Steroids elution profiles from IP-W columns exhibited broader peaks with long retention times, because relatively longer PIPAAm chain was grafted on silica bead surfaces, and steroids would diffuse more into these brush layers [19], compared to shorter PIPAAm brush layers prepared using DMF and 2-propanol. Thus, the peak shapes observed for steroids on IP-W columns became broader than those on IP-P and IP-D columns.

On the other hand, IP-P and IP-D columns separated steroids at high temperatures. In addition, different elution profiles were observed between IP-P and IP-D columns, although the similar amount

of PIPAAm were grafted on silica bead surfaces. For investigating the detailed retention mechanism of analytes on IP-P and IP-D columns, the van't Hoff plots of these analytes were obtained (Fig. 5), and exhibited the relationship between the analyte retention and the column temperature. On IP-P columns, prepared using 2-porpanol in ATRP, change in retention times was relatively small compared to that on IP-D columns using DMF. Large retention-time change was observed around lower critical solution temperature (LCST) of PIPAAm on IP-P columns, while it was observed below LCST (larger 1/T region) on IP-D columns. Fig. 6 shows the peak width of steroids on various temperatures. Peak width decreased with increasing temperature, because the PIPAAm brush layer was dehydrated and shrunken at high temperature, leading the prevention of analyte diffusion into PIPAAm brush layer. Additionally, steroid solubility in the mobile phase increased with increasing temperature, leading to sharp peak shapes. Small peak widths were observed on IP-P columns compared to those on IP-D columns. For investigating the separation efficiencies of these steroids using the columns, resolution between two steroids was obtained (Fig. 7). Resolution between two steroids increased with increasing temperature, because the hydrophobic interaction increased, and peak width decreased with increasing column temperature. Additionally, a higher resolution was observed on IP-P columns compared to that on IP-D columns, suggesting the separation efficiency on IP-P columns was higher than that on IP-D columns.

For investigating the rapid thermoresponsive alteration property of the prepared stationary phase, step-temperature gradient was applied to the prepared columns. Fig. 8 shows the effects of a step-temperature gradient on steroid elution from the prepared columns. First, adenosine and hydrocortisone a relatively hydrophilic steroid, were separated by 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. Relatively larger reduction in retention time was observed on IP-D columns compared to that on IP-P columns, suggesting that the different graft

configuration of PIPAAm brush. This is also attributed to the larger retention time change of hydrophobic steroids on IP-D columns (Fig.4).

3.3 Investigation of retention mechanism of steroids. To evaluate the diffusion of analytes into the pores of PIPAAm grafted silica beads, the calibration curves of thermo-responsive elution profiles of glucose and pullulan standards on the PIPAAm brush-grafted beads-packed columns were obtained (Fig. 9). Calibration curve shifted to right with increasing column temperature on all columns, while the curve on unmodified control columun scarcely shift with temperature [41]. With increasing temperature, grafted PIPAAm brush on silica beads collapsed due to the dehydration and aggregation of PIPAAm. Thus, the effective pore diameter of the prepared beads was slightly increased with changing the column temperature. Larger shift was observed on IP-P columns compared to that on IP-D columns, although the molecular weight and graft density of PIPAAm of both columns were similar to each other. Especially, the retention volume of small molecules increased with increasing temperature on IP-P columns. This result suggested that IP-P columns tend to enhance analyte diffusion into their pores at high temperatures.

For investigating the actual pore diameter of the prepared beads, N_2 adsorption-desorption measurement and calculation using Brunauer-Emmett-Teller (BET) method [42,43] and Barrett-Joyner-Halenda (BJH) method [43,44] were performed. N_2 adsorption-desorption isotherm and pore size distribution were shown in supplementary materials in Fig. S2 and S3. Table 5 summarized the surface area, the total pore volume, and the pore diameter of the IP-P-3.48 and IP-D-3.48. These data also suggested that nearby all pore on IP-D-3.48 were almost clogged by grafted PIPAAm on outer surfaces, while IP-P-3.48 kept their pore intact for analyte diffusion. The clogging on IP-D-3.48 was induced by the higher grafting rate of PIPAAm. In the grafting of PIPAAm using 2-propanol, the polymerization reaction proceeded with relatively low reaction rate. Thus, the reaction solution including monomer and catalyst had an enough time to penetrate inside pores from bulk reaction

solution, leading relatively uniform grafting of PIPAAm inner and outer bead surfaces. The GPC chart of cleaved PIPAAm of IP-P-3.48 exhibited two overlapped peak, indicating that there was a small difference in molecular weight between inside and outside pores. (Supplementary Materials, Fig.S1) On the contrary, in the grafting of PIPAAm using DMF, the polymerization proceeded with a relatively high reaction rate, which allowed the grafting amount of PIPAAm to increase on the outer surface of silica beads than the inner surfaces, leading to the difference of molecular weight of PIPAAm between inside and outside pores. The GPC chart of cleaved PIPAAm of IP-D-3.48 showed a relatively divided peak compared to that of IP-P-3.48 (Supplementary Materials, Fig. S1), indicating that there was a relatively large difference in molecular weight between inside and outside pores. The longer PIPAAm on outer surface would lead to clogging the pores of IP-D-3.48 (schematically drawn in Fig. S.4).

Thus, these results indicated that PIPAAm grafting configuration on porous silica bead surfaces was remarkably influenced by the properties of reaction solvent in surface-initiated ATRP. The resulted PIPAAm graft configuration affected the separation efficiency of bioactive compounds. Thus, the selection of reaction solvent was important for preparing a thermoresponsive stationary phase with a high resolution separation character.

CONCLUSIONS

Thermo-responsive polymer brush grafted silica beads were prepared through surface-initiated ATRP using 2-propanol, DMF, or water as reaction solvents. Similar amount of PIPAAm were grafted on silica bead surfaces using each solvent by modulating the polymerization duration. However, different elution behaviors were observed from these columns. Measurements of pore diameters and the calibration curves of the columns revealed that the beads prepared using 2-propanol enhance analyte retention into pores compared to that using DMF, leading the high separation efficiency of hydrophobic steroids. Thus, the selection of reaction solvent was important for preparing an effective thermoresponsive stationary phase.

Acknowledgement

Part of the present research was financially supported by the Development of New Environmental Technology Using Nanotechnology Project of the National Institute of Environmental Science (NIES), commissioned from the Ministry of Environment, Japan, Grants-in-Aid for Scientific Research (B) no. 20300169 from the Japan Society for the Promotion of Science. We would appreciate to Dr. Norio Ueno for English editing.

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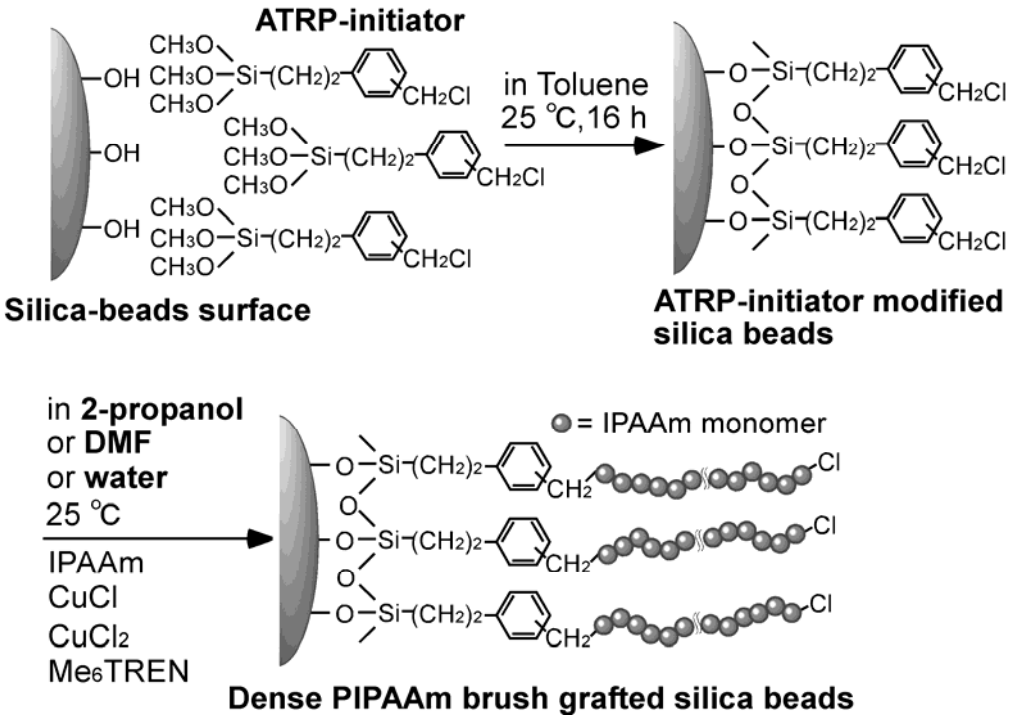

Fig. 1. Scheme of the preparation of poly(N-isopropylacrylamide) (PIPAAm) brush grafted silica beads using a surface-initiated atom transfer radical polymerization (ATRP) with various solvents.

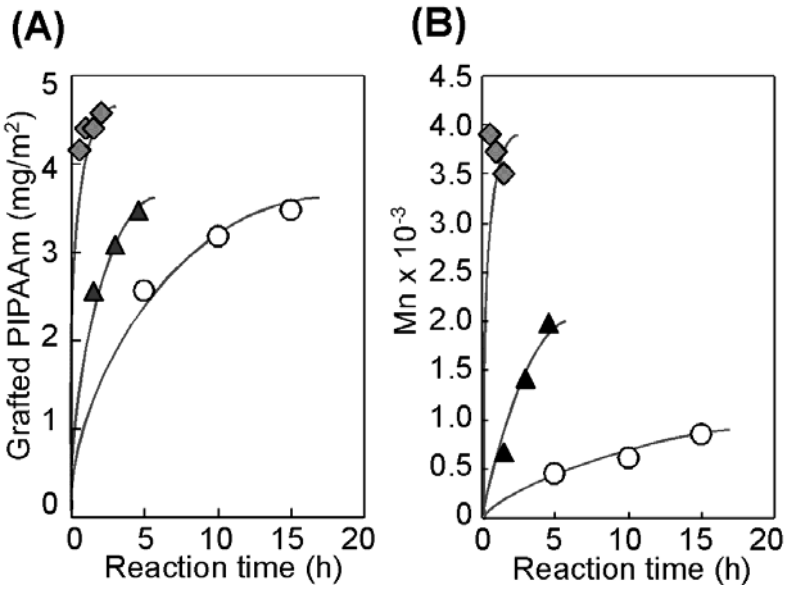

Fig. 2. Reaction time-course changes in (A) the amount of grafted PIPAAm on silica bead surfaces prepared by surface-initiated ATRP and (B) the molecular weight prepared by ATRP in solution using various solvents. The open circles represent 2-propanol; the closed triangles, DMF; the closed diamond, water as a reaction solvent of ATRP.

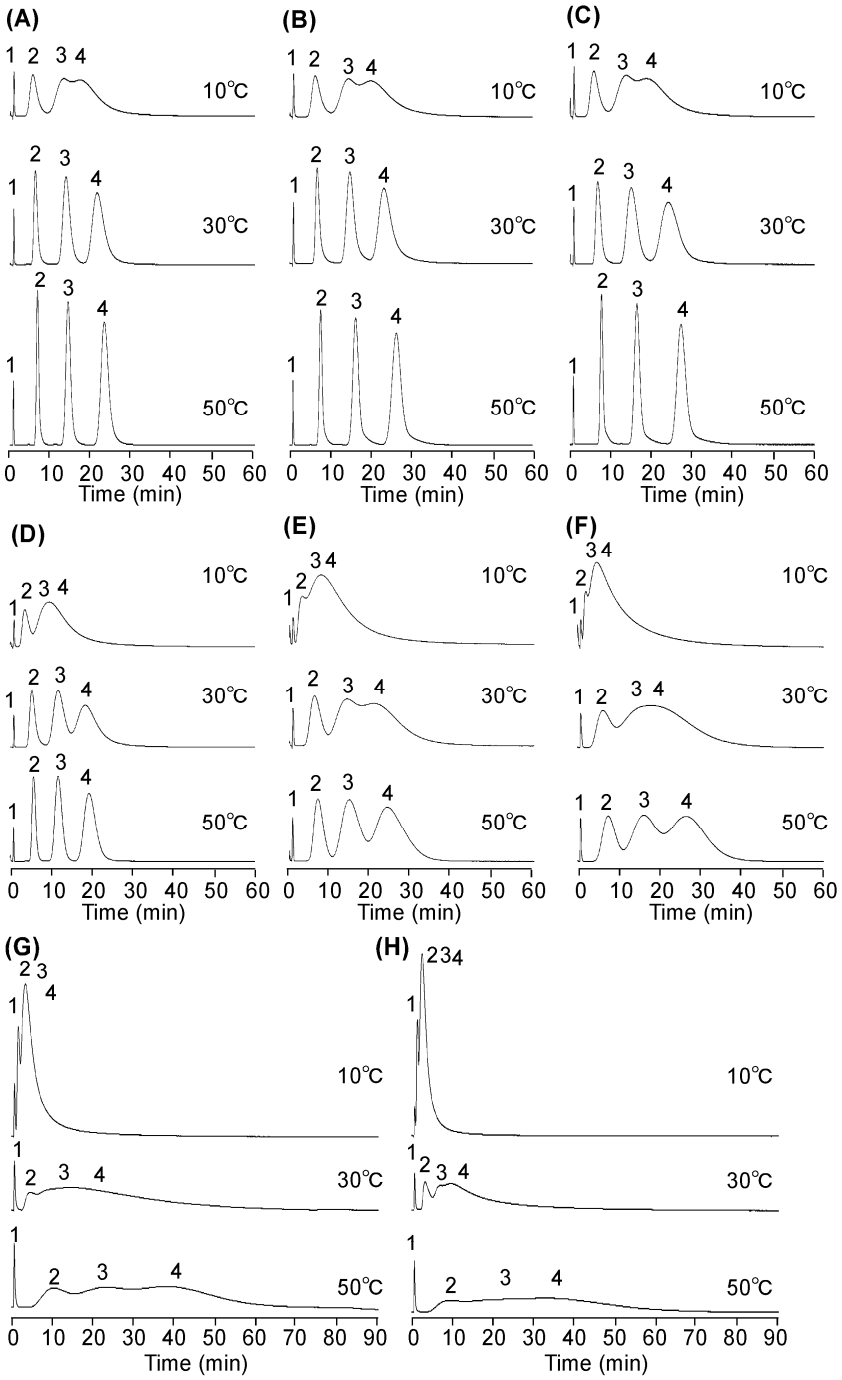

Fig. 3. Chromatograms of steroids separated on HPLC of which packing materials were PIPAAm brush grafted silica beads at various temperatures: (A)IP-P-2.56, (B) IP-P-3.18, (C) IP-P-3.48, (D) IP-D-2.56, (E) IP-D-3.09, (F) IP-D-3.48, (G) IP-W-4.16, and (H) IP-W-4.40 columns (The column abbreviations are given in Table 3). Mobile phase is Milli-Q water. Analytes were monitored at 254 nm by a UV detector with a flow rate of 1.0 mL/min. The peak No.1 represents adenosine; No.2, hydrocortisone; No.3, dexamethasone; No.4, hydrocortisone acetate.

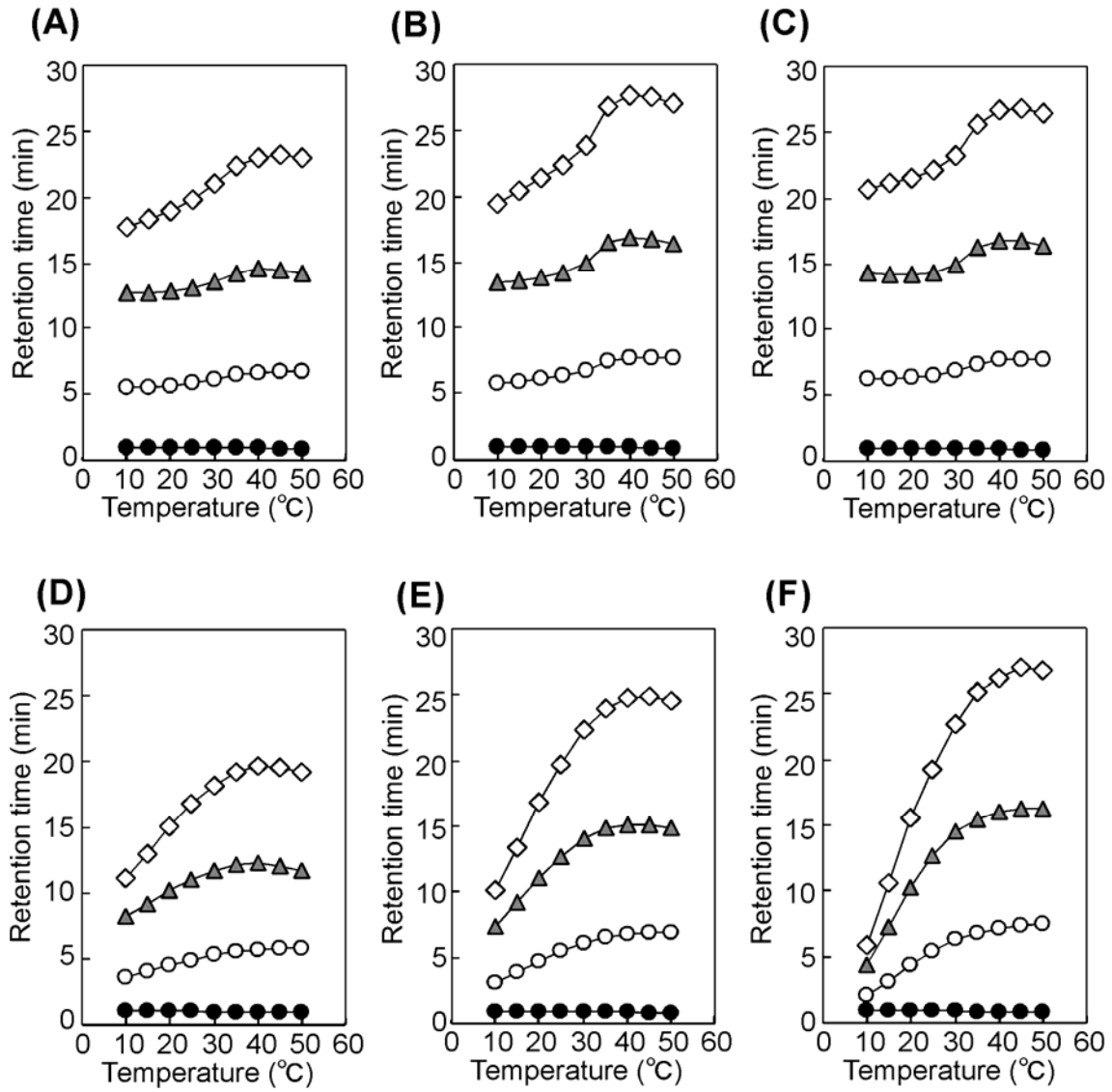

Fig. 4. Temperature-dependent retention time changes of hydrophobic steroids on (A) IP-P-2.56, (B) IP-P-3.18, (C) IP-P-3.48, (D) IP-D-2.56, (E) IP-D-3.09, and (F) IP-D-3.48 columns (The column abbreviations are given in Table 3). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydrocortisone acetate.

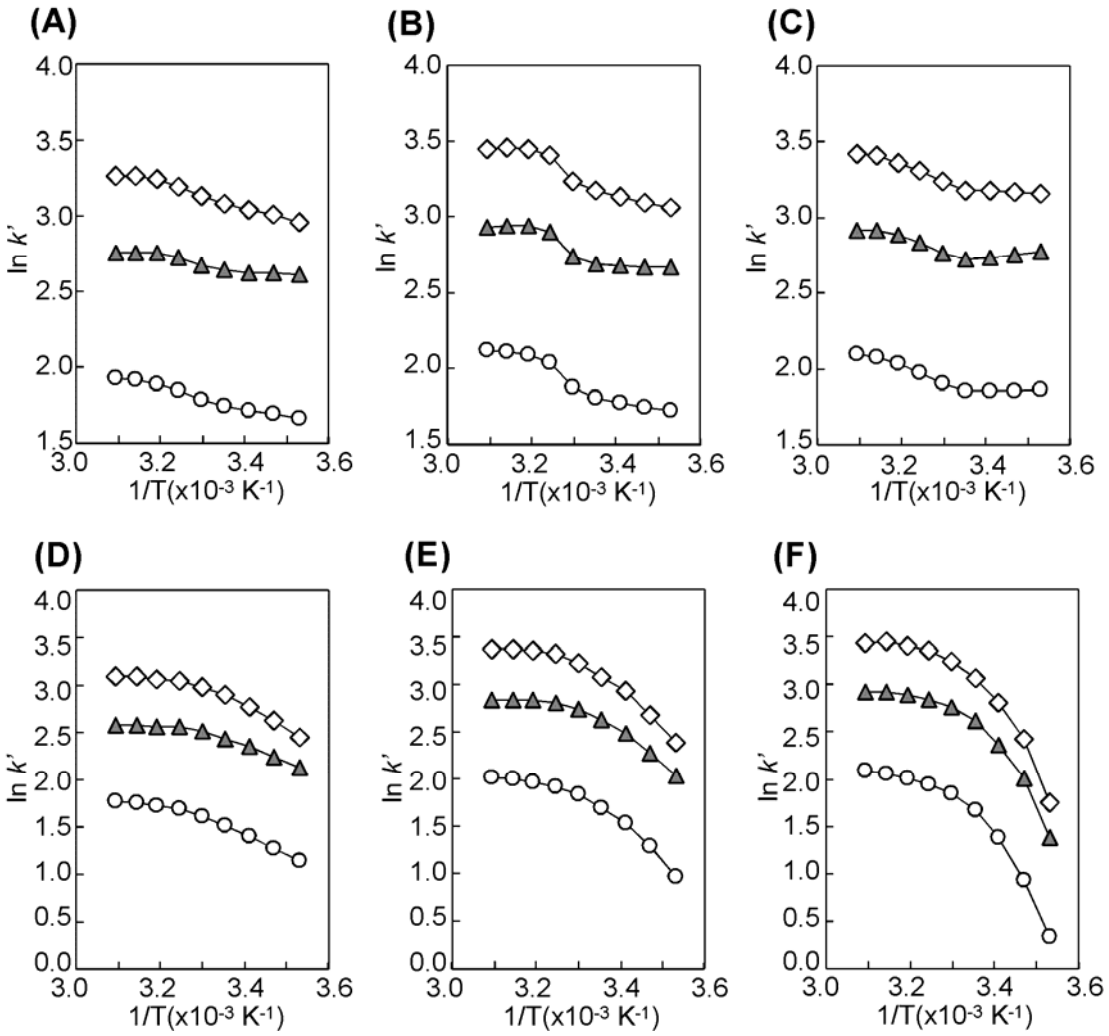

Fig. 5. The van't Hoff plots of hydrophobic steroids on (A) IP-P-2.56, (B) IP-P-3.18, (C) IP-P-3.48, (D) IP-D-2.56, (E) IP-D-3.09, and (F) IP-D-3.48 columns (The column abbreviations are given in Table 3). The open circles represent hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydrocortisone acetate.

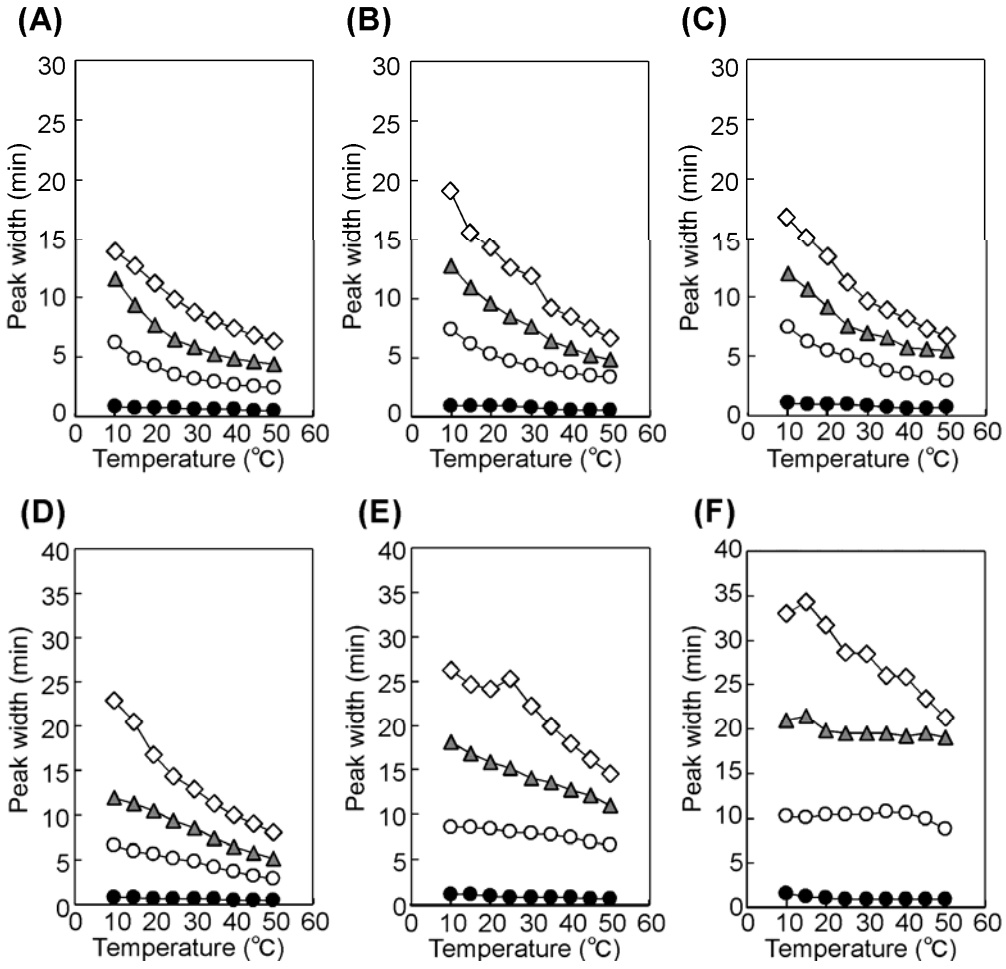

Fig. 6. Temperature-dependent peak width changes of hydrophobic steroids on (A) IP-P-2.56, (B) IP-P-3.18, (C) IP-P-3.48, (D) IP-D-2.56, (E) IP-D-3.09, and (F) IP-D-3.48 columns (The column abbreviations are given in Table 3). The closed circles represent adenosine; the open circles, hydrocortisone; the closed triangles, dexamethasone; the open diamonds, hydrocortisone acetate.

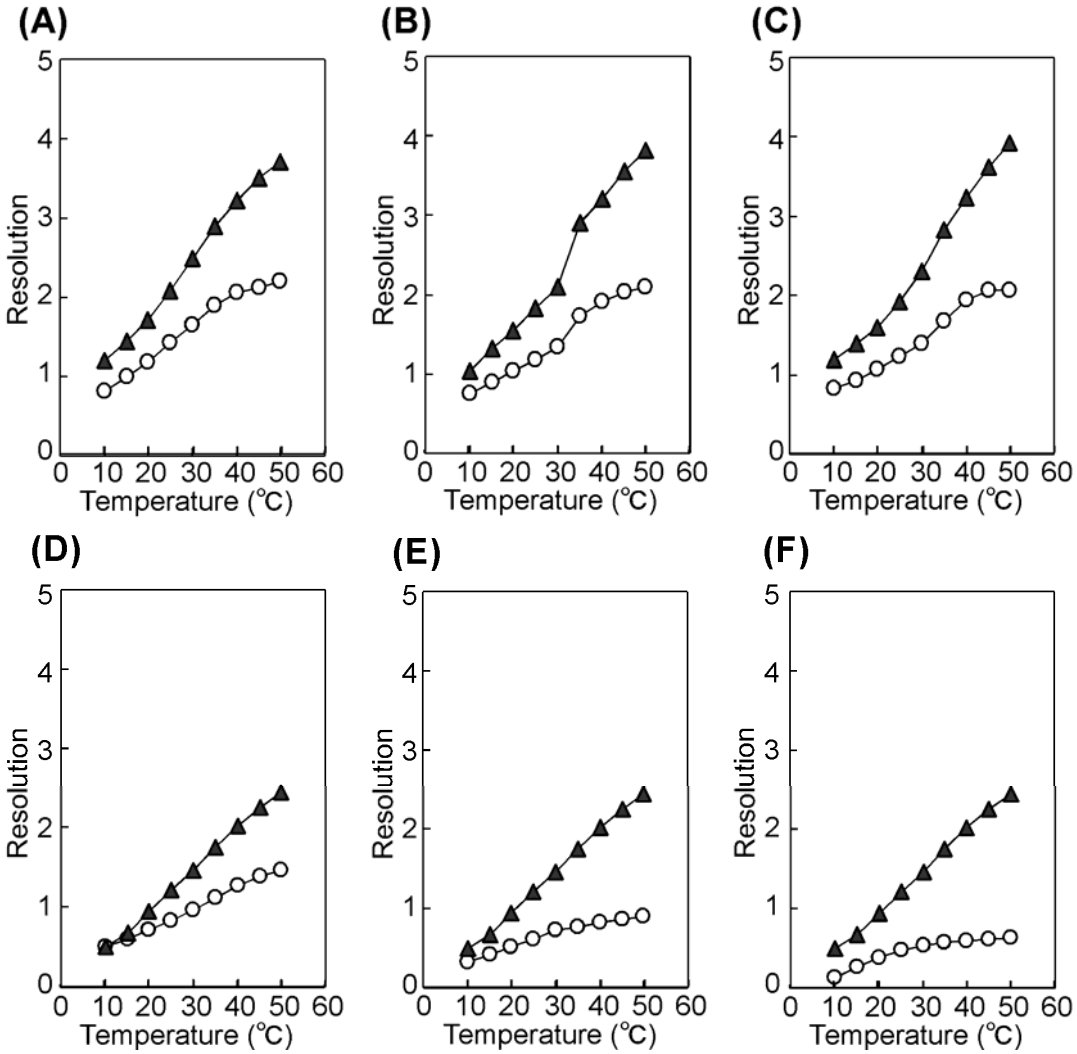

Fig. 7. Temperature-dependent resolution changes of hydrophobic steroids on (A) IP-P-2.56, (B) IP-P-3.18, (C) IP-P-3.48, (D) IP-D-2.56, (E) IP-D-3.09, and (F) IP-D-3.48 columns (The column abbreviations are given in Table 3). The open circles represent resolution between hydrocortisone and dexamethasone; the closed triangles, between hydrocortisone and hydrocortisone acetate.

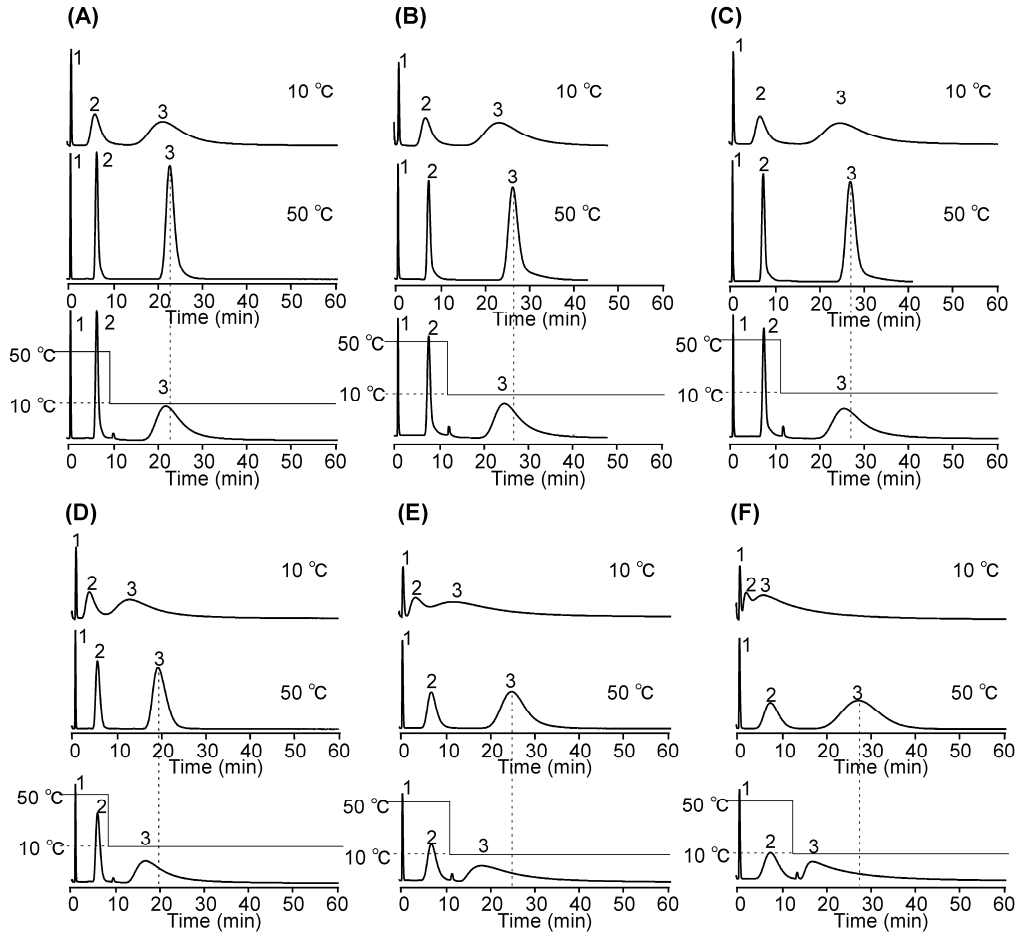

Fig. 8. Step-temperature gradient on steroids elution from (A) IP-P-2.56, (B) IP-P-3.18, (C) IP-P-3.48, (D) IP-D-2.56, (E) IP-D-3.09, and (F) IP-D-3.48 columns (The column abbreviations are given in Table 3). Mobile phase is Milli-Q water. Analytes were monitored at 254 nm by a UV detector with a flow rate of 1.0 mL/min. The peak No.1 represents adenosine; No.2, hydrocortisone; No.3, hydrocortisone acetate. Chromatograms of steroids at 10 and 50 °C are represented for comparing their retention times.

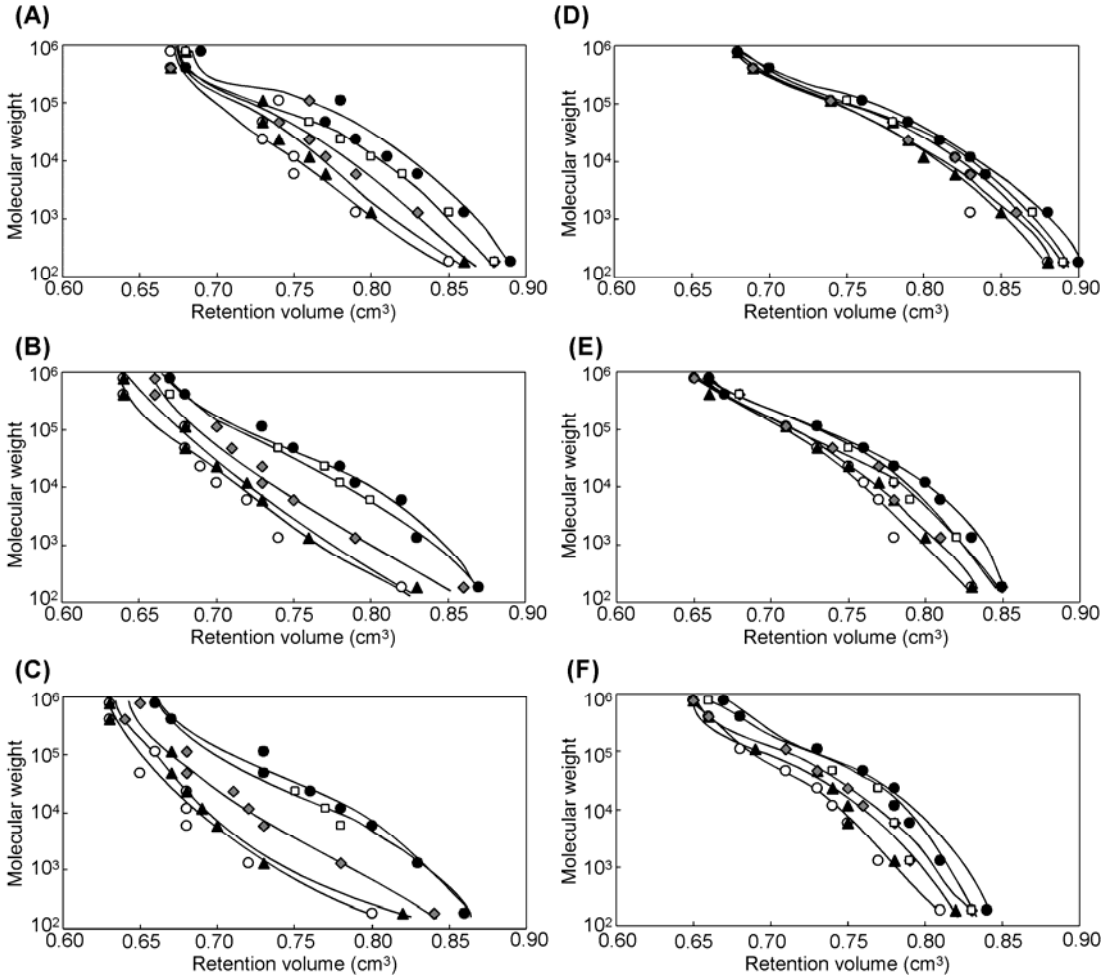

Fig. 9. Plots of molecular weight versus retention volume for glucose and pullulan standards eluted through (A) unmodified control column, (B) IP-P-2.56, (C) IP-P-3.18, (D) IP-P-3.48, (E) IP-D-2.56, (F) IP-D-3.09, and (G) IP-D-3.48 columns (The column abbreviations are given in Table 3). Mobile phase is Milli-Q water. Glucose and pullulan standards were monitored by RI detection with a flow rate of 1.0 mL/min. The open circles, the closed triangles, the closed diamonds, the open squares and the closed circles represent the data taken at 10, 20, 30, 40, and 50 °C, respectively.

Supplementary Materials

Effect of Reaction Solvent on the Preparation of Thermoresponsive Stationary Phase through a Surface Initiated Atom Transfer Radical Polymerization

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Table S1
Properties of solvents.

Solvent	Molecular weight	Specific gravity	Viscosity coefficient (cP)	Dipole moment (D)
2-propanol	60.09	0.7683	2.431	1.68
DMF	73.10	0.94397	0.802	3.86
Water	18.02	0.99821 [a]	1.0019 [b]	1.94

[a] Density (g/cm3) at 20 °C
[b] Viscosity coefficient (cSt) at 20 °C

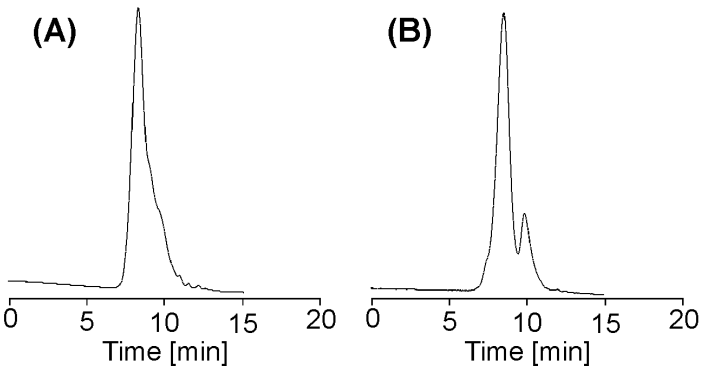

Fig. S2 GPC chart of the retrieved PIPAAm for obtaining the molecular weight (A) IP-P-3.48 and (B) IP-D-3.48. Mobile phase is DMF containing 50 mmol/L LiCl. Two narrow peaks overlapped in GPC chromatograms, probably due to the porous geometry of silica beads.

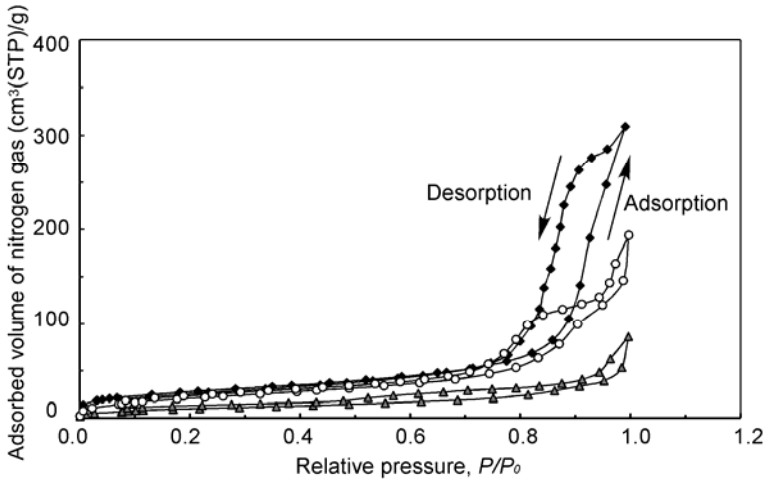

Fig. S2 N_2 adsorption–desorption isotherms of the control unmodified silica (the closed diamonds), IP-P-3.48 (the open circles), and IP-D-3.48 (the closed triangles). (The column abbreviations are given in Table 3)

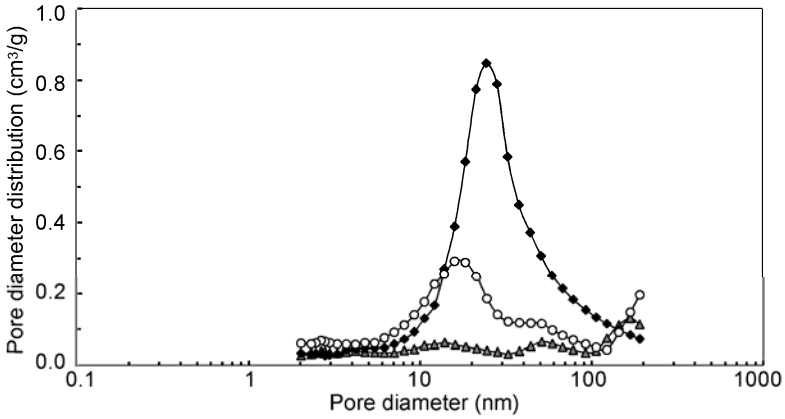

Fig. S3 Pore size distribution of the control unmodified silica (the closed diamonds), IP-P-3.48 (the open circles), and IP-D-3.48 (the closed triangles). (The column abbreviations are given in Table 3)

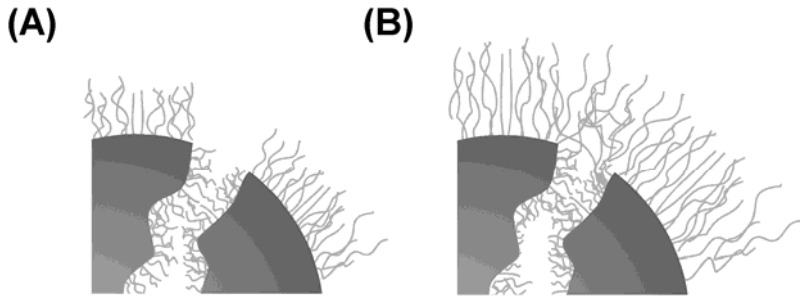

Fig. S4 Schematic illustration of PIPAAm grafting on (A) IP-P-3.48 and (B)IP-D-3.48. (The column abbreviations are given in Table 3)