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Preparation of thermo-responsive polymer brushes on hydrophilic polymeric beads by surface-initiated atom transfer radical polymerization for a highly resolutive separation of peptides

Aya Mizutani[a,b,1], Kenichi Nagase[b], Akihiko Kikuchi[c], Hideko Kanazawa[a], Yoshikatsu Akiyama[b], Jun Kobayashi[b], Masahiko Annaka[d], Teruo Okano[b,*]

[a] Graduate School of Pharmaceutical Sciences, Keio University, 1-5-14 Shibakoen, Minato, Tokyo 105-0011, Japan
[b] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, TWIns, 8-1 Kawadacho, Shinjuku, Tokyo 162-8666, Japan
[c] Department of Materials Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan
[d] Department of Chemistry, Kyushu University, 6-10-1 Hakozaki, Higashi, Fukuoka 812-8581, Japan

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ABSTRACT

Poly(*N*-isopropylacrylamide-*co*-*N*-*tert*-butylacrylamide) [P(IPAAm-*co*-tBAAm)] brushes were prepared on poly(hydroxy methacrylate) (PHMA) [hydrolyzed poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate)] beads having large pores by surface-initiated atom transfer radical polymerization (ATRP) and applied to the stationary phases of thermo-responsive chromatography. Optimized amount of copolymer brushes grafted PHMA beads were able to separate peptides and proteins with narrow peaks and a high resolution. The beads were found to have a specific surface area of 43.0 m^2/g by nitrogen gas adsorption method. Copolymer brush of P(IPAAm-*co*-tBAAm) grafted PHMA beads improved the stationary phase of thermo-responsive chromatography for the all-aqueous separation of peptides and proteins.

1. Introduction

Thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm) has been widely applied to biomedical field such as controlled drug and gene delivery systems [1,2], enzyme bioconjugates [3,4], affinity precipitation [5], microfluidics [6,7], cell culture substrates [8,9], and tissue engineering for regenerative medicine [10–13]. Furthermore, column separation method in all-aqueous system using PIPAAm grafted surfaces as stationary phases have been developed and became a powerful tool for biomedical separations [14–19]. PIPAAm is well-known to exhibit thermo-responsive soluble–insoluble change across its lower critical solution temperature (LCST) at 32 °C in aqueous solution [20]. Its solubility change can be explained by the reversible hydration/dehydration property of its isopropyl side chains. The hydrating allows the polymer to expand its chains in water below the LCST and become hydrophilic, while the dehydrating allows the polymer to become compact and hydrophobic above the LCST. Thermo-responsive chromatography using PIPAAm grafted silica or polystyrene beads with all-aqueous mobile phase are highly useful for controlling both stationary phase function and properties for high performance liquid chromatography (HPLC) by changing only column temperature, with the advantages of preserving the biological activity of peptides and proteins and eliminating the use of organic mobile phases commonly used in reversed-phase chromatography for reducing environmental load.

For the preparation of PIPAAm grafted stationary phases, silica beads [14–17] and polystyrene beads [18,19] have been used as starting materials. Silica-based stationary phases are widely used in HPLC systems because of their good performance as chromatographic stationary phase. However, silica beads become instable under aqueous alkaline conditions (pH higher than 8.0) due to the hydrolysis of silica [21]. Therefore, thermo-responsive chromatography using polystyrene beads as starting materials have developed for improving stability under all-aqueous mobile phase [18,19]. Using PIPAAm grafted polystyrene beads, the thermally induced all-aqueous separation of peptides were achieved [18,19]. However, there are several issues in the use of polystyrene as a starting material for a PIPAAm grafted stationary phase. Since polystyrene has a strong hydrophobicity [22–24], peak tailing was

* Corresponding author. Tel.: +81 3 5367 9945x6201; fax: +81 3 3359 6046.
E-mail address: tokano@abmes.twmu.ac.jp (T. Okano).
[1] Present address: RIKEN (The Institute of Physical and Chemical Research), 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan.

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observed at high temperature. In addition, the specific surface area of PIPAAm grafted polystyrene beads remarkably decreased after PIPAAm grafting, because the previously used beads has relatively small pore size (the average pore size: 250 Å) [18,19]. This factor reduces the resolution of peptide separation.

For improving the resolution of peptide separation and the stability of the stationary phase, hydrophilic beads, poly(hydroxy methacrylate) (PHMA) [hydrolyzed poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate)] [25] having larger pore (600 Å) was chosen as a starting material. Additionally, polymer grafting on the beads was performed using surface-initiated atom transfer radical polymerization (ATRP). Surface-initiated ATRP is an attractive polymer grafting method allowing the surface of beads to receive well-defined polymer brushes by surface immobilized ATRP initiators [16,26–28]. Control of PIPAAm graft amounts and densities on porous polystyrene beads by ATRP is found to be a key factor in facilitating the separation of hydrophobic peptides in aqueous mobile phase [18].

In the present study, P(IPAAm-*co*-*N*-*tert*-butylacrylamide) (tBAAm), a thermo-responsive copolymer with hydrophobic group, brushes were prepared on hydrophilic PHMA beads having large pores by surface-initiated ATRP and applied to the stationary phases of thermo-responsive chromatography.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (IPAAm) was kindly provided by Kohjin (Tokyo, Japan) and purified by recrystallization from *n*-hexane, followed by thorough drying in vacuo at 25 °C. *N*-*tert*-Butylacrylamide (tBAAm) was obtained from Wako Pure Chemicals (Osaka) and purified by recrystallization from acetone, followed by through drying in vacuo at 25 °C. [Hydrolyzed poly(glycidyl methacrylate-*co*-ethylene glycol dimethacrylate)] (poly(hydroxy methacrylate)) beads (CQP30 of MCI GEL, the average diameter, 10 μm; the average pore size, 600 Å; the specific surface area, 58.4 m^2/g) were obtained from Mitsubishi Chemical Corporation (Tokyo). 2-Bromoisobutyryl bromide (98%) was obtained from Aldrich Chemical (St Louis, MO, USA). Tris(2-aminoethyl)amine was obtained from Acros Organics (Pittsburgh, PA). Formaldehyde, formic acid, sodium hydroxide, chloroform, anhydrous magnesium sulfate, copper(I) bromide (CuBr), copper(II) bromide ($CuBr_2$), ethylendiamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt dehydrate (EDTA·2Na), sodium hydroxide (NaOH), acetonitrile, 2-propanol, methanol, acetone, uracil, glucose, and phosphate buffer powder (pH 7.0, 1/15 mol/L) were obtained from Wako Chemicals. Pullulan standards (the molecular weight (M_w), 1300–88,000) were obtained from Showa Denko (Tokyo). Insulin chain A (oxidized ammonium salt) from bovine insulin, insulin chain B (oxidized) from bovine insulin, insulin from bovine pancreas, albumin from chicken egg white (ovalbumin), and aprotinin from bovine lung were obtained from Sigma Chemicals (St. Louis, MO). Water used in this study was purified by an ultrapure water purification system (Milli-Q Synthesis A10) (Millipore, Billerica, MA) unless otherwise mentioned.

2.2. Preparation of ATRP initiator-immobilized beads

ATRP initiator-immobilized beads were prepared using 2-bromoisobutyryl bromide (Fig. 1). PHMA beads (10.0 g) were placed into a 300-mL cleaned three-neck flask, followed by the addition of 200 mL hydrated acetonitrile at 25 °C under nitrogen atmosphere. Then, 2-bromoisobutyryl bromide (19.5 mL, 157.8 mmol) was slowly dropped to the suspensions at 4 °C. The suspensions were stirred for 1 h at 0 °C, then temperature was changed to 25 °C, and the suspensions were additionally stirred for 3 h. During the reaction, HBr, a side reaction product was absorbed in NaOH solution. Prepared beads were filtered and rinsed repeatedly with acetonitrile and acetone, then dried at 40 °C for 3 h under vacuum. ATRP initiator-immobilized PHMA beads are abbreviated as PHMA-Br.

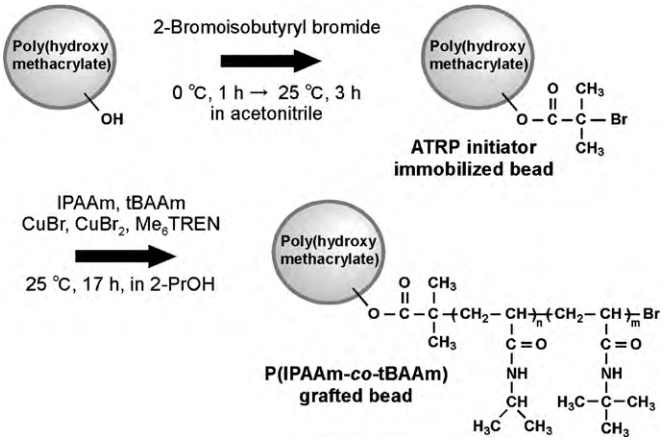

Fig. 1. Scheme for the preparation of poly(*N*-isopropylacrylamide-*co*-*tert*-butylacrylamide) (P(IPAAm-*co*-tBAAm)) brush-grafted poly(hydroxy methacrylate) (PHMA) beads by surface-initiated atom transfer radical polymerization (ATRP).

2.3. Preparation of P(IPAAm-co-tBAAm) brush-grafted beads

P(IPAAm-*co*-tBAAm) brush-grafted PHMA beads were prepared by surface-initiated ATRP on initiator-immobilized PHMA beads in 2-propanol (Fig. 1). In the present study, P(IPAAm-*co*-tBAAm) brush was modified on PHMA beads, because the previous report revealed that PIPAAm chains covalently grafted on hydrophilic substrates are hard to aggregated and dehydrated, compared with that on hydrophobic substrates [29]. Therefore, tBAAm, a hydrophobic monomer, were incorporated into PIPAAm copolymer for promoting the dehydration and aggregation of the grafted polymer. Tris(2-(dimethylamino)ethyl)amine (Me_6TREN) as an ATRP ligand was synthesized using a previously reported method [7,30]. IPAAm and tBAAm monomers (IPAAm/tBAAm: 90.0/10.0 (molar ratio)) were dissolved in 100 mL dried 2-propanol and deoxygenated with nitrogen gas bubbling for 1 h at 25 °C. CuBr (573.8 mg, 4.0 mmol), $CuBr_2$ (89.3 mg, 0.4 mmol), and Me_6TREN (1026.1 mg, 4.4 mmol) were added under nitrogen atmosphere, and the solution was stirred for 15 min to form CuBr/$CuBr_2$/Me_6TREN catalytic system [31]. ATRP initiator-immobilized beads were placed into a 150-mL clean dry sample bottle. Both the solution containing monomers with the catalytic system and the initiator-immobilized beads were placed into a glove bag purged with dry nitrogen gas by repeated vacuum and nitrogen flush three times to remove adventitious oxygen. Monomers solution was then poured into the sample bottle containing the beads, and the sample bottle was sealed under nitrogen. ATRP reactions proceeded for 17 h at 25 °C under continuous shaking on a shaker (SN-M40S) (NISSIN, Tokyo). P(IPAAm-*co*-tBAAm) brush-grafted beads were filtered and rinsed repeatedly with methanol, 50 mmol/L EDTA solution, water, and acetone, consecutively, followed by thorough drying at 40 °C for 3 h under vacuum. The reaction conditions for P(IPAAm-*co*-tBAAm) brush-grafted beads prepared by ATRP are shown in Table 1. Copolymer brush-grafted PHMA beads are abbreviated as PHMA-IPtB-*X*, where *X* is the amount of grafted copolymer in mg/m^2.

Table 1
Characterizations of atom transfer radical polymerization (ATRP) initiator-immobilized and copolymer brush-grafted poly(hydroxy methacrylate) (PHMA) beads by elemental analyses.

Sample[a]	$[IPAAm]_0$ (mmol/L)	$[tBAAm]_0$ (mmol/L)	Elemental composition (%)[b]			Immobilized initiator (μmol/m^2)[c]	Grafted copolymer (mg/m^2)
			C	H	N		
PHMA-Br	–	–	51.0	7.7	1.0	28.05	–
PHMA-IPtB-1.6	225	25	52.9	7.8	2.0	–	1.57
PHMA-IPtB-5.1	900	100	54.7	9.7	3.8	–	5.07

[a] PHMA-Br indicates ATRP initiator-immobilized PHMA beads. PHMA-IPtB-*X* where *X* is the amount of grafted poly(*N*-isopropylacrylamide-*co*-*tert*-butylacrylamide) [P(IPAAm-*co*-tBAAm)].
[b] Determined by CHN elemental analyses ($n = 3$).
[c] Determined by Br elemental analyses ($n = 2$).

2.4. Elemental analyses

Elemental analyses of ATRP initiator-immobilized PHMA beads were performed by an organic halogens and a sulfur analyzer (Yanako, Kyoto) with an ion-chromatography system (ICA-2000) (TOA DKK, Tokyo). Immobilized ATRP initiator moieties [$-OCOC(CH_3)_2Br$] on PHMA beads were calculated from the bulk bromide composition of initiator-immobilized PHMA beads using the following equation:

$$\text{Immobilized ATRP initiator}\,(\mu\text{mol/m}^2) = \frac{\%\text{Br}(10^6)}{\%\text{Br (calcd.)}[(1-\%\text{Br})/\%\text{Br (calcd.)}]MS} \quad (1)$$

where %Br is bromide content (%) determined by elemental analysis, %Br (calcd.) is the theoretically calculated weight percent of bromide in the initiator, *M* is the formula weight of the initiator (mol/g), and *S* is the specific surface area of the original PHMA bead support in m^2/g [58.4 m^2/g, measured by nitrogen gas sorption (the detail appears in subsection 3.2)].

Elemental analyses of ATRP initiator-immobilized and P(IPAAm-*co*-tBAAm) brush-grafted PHMA beads were performed with a PE 2400 series II CHNS/O analyzer (PerkinElmer, Shelton, CT). The amount of grafted copolymer on PHMA beads (mg/m^2) was calculated using the following equation:

$$\text{Grafted copolymer}\,(\text{mg/m}^2) = \frac{\%N\,(10^3)}{\%N_p\ \text{(calcd.)}[((1-\%N_p)/\%N_p\,\text{(calcd.)}) - \%N_i/\%N_i\,\text{(calcd.)}]S} \quad (2)$$

where $\%N_p$ is the percent increase of nitrogen from that of the initiator-immobilized beads determined by elemental analysis, $\%N_p$ (calcd.) is the theoretically calculated weight percent of nitrogen in monomer, $\%N_i$ is the nitrogen composition of initiator-immobilized beads determined by elemental analysis, and $\%N_i$ (calcd.) is the calculated weight percent of nitrogen in initiator moiety.

2.5. Nitrogen sorption

Adsorption and desorption isotherm analyses of ATRP initiator-immobilized and P(IPAAm-*co*-tBAAm) brush-grafted PHMA 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 bioactive compounds

P(IPAAm-*co*-tBAAm) brush-grafted beads (PHMA-IPtB-1.6 and PHMA-IPtB-5.1) were packed into a stainless steel column (4.6 mm i.d. × 150 mm) from their slurry suspended in water/methanol/2-propanol mixed solvent (1:1:1) using a slurry-packing apparatus (model 124A) (Chemco, Osaka) and auto-pressure programmer (model 124PP) (Chemco) at 14.7 MPa. Copolymer brush-grafted beads-packed columns were connected to an HPLC system (DGU-$20A_3$, LC-20AD, CBM-20A, SIL-20AC, RID-10A, and SPD-20A) (Shimadzu, Kyoto) controlled by a personal computer with LC solution analysis software (Shimadzu) on Windows XP.

To investigate the diffusion of analyte into the pores, PHMA-IPtB-1.6 and PHMA-IPtB-5.1 packed columns were calibrated with glucose and pullulan standards at 10–40 °C. Glucose and pullulan standards were dissolved in phosphate buffer (PB) (pH 7.0, 66.7 mmol/L) at a concentration of 0.5 mg/mL. Molecular weight, M_w and radius of gyration, R_g of glucose and pullulan standards are shown in Table 2. R_g was calculated according to the following equation [32]:

$$R_g = 1.47 \times 10^{-2} {M_w}^{0.58} \quad (3)$$

Thermo-responsive elution behaviors for glucose and pullulan standards were monitored by an RI detector (RID-10A) (Shimadzu) with a flow rate of 1.0 mL/min. Column temperature was controlled with a deviation of ±0.1 °C using a low temperature circulating bath (CA-1112) (EYELA, Tokyo) and a thermostated water bath (Thermomeca NT-202D) (NISSIN).

Insulin chain A (0.5 mg/mL), insulin chain B (0.5 mg/mL), insulin (0.5 mg/mL), ovalbumin (0.2 mg/mL), and aprotinin (0.5 mg/mL) were dissolved in PB (pH 7.0, 66.7 mmol/L). Three peptides, insulin chain A, insulin chain B, and insulin were mixed and injected into the HPLC. Two proteins, aprotinin and ovalbumin were also mixed and injected into the HPLC. PB (pH 7.0, 66.7 mmol/L) was used as the mobile phase. Thermo-responsive elution of peptides and proteins were monitored by a UV detector (SPD-20A) (Shimadzu) at 210 or 240 nm and 280 nm with a flow rate of 1.0 mL/min, respectively. Uracil was used for measuring the column void volume. Column temperature was controlled with a deviation of ±0.1 °C using the low temperature circulating bath and the thermostated water bath. Elution profile with temperature-gradient was measured using an AquaWay gradienter (AW301) (CellSeed, Tokyo).

Table 2
Molecular weight, M_w and radius of gyration, R_g for analytes.

Analyte	M_w[a]	R_g (nm)[b]
Glucose	180	0.3
P-1	1300	0.9
P-5	5900	2.3
P-10	11800	3.4
P-20	22800	5.0
P-50	47300	7.6
P-100	112000	12.5
P-200	212000	18.1
P-400	404000	26.2
P-800	788000	38.7

[a] Manufacture's data.
[b] Calculated using the relation between the M_w and R_g of pullulan [32].

Table 3
Properties of peptides.

Analyte	M_w[a]	Hydrophobicity (Σf)[b]
Insulin chain A	2530	11.04
Insulin chain B	3496	24.05
Insulin	5750	32.8
Aprotinin	6511	
Ovalbumin	45000	

[a] Manufacture's data.
[b] Calculated using the relative hydrophobicity values of amino acids [33].

M_w and hydrophobicity, Σf [33] for peptides are summarized in Table 3.

Retention factor, k was calculated using the following equation [34,35]:

$$k = \frac{t_R - t_0}{t_0} \quad (4)$$

where t_R and t_0 are the retention times of analyte and uracil, respectively. Resolution, R_s was calculated according to the following equation [36,37]:

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

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

3. Results and discussion

3.1. Elemental analyses

ATRP initiator-immobilized PHMA beads were characterized by Br elemental analysis. Amount of immobilized $-OCOC(CH_3)_2Br$ moiety, an ATRP initiator, on PHMA beads were summarized in Table 1. Elemental analyses of CHN were performed on ATRP initiator-immobilized and P(IPAAm-*co*-tBAAm) brush-grafted PHMA beads. CHN compositions and the amount of grafted copolymer on PHMA beads were summarized in Table 1. The amount of grafted copolymer increased with increasing the initial monomer concentration of ATRP. A previous report indicated that polymerization rate and grafted polymer length increased with increasing initial monomer concentration [27]. Therefore, the length of grafted copolymer on PHMA was speculated to increase with increasing initial monomer concentration.

3.2. P(IPAAm-co-tBAAm)-grafted surface area for interaction with analytes

N_2 adsorption–desorption measurement at a liquid N_2 temperature of −196 °C was used for obtaining the specific surface area and the porosity of the prepared beads. Fig. 2 shows the N_2 adsorption–desorption isotherms. According to the Brunauer-Emmett-Teller (BET) classification, the observed isotherms were found to be classified as the type III isotherms describing the process of physical adsorption of nitrogen [38]. Remarkable hysteresis loops in the relative pressure ranges from approximately 0.7 to 1.0 within isotherms implied that the pores were mostly mesoporous [39].

The specific surface areas, the total pore volume, and the pore diameters of ATRP initiator-immobilized and copolymer brush-grafted PHMA beads appeared in Table 4. The pore size distribution curves of the beads are shown in Fig. 3. The specific surface areas were estimated using BET method [40,41], and the total pore volume and the pore size distribution were estimated using the Barrett-Joyner-Halenda (BJH) method [41–43]. As shown in Table 4, the specific surface areas of PHMA-IPtB-1.6 and PHMA-IPtB-5.1 were 43.0 and 24.8 m^2/g (the relative value: 74 and 42%), respectively, and the total pore volumes of PHMA-IPtB-1.6 and PHMA-IPtB-5.1 were 0.13 and 0.06 cm^2/g (the relative value: 81 and 38%), respectively. For comparing these data with those of unmodified PHMA, the percentage ratio of the data were calculated with the corresponding data of the unmodified PHMA as 100%. Fig. 3 shows that the pore size distribution curves of the beads moved to the left with increasing the amount of grafted copolymer, because sterically-bulky copolymer grafting reduced the pore sizes of the beads and surface area.

To evaluate the diffusion of analytes into the pores of P(IPAAm-*co*-tBAAm)-grafted beads, the calibration curves of thermo-responsive elution profiles of glucose and pullulan standards on the copolymer brush-grafted beads-packed columns were obtained (Fig. 4). To observe the influence of PHMA porosity, calibration curves on unmodified PHMA beads were used as the control data. Retention volume, V_r, for copolymer brush-grafted beads increased with increasing temperature, because of the dehydration and collapse of PIPAAm brushes on PHMA bead surfaces.

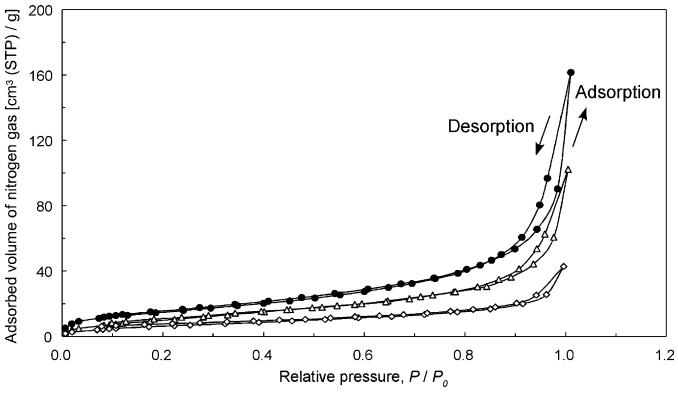

Fig. 2. N_2 adsorption–desorption isotherms of the control unmodified PHMA (the closed circles), PHMA-IPtB-1.6 (the open triangles), and PHMA-IPtB-5.1 (the open diamonds) ($n = 2$).

Table 4
Beads characterizations for ATRP initiator-immobilized and copolymer brush-grafted PHMA beads by the N_2 adsorption–desorption measurement.

Sample	Surface area (m^2/g)[a]	Total pore volume (cm^2/g)[b]	Peak pore diameter (nm)[b]
PHMA	58.4	0.16	168
PHMA-IPtB-1.6	43.0	0.13	106
PHMA-IPtB-5.1	24.8	0.06	68

[a] Calculated using Brunauer-Emmett-Teller (BET) method [41,42] ($n = 2$).
[b] Calculated using Barrett-Joyner-Halenda (BJH) method [42,43] ($n = 2$).

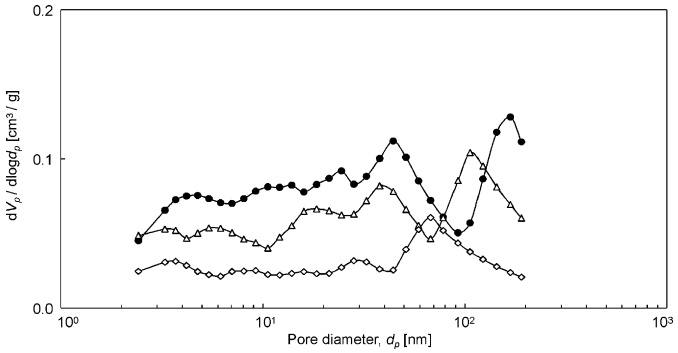

Fig. 3. Pore size distribution curves of the control unmodified PHMA (the closed circles), PHMA-IPtB-1.6 (the open triangles), and PHMA-IPtB-5.1 (the open diamonds) ($n = 2$).

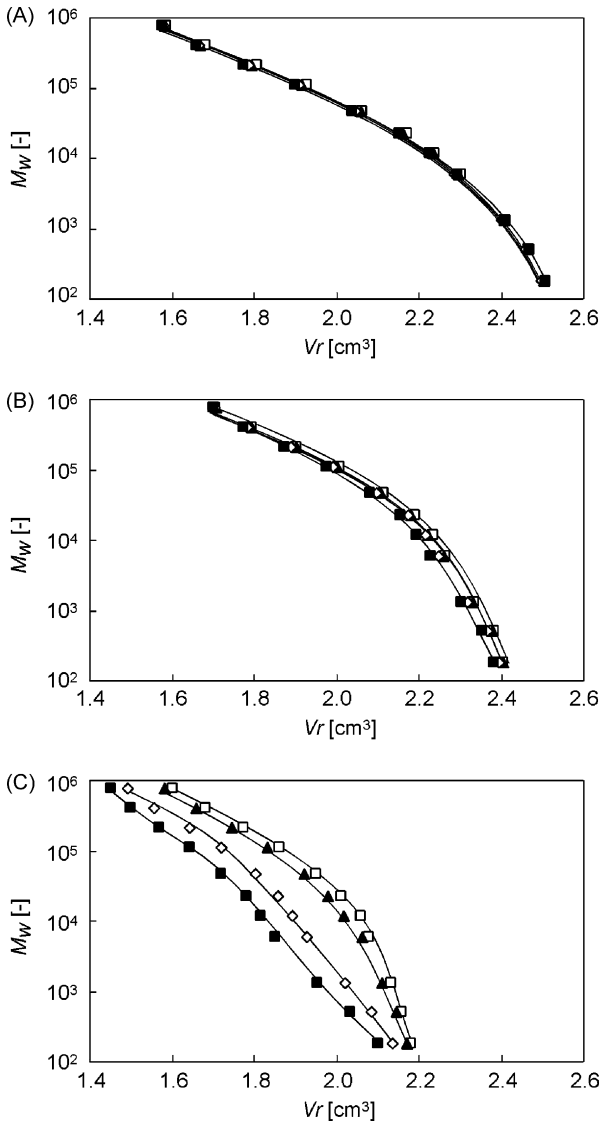

Fig. 4. Plots of molecular weight, M_w versus retention volume, V_r for glucose and pullulan standards eluted through (A) the control unmodified PHMA, (B) PHMA-IPtB-1.6, and (C) PHMA-IPtB-5.1. Mobile phase is phosphate buffer (PB) (pH 7.0, 66.7 mmol/L). Glucose and pullulan standards were monitored by RI detection with a flow rate of 1.0 mL/min. The closed squares, the open diamonds, the closed triangles, and the open squares represent the data taken at 10, 20, 30, and 40 °C, respectively.

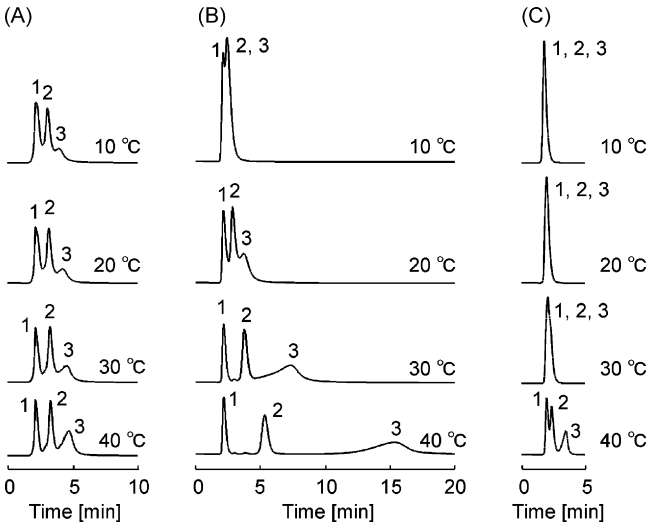

Fig. 5. Chromatograms of insulin chain A, insulin chain B, and insulin in PB (pH 7.0, 66.7 mmol/L) on (A) the control unmodified PHMA, (B) PHMA-IPtB-1.6, and (C) PHMA-IPtB-5.1 at various temperatures. Mobile phase is PB (pH 7.0, 66.7 mmol/L). Peptides were monitored at 210 nm by a UV detector with a flow rate of 1.0 mL/min. Peaks No. 1, 2, and 3 represent insulin chain A, insulin chain B, and insulin, respectively.

The V_r values of PHMA-IPtB-1.6 were almost the same as those of the control unmodified PHMA. Therefore, analytes could diffused into the pores of PHMA-IPtB-1.6 and unmodified PHMA, equally. In contrast, the V_r values of PHMA-IPtB-5.1 were approximately 80–90% of those of the control. Grafted copolymer on PHMA-IPtB-5.1 substantially reduced the pore sizes as shown in Table 4 and Fig. 4 and was speculated to prevent analytes diffusion into the pores.

3.3. Temperature-modulated elution of peptides and proteins

Temperature-dependent elution profiles of a mixture of insulin chain A, insulin chain B, and insulin on PHMA-IPtB-1.6 and PHMA-IPtB-5.1 packed columns were observed. PB (pH 7.0, 66.7 mmol/L) was used as a mobile phase. Chromatograms of insulin and its fragments at various temperatures are shown in Fig. 5. Thermo-responsive retention factor and resolution changes are shown in Figs. 6 and 7, respectively. As shown in Figs. 5 and 6, the retention times of insulin and its fragments increased with their hydrophobicity, represented by Σf values in Table 3 [33]. These results showed that the retention of insulin and its fragments was per-

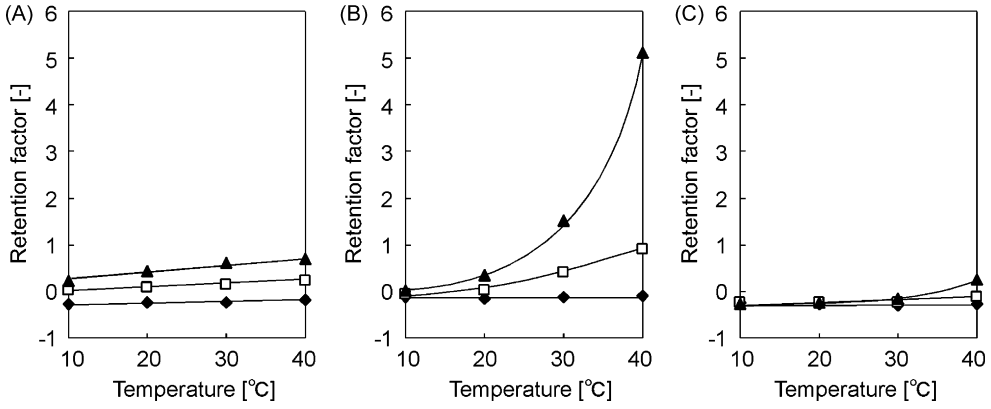

Fig. 6. Temperature-dependent changes in the retention factors of insulin and its fragments on (A) the control unmodified PHMA, (B) PHMA-IPtB-1.6, and (C) PHMA-IPtB-5.1 columns ($n = 3$). The closed diamonds, the open squares, and the closed triangles represent insulin chain A, insulin chain B, and insulin, respectively. Error bars are unrecognized, because the bars were smaller than the size of each symbol.

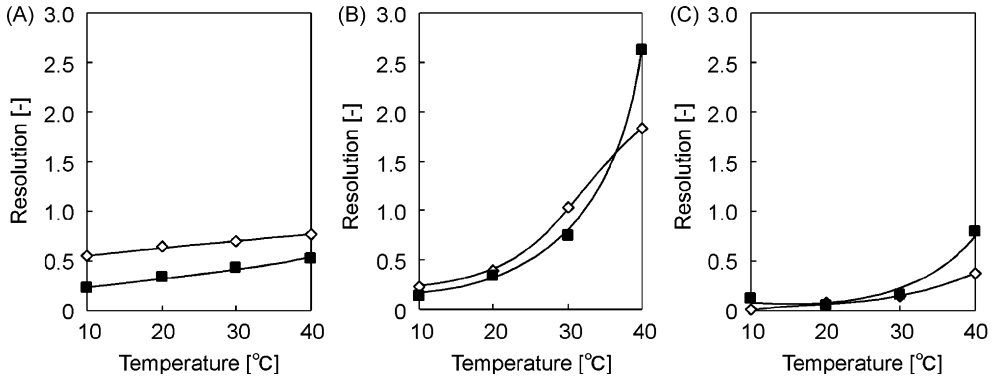

Fig. 7. Temperature-dependent changes in the resolution between insulin chain A and insulin chain B (the open diamonds), insulin chain B and insulin (the closed squares) on (A) the control unmodified PHMA, (B) PHMA-IPtB-1.6, and (C) PHMA-IPtB-5.1 columns.

formed with the hydrophobic interaction between the grafted copolymer on stationary phase surfaces and analytes.

In addition, the retention times of insulin and its fragments on the copolymer brush-grafted beads packed column increased with the increase of column temperature, explained by hydrophobically driven partitioning between hydrophobic dehydrated copolymer chains and peptides, although those on the control unmodified PHMA showed almost the same values at all temperatures because of the lack of thermo-responsive polymer coating on the stationary phase. Retentions and separations of peptides on the control unmodified PHMA were speculated to be performed through hydrophobic interactions between peptides and alkyl chains of PHMA.

As shown in Fig. 5, the thermo-responsive retention profiles of insulin and its fragments were quite different between PHMA-IPtB-1.6 and PHMA-IPtB-5.1. Moreover, Figs. 6 and 7 showed that the values for the retention factors and the resolutions of peptides on PHMA-IPtB-1.6 were larger than those on the control unmodified PHMA at above 30 °C, although almost all of those on PHMA-IPtB-5.1 were smallest amongst all of the beads at all temperature. The reasons for the different separation profiles on copolymer brush-grafted beads could be explained by the hydrophobicity and the effective surface area of the beads. As indicated in the previous reports [9,18], surface hydrophilicity increased with increasing the amount of grafted PIPAAm at both below and above phase transition temperature. Therefore, PHMA-IPtB-5.1, a large amount of thermo-responsive copolymer grafted PHMA showed to suppress the hydrophobic interaction between the stationary phase and peptides. Moreover, as concluded in the previous report [18], an important factor for effective peptide separation is the thermo-responsive polymer-grafted surface area, leading to large hydrophobic partitioning with peptides. In the subsection 3.2, the copolymer-grafted surface area was revealed that the specific surface area and the total pore volume of PHMA-IPtB-5.1 were below 50% of those of the control unmodified PHMA, although those of PHMA-IPtB-1.6 were approximately 80%. Moreover, Fig. 4 showed the thermo-responsive changes of pore sizes. As discussed in subsection 3.1, the length of grafted copolymer on PHMA-IPtB-5.1 was larger than that on PHMA-IPtB-1.6. Therefore, in the case of PHMA-IPtB-5.1, aggregation/expansion behavior of the grafted copolymer according to the temperature changes largely concerned the change of pore size of the beads, leading to large shifts on calibration curves. As a result, grafted copolymer on PHMA-IPtB-5.1 was speculated to prevent analytes diffusion into the pores. In the case of PHMA-IPtB-1.6, the thermo-responsive dynamics of the grafted short copolymer was speculated to scarcely concern the changes of pore size of the beads. Therefore, PHMA-IPtB-1.6 remains relatively large thermo-responsive polymer-grafted surface area, and allows analytes to interact with the grafted copolymer, leading to large hydrophobic partitioning with peptides.

The most attractive features of the copolymer brush-grafted PHMA beads are narrower peaks and high resolution, compared with the previous reports [15,18]. Resolution between insulin chain A and insulin at 40 °C for PHMA-IPtB-1.6 was 3.61, which was approximately nine times larger than that for PIPAAm brush-grafted polystyrene beads (0.39, unpublished result). In addition, in the previous report [15] using PIPAAm sparsely grafted silica beads, the half-value width of the peak of insulin chain B was approxi-

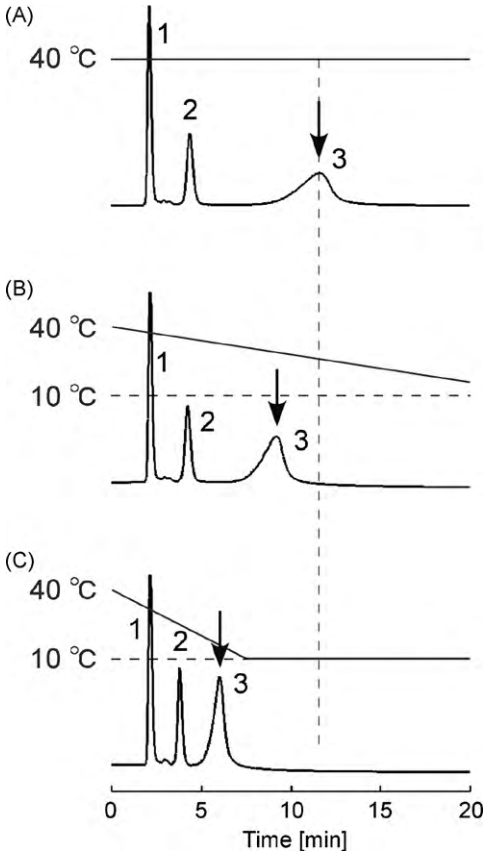

Fig. 8. Effect of a temperature change (linear gradient) on the retention times of insulin chain A, insulin chain B, and insulin mixed solutions using PHMA-IPtB-1.6 column (A) at 40 °C, (B) from 40 °C to 10 °C (1.0 °C/min), and (C) from 40 °C to 10 °C (4.0 °C/min). Peptides were monitored at 240 nm with a flow rate of 1.0 mL/min. Peaks No. 1, 2, and 3 represent insulin chain A, insulin chain B, and insulin, respectively.

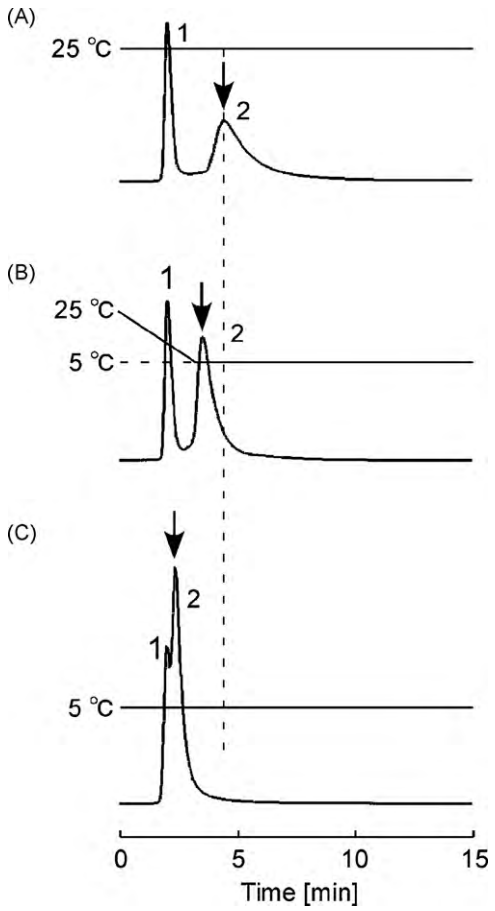

Fig. 9. Effect of temperature change (linear gradient) on the retention times of ovalbumin and aprotinin mixed solution on PHMA-IPtB-1.6 column (A) at 25 °C, (B) from 25 °C to 5 °C (6.0 °C/min), and (C) at 5 °C. Proteins were monitored at 280 nm with a flow rate of 1.0 mL/min. Peaks No. 1 and 2 represent ovalbumin and aptotinin, respectively.

mately three times larger than that of PHMA-IPtB-1.6. These results indicated that PHMA-IPtB-1.6 beads are able to perform a high-resolution peptide separation. Hydrophilic properties of PHMA beads much more suppressed the interaction between peptides and the surface of starting material than hydrophobic polystyrene beads and silica beads, leading to analyte interaction with only grafted copolymer. Additionally, the dense P(IPAAm-*co*-tBAAm) brush structure, prepared by surface-initiated ATRP, also prevent the interaction between peptides and PHMA surface. These factors are supposed to give a high-resolution peptide separation.

Additionally, using a column oven for changing temperature, the effect of separation of linear-temperature gradient analyses by changing column temperature was observed on peptides and proteins on PHMA-IPtB-1.6 (Figs. 8 and 9). Programmed temperature changes of stationary phase shortened the retention times of insulin and its fragments with narrower peak widths (Fig. 8(B, C)), compared to that monitored at 40 °C (Fig. 8(A)). The programmed temperature changes of stationary phase adjusted also the separation capacity of ovalbumin and aprotinin (Fig. 9). These results indicated that the surface property of the stationary phase could be arbitrarily and rapidly regulated from hydrophobic to hydrophilic by programmed linear-temperature gradient analysis with a single aqueous mobile phase. Since thermo-responsive hydrophobic/hydrophilic switching of P(IPAAm-*co*-tBAAm) brush is very fast in single aqueous mobile phase, linear-temperature gradient analyses were speculated to have several advantages, compared with conventional solvent gradient analyses in reversed-phase chromatography. Smart switching of the surface in all-aqueous solution was supposed to allow us to perform study for finding separation conditions and the continuous analyses of bioactive compounds in a short time preserving the biological activity.

PHMA beads with the proper amount of P(IPAAm-*co*-tBAAm) performed the separation of peptides with high resolution, because of their large surface area for the interaction and hydrophilic property of graft interface. Additionally, the prepared beads exhibited thermo-responsive hydrophobic/hydrophilic alteration with changing temperature, leading to the modulation of retention time. Therefore, hydrophilic polymeric beads having large pores are an appropriate starting material for obtaining improved stationary phases with thermo-responsive chromatography.

4. Conclusions

P(IPAAm-*co*-tBAAm) brushes were prepared on hydrophilic PHMA bead having large pores by surface-initiated ATRP for improving thermo-responsive chromatography matrices. Using the proper amount of copolymer grafted PHMA beads, peptides and proteins were effectively separated with high-resolution. Possible factors for high resolutive analyses were the hydrophilicity of the starting material with large surface area where copolymer grafted bead. The prepared thermo-responsive copolymer grafted PHMA beads should be useful as a new alternative to the thermo-responsive polymer grafted silica/polystyrene beads for all-aqueous chromatography with high-resolution.

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