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Thermo-responsive polymer brush-grafted porous polystyrene beads for all-aqueous chromatography

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ABSTRACT

Poly(*N*-isopropylacrylamide) (PIPAAm) brush-grafted porous polystyrene beads with variable grafted polymer densities were prepared using surface-initiated atom transfer radical polymerization (ATRP) for applications in thermo-responsive chromatography. Utilization of these grafted beads as a stationary phase in aqueous chromatographic analysis of insulin provides a graft density-dependent analyte retention behavior. The separations calibration curve on PIPAAm-grafted polystyrene was obtained using pullulan standards and exhibited inflection points attributed to analyte diffusion into bead pores and partitioning into grafted PIPAAm brush surfaces. Presence of these inflection points supports a separation mechanism where insulin penetrates pores in polystyrene beads and hydrophobically interacts with PIPAAm brushes grafted within the pores. Control of PIPAAm brush graft density on polystyrene facilitates effective aqueous phase separation of peptides based on thermally modulated hydrophobic interactions with grafted PIPAAm within stationary phase pores. These results indicated that PIPAAm brush-grafted porous polystyrene beads prepared by surface-initiated ATRP was effective stationary phase of thermo-responsive chromatography for aqueous phase peptide separations.

1. Introduction

Poly(*N*-isopropylacrylamide) (PIPAAm), a popular stimuli-sensitive polymer, exhibits temperature-responsive soluble–insoluble change across its lower critical solution temperature (LCST) at 32 °C in aqueous solution [1]. Its solubility change can be explained by reversible hydration/dehydration of polymer isopropyl side chains, hydrating to expand chains in water below the LCST, while dehydrating to form compact, insoluble conformations above the LCST [1,2]. This intrinsic thermo-responsive property is widely exploited in cell-related biomedical applications, including cell culture substrates [3,4], and tissue engineering for regenerative medicine [5]. Additionally, the property has been exploited in all-aqueous hydrophobic chromatographic separations of peptides and bioactive mixtures [6,7]. This system is highly useful to control both stationary phase function and properties for aqueous mobile phase high performance liquid chromatography (HPLC) by changing only column temperature. Performance advantages include maintenance of biological activity of peptides and proteins, and reduced waste from organic mobile phases commonly used in reversed-phase chromatography.

Nonetheless, silica beads exhibit instability under alkaline conditions, and are best used in neutral pH mobile phases [8]. Experimentally, separation reproducibility decreases with repeated use of the silica-based stationary phases even at neutral condition. Thus, base materials with aqueous stability at neutral-high pH are required for improved aqueous separations reproducibility. We chose highly cross-linked porous polystyrene beads as the base materials of novel thermo-responsive chromatography matrices due to their high stability [9]. However, the use of polystyrene beads for the separation of bioactive compounds would be limited, since strong hydrophobicity of polystyrene causes non-specific adsorption of analytes [10].

In order to use polystyrene beads as improved chromatography stationary phases, we prepared dense PIPAAm brush-grafted porous polystyrene beads by surface-initiated atom transfer radical polymerization (ATRP). Surface-initiated ATRP is an attractive polymer grafting method since it allows for the preparation of dense polymer brushes using surface-immobilized ATRP initiators [11–13]. Recently, some research groups had applied polymer brush-grafted silica beads prepared by ATRP to the stationary phases of reversed-phase chromatography [14,15] and thermo-

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responsive chromatography [16]. Dense polymer brush-grafted beads enhanced the retention of hydrophobic compounds compared to that using sparsely grafted beads [14–16]. These results revealed that modification of dense polymer brush on beads surface remarkably improved the performance of the beads as chromatography stationary phase. In the preparation of PIPAAm-grafted polystyrene beads, surface-initiated ATRP can be a promising technique to restrict non-specific adsorption of bioactive compounds by densely grafting of PIPAAm [17]. Furthermore, chain length [18–20] and densities [21] of grafted polymer products can be regulated by varying the ATRP conditions. In the previous reports of thermo-responsive chromatography using silica beads as the base material, PIPAAm graft length and densities remarkably influence thermo-responsive hydrophobicity changes and the elution behavior of aqueous phase analytes, factors that can be modulated for the effective separation of bioactive compounds in water [16,22].

In the present study, we investigated polymer grafting of PIPAAm on porous polystyrene beads as the novel highly stable stationary phase for thermo-responsive chromatography. Dense PIPAAm brush-grafted polystyrene beads with varying graft amounts were prepared using surface-initiated ATRP. To investigate the appropriate PIPAAm grafting condition for thermo-responsive chromatography stationary phases, the prepared beads were evaluated by chromatographic analysis using standard pullulans and peptides.

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. Poly(styrene-divinylbenzene) beads (MCI GEL, CHP5C, average diameter 10 μm; pore size 250 Å; specific surface area, 600 m^2/g) were obtained from Mitsubishi Chemical Corporation (Tokyo). Chloromethyl methyl ether (CME) and dioxane were purchased from Wako Pure Chemicals Industries, Co. Ltd. (Osaka). Zinc(II) chloride in diethyl ether (1 M $ZnCl_2$ solution) was purchased from Sigma Chemicals (St. Louis, MO, USA). Tris(2-aminoethyl)amine was obtained from Acros Organics (Pittsburgh, PA). Formaldehyde, formic acid, sodium hydroxide, chloroform, and anhydrous magnesium sulfate were purchased from Wako Chemicals. Copper(I) chloride (CuCl), copper(II) chloride ($CuCl_2$), ethylendiamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt dehydrate (EDTA·2Na), dehydrated 2-propanol, methanol, and acetone were obtained from Wako Chemicals. Phosphate buffer powder (1/15 mol/l, pH 7.0) was purchased from Wako Chemicals. Insulin chain A (oxidized, ammonium salt) from bovine insulin, insulin chain B (oxidized) from bovine insulin, and insulin from bovine pancreas were obtained from Sigma. Glucose was purchased from Wako Chemicals. Standard pullulans (M_w 1300–788,000) were obtained from Showa Denko K.K. (Tokyo).

2.2. Preparation of ATRP initiator-immobilized polystyrene beads

Chloromethylated polystyrene beads, immobilized ATRP-initiating group, were prepared by Friedel-Crafts reaction as shown in Fig. 1(a) [23]. Polystyrene beads (5.0 g) were placed into a cleaned three-neck flask, followed by the addition of predetermined amount of CME at 0 °C under nitrogen atmosphere. We treated CME use with a protective face mask to prevent inhalation since CME is a known carcinogen and via inhalation can cause pneumonia or lung cancer. This suspension was stirred at 0 °C for 2 h to swell beads, followed by the addition of $ZnCl_2$. The reaction then proceeded at 30 or 40 °C under continuous stirring for predetermined times. The reaction mixture gradually became a red color. Dioxane was then added to bleach the red color from the reaction mixture. Chloromethylated beads were filtered and rinsed repeatedly with dioxane and acetone, then dried at 50 °C for 3 h under vacuum. ATRP initiator-immobilized polystyrene beads are abbreviated as CM-*X* where *X* is the amount of modified chloromethyl units in μmol/m^2.

2.3. Preparation of PIPAAm brush grafts on porous polystyrene beads

PIPAAm brush-grafted polystyrene beads were prepared by surface-initiated ATRP on initiator-immobilized polystyrene beads in 2-propanol as shown in Fig. 1 (b). Tris(2-(dimethylamino)ethyl)amine (Me_6TREN) as an ATRP ligand was synthesized using a previously reported method [24]. IPAAm monomer was dissolved in dried 2-propanol to a set initial con-

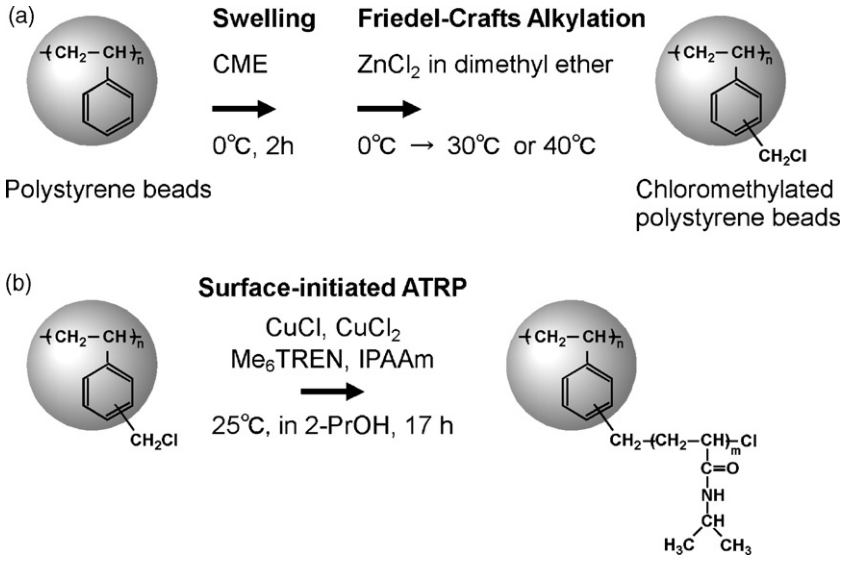

Fig. 1. Scheme for (a) the preparation of ATRP initiator-immobilized porous polystyrene beads by Friedel-Crafts reaction, and (b) the preparation of PIPAAm brush-grafted porous polystyrene beads by surface-initiated ATRP.

centration ($[IPAAm]_0$) at 1 or 2 M, and deoxygenated with nitrogen gas bubbling for 1 h at 25 °C. Reagents CuCl (115.2 mg, 1.2 mmol), $CuCl_2$ (15.7 mg, 0.1 mmol), and Me_6TREN (298.6 mg, 1.3 mmol) were added under nitrogen atmosphere, and the solution was stirred for 15 min to form the $CuCl/CuCl_2/Me_6TREN$ catalytic system. ATRP initiator-immobilized beads (1.0 g) were placed into a clean dry glass vessel. Both the monomer solution 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. IPAAm monomer solution was then poured into the glass vessel containing the beads, and sealed under nitrogen. ATRP reactions proceeded for 17 h at 25 °C under continuous shaking on a shaker (SN-M40S, NISSIN, Tokyo). PIPAAm brush-grafted beads were filtered and rinsed repeatedly with methanol, 50 mM EDTA solution, Milli-Q water (prepared from an ultrapure water purification system, synthesis A10, Millipore, Billerica, MA), and acetone, consecutively, followed by thorough drying at 50 °C for 3 h under vacuum. PIPAAm brush-grafted polystyrene beads are abbreviated as IP-X-Y, where X is the amount of modified ATRP initiator in μmol/m^2, and Y is the amount of grafted PIPAAm in mg/m^2.

2.4. Characterization of ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene

Elemental analysis of ATRP initiator-immobilized polystyrene beads were performed by organic halogens and a sulfur analyzer (Yanako) and with the ion chromatography system ICA-2000 (TOA DKK, Tokyo). Immobilized ATRP initiator units ($-CH_2Cl$) on polystyrene beads (μmol/m^2) were calculated from the bulk chloride composition of initiator-immobilized polystyrene beads using the following equation:

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

where %Cl is the percent chloride as determined by elemental analysis, %Cl (calcd.) is the calculated weight percent of chloride in initiator unit, M is the formula weight of initiator unit (mol/g), and S is the specific surface area of the original polystyrene bead support in m^2/g (per manufacture's data, 600 m^2/g).

Elemental analyses of ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene beads were performed with a PE 2400 series II CHNS/O analyzer (PerkinElmer, Inc., Waltham, MA). Amount of grafted PIPAAm on polystyrene beads (mg/m^2) was calculated using the following equation:

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

where $\%N_p$ is the percent nitrogen increase over that of the initiator-immobilized beads as determined by elemental analysis, $\%N_p$ (calcd.) is the calculated weight percent of nitrogen in IPAAm, $\%N_i$ is the nitrogen composition of initiator-immobilized polystyrene beads as determined by elemental analysis, and $\%N_i$ (calcd.) is the calculated weight percent of nitrogen in initiator unit.

Surface morphology of osmium tetroxide (OsO_4)-stained ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene beads were observed using scanning electron microscopy (SEM) S-4300 (Hitachi, Tokyo) operated at 2.0 keV (8000×, 20,000×, 30,000×) and VE-9800 (Keyence, Osaka) operated at 1.0 keV (1500×). Transmission electron microscopy (TEM) observations of ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene beads were performed by H-7000B (Hitachi, Tokyo) using TEM grids (STEM 100 Cu grid, Okenshoji, Tokyo).

Table 1
M_w and 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	11,800	3.4
P-20	22,800	5.0
P-50	47,300	7.6
P-100	112,000	12.5
P-200	212,000	18.1
P-400	404,000	26.2
P-800	788,000	38.7
Insulin chain A	2530	
Insulin chain B	3496	
Insulin	5750	2.6 ± 1[c]
		9.7 ± 5[d]

[a] Manufacture's data.
[b] Calculated using the relation between the M_w and R_g of pullulan [26].
[c] R_g at 20 °C in 0.1 M NaCl aqueous Tris buffer (pH 7.5) [25].
[d] R_g at 40 °C in 0.1 M NaCl aqueous Tris buffer (pH 7.5) [25].

2.5. Temperature-modulated elution of peptides and standard pullulans

PIPAAm brush-grafted beads (IP-1.9-1.7, IP-3.0-1.8, IP-3.9-2.0, IP-1.9-2.4, IP-3.0-2.6, and IP-3.9-2.5) were packed into stainless steel columns (150 mm × 4.6 mm i.d.) from a slurry of beads suspended in water/methanol/2-propanol mixed solvents (1:1:1) using slurry-packing apparatus model 124A (Chemco, Osaka) and auto-pressure programmer model 124PP (Chemco) at 150 kg/cm^2 under flow. PIPAAm brush-grafted bead-packed columns were connected to an HPLC system ($DGU\text{-}20A_3$, LC-20AD, CBM-20A, SIL-20AC, RID-10A, and SPD-20A, Shimadzu Corporation, Kyoto) controlled by a personal computer with LC solution analysis software (Shimadzu) on Windows XP.

Insulin chain A, insulin chain B, and insulin were dissolved in pH 7.0 phosphate buffer solution (PBS, 66.7 mM). Sample concentrations were 0.2 mg/mL for insulin chain A, 0.4 mg/mL for insulin chain B, and 1.0 mg/mL for insulin. Insulin chain A, insulin chain B, and insulin solutions were mixed together to separate and produce chromatograms. Molecular weights (M_w) of insulin chain A and insulin chain B, M_w and radius of gyrations (R_g) of insulin [25] are shown in Table 1. PBS (pH 7.0, 66.7 mM) was used as the mobile phase. Thermo-responsive elution of peptides was monitored by UV detector at 210 nm 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).

To investigate diffusion of analyte into pores, IP-1.9-1.7, IP-3.0-1.8, and IP-3.9-2.0 packed columns were calibrated with glucose and standard pullulans at 10–50 °C. Glucose and standard pullulans were dissolved in PBS at a concentration of 0.5 mg/mL. M_w and R_g of glucose and standard pullulans are shown in Table 1. R_g was calculated according to the following equation [26]:

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

Thermo-responsive elution behavior for glucose and standard pullulans was monitored by RI detector 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) and a thermostated water bath (Thermomeca NT-202D, NISSIN).

3. Results and discussion

3.1. Characterization of ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene

ATRP initiator-immobilized polystyrene beads were characterized by Cl elemental analyses. Chlorine compositions and amount of immobilized $-CH_2Cl$ units on polystyrene beads were summarized in Table 2. Amount of immobilized-CH_2Cl units increased with increasing amount of CME, reaction time, and reaction temperature. These results indicate that amounts of immobilized ATRP initiator on polystyrene beads are modulated by changing Friedel-Crafts reaction conditions. Elemental analyses of CHN were performed on ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene beads, and these data were summarized in Table 3. Amount of grafted PIPAAm tended to increase with increasing initial monomer concentration ($[IPAAm]_0$). A previous report indicated that polymerization rate and grafted polymer length increased with increasing initial monomer concentration [20]. Thus, the PIPAAm graft length on polystyrene would be increased with increasing IPAAm initial concentration.

To observe the surface morphology of the prepared beads, SEM and TEM observations were performed. SEM images of ATRP initiator-immobilized (CM-1.9) and PIPAAm brush-grafted (IP-1.9-1.7) beads are shown in Fig. 2. These SEM images indicated that PIPAAm grafting by ATRP provided a homogeneous coating on polystyrene bead surfaces. Additionally, SEM images measured at 1500× and 8000× magnifications showed monodispersity of CM-1.9 and IP-1.9-1.7 (Fig. S.1, as supplementary material available online). TEM images suggested that polystyrene beads surface was modified by grafted PIPAAm (Fig. S.2).

Additionally, in order to confirm PIPAAm-grafted beads utility as a stationary phase in aqueous chromatography, dispersibility of these beads in water was monitored. IP-1.9-1.7 uniformly dispersed in water at 25 °C, while CM-1.9 beads aggregated (appearance of dispersed IP-1.9-1.7 beads in water at 25 °C is shown in Fig. S.3). This indicated that grafted chains of PIPAAm impart the required hydrophilicity to polystyrene beads surfaces for applications as stationary phases for aqueous chromatography.

3.2. Temperature-modulated elution of peptides

To investigate the influence of PIPAAm graft amounts on temperature-dependent surface property alterations of the modified bead surfaces, we observed the elution behavior of insulin from PIPAAm-grafted bead-packed columns at 10 and 50 °C. PBS (pH 7.0, 66.7 mM) was used as the mobile phase. Chromatograms for insulin are shown in Fig. 3. Retention times for insulin on IP-1.9-1.7, IP-3.0-1.8, and IP-3.9-2.0 increased with increasing column temperature, explained by hydrophobically driven partitioning between dehydrated PIPAAm chains and insulin. Retention times for insulin at 10 and 50 °C on IP-1.9-2.4, IP-3.0-2.6, and IP-3.9-2.5 were unchanged. In addition, retention times for insulin at 50 °C increased with decreasing amounts of grafted PIPAAm for beads having 1.7–2.0 mg/m^2 densities of PIPAAm, though there were no differences for beads having more than 2.4 mg/m^2 PIPAAm densities. These results suggested that interactions between PIPAAm-grafted beads and insulin can be controlled by the optimization of the amounts of grafted PIPAAm on beads.

Furthermore, we observed thermo-responsive elution behavior for mixtures of insulin chain A, insulin chain B, and insulin from IP-1.9-1.7, IP-3.0-1.8 and IP-3.9-2.0 columns, respectively. Unmodified polystyrene beads-packed columns were used for comparison. Chromatograms of insulin and its fragments are shown in Fig. 4, and recovery rates, estimated by ratio of peak area of chromatograms, are summarized in Table 4. Recovery rates of

Table 2
Amount of modified $-CH_2-Cl$ on polystyrene beads by Friedel-Crafts reaction.

Sample	Reaction conditions				Chloride composition [%][a]	Amount of immobilized ATRP initiator unit ($-CH_2-Cl$) [μmol/m^2]
	CME [mL]	$ZnCl_2$ [μmol]	Reaction time [h]	Temperature [°C]		
CM-1.9	30	200	6	30	3.9	1.94
CM-2.2	30	200	12	30	4.3	2.15
CM-3.0	30	200	24	40	5.9	3.03
CM-3.5	30	200	48	40	6.8	3.54
CM-3.9	60	400	24	40	7.5	3.94

[a] Measured by elemental analysis of halogen. Data from two separate experiments.

Table 3
Elemental analyses of CHN for ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene beads.

Sample	$[IPAAm]_0$ [M]	2-Propanol [mL]	Elemental composition [%][a]			Amount of grafted PIPAAm [mg/m^2]
			C	H	N	
CM-1.9	–	–	80.67 ± 3.30	7.73 ± 0.32	0.65 ± 0.05	–
CM-2.2	–	–	79.40 ± 4.00	7.72 ± 0.47	0.65 ± 0.04	–
CM-3.0	–	–	80.63 ± 2.90	7.78 ± 0.32	0.59 ± 0.04	–
CM-3.5	–	–	78.76 ± 2.27	7.53 ± 0.21	0.59 ± 0.01	–
CM-3.9	–	–	81.45 ± 0.38	7.77 ± 0.04	0.60 ± 0.01	–
IP-1.9-1.7	1.0	58.2	73.48 ± 0.14	7.17 ± 0.21	6.97 ± 0.03	1.72 ± 0.04
IP-2.2-1.8	1.0	64.5	73.13 ± 0.03	7.16 ± 0.02	7.06 ± 0.02	1.79 ± 0.01
IP-3.0-1.8	1.0	90.9	72.47 ± 0.15	8.83 ± 0.48	7.02 ± 0.01	1.80 ± 0.01
IP-3.5-1.9	1.0	106.2	71.63 ± 0.11	8.75 ± 0.18	7.25 ± 0.09	1.94 ± 0.06
IP-3.9-2.0	1.0	118.2	71.51 ± 0.11	8.51 ± 0.15	7.3 ± 0.05	1.97 ± 0.03
IP-1.9-2.4	2.0	58.2	71.81 ± 0.04	7.33 ± 0.01	8.00 ± 0.02	2.43 ± 0.02
IP-2.2-2.4	2.0	64.5	71.36 ± 0.06	8.29 ± 0.11	8.00 ± 0.04	2.44 ± 0.03
IP-3.0-2.6	2.0	90.9	70.54 ± 0.11	9.12 ± 0.09	8.10 ± 0.04	2.57 ± 0.04
IP-3.5-2.4	2.0	106.2	70.80 ± 0.07	8.59 ± 0.15	7.93 ± 0.03	2.43 ± 0.02
IP-3.9-2.5	2.0	118.2	73.90 ± 3.63	9.32 ± 1.54	8.07 ± 0.29	2.54 ± 0.25

[a] Measured by elemental analysis of CHN. Data from three separate experiments, expressed as mean ± SD.

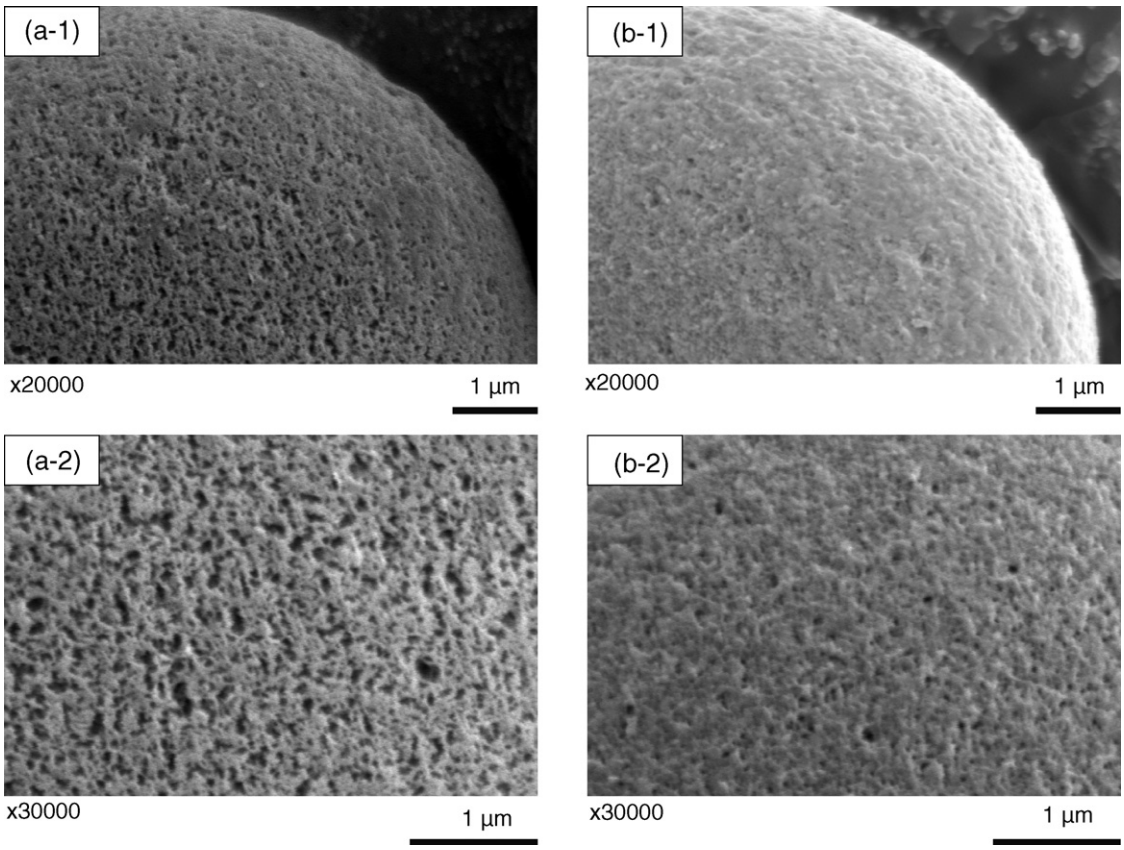

Fig. 2. SEM images of (a-1) (a-2) CM-1.9, and (b-1) (b-2) IP-1.9-1.7. Measured at 2.0 keV.

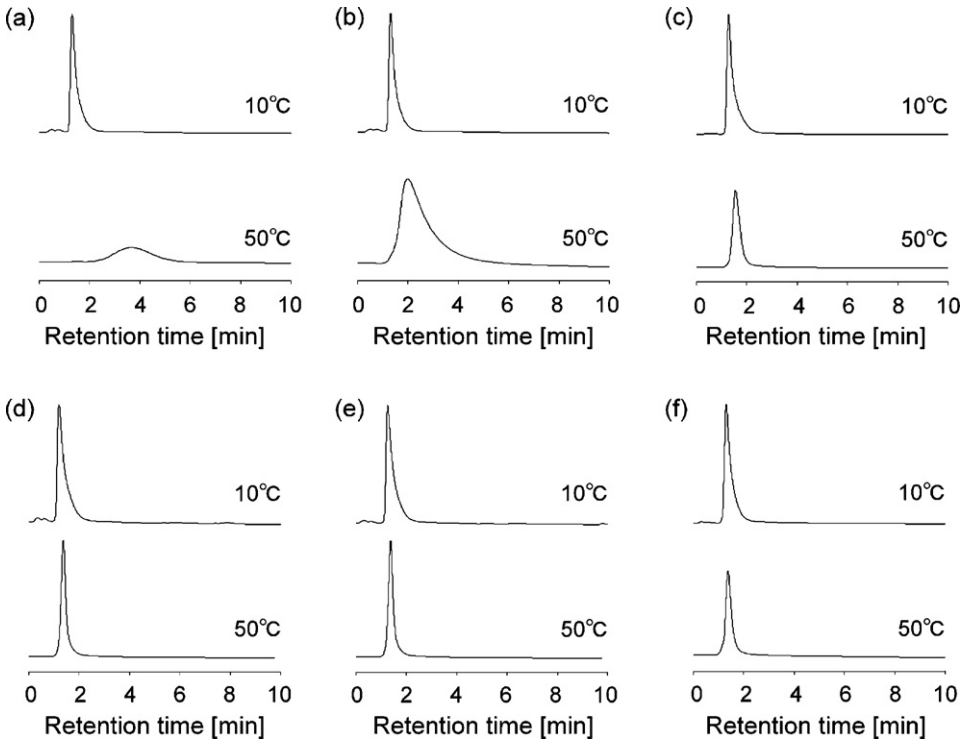

Fig. 3. Chromatograms of insulin on PIPAAm brush-grafted polystyrene beads at 10 and 50 °C: (a) IP-1.9-1.7, (b) IP-3.0-1.8, (c) IP-3.9-2.0, (d) IP-1.9-2.4, (e) IP-3.0-2.6, and (f) IP-3.9-2.5. Mobile phase is 66.7 mM phosphate buffer (pH 7.0). Insulin was monitored by UV detection at 210 nm with a flow rate of 1.0 mL/min PBS.

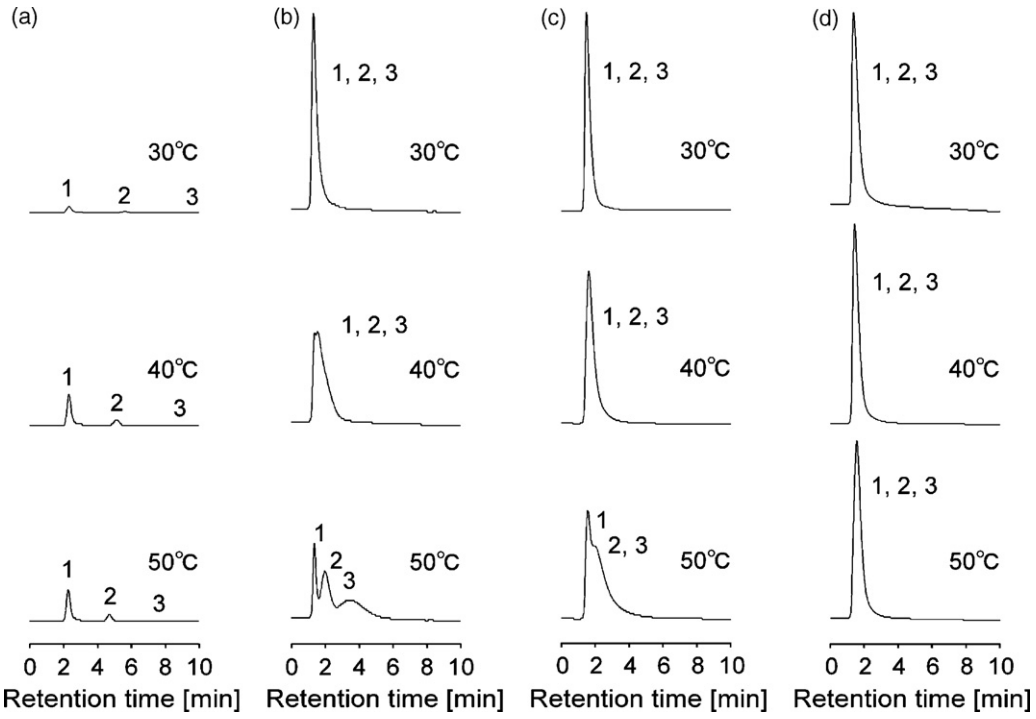

Fig. 4. Chromatograms of insulin chain A, insulin chain B, and insulin mixed solutions in PBS eluted through (a) control ungrafted polystyrene beads, (b) IP-1.9-1.7, (c) IP-3.0-1.8, and (d) IP-3.9-2.0 at different temperatures. Mobile phase is 66.7 mM phosphate buffer solution (PBS) (pH 7.0). Peptides were monitored by UV detection at 210 nm with a flow rate of 1.0 mL/min PBS. Peaks: 1, insulin chain A; 2, insulin chain B; and 3, insulin.

insulin and its fragments from PIPAAm brush-grafted beads were 93–99% regardless of grafting condition as well as temperature, while values for naked polystyrene beads were 6–8%. These results suggest that PIPAAm brush-grafted beads prevented non-specific adsorption compared to unmodified polystyrene beads, and the PIPAAm brush layer homogeneously coated the polystyrene bead surface via surface-initiated ATRP. Moreover, insulin and its fragments were effectively separated using IP-1.9-1.7 as the stationary phase at 50 °C. These results suggested that hydrophobicity of grafted PIPAAm brushes on IP-1.9-1.7 increased with increasing temperature, and the insulin separation from its fragments scaled with this hydrophobicity, represented by the Σf values of insulin chain A, insulin chain B, and insulin (11.0, 24.1, and 32.8, respectively) [27]. Insulin and its fragments were also separated using unmodified polystyrene stationary phases; however, peak area was remarkably small compared to the IP-1.9-1.7 column, indicating non-specific analyte adsorption onto the untreated polystyrene surface. Thus, homogeneous grafting of PIPAAm onto polystyrene permits the efficient aqueous separation of insulin and its fragments at 50 °C.

Table 4
Recovery rates of insulin chain A, insulin chain B, and insulin mixed solutions using PIPAAm brush-grafted beads as stationary phases at 30, 40, and 50 °C ($n = 3$).

Stationary phases	Recovery rates [%][a]		
	30 °C	40 °C	50 °C
Polystyrene	6.9 ± 5.9	7.8 ± 7.2	7.9 ± 7.2
IP-1.9-1.7	98.5 ± 0.4	98.6 ± 0.3	99.1 ± 2.5
IP-3.0-1.8	96.7 ± 2.9	98.1 ± 3.1	98.6 ± 3.8
IP-3.9-2.0	93.1 ± 2.6	96.1 ± 4.4	96.4 ± 5.3

[a] Calculated from the peak areas of eluted insulin. The control values of peak area were measured without connecting columns. Data from three separate experiments, expressed as mean ± SD.

3.3. Peptide retention mechanism by PIPAAm brush-grafted polystyrene beads

To confirm the diffusion of analytes into pores of PIPAAm-grafted beads, thermo-responsive elution behavior of glucose and standard pullulans for PIPAAm brush-grafted bead columns were used to obtain calibration curves shown in Fig. 5. To observe influences of polystyrene bead porosity, calibration curves from unmodified polystyrene beads were compared as control data. Retention volume (V_r) values for the PIPAAm brush-grafted beads are smaller than those for the unmodified beads (26–40%). The V_r values increase with increasing temperature, explained by dehydration and collapse of PIPAAm brushes on polystyrene bead surfaces. By contrast, the calibration curve for IP-3.9-2.0 is almost straight, since grafted PIPAAm chains hinder analyte diffusion into pores. Moreover, the width of the calibration curve for IP-3.9-2.0 increases with decreasing temperature, explained by analyte diffusion into expanded PIPAAm chains. Inflection points are observed in calibration curves for IP-1.9-1.7 at 4.7×10^4 in M_w (radius of gyration; R_g: 7.6 nm) at 40–50 °C, and at 5.9×10^3 in M_w (R_g: 2.3 nm) at 10–30 °C, both different than that from unmodified polystyrene standard curves. These two inflection points are attributed to analyte diffusion into bead pores. Analytes with smaller molecular weights than the inflection points tend to diffuse into pores and partitioned into PIPAAm brush layers. Additionally, at low temperature regions, effective pore size was reduced due to hydrated and expanded PIPAAm chains, and inflection points then decreased in these low temperature regions. Therefore, analytes smaller than 7.6 nm in R_g at 40–50 °C or 2.3 nm in R_g at 10–30 °C diffused into pores and partitioned into IP-1.9-1.7 PIPAAm brush layers. On the contrary, in case of IP-3.9-2.0, we assume that analytes did not tend to diffuse into pores, but they partitioned into PIPAAm brush layers at low temperature.

As shown in Fig. 3, a large amount of PIPAAm-grafted beads, prepared with high initial monomer concentration in ATRP, did

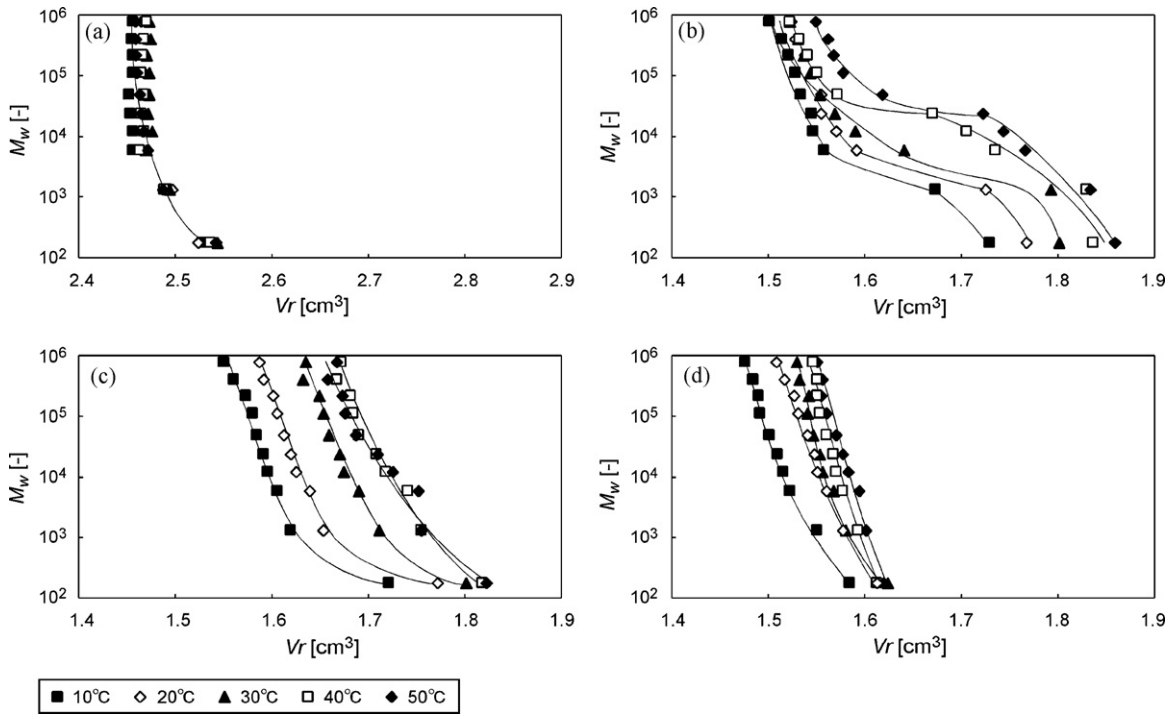

Fig. 5. Plots of M_w versus V_r for glucose and standard pullulans eluted through (a) polystyrene control beads, (b) IP-1.9-1.7, (c) IP-3.0-1.8, and (d) IP-3.9-2.0. Mobile phase is 66.7 mM phosphate buffer solution (PBS) (pH 7.0). Glucose and standard pullulans were monitored by RI detection with a flow rate of 1.0 mL/min PBS. Symbols: closed squares, 10 °C; open diamonds, 20 °C; closed triangles; 30 °C; open squares, 40 °C; closed diamonds, 50 °C.

not interact with insulin, whereas insulin elution was retarded on PIPAAm-grafted beads with relatively low grafting amounts at 50 °C. We assumed that insulin retention was attributed to analyte diffusion into pores and interactions with grafted PIPAAm brushes inside the pores. The R_g for insulin at 40 °C is about 10 nm [25], which is a similar size at the inflection points (R_g: 7.6 nm) of the calibration curves of IP-1.9-1.7 at 40–50 °C observed. Insulin diffused into pores of PIPAAm brush-grafted polystyrene, leading to increases in the effective surface area for hydrophobic interaction with dehydrated PIPAAm (Schematically drawn in Fig. S.4.). At 10 °C, all of PIPAAm brush-grafted beads did not interact with insulin because grafted PIPAAm brushes were hydrated, and hydrophobic interaction between the PIPAAm brushes and insulin was weak, although insulin (R_g: 2.6 nm at 20 °C [25]) can diffuse into pores of IP-1.9-1.7 (R_g of inflection points at 10–30 °C: 2.3 nm) (Fig. S.4). Thus, grafting appropriate amounts of PIPAAm onto polystyrene beads is important to allow analyte diffusion into pores for the effective separation of insulin and its fragments.

These results demonstrated that PIPAAm brush-grafted polystyrene beads, prepared by grafting PIPAAm, permitted analyte diffusion into pores to impart increased column retention to effectively separate peptides in aqueous milieu at elevated temperatures. This analyte-surface partitioning is mediated by strong hydrophobic interactions from dehydrated PIPAAm inside stationary phase pores. Control of PIPAAm graft amounts and densities on porous polystyrene beads by ATRP is a key factor in facilitating separation of these peptides in aqueous HPLC.

4. Conclusions

Porous polystyrene beads grafted with PIPAAm brush prepared by surface-initiated ATRP exhibit strong thermo-responsive hydrophobic interactions with peptides as stationary phases in aqueous chromatographic analysis. Amounts of immobilized ATRP initiator and grafted PIPAAm on polystyrene surfaces were modulated by changing Friedel-Crafts reaction conditions and initial IPAAm feed concentration in grafting. Thermally induced separation of insulin chain A, insulin chain B, and insulin is achieved using PIPAAm brush-grafted polystyrene beads with optimized amounts of PIPAAm grafts. Calibration curves obtained using glucose and standard pullulans indicate that analytes diffuse into pores of low density PIPAAm-grafted beads. These results suggest that an important factor for effective peptide separation is the PIPAAm-grafted surface area, leading to large hydrophobic partitioning interactions with analyte.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2009.11.073.

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