Journal of Chromatography B, 878 (2010) 2191–2198

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Effective separation of peptides using highly dense thermo-responsive polymer brush-grafted porous polystyrene beads

Aya Mizutani[a,b], 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

ARTICLE INFO

Article history:
Received 20 January 2010
Accepted 20 June 2010
Available online 1 July 2010

Keywords:
Thermo-responsive polymer
Poly(*N*-isopropylacrylamide)
Atom transfer radical polymerization
Polymer brush
Poly(styrene-*co*-divinylbenzene)
Thermo-responsive chromatography
Peptide

ABSTRACT

For the development of well-defined highly dense thermo-responsive polymer grafted surface as an improved stationary phase for thermo-responsive chromatography, poly(*N*-isopropylacrylamide) (PIPAAm) brush-grafted porous polystyrene beads were prepared by surface-initiated atom transfer radical polymerization (ATRP). The PIPAAm grafted region of polystyrene beads was adjusted by the addition of isooctane as a poor solvent for polystyrene upon the reaction of ATRP initiator immobilization. Using a thermo-responsive HPLC column containing the prepared beads with PIPAAm brush grafted on the inside pores nearby the outer surfaces, angiotensin subtypes were effectively separated with aqueous mobile phase, because the densely grafted PIPAAm on nearby the outer surface effectively interacted with the peptides hydrophobically. Retention of basic peptide was achieved by the beads with basic mobile phase. These results indicated that the prepared beads with grafted PIPAAm nearby the outer surface became an effective chromatographic stationary phase for retaining basic peptides using wide pH range of mobile phase.

1. Introduction

Since efficient biomedical separations that can purify useful peptides and proteins are constantly demanded, various separation methods have been developed and used for purifying them. Among these, an attractive column separation method in all-aqueous system is a method using a column containing base materials covered with a thermo-responsive polymer, poly(*N*-isopropylacrylamide) (PIPAAm) which is well known to exhibit thermo-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 a reversible hydration/dehydration property of its isopropyl side chains, which hydrate themselves and expand their chains in water below the LCST, and dehydrate themselves, become compact, and insoluble above the LCST [1,2]. Since the LCST is near human body temperature, the polymer is widely used in biomedical applications such as controlled drug and gene delivery systems [3,4], enzyme bioconjugates [5,6], affinity precipitation [7], microfluidics [8,9], cell culture substrates [10,11], and tissue engineering for regenerative medicine [12–15]. Furthermore, thermo-responsive chromatography using PIPAAm grafted silica beads as a stationary phase has been developed for a thermally induced separation for bioactive compounds in aqueous mobile phase without organic phase [16–19]. This system is highly useful to control both the stationary phase function and properties for high performance liquid chromatography (HPLC) by changing only column temperature, with the advantages of preserving the biological activities of peptides and proteins and eliminating the use of organic mobile phases commonly used in reversed-phase chromatography for reducing environmental load.

Silica beads are instable under high pH condition, and the instability always limits the application and requires neutral pH mobile phases [20]. Even though silica-based stationary phases are used in neutral condition, the reproducibility of separation decreases with repeated use due to the hydrolysis of silica surface. Thus, to improve the stability in the wide pH range of mobile phases, our laboratory has prepared PIPAAm brush surfaces using polystyrene beads, which show a high aqueous stability [21,22], as a starting material for surface-initiated atom transfer radical polymerization (ATRP) [23]. ATRP is an attractive polymer grafting method allowing the surface of beads to receive well-defined polymer brushes by surface immobilized ATRP initiators [18,24–26]. Control of PIPAAm

* Corresponding author. Tel.: +81 3 5367 9945x6201; fax: +81 3 3359 6046.
E-mail address: tokano@abmes.twmu.ac.jp (T. Okano).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.06.026

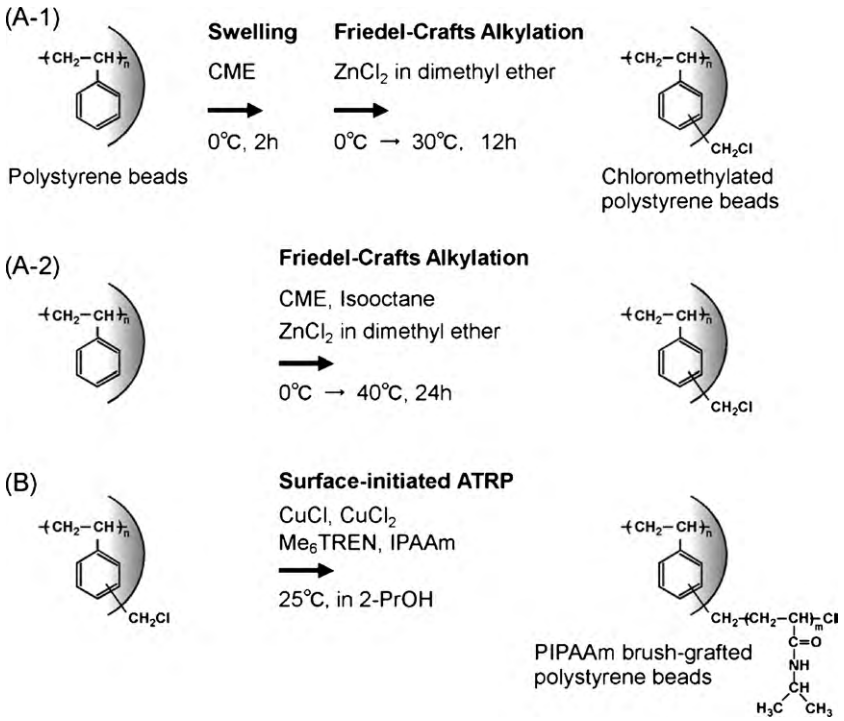

Fig. 1. (A-1 and A-2) Schemes for the preparation of atom transfer radical polymerization (ATRP) initiator-immobilized porous polystyrene beads by the Friedel–Crafts reaction using chloromethyl methyl ether (CME) (A-1) and a mixture solution of CME and isooctane (A-2) as reaction solvents. (B) Scheme for preparation of PIPAAm brush-grafted porous polystyrene beads by surface-initiated ATRP.

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 [23]. Moreover, the thermally induced separation of insulin and its fragments is achieved at 50 °C using PIPAAm brush-grafted porous polystyrene beads with the proper amounts of PIPAAm grafts [23]. However, the separation at high temperature such as 50 °C may deteriorate their biological activities. Therefore, further improved PIPAAm grafted stationary phase, which can separate bioactive compounds at lower temperature, is desired for broader applications. To prepare more effective stationary phase, our research group has paid attention to following two points; (1) the effective immobilization of ATRP initiator on the outer surface of porous polystyrene, and (2) the use of basic buffer solution as a mobile phase for enhancing the hydrophobic interaction between PIPAAm and analytes.

Since chloromethyl methyl ether (CME) is a good solvent for polystyrene, can swell porous polystyrene beads well [27], widen the pores, and allow ATRP initiator to penetrate into the bottoms of pores, CME was selected upon the immobilization of ATRP initiator for obtaining the high densely immobilization of ATRP. However, this initiator-immobilization procedure is unsuitable, because the inner part of beads is hardly used for interaction with analytes [28]. When ATRP initiator can be immobilized selectively the outer surface regions of beads, the grafting of PIPAAm is speculated to be localized to the limited regions, giving a highly effective retention. Moreover, the use of basic buffer solution as a mobile phase can provide a strong hydrophobic interaction, because an ionization of basic analytes is suppressed, resulting in enhancing their hydrophobicity and offering an effective retention on the stationary phase.

In this study, PIPAAm brush-grafted polystyrene beads were prepared by surface-initiated ATRP through the grafting of PIPAAm on the outer surface regions of beads. The prepared beads were evaluated in thermo-responsive aqueous separation and the retention of basic peptides using basic buffer solution.

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-*co*-divinylbenzene) beads (CHP5C of MCI GEL) (the 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), isooctane, and dioxane were purchased from Wako Pure Chemicals Industries (Osaka).

Table 1
Amount of immobilized $-CH_2Cl$ of polystyrene beads by the Friedel–Crafts reaction.

Sample[a]	Reaction conditions				Chloride composition (%)	Amount of immobilized ATRP initiator moieties ($-CH_2Cl$) (μmol/m^2)[b]
	Beads (g)	CME (mL)	Isooctane (mL)	$ZnCl_2$ (μmol)		
CM-2.2	5.0	30	0	200	4.3	2.15
CMIS-2.4	5.0	120	120	200	4.7	2.36
CMIS-1.0	10.0	60	60	200	2.1	1.02

[a] The sample names, CM-*X* and CMIS-*X* where *X* is the amount of immobilized chloromethyl moieties in μmol/m^2.
[b] Determined by the elemental analyses of halogen. Data were obtained from two separate experiments.

Zinc(II) chloride in diethyl ether (1 mol/L $ZnCl_2$ 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. Premixed phosphate buffer powder (pH 7.0, 1/15 mol/L) was purchased from Wako Chemicals. Angiotensin I human acetate hydrate, angiotensin II human acetate hydrate, angiotensin III human acetate hydrate, and bradykinin acetate were obtained from Sigma. Uracil was purchased from Wako Chemicals. Water used in this study was purified by an ultrapure water purification system (Synthesis A10) (Millipore, Billerica, MA) unless otherwise mentioned.

2.2. Preparation of ATRP initiator-immobilized polystyrene beads: using CME as a reaction solvent

ATRP initiator-immobilized polystyrene beads were prepared by the Friedel–Crafts reaction as shown in Fig. 1(A-1) [27]. Polystyrene beads (5.0 g) were placed into a cleaned three-neck flask, followed by addition of 30 mL CME (a good solvent for polystyrene [27]) at 0 °C under a nitrogen atmosphere. CME can play two roles as an electrophile agent and a solvent for the Friedel–Crafts reaction. (*Caution*: Since CME is a known carcinogen and via inhalation can cause pneumonia or lung cancer, using CME always requires a protective face-mask to prevent inhalation.) This suspension was stirred at 0 °C for 2 h to swell the beads, followed by the addition of a 200 μmol of $ZnCl_2$. Friedel–Crafts reaction then proceeded at 30 °C under continuous stirring for 12 h. The reaction mixture gradually became red. Dioxane was then added to bleach the red color in the reaction mixture. Chloromethylated beads were filtered and rinsed repeatedly with dioxane and acetone, and then dried at 50 °C for 3 h under vacuum. ATRP initiator-immobilized polystyrene beads prepared using CME as a reaction solvent are abbreviated as CM-*X* where *X* is the amount of immobilized chloromethyl moieties in μmol/m^2.

2.3. Preparation of ATRP initiator-immobilized polystyrene beads: using a mixture solution of CME and isooctane as a reaction solvent

In order to immobilize ATRP initiator on the outer surface of polystyrene of which surface areas were mainly used for interacting with analyte [28], polystyrene beads were treated with a mixture of CME and isooctane (a poor solvent for polystyrene [29]) by the Friedel–Crafts reaction as shown in Fig. 1(A-2). Polystyrene beads were placed into a cleaned three-neck flask, followed by the addition of a mixture of CME and isooctane at 0 °C under nitrogen atmosphere. The use of isooctane in the solution could prevent the beads from swelling. $ZnCl_2$ was then added to this suspension and stirred at 0 °C, and the reaction proceeded at 40 °C under continuous stirring for 24 h. The reaction mixture gradually became red. Dioxane was then added to bleach the red color of the reaction mixture. After being filtered and rinsed repeatedly with dioxane and acetone, chloromethylated polystyrene beads were dried at 50 °C for 3 h under vacuum. The detailed conditions of the Friedel–Crafts reaction are shown in Table 1. ATRP initiator-immobilized polystyrene beads prepared by a mixture of CME and isooctane are abbreviated as CMIS-*X* where *X* is the amount of immobilized chloromethyl moieties in μmol/m^2.

Table 2
Elemental analyses of CHN for ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene beads.

Sample[a]	$[IPAAm]_0$ (mmol/L)	Reaction time of ATRP (h)	$[IPAAm]_0/[Immobilized\ initiator]_0/[CuCl]_0/[CuCl_2]_0/[Me_6TREN]_0$	Elemental composition (%)[b]			Amount of grafted PIPAAm (μg/m^2)	Conversion (%)[c]
				C	H	N		
CM-2.2	–	–	79.40 ± 4.00	7.72 ± 0.47	0.65 ± 0.04	–		
CMIS-2.4	–	–	81.17 ± 1.96	8.02 ± 0.17	0.13 ± 0.07	–		
CMIS-1.0	–	–	83.00 ± 0.28	8.92 ± 0.55	1.43 ± 0.04	–		
IP-2.2-0.8	250	17.0	25:1.3:4.0:0.4:4.4	75.82 ± 0.26	7.44 ± 0.21	4.54 ± 0.06	762.9 ± 0.02	16.2
IPIS-2.4-0.9	500	4.0	50:1.4:4.0:0.4:4.4	76.38 ± 0.03	7.76 ± 1.37	4.41 ± 0.42	881.9 ± 0.03	9.4
IPIS-1.0-0.5	200	17.0	20:0.6:4.0:0.4:4.4	76.96 ± 0.23	7.10 ± 0.46	4.69 ± 0.04	595.1 ± 0.01	15.8

[a] The sample names, CM-*X* and CMIS-*X* where *X* is the amount of immobilized chloromethyl moieties in μmol/m^2. The sample names, IP-*X*-*Y* and IPIS-*X*-*Y* where *X* is the amount of immobilized ATRP initiator in μmol/m^2, and *Y* is the amount of grafted PIPAAm in mg/m^2.
[b] Measured by the elemental analyses of CHN. Data were obtained from three separate experiments, expressed as mean ± SD.
[c] Calculated from the grafted PIPAAm amount and the fed monomer amount.

Table 3
Properties of peptides.

Analyte	Primary structure	Hydrophobicity (Σf)[a]	pI[b]	*M*w[b]
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	10.61	7.7	1297
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	8.85	7.75	1046
Angiotensin III	Arg-Val-Tyr-Ile-His-Pro-Phe	8.87	9.85	931
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Arg	8.35	12.5	1060

[a] Calculated using the relative lipophilicity values of amino acids [33].
[b] Data taken from reference [34].

2.4. Preparation of PIPAAm brush grafts on porous polystyrene beads

PIPAAm brush-grafted polystyrene beads were prepared by surface-initiated ATRP on the initiator-immobilized polystyrene beads in 2-propanol (Fig. 1(B)). Tris(2-(dimethylamino)ethyl)amine (Me_6TREN) as an ATRP ligand was synthesized by a previously reported method [11,30]. IPAAm monomer was dissolved in dried 2-propanol and deoxygenated by nitrogen gas bubbling for 1 h at 25 °C. Reagents CuCl (396.0 mg, 4.0 mmol), $CuCl_2$ (53.8 mg, 0.4 mmol), and Me_6TREN (1026.1 mg, 4.4 mmol) were added under a nitrogen atmosphere, and the solution was stirred for 15 min for forming $CuCl/CuCl_2/Me_6$TREN catalytic system. ATRP initiator-immobilized beads were placed into a 150-mL clean dry glassware. 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 for removing adventitious oxygen. IPAAm monomer solution was then poured into the glassware containing the beads, and the glassware was sealed under nitrogen. ATRP reactions proceeded for a specific time 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 mmol/L EDTA solution, water, and acetone, consecutively, followed by thorough drying at 50 °C for 3 h under vacuum. The reaction conditions for PIPAAm brush-grafted beads prepared by ATRP are shown in Table 2. PIPAAm brush-grafted polystyrene beads prepared using CME and CME/isooctane are abbreviated as IP-*X*-*Y* and IPIS-*X*-*Y*, respectively, where *X* is the amount of immobilized ATRP initiator in μmol/m^2, and *Y* is the amount of grafted PIPAAm in mg/m^2.

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

Elemental analyses of ATRP initiator-immobilized polystyrene beads were performed by an organic halogens and sulfur analyzer (Yanako, Kyoto) with an ion chromatography system (ICA-2000) (TOA DKK, Tokyo). Immobilized ATRP initiator moieties (–CH_2Cl) on polystyrene beads (μmol/m^2) were calculated from the bulk

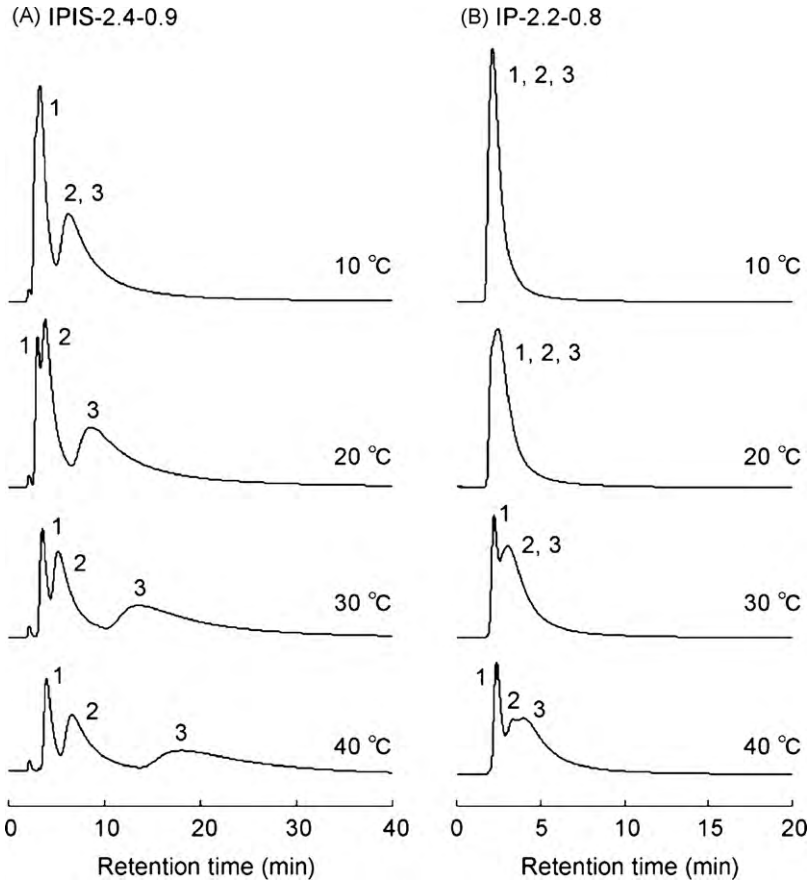

Fig. 2. Chromatograms of angiotensin I, angiotensin II, and angiotensin III in phosphate buffer (PB) eluted through (A) IPIS-2.4-0.9 and (B) IP-2.2-0.8 columns at various temperatures. Mobile phase is PB (pH 7.0, *I* = 0.15). The peptides were monitored at 210 nm with a flow rate of 1.0 mL/min. The peaks No.1, 2, and 3 represent angiotensin II, angiotensin III, and angiotensin I, respectively.

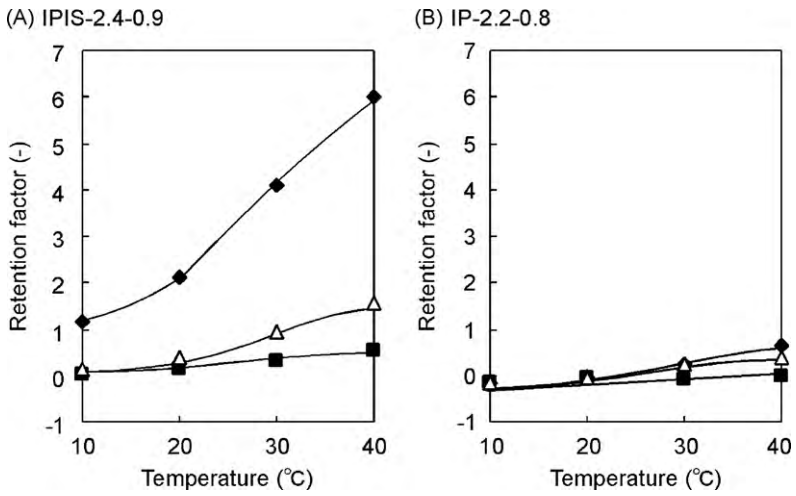

Fig. 3. Temperature-dependent changes in the retention factors of angiotensin subtypes on (A) IPIS-2.4-0.9 and (B) IP-2.2-0.8 columns. The closed diamonds, the open triangles, and the closed squares represent the retention factors of angiotensin I, angiotensin III, and angiotensin II, respectively.

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

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

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

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

2.6. Temperature-modulated elution of peptides

PIPAAm brush-grafted beads (IP-2.2-0.8, IPIS-2.4-0.9, and IPIS-1.0-0.5) were packed into stainless steel columns (4.6 mm i.d. × 150 mm) from their slurry suspend in water/methanol/2-propanol mixed solvents (1:1:1) by a slurry-packing apparatus (model 124A) (Chemco, Osaka) with an auto-pressure programmer (model 124PP) (Chemco) at 14.7 MPa. PIPAAm brush-grafted beads-packed columns were connected to an HPLC system (DGU-$20A_3$, LC-20AD, CBM-20A, SIL-20AC, and SPD-20A) (Shimadzu, Kyoto) controlled by a personal computer with LC solution analysis software (Shimadzu) on Windows XP. Angiotensin I, angiotensin II, and angiotensin III were dissolved in pH 7.0 phosphate buffer (PB) (I = 0.15). The sample concentrations were 0.5 mg/mL. Angiotensin I, angiotensin II, and angiotensin III were mixed and injected into the HPLC. PB (pH 7.0, I = 0.15) was used as a mobile phase. Bradykinin was dissolved in three different Miller and Golder buffer solution [31] (pH 7.0, 10.0, and 12.0) with the same ionic strength (I = 0.1). The sample concentrations were 0.5 mg mL^{-1}. Miller and Golder buffer solutions (pH 7.0, 10.0, and 12.0) (I = 0.1) were used as mobile phases. Thermo-responsive elution of peptides was monitored by a UV detector (SPD-20A) (Shimadzu) at 210 nm with a flow rate of 1.0 mL/min. Uracil was used for measuring the column void volume. 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).

3. Results and discussion

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

ATRP initiator-immobilized polystyrene beads were characterized by Cl elemental analyses. Chlorine composition and the

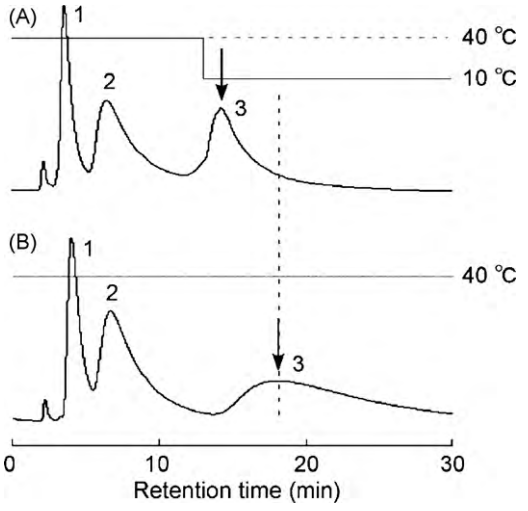

Fig. 4. Chromatograms of angiotensin I, angiotensin II, and angiotensin III in phosphate buffer (PB) eluted through IPIS-2.4-0.9 (A) at 40 °C (0–13 min) and 10 °C (13–30 min) (B) at 40 °C. Mobile phase is PB (pH 7.0, I = 0.15). Peptides were monitored at 210 nm with a flow rate of 1.0 mL/min. The peaks No. 1, 2, and 3 represent angiotensin II, angiotensin III, and angiotensin I, respectively.

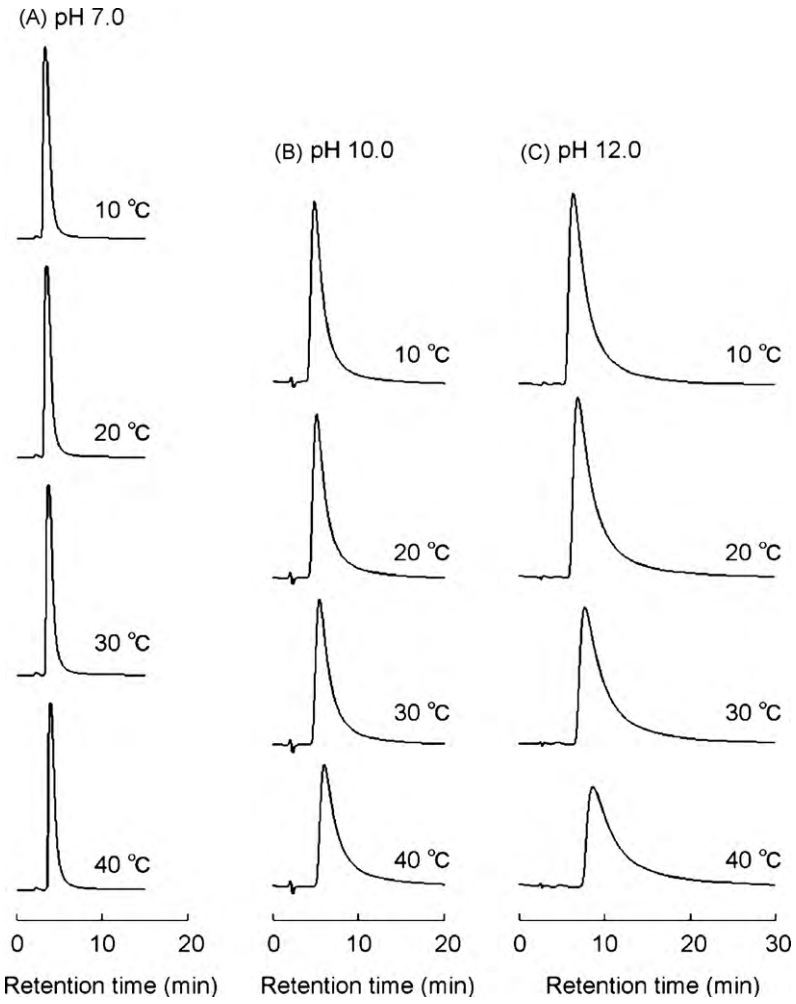

Fig. 5. Chromatograms of bradykinin in Miller and Golder buffer solution (A) pH 7.0, (B) pH 10.0, and (C) pH 12.0 eluted through IPIS-1.0-0.5 at various temperatures. Mobile phases are Miller and Golder buffer solutions (pH 7.0, 10.0, and 12.0) (I = 0.1). The peptide was monitored at 210 nm with a flow rate of 1.0 mL/min.

amount of immobilized $-CH_2Cl$ moieties of ATRP initiators on polystyrene beads were summarized in Table 1. In the immobilization of $-CH_2Cl$ on porous polystyrene beads, the amount of immobilized $-CH_2Cl$ increases with the increase in CME composition [23]. However, the immobilized $-CH_2Cl$ moieties of CM-2.2 and CMIS-2.4 were almost the same, although the CME amount for preparing CMIS-2.4 were larger than that of CM-2.2. This was due to the addition of isooctane upon the preparation of CMIS-2.4. CME (a good solvent for polystyrene [27]) and a mixture solution of CME and isooctane (a poor solvent for polystyrene [29]) were used as the reaction solvent for immobilizing ATRP initiator. Polystyrene beads cross-linked with divinylbenzene can swell in CME and shrink in isooctane. When isooctane was added to the reaction solvent of ATRP initiator immobilizing, the diameter of the pores of polystyrene beads was speculated to decrease, resulting in the suppression of ATRP-initiator diffusion into the pores [32]. Therefore, the immobilized $-CH_2Cl$ moieties of CMIS-2.4 were thought to exist on predominantly the outer surface of the beads.

Elemental analyses of CHN were performed on ATRP initiator-immobilized and PIPAAm brush-grafted polystyrene beads. CHN compositions and the amount of grafted PIPAAm on polystyrene beads were summarized in Table 2. Amount of grafted PIPAAm slightly increased with the increase in initial IPAAm concentration in ATRP. A previous report indicated that the polymerization rate and the grafted polymer length increase with increase in the initial monomer concentration [25]. Thus, PIPAAm graft length on polystyrene was speculated to increase with increasing IPAAm initial concentration.

3.2. Temperature-modulated elution of angiotensin subtypes

For investigating the influence of the hydrophobicity of analytes on thermo-responsive retention, three types of angiotensin subtypes were used as models, because they have almost the same molecular weight and different hydrophobicity (Table 3). Temperature-dependent elution profiles of a mixture of angiotensin I, angiotensin II, and angiotensin III on IP-2.2-0.8 and IPIS-2.4-0.9 packed columns were observed. PB (pH 7.0, I = 0.15) was used as a mobile phase. Chromatograms of angiotensin subtypes and the thermo-responsive retention factor changes are shown in Figs. 2 and 3, respectively. Retention times of angiotensin subtypes increased with their hydrophobicity, represented by Σf values in Table 3 [33]. This indicated that the retention of angiotensin subtypes was performed though the hydrophobic interaction between the stationary phase and the analytes. Additionally, the retention times of angiotensin subtypes increased with the increase in column temperatures, indicating the increase in hydrophobic interaction between the grafted PIPAAm and the angiotensin peptides. The grafted PIPAAm on the porous polystyrene beads surface dehydrated and increased its hydrophobicity with the increase in temperature, and the

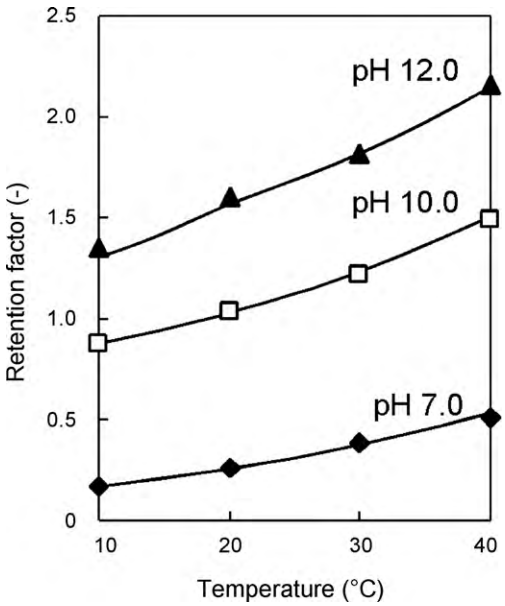

Fig. 6. Temperature-dependent changes in the retention factors of bradykinin on IPIS-1.0-0.5 column. The closed diamonds, the open squares, and the closed triangles represent the retention factors at pH 7.0, 10.0, and 12.0, respectively.

hydrophobicity of angiotensin subtypes at high temperatures gave a higher resolution to the chromatograms.

As shown in Fig. 2, the thermo-responsive retention profiles of the angiotensin subtypes were quite different between IP-2.2-0.8 and IPIS-2.4-0.9, although the amount of immobilized ATRP initiator and the amount of grafted PIPAAm of IP-2.2-0.8 and IP-2.4-0.9 were almost the same. IPIS-2.4-0.9 beads-packed column separated angiotensin subtypes at high temperatures (Fig. 2 (A)), while angiotensin subtypes were unable to be separated on IP-2.2-0.8 even at 40 °C (Fig. 2 (B)). These different elution profiles were probably due to the grafted PIPAAms' characteristic differences that were caused by the conditions of Friedel–Crafts reaction for immobilizing an ATRP initiator. As mentioned in Section 3.1, the preparation of initiator-immobilized beads using a good or poor solvent for polystyrene could influence the immobilized region of ATRP initiator on the porous polystyrene surface. On CMIS-2.4, ATRP initiator was speculated to be immobilized on mainly the outer surface on porous polystyrene, resulting in the restricted graft of PIPAAm on only the outer surface. On the contrary, on CM-2.2, ATRP initiator was immobilized both on the outer surface and the inside pores, leading to a homogenous grafting of PIPAAm on both the inside and the outside pores. Thus, PIPAAm graft density on the outer surface of IPIS-2.4-0.9 was higher than that on IP-2.2-0.8, resulting in a stronger hydrophobic interaction with the angiotensin peptides.

Moreover, the effect of a step-temperature gradient on the separation by changing column temperature was also investigated with the angiotensin subtypes on IPIS-2.4-0.9 (Fig. 4). After the elution of angiotensin II and angiotensin III through a strong hydrophobic interaction at 40 °C, the column temperature was reduced to 10 °C by immersing the column in the thermostated water bath set at 10 °C. The retention time of angiotensin I shortened with its narrower peak width (Fig. 4(A)) compared to that monitored at 40 °C (Fig. 4(B)). The results indicated that the hydrophobic interaction between angiotensin I and IPIS-2.4-0.9 immediately decreased after decreasing column temperature because of the hydrophobic/hydrophilic alteration of the prepared beads surface. Therefore, IPIS-2.4-0.9 has an effective separation surface, rapidly altering their hydrophobic properties solely with temperature.

3.3. Temperature and pH-dependent retention of basic peptides

To investigate the retention profile of basic peptide on the prepared beads using basic aqueous solution as a mobile phase, the temperature-dependent elution profiles of bradykinin on IPIS-1.0-0.5 packed column were monitored at 10–40 °C. Since a hydrophobic interaction between the analytes and PIPAAm brush-grafted polystyrene beads increase with decreasing the amount of grafted PIPAAm [23], IPIS-1.0-0.5, which has the least amount of grafted PIPAAm, was used as a stationary phase. Miller and Golder buffer solutions (pH 7.0, 10.0, and 12.0) (I = 0.1) were used as mobile phases. Chromatograms of bradykinin and their thermo-responsive retention factor changes are shown in Figs. 5 and 6, respectively. Fig. 6 shows that the retention factors of bradykinin increased with increasing pH, because the hydrophobicity of bradykinin increased with increasing pH close to its pI 12.5 (Table 3) [34]. In general, it is difficult to retain basic analytes by reversed-phase chromatography with isocratic elution, because silica beads as a base material are instable at high pH condition [20]. Moreover, upon the use of conventional polymer beads as a base material, the gradient elution of organic solvent must be able to work for retaining basic peptides [35,36]. On the contrary, since the prepared PIPAAm brush-grafted polystyrene beads are stable in basic condition, basic buffer solution can be used as a mobile phase. In basic solution, the ionization of basic compounds was suppressed, expecting in a stronger hydrophobic interaction between stationary phase and analytes. Moreover, the strong hydrophobic interaction can be modulated only changing column temperature without organic solvents. Therefore, the prepared PIPAAm porous polystyrene beads are an effective stationary phase, which can retain basic bio-molecules with basic mobile phase.

4. Conclusions

PIPAAm brush-grafted porous polystyrene beads were prepared by the effectively grafting of PIPAAm by surface-initiated ATRP for obtaining improved thermo-responsive chromatography matrices. ATRP initiator is effectively immobilized only on the outer surface of porous polystyrene beads using isooctane as a poor solvent in the reaction. Using the prepared beads, angiotensin subtypes were effectively separated, because densely grafted PIPAAm on the outer bead surface effectively interacted with the peptides hydrophobically. In addition, bradykinin, a basic peptide was strongly retained by the prepared beads with a basic aqueous mobile phase (pH 10.0 and 12.0), because the ionization of bradykinin was suppressed, resulting in the formation of its stronger hydrophobicity. Therefore, the prepared PIPAAm grafted porous polystyrene beads can give an effective stationary phase for separating peptides with an all-aqueous mobile phase (pH 7.0–12.0) without the use of organic solvent, unfriendly material for environment.

Acknowledgments

Present research was supported in part 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, and Grants-in-Aid for Scientific Research (B) No. 19591568 from the Japan Society for the Promotion of Science. The authors are grateful to Dr. Norio Ueno, Tokyo Women's Medical University for English editing.

References

[1] Y.H. Bae, T. Okano, S.W. Kim, J. Polym. Sci. Part B, Polym. Phys. 28 (1990) 923–936.
[2] M. Heskins, J.E. Guillet, E. James, J. Macromol. Sci. Chem. A 2 (1968) 1441–1445.

[3] S. Cammas, K. Suzuki, C. Sone, Y. Sakurai, K. Kataoka, T. Okano, J. Controlled Release 48 (1997) 157–164.
[4] C. Alvarez-Lorenzo, A. Concheiro, J. Chromatogr. B 804 (2004) 231–245.
[5] Q.Z. Zhu, F. Liu, D.H. Li, J.G. Xu, W. Su, J. Huang, Anal. Chim. Acta 375 (1998) 177–185.
[6] M. Matsukata, T. Aoki, K. Sanui, N. Ogata, A. Kikuchi, Y. Sakurai, T. Okano, Bioconjug. Chem. 7 (1996) 96–101.
[7] F. Hilbrig, R. Freitag, J. Chromatogr. B 790 (2003) 79–90.
[8] T. Saitoh, A. Sekino, M. Hiraide, Anal. Chim. Acta 536 (2005) 179–182.
[9] N. Idota, A. Kikuchi, J. Kobayashi, Y. Akiyama, K. Sakai, T. Okano, Langmuir 22 (2006) 425–430.
[10] N. Yamada, T. Okano, H. Sakai, F. Karikusa, Y. Sawasaki, Y. Sakurai, Makromol. Chem. Rapid Commun. 11 (1990) 571–576.
[11] A. Mizutani, A. Kikuchi, M. Yamato, H. Kanazawa, T. Okano, Biomaterials 29 (2008) 2073–2081.
[12] K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, K. Yamamoto, E. Adachi, S. Nagai, A. Kikuchi, N. Maeda, H. Watanabe, T. Okano, Y. Tano, N. Engl. J. Med. 351 (2004) 1187–1196.
[13] M. Hasegawa, M. Yamato, A. Kikuchi, T. Okano, I. Ishikawa, Tissue Eng. 11 (2005) 469–478.
[14] T. Ohki, M. Yamato, D. Murakami, R. Takagi, J. Yang, H. Namiki, T. Okano, K. Takasaki, Gut 55 (2006) 1704–1710.
[15] K. Ohashi, T. Yokoyama, M. Yamato, H. Kuge, H. Kanehiro, M. Tsutsumi, T. Amanuma, H. Iwata, J. Yang, T. Okano, Y. Nakajima, Nat. Med. 13 (2007) 880–885.
[16] H. Kanazawa, K. Yamamoto, Y. Matsushima, N. Takai, A. Kikuchi, Y. Sakurai, T. Okano, Anal. Chem. 68 (1996) 100–105.
[17] H. Kanazawa, Y. Kashiwase, K. Yamamoto, Y. Matsushima, A. Kikuchi, Y. Sakurai, T. Okano, Anal. Chem. 69 (1997) 823–830.
[18] K. Nagase, J. Kobayashi, A. Kikuchi, Y. Akiyama, H. Kanazawa, T. Okano, Langmuir 23 (2007) 9409–9415.
[19] K. Nagase, J. Kobayashi, A. Kikuchi, Y. Akiyama, H. Kanazawa, T. Okano, Langmuir 24 (2008) 511–517.
[20] N.T. Miller, J.M. Dibussolo, J. Chromatogr. 499 (1990) 317–332.
[21] L.L. Lloyd, M.I. Millichip, J.M. Watkins, J. Chromatogr. A 944 (2002) 169–177.
[22] Y.B. Yang, M. Verzele, J. Chromatogr. 387 (1987) 197–205.
[23] A. Mizutani, K. Nagase, A. Kikuchi, H. Kanazawa, Y. Akiyama, J. Kobayashi, M. Annaka, T. Okano, J. Chromatogr. A 1217 (2010) 522–529.
[24] S. Edmondson, V.L. Osborne, W.T.S. Huck, Chem. Soc. Rev. 33 (2004) 14–22.
[25] D. Xiao, M.J. Wirth, Macromolecules 35 (2002) 2919–2925.
[26] H. Tu, C.E. Heitzman, P.V. Braun, Langmuir 20 (2004) 8313–8320.
[27] G.D. Jones, Ind. Eng. Chem. 44 (1952) 317–332.
[28] L. Hong, A. Felinger, K. Kaczmarski, G. Guiochon, Chem. Eng. Sci. 59 (2004) 3399–3412.
[29] W. Jin, H. Fu, X. Huang, H. Xiao, H. Zou, Electrophoresis 24 (2003) 3172–3180.
[30] M. Ciampolini, N. Nardii, Inorg. Chem. 5 (1966) 41–44.
[31] G.L. Miller, R.H. Golder, Arch. Biochem. 29 (1950) 420–423.
[32] M.M. Nasef, E.S.A. Hegazy, Prog. Polym. Sci. 29 (2004) 499–561.
[33] R.F. Rekker, The Hydrophobic Fragmental Constant, Elsevier, Amsterdam, 1977.
[34] The Japanese Biochemical Society, Data Book in Biochemistry, Kagaku Dojin, Tokyo, 1979.
[35] S. Xie, R.W. Allington, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 865 (1999) 169–174.
[36] J. Mohammad, B. Jäderlund, H. Lindblom, J. Chromatogr. A 852 (1999) 255–259.