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Improvement of separation efficiencies of anion-exchange chromatography using monolithic silica capillary columns modified with polyacrylates and polymethacrylates containing tertiary amino or quaternary ammonium groups

Yuta Watanabe [a], Tohru Ikegami [a,*], Kanta Horie [a,1], Takeshi Hara [a,2], Jafariah Jaafar [b], Nobuo Tanaka [a]

[a] Department of Biomolecular Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan
[b] Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM, Skdai, Johor, Malaysia

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ABSTRACT

Anion-exchange (AEX) columns were prepared by on-column polymerization of acrylates and methacrylates containing tertiary amino or quaternary ammonium groups on monolithic silica in a fused silica capillary modified with anchor groups. The columns provided a plate height (H) of less than 10 μm at optimum linear velocity (u) with keeping their high permeability ($K = 9–12 \times 10^{-14}$ m^2). Among seven kinds of AEX columns, a monolithic silica column modified with poly(2-hydroxy-3-(4-methylpiperazin-1-yl)propyl methacrylates) (HMPMA) showed larger retentions and better selectivities for nucleotides and inorganic anions than the others. The HMPMA column of 410 mm length produced 42 000–55 000 theoretical plates (N) at a linear velocity of 0.97 mm/s with a backpressure of 3.8 MPa. The same column could be employed for a fast separation of inorganic anions in 1.8 min at a linear velocity of 5.3 mm/s with a backpressure of 20 MPa. In terms of van Deemter plot and separation impedance, the HMPMA column showed higher performance than a conventional particle-packed AEX column. The HMPMA column showed good recovery of a protein, trypsin inhibitor, and it was applied to the separation of proteins and tryptic digest of bovine serum albumin (BSA) in a gradient elution, to provide better separation compared to a conventional particle-packed AEX column.

1. Introduction

The separation of ionic species is very important in the field of separation science because it relates to a wide variety of fields of science, including biological, pharmaceutical, medical, agricultural, environmental chemistry and so on. Small ionic compounds show no or small retention on reversed phase liquid chromatography (RPLC), and it is customary to add ion-pairing cationic reagent such as tetrabutylammonium salts [1] or ion-pairing anionic reagent, chaotropic counter anions [2,3] to the mobile phases. Generally, due to the slow equilibration of these chromatographic systems, chromatographers tend to avoid the use of ion-pair mode. Additionally, the ion-pairing chromatography has a drawback when it is connected to electrospray ionization system of mass spectrometry due to the ion suppression by the ion-pairing agents. Capillary electrochromatography [4] and micellar electrokinetic chromatography [5] are powerful tools for the separation of ionic species, but the examples of their practical applications is limited, probably due to the problem of reproducibility of the separation. Anion- and cation-exchange (CEX) chromatography are also useful for the separation of small ions and peptides, proteins, nucleosides, nucleotides and so on [6]. But separation efficiencies in ion-exchange chromatography are generally lower than that of RPLC, and the fact is problematic for the comprehensive separation of very complex mixtures, for genomics, proteomics, metabolomics and related fields.

Recently, the use of monolithic silica columns has attracted great attention [7], because these columns enable highly efficient separations with theoretical plate number of up to $N = 10^6$, using long columns [8], or very fast separation using short columns [9,10] in the RPLC mode. The application of monolithic columns based on silica and polymer resin for supports of AEX and CEX have been reviewed recently [11]. At the moment, the separation efficiency, in terms of a height equivalent to a theoretical plate (H) is in the range of $H = 10.9–83.8$ μm in isocratic modes, and highly efficient separations with $H = 6.2$ μm are also possible in the capillary electrochromatography (CEC) separation mode. The methodology to prepare monolithic columns for ion-exchange chromatography often incorporate a coating procedure with cationic or anionic surfactant reagents [12,13], or amphoteric surfactants [14] on a

* Corresponding author. Tel.: +81 75 724 7801; fax: +81 75 724 7710.
E-mail address: ikegami@kit.ac.jp (T. Ikegami).
[1] Present address: Eisai Co. Ltd., Kagamigahara, Japan.
[2] Present address: Department of Physical Chemistry, Giessen University, Giessen, Germany.

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RP monolithic columns (Chromolith RP from Merck, Darmstadt, Germany). Additionally, monolithic silica columns modified with iminodiacetic acid [15], lysine [16] and a column electrostatically adhered with latex [17] are known.

Different from aforementioned bonded phase, our group reported the preparation of monolithic silica columns by on-column polymerization reactions to give AEX and CEX-type columns, which resulted in the separation of nucleic bases with H = 20–47 μm [18]. Under a CEC conditions, a similar column provided highly efficient separation with H = 3–5 μm for inorganic anions [19]. Recent progress in the field of ion-exchange chromatography contains monolithic columns and particle-packed columns; the former includes polymer-based disk monolithic columns [20], methacrylate-based mixed mode monolithic column [21], grafted weak anion-exchange methacrylate monolithic columns [22], monolithic column based on poly(glycidyl methacrylate–co-divinylbenzene) [23], silica-based disk monolithic columns [24], monolithic silica columns with zwitter-ionic functionality [25], and with mixed mode RP-AEX column [26], and the latter includes columns with tentacle type stationary phase [27], perfusion ion-exchange chromatography [28], cored anion-exchange chromatography media [29], surfactant coated graphitic carbon phases [30], hyper-branched condensation polymers [31], anion-, cation-, and zwitterion-exchange modes [32], and anion-exchange cryogel [33]. Non-porous anion-exchange silica fibers have also applied to the separation [34]. Although there are many ways of the stationary phase preparation, and several column bed structures, it is not easy to obtain AEX columns that can provide H < 10 μm for isocratic separations. A commercially available methacrylate-based monolithic AEX column (50 mm × 4.6 mm I.D.) provided N = 7300–40 000/m at the optimum flow rate, so the lowest plate height was ca. 25 μm [35].

Here we report on the preparation of monolithic silica columns modified with acrylate and methacrylate polymers that possess anion-exchange functionalities, and their chromatographic characterization based on separations of inorganic anions, nucleotides, and proteins.

2. Experimental

2.1. Materials

Water was purified with an Arium 611DI/611UV system (Sartorius, Göttingen, Germany). Toluene (Nacalai Tesque, Kyoto, Japan) was distilled from calcium hydride after refluxing the mixture for 2 h. *N*-(3-Triethoxysilylpropyl)methacrylamide was prepared by a reported procedure [36]. *N*,*N*-Dimethylaminopropylacrylamide methyl chloride quaternary salt (DMAPAA-Q), *N*,*N*-Dimethylaminoethyl acrylate methyl chloride quaternary salt (DMAEA-Q), *N*,*N*-Dimethylaminopropylacrylamide (DMAPAA), *N*,*N*-Dimethylaminoethyl acrylate (DMAEA) were purchased from Kohjin (Tokyo, Japan), and used without further purification. 3-*N*,*N*-Diethylamino-2-hydroxypropyl methacrylate (DAHMA) was prepared by a reported procedure [18]. 2-Hydroxy-3-(4-methylpiperazin-1-yl)propyl methacrylates) (HMPMA) was prepared as follows: a mixture of glycidyl methacrylate (1.43 g, 10.0 mmol) and *N*-methylpiperazine (1.01 g, 10.0 mmol) in toluene (4 mL) was heated at 60 °C for 3 h, then the solvent was removed in vacuo to yield the monomer. Ammonium persulfate (APS) and azobis(isobutyronitrile) (AIBN) were obtained from Wako (Osaka, Japan), and used without purification.

Sample solutes; sodium nitrite, sodium bromide, potassium nitrate, sodium iodide, sodium bromate were obtained from Nacalai, adenosine-5′-monophosphate (AMP), cytidine-5′-monophosphate (CMP), guanosine-5′-monophosphate (GMP), uridine-5′-monophosphate (UMP), adenosine-5′-diphosphate (ADP), cytidine-5′-diphosphate (CDP), guanosine-5′-diphosphate (GDP), uridine-5′-diphosphate (UDP), adenosine-5′-triphosphate (ATP), cytidine-5′-triphosphate (CTP), guanosine-5′-triphosphate (GTP), uridine-5′-triphosphate (UTP) were purchased from Wako. Cytochrome *c*, myoglobin, ovalbumin, and trypsin inhibitor were obtained from Sigma (St. Louis, MO, USA). Salts for buffer solutions; analysis grade phosphoric acid, disodium hydrogenphosphate dodecahydrate, sodium dihydrogenphosphate dihydrate, Tris(hydroxymethylamino)methane hydrochloric acid salt (Tris–HCl), sodium perchlorate, acetic acid, ammonium acetate, and sodium chloride were obtained from Nacalai.

2.2. Functionalization of monolithic silica columns

Hybrid-type monolithic silica capillary columns were prepared by a reported procedure using a mixture of tetramethoxysilane and methyltrimethoxysilane (3:1, v/v, Shin-Etsu Chemical, Tokyo, Japan) and fused silica capillary of 200 μm I.D., 350 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) [8,37]. These columns were modified with an anchor group, 3-methacrylamidopropyltriethoxysilane (MAS) [36]. The bonding procedure was reported previously [36]. Then each monomer solution summarized in Table 1 was charged into the MAS bonded column. Then both ends of the column were dipped in the reaction mixture in a 1 mL vial tube through a septum (500X8-ST15, Chromacol, Welwyn Garden City, UK) and allowed to react at 60 or 70 °C in a water bath for 2 h. After the reaction, the column was washed quickly using the same solvent for the polymerization reaction, using a HPLC pump at 15 MPa for 6 h. The washing process was repeated twice to obtain AEX monolithic silica capillary columns. In this report, 200H stands for a 200 μm I.D. capillary column prepared from the hybrid silane mixture, and the abbreviation of each monomer is added, as 200H-DMAPAA-Q.

To obtain a 200H-DEAP column, a mixture of (3-*N*,*N*-diethylaminopropyl)triethoxysilane (DEAP), toluene, pyridine (1:1:1, v/v/v) was passed through a monolithic silica capillary column using N_2 gas pressure (1 MPa) for 24 h at 80 °C, followed by a wash with toluene (1 MPa for 24 h). The bonding procedure was repeated once to obtain a 200H-DEAP column. This column name was abbreviated as 200H-DEAP though it was prepared without employing a polymerization reaction.

2.3. Instrument and chromatographic measurements

The HPLC system consisted of Shimadzu LC-10AD VP (Kyoto, Japan), PU611 and PU610 (GL Science, Tokyo, Japan) pumps and a MU701 detector (GL Science) with a capillary flow cell (GL Science, cell volume 18 nL). Inorganic anion and nucleotide samples were injected using a 20 μL loop sample injector (Rheodyne, 7725, Park Court, CA, USA) using a split-flow mode. Protein samples were directly injected by an injector with nL loops (Rheodyne, 7520, 200 nL or 500 nL) with a split flow of the mobile phase prior to the injector.

Throughout these experiments, capillary columns were evaluated in an air bath maintained at 30 °C. For processing the chromatographic data, EZChrom Elite-2.61 data processor (GL Science) was employed. For a reference, a HPLC system with a particle-packed AEX column, Inertsil AX (5 μm, 100 Å, 250 mm × 3.0 mm I.D.), a PU610 pump, an injector (Rheodyne, 7725, 20 μL loop), and an UV 620 detector was employed.

2.4. Samples

Twelve nucleotides were dissolved in water at 0.2 mg/mL concentration of each component, and the four nucleotides (monophosphates) were dissolved in water at 1.25 mg/mL con-

Table 1
Polymerization conditions for AEX monolithic silica columns.

Monomer	Feed composition monomer (mL)	Initiator (mg)	Solvent (mL)	Temperature (°C)
DMAPAA-Q	0.2	APS (5)	Water (1)	60
DMAEA-Q	0.2	APS (5)	Water (1)	60
DMAPAA	0.2	AIBN (1.8)	Toluene (1)	70
DMAEA	0.2	AIBN (1.8)	Toluene (1)	70
DAHMA	0.2	AIBN (1.8)	Toluene (1)	70
HMPMA	0.2	AIBN (1.8)	Toluene (1)	70

centration of each component. Inorganic salts were dissolved in water at 1 mg/mL for sodium nitrite and potassium nitrate, 2 mg/mL for sodium iodide, 3 mg/mL for sodium bromide, and 1 mg/mL for sodium bromate to obtain the samples. Proteins were dissolved in water at 1 mg/mL concentration for trypsin inhibitor, and 2 mg/mL concentration for other proteins to prepare the sample solutions.

3. Results and discussion

3.1. Preparation of seven AEX monolithic silica capillary columns

The basic chromatographic characteristics of the seven AEX monolithic silica capillary columns were examined using 50 mM phosphate buffer (pH 2.8) and four nucleotides, AMP, CMP, GMP, and UMP as samples. The sample solution was injected by a split system with an isocratic conditions. Fig. 1 shows the separation of the nucleotides on 200H-DEAP, 200H-DMAPAA-Q, 200H-DMAEA-Q, 200H-DMAPAA, 200H-DMAEA, 200H-DAHMA, and 200H-HMPMA, possessing tertiary amino or quaternary ammonium functionalities, with the dimensions of 300 mm × 200 μm I.D. in all cases. All columns separated the nucleotides completely. Under the conditions, CMP and AMP are protonated, while UMP and GMP are in their neutral state, thus the former pair is more retained than the latter pair. Among the protonated and neutral pairs, nucleotides with a purine base (AMP, GMP) possess greater retentions than those with pyrimidine (CMP, UMP) bases. This result can be explained by the difference in the hydrophobic nature of these nucleic bases. Since a monolithic silica column modified with only MAS could not separate these nucleotides as reported [18], it was apparent that AEX-type columns were prepared by on-column polymerization reactions of acrylate and methacrylate monomers with tertiary amino and quaternary ammonium functionalities.

As shown in Fig. 1, columns with quaternary ammonium functionalities, and 200H-DEAP column resulted in sharp peaks with small retentions less than about $k = 1$ for all test solutes, while columns immobilized with monomers possessing tertiary amino groups provided larger retentions of up to $k = 4.0$, and a significant tailing was observed for AMP. It is interesting that the 200H-HMPMA column with a cyclic diamine moiety resulted in the largest retention for all analytes, realizing high separation efficiency and good permeability simultaneously, as shown later. This tendency would be consistent with an expected greater ion-exchange capacity of the diamine moiety.

Fig. 2 shows chromatograms of the separation of five inorganic anions, BrO_3^-, NO_2^-, Br^-, NO_3^-, and I^- using the seven AEX monolithic silica columns. It was found that 200H-DMAPAA-Q and 200H-DMAEA-Q were not able to separate these anions completely, though the separation efficiencies were high ($N = 34\,000$ for iodide ion). The 200H-DMAPAA, 200H-DMAEA and 200H-DAHMA columns possessed better selectivity for these anions than the former ones, but the bromide ion overlapped with nitrite or nitrate, which resulted in incomplete separation, while the 200H-HMPMA column achieved complete separation of the five anions. *N*-Methylpiperazine unit seems to be most useful for the functionalization of AEX column among employed monomers. The greater ion-exchange capacity of the cyclic diamine functionality may have relation to the results. Fig. 3a shows a separation of the five inorganic anions using a 200H-HMPMA column (410 mm × 200 μm I.D.). The separation efficiency was in the range of $N = 42\,000$–$55\,000$ (per column), thus, $H = 7.5$–9.8 μm was real-

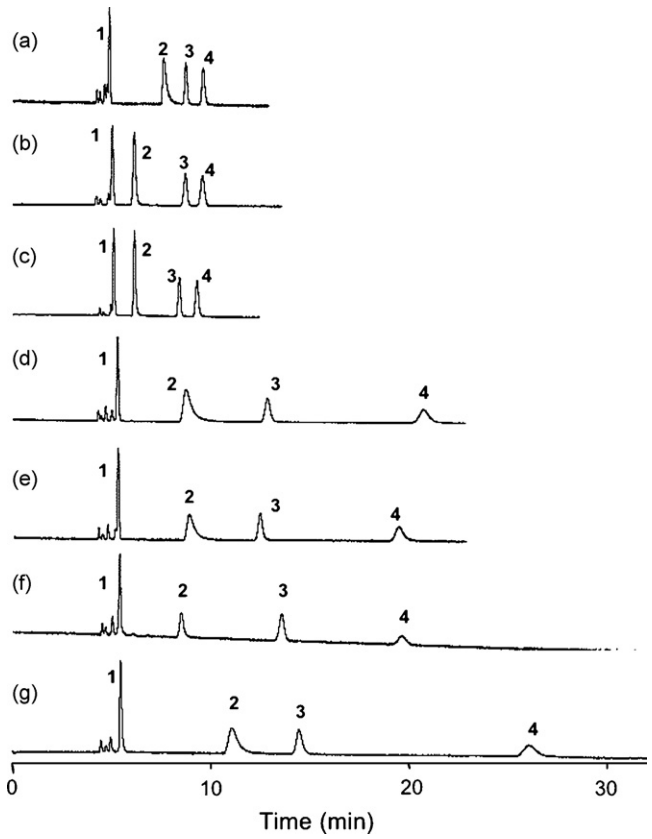

Fig. 1. Separation of four nucleotides using seven AEX-type monolithic silica capillary columns. Column: (a) 200H-DEAP column, (b) 200H-DMAPAA-Q column, (c) 200H-DMAEA-Q column, (d) 200H-DMAPAA column, (e) 200H-DMAEA column, (f) 200H-DAHMA column, (g) 200H-HMPMA column, 300 mm × 200 μm I.D. for each column. Mobile phase: 50 mM phosphate buffer solution (pH 2.8). Solute: 1: CMP^-, 2: AMP, 3: UMP, 4: GMP. Detection: UV at 260 nm. Temperature: 30 °C. Column backpressure and linear velocity of each column: (a) ΔP = 2.0 MPa, u = 1.02 mm/s, (b) ΔP = 2.5 MPa, u = 1.00 mm/s, (c) ΔP = 2.3 MPa, u = 1.00 mm/s, (d) ΔP = 2.5 MPa, u = 1.00 mm/s, (e) ΔP = 2.1 MPa, u = 0.98 mm/s, (f) ΔP = 2.5 MPa, u = 0.98 mm/s, (g) ΔP = 2.4 MPa, u = 1.01 mm/s.

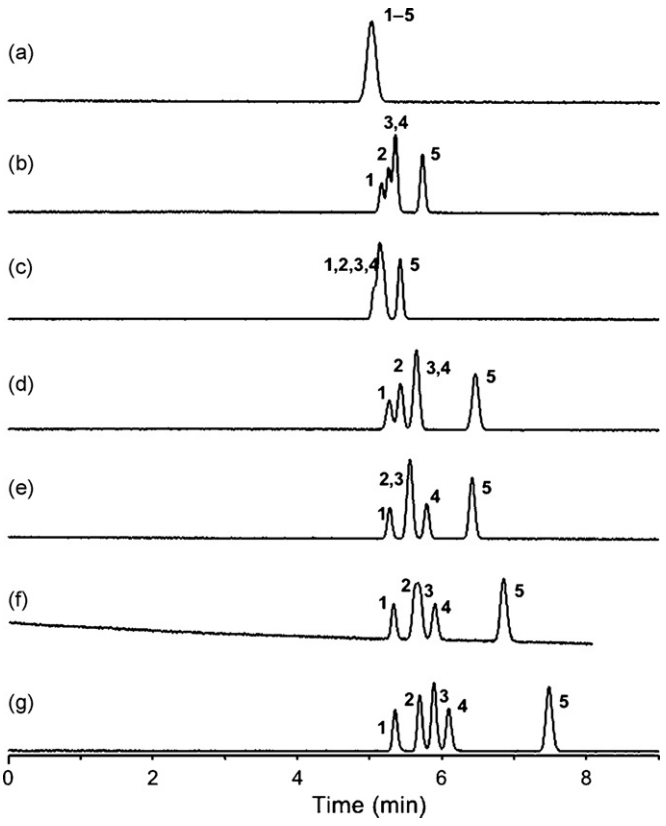

Fig. 2. Separation of five inorganic anions using seven AEX-type monolithic silica capillary columns. Column: (a) 200H-DMAPAA-Q column, (b) 200H-DMAEA-Q column, (c) 200H-DMAPAA column, (d) 200H-DMAEA column, (e) 200H-DAHMA column, (f) 200H-HMPMA column, 300 mm × 200 μm I.D. for each column. Mobile phase: 1.79 g/L $Na_2HPO_4 \cdot 12H_2O$ + 0.78 g/L $NaH_2PO_4 \cdot 2H_2O$ + 14.04 g/L $NaClO_4$ (pH 6.6). Solute: 1: BrO_3^-, 2: NO_2^-, 3: Br^-, 4: NO_3^-, 5: I^-. Detection: UV at 210 nm. Temperature: 30 °C. Column backpressure and linear velocity of each column: (a) ΔP = 1.9 MPa, u = 1.02 mm/s, (b) ΔP = 2.4 MPa, u = 0.97 mm/s, (c) ΔP = 2.3 MPa, u = 1.00 mm/s, (d) ΔP = 2.4 MPa, u = 0.98 mm/s, (e) ΔP = 2.1 MPa, u = 0.98 mm/s, (f) ΔP = 2.4 MPa, u = 0.97 mm/s, (g) ΔP = 2.3 MPa, u = 0.98 mm/s.

ized at a linear velocity of 0.97 mm/s. Under the same conditions, a particle-packed column, Inertsil AX (250 mm × 3.0 mm I.D.) could not separate NO_2^- and Br^- ions completely as shown in Fig. 3b, and the separation efficiency was in the range of N = 13 000–18 000 (per column), thus, H = 14–19 μm, though the linear velocity 1.4 mm/s, employed in the test, was slightly higher than optimal conditions (1.0 mm/s).

3.2. Permeability of AEX monolithic silica columns

The modification method by an on-column polymerization can control the amount of stationary phase, i.e. phase ratio, by changing the composition of reaction mixtures and reaction conditions [38]. With the immobilization of a functional monomer, octadecyl methacrylate, column backpressure increases only slightly. The high permeability values reflect the skeleton structure of the mono-

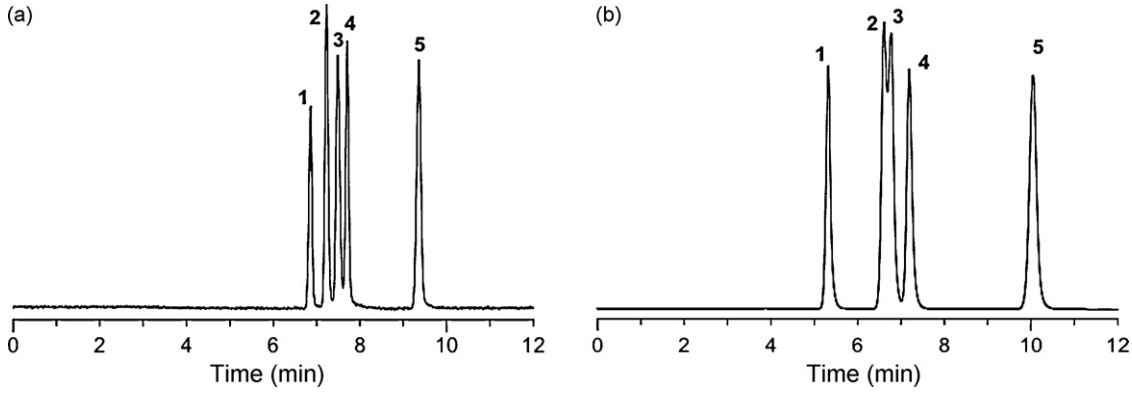

Fig. 3. Separation of 5 inorganic anions using an AEX-type monolithic silica capillary and a particle-packed column. Mobile phase, solutes, and detection; see the conditions of Fig. 2. (a) 200-HMPMA column: Column size: 410 mm × 200 μm I.D. Temperature: 23 °C. Linear velocity: 0.97 mm/s. Column backpressure: 3.8 MPa. (b) Inertsil AX column: column size: 5 μm, 250 mm × 3 mm I.D. Temperature: 30 °C. Flow rate: 0.40 mL/min. Column backpressure: 8.6 MPa. Linear velocity: 1.4 mm/s.

Table 2
Permeability of AEX columns.

Column	Permeability, K ($\times 10^{-14}$ m^2)
200H-DEAP	11.9
200H-DMAPAA-Q	9.1
200H-DMAEA-Q	9.6
200H-DMAPAA	9.2
200H-DMAEA	10.6
200H-DAHMA	8.9
200H-HMPMA	9.4
Inertsil AX (5 μm)	2.0

lithic silica, having a greater through-pore size than particle-packed columns, and they indicate that the influence of polymer coating of the silica skeleton on the liquid transport is not significant. In the case of excessive polymer coating, or the formation of plugs by the polymer in the through-pores, the column is clogged, and the polymers cannot be washed out. In other words, "washable" columns after the polymerization are supposed to keep the good liquid transport characteristics of the monolithic columns. Table 2 lists permeability of AEX monolithic silica columns. Permeability, K, was calculated by Eq. (1), where u, η, L, ε, and ΔP stand for the linear velocity of the mobile phase, the viscosity of the mobile phase, the column length, the porosity of the column, and the column backpressure, respectively [39].

$$K = \frac{u\eta L\varepsilon}{\Delta P} \quad (1)$$

The total porosities of these columns were estimated to be $\varepsilon = 0.9$ for the monolithic columns, and $\varepsilon = 0.65$ for the particle-packed column [40]. The viscosity of the mobile phase (phosphate buffer) was assumed to be similar to that of pure water. All the monolithic silica columns possess a permeability greater than 9×10^{-14} m^2, while the particle-packed column (silica base, 5 μm particle modified with diethylamino group) has a permeability of 2.0×10^{-14} m^2, and the monolithic silica columns had 4.3–5.0 times the permeability of the particle-packed column.

Using (3-*N*,*N*-diethylaminopropyl)triethoxysilane and the same bare monolithic silica column, another AEX column, 200H-DEAP was obtained. It possesses $K = 11.9 \times 10^{-14}$ m^2, and the value is slightly greater than those of the polymer-coated monolithic silica columns. Thus, the polymerization lowers the permeability of the monolith, but the decrease is not significant. A monolithic silica column modified with the polymerization of octadecyl methacrylate showed $K = 12.2 \times 10^{-14}$ m^2 in 80% acetonitrile [38]. Thus the AEX monolithic silica columns are also expected to be useful for fast separation at high linear velocity, or highly efficient separation using long columns.

3.3. Column efficiency of AEX monolithic silica columns

The efficiency of the 200H-HMPMA column was evaluated by a Van Deemter plot for iodide ion at 23 °C. A similar measurement was done for Inertsil AX at 30 °C, and summarized in Fig. 4. At the optimal linear velocity, $u = 0.82$ mm/s, the 200H-HMPMA column provided $H = 8.8$ μm (7.2 μm for nitrate ion), while the Inertsil AX showed $H = 12.5$ μm at $u = 0.94$ mm/s. In the case of a particle-packed column, the state of packing can be checked by reduced plate height, h, given by Eq. (2), where d_p stands for particle size.

$$h = \frac{H}{d_p} \quad (2)$$

Generally, commercially available columns have $h = 2.5$–3.5, and the chance to have a column which has h less than 2, is limited [41]. Therefore, the Inertsil AX column can be regarded as a "good" column, and if a similar column is prepared by 3–4 μm particles, it will provide highly efficient separation media close to the performance of the 200H-HMPMA column.

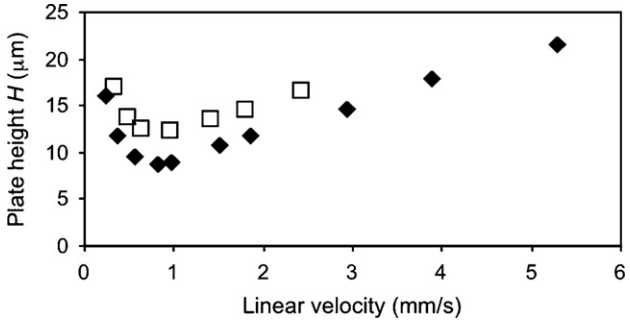

Fig. 4. Van Deemter plots of an AEX monolithic silica capillary column and a particle-packed column using I^- as a solute. Chromatographic conditions are similar to Figs. 2 and 3. (♦) 200H-HMPMA, Temperature: 23 °C. (□): Inertsil AX, Temperature: 30 °C.

Separation impedance, E is given by Eq. (3), where t_0, ΔP, η, N, H, and K stand for the elution time of an unretained solute, the column backpressure, the viscosity of the mobile phase, the theoretical plate number, the plate height, and the permeability, respectively [42]. This value is useful to describe the total performance of columns, based on the required time and column backpressure to produce one theoretical plate.

$$E = \frac{t_0 \Delta P}{N^2 \eta} = \left(\frac{\Delta P}{N}\right) \times \left(\frac{t_0}{N}\right) \times \left(\frac{1}{\eta}\right) = \frac{H^2}{K} \quad (3)$$

In Fig. 5, E values for the separation of iodide ion are plotted against linear velocity, u, for 200H-HMPMA and Inertsil AX. At optimal u, Inertsil AX gave $E = 6\,500$, while 200H-HMPMA provided $E = 800$, probably due to the high permeability of the monolithic silica column based on the larger through-pore/skeleton size ratio compared to particle-packed columns. The E value of 200H-HMPMA reaches to 4 000 at around $u = 4$ mm/s, and the E values are slightly larger than those of a hybrid-type monolithic silica column modified with octadecyldimethylsilyl group, that gives $E = 4\,000$ at $u = 4.5$ mm/s [37]. In summary, the 200H-HMPMA column can provide higher separation efficiency and permeability at lower column backpressure than a column packed with 5 μm particles.

3.4. Relationship between selectivity for anions and stationary phases

Anions are separated by ion-exchange due to the Coulombic interaction on AEX columns, but the contribution of hydrophobic or

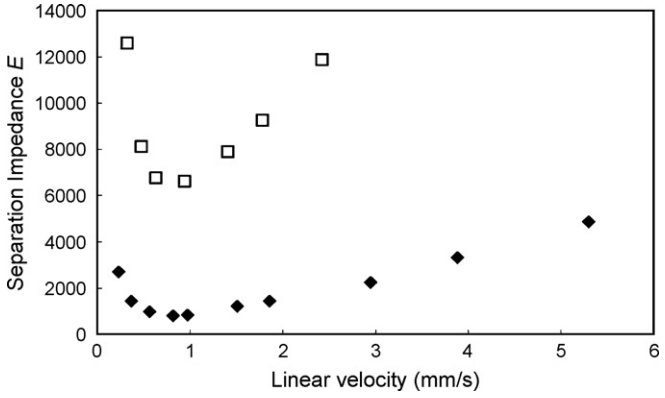

Fig. 5. E–u plots of an AEX monolithic silica capillary column and a particle-packed column using I^- as a solute. Chromatographic conditions are similar to Figs. 2 and 3. (♦) 200H-HMPMA, Temperature: 23 °C. (□): Inertsil AX, Temperature: 30 °C.

Table 3
Selectivity of anions on different stationary phases.

	DMAPAA	DMAEA	DAHMA	HMPMA
log *P* of monomer	0.07	0.64	1.13	0.21
$\alpha(k_{BrO_3^-}/k_{Br^-})$	0.34	0.38	0.39	0.30
$\alpha(k_{NO_2^-}/k_{Br^-})$	0.62	0.94	0.89	0.74
$\alpha(k_{NO_3^-}/k_{Br^-})$	1.04	1.51	1.40	1.26
$\alpha(k_{I^-}/k_{Br^-})$	2.54	2.91	3.11	3.05

hydrophilic interaction cannot be ruled out. But in the case of inorganic anions, the side interaction such as hydrophobicity should be limited, and the retentivity of such ions depends on the valence of the ions and the selectivity of the ions at the ion-exchange reaction. Here, the relationship between the structure of AEX functionalities of monomers employed for the column preparation, and the selectivity for ions is discussed.

Table 3 shows selectivity, α, of anions on different stationary phases; α values are calculated using K_{Br^-} value as a standard. On all columns, iodide possessed larger interaction with the stationary phases compared to bromide. The larger ionic radius results in the lower charge density, that causes weaker solvation. As a result, the solvated ionic radius becomes smaller in the case of iodide, which makes it easier for the ion to contact the stationary phase. The $\alpha(k_{I^-}/k_{Br^-})$ can be an index for selectivity of ion size, and it increased in the order of 200H-DMAPAA, 200H-DMAEA, 200H-HMPMA, and 200H-DAHMA. In the order, the hydrophobicities of monomers in terms of log *P* also increase as 200H-DMAPAA (0.07), DMAEA (0.64), HMPMA (0.21), and DAHMA (1.13) [43], therefore the stationary phases modified with more hydrophobic monomers should have better selectivity of ionic size. The order may also be the polarizability of the monomers. To prepare stationary phases possessing good selectivity of ionic sizes, the use of monomers including tertiary amino groups should be better than to employ monomers including quaternary ammonium functionality. The ion selectivity might have a relationship to the Hofmeister series [44].

3.5. Separation of nucleotides with a 200H-HMPMA column

As shown in Fig. 4, the 200H-HMPMA column can provide high separation efficiency even at high linear velocity. This column was applied to fast separation of 12 nucleotides and five anions as shown in Fig. 6. Twelve nucleotides were separated by a 200H-HMPMA column (520 mm × 200 μm I.D.) using a 500 nL volume injector with a split flow of linear gradient elution of 0–1.0 M NaCl in 20 mM phosphate buffer (pH 6.9), in 18 min. The chromatogram in Fig. 6a (u = 0.97 mm/s) showed good separation of nucleotides, and the peak capacity (PC) [39] of the separation was estimated as 73 for an 18 min separation. This value should be large for an ion-exchange separation mode. For example, cation-exchange separation gave PC = 51 for 30 min separation, and it is smaller than that of RPLC, which provided PC = 115 for 40 min separation [45]. The gradient time was shortened to 10 min by increasing the linear velocity to u = 1.9 mm/s, to obtain the chromatogram in Fig. 6b. The PC was estimated as 62 in the fast separation.

Using the same conditions of the separation of the five anions on a 200H-HMPMA column (410 mm × 200 μm I.D.) in Fig. 2, the flow rate was increased to generate a linear velocity of 5.3 mm/s. The five anions were eluted within 2 min as shown in Fig. 6c. The resolution of bromide and nitrate became slightly worse in the fast separation, but still the efficiency was kept as high as N = 19 000

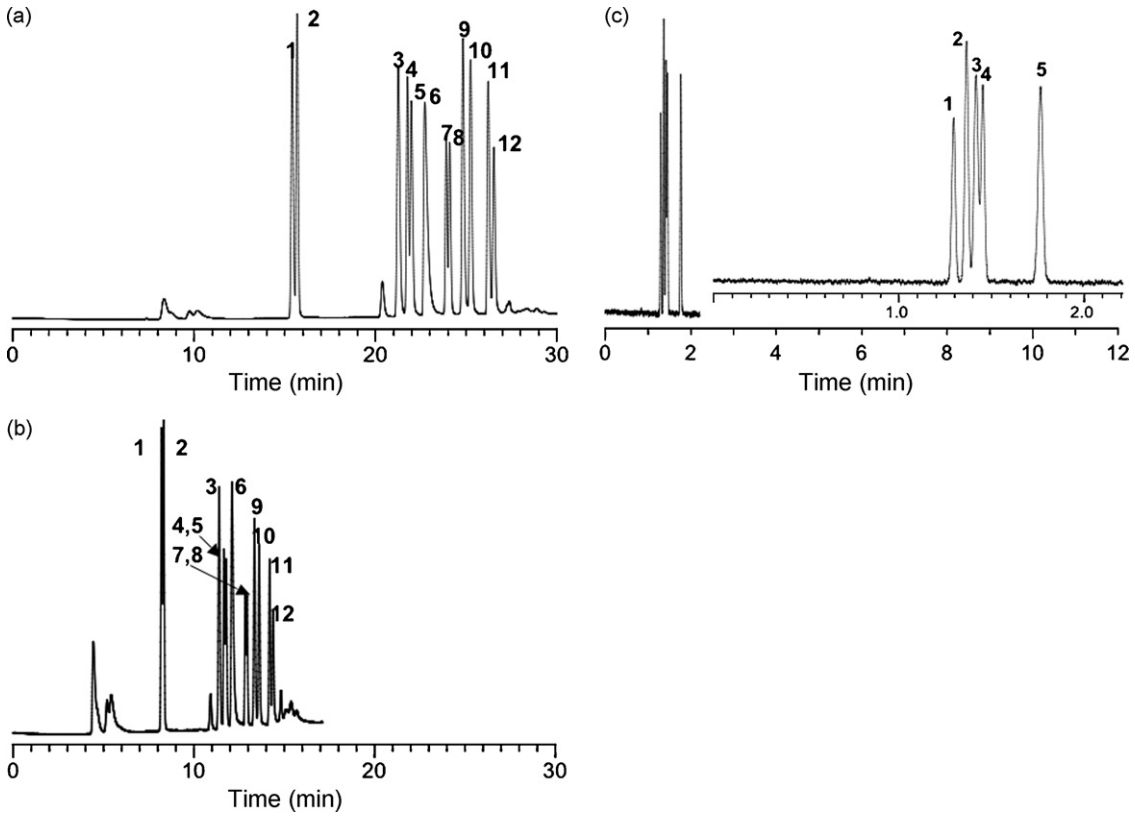

Fig. 6. Gradient separation of nucleotides and anions on an AEX monolithic silica capillary column. and (b), Column: 200H-HMPMA, 520 mm × 200 μm I.D. Mobile phase: (A) 20 mM sodium phosphate (pH 6.9), (B) 1.0 M NaCl dissolved in A, Linear gradient, (B) 0–100% (t_G = 18 min for (a), and 10 min for (b)). Detection: UV at 260 nm. Temperature: 23 °C, Column backpressure: 7.1 MPa for (a), 14 MPa for (b), Sample: 1: CMP, 2: UMP, 3: GMP, 4: CDP, 5: UDP, 6: AMP, 7: CTP, 8: UTP, 9: GDP, 10: ADP, 11: GTP, 12: ATP. (c) Column, column size, mobile phase, detection, and temperature were similar to Fig. 3(a). Solute: 1: BrO_3^-, 2: NO_2^-, 3: Br^-, 4: NO_3^-, 5: I^-. Linear velocity: 5.3 mm/s. Column backpressure: 20 MPa.

Table 4
Peak area of trypsin inhibitor after passing through a 200H-HMPMA column and an open fused silica capillary tube.

	1st	2nd	3rd	4th	5th	Average
200H-HMPMA	214 100	212 800	213 900	211 400	214 700	213 380 ± 1200
Open tube	230 400	221 100	220 300	227 000	214 200	222 600 ± 5600

for the iodide ion. Hatsis and Lucy employed a Chromolith Speed Rod (50 mm × 4.6 mm I.D.) coated with didodecyldimethylammonium bromide for a fast separation, and separated seven anions in 1 min (nitrate ion was eluted at around 22 s) [46]. The separation provided H = 27–40 μm at 17 mm/s (flow rate 10 mL/min), and the efficiency was less than N = 2 000. The long column employed here can be driven by a common HPLC pump, and it can provide significantly higher separation efficiency even at u = 5.3 mm/s compared to the reported results [11,46]. Monolithic silica columns have an advantage in their high separation efficiency and low column backpressure, but simultaneously have a disadvantage of a lower column phase ratio compared to particle-packed columns generally. For example, k_{I^-} is 0.58 on a 200H-HMPMA column, while Inertsil AX gives k_{I^-} = 2.39, although ion-exchange capacity is important factor in the case of ion-exchange separation. Smaller retentions may have an advantage for fast separation, if the stationary phases possess enough selectivity, α, and separation efficiency, N for the target compounds.

3.6. Separation of proteins with a 200H-HMPMA column

Separation of proteins often employs affinity, size exclusion, and ion-exchange chromatographic techniques, and the ion-exchange chromatography should be a better way for a fine separation of mixtures of proteins. In RPLC mode using organic modifiers and acids such as formic acid or trifluoroacetic acid, denaturation of proteins is difficult to avoid. Under such conditions, the irreversible adsorption of protein sometimes becomes a serious problem in the separation and analysis of proteins in RP mode. The recovery of a protein, trypsin inhibitor was examined using a 200H-HMPMA column (300 mm × 200 μm I.D.) and a open fused silica capillary (300 mm × 200 μm I.D.) in a 0.5 M NaCl/20 mM Tris–HCl buffer (pH 8.0). A split flow was introduced to an injector equipped with a 200 nL loop, and the volume of the protein sample at a 1 mg/mL concentration was directly charged to the column or the open tube. Table 4 shows the peak areas of the trypsin inhibitor peak for five measurements. On an average, the 200H-HMPMA column and the open tube gave peak areas of 213 380 and 222 600, respectively. Judging from the result, 95.9% of the protein passed through the 200H-HMPMA column, compared to the open fused silica tubing. Polymer coating on monolithic silica columns can suppress silanol effect [38], and this method of column functionalization should be promising for the preparation of columns to separate proteins. Fig. 7a–d show chromatograms of the gradient separation of four proteins, Cytochrome *c*, myoglobin, ovalbumin, and trypsin inhibitor on four columns, 200H-HMPMA, 200H-DMAPAA-Q, 200H-DEAP, and Inertsil AX. The column modified with polymeric silylating reagent, 200H-DEAP, could not retain

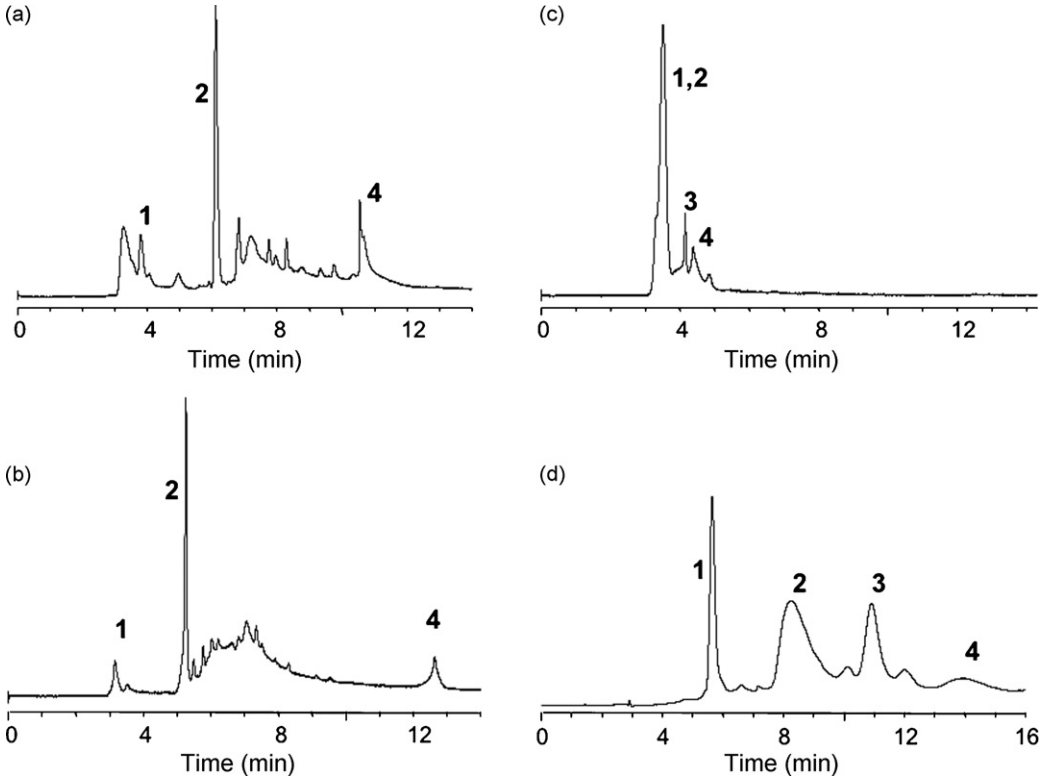

Fig. 7. Separation of four proteins using AEX monolithic silica capillary columns, and a particle-packed column. (a)–(d), Detection: UV at 280 nm. Temperature: 30 °C. Solute: 1: Cytochrome *c*, 2: myoglobin, 3: ovalbumin, 4: trypsin inhibitor. In (a) and (b), the identification of ovalbumin was not done. (a)–(c), Mobile phase: (A) 20 mM Tris–HCl (pH 8.0), (B) 0.5 M NaCl in (A). Linear gradient: (B) 0–100% (t_G = 10 min). (a) 200H-HMPMA, Column size: 300 mm × 200 μm I.D., (b) 200H-DMAPAA-Q, Column size: 300 mm × 200 μm I.D., (c) 200H-DEAP, Column size: 300 mm × 200 μm I.D., (d), Inertsil AX, Column size: 5 μm, 250 mm × 3.0 mm I.D. Mobile phase: (A) 20 mM Tris–HCl (pH 8.4), (B) 0.5 M NaCl in (A). Linear gradient: (B) 0–100% (t_G = 15 min). Flow rate: 0.4 mL/min. Column backpressure and linear velocity: (a) ΔP = 5.3 MPa, u = 1.6 mm/s, (b) ΔP = 5.5 MPa, u = 1.6 mm/s, (c) ΔP = 4.3 MPa, u = 1.6 mm/s, (d) ΔP = 8.9 MPa, u = 1.4 mm/s.

these proteins under the separation conditions (Fig. 7c). Inertsil AX resulted in broad peaks of proteins especially for myoglobin (Fig. 7d), while the protein was separated as a sharp peak on 200H-HMPMA and 200H-DMAPAA-Q columns (Fig. 7a, b) with a 10 min gradient of NaCl concentration from 0 to 0.5 M in 20 mM Tris–HCl buffer (pH 8.0). Additionally, these monolithic columns separated many sub-peaks of ovalbumin, probably due to the higher separation efficiencies than the particle-packed column. Considering the p*I* value of these proteins, basic protein such as Cytochrome *c* (p*I* = 10.6) would not be retained by these columns. Acidic proteins such as ovalbumin (p*I* = 4.6) and trypsin inhibitor (p*I* = 4.5) were retained, and eluted at higher concentration of NaCl. Since the monolithic silica columns employed in this experiment were not designed as wide-pore columns for protein separation, the degree of the separation was not good as those of small compounds. PC was estimated for the separation by 200H-HMPMA column as 46 for a 10 min separation (peak width of myoglobin was employed for calculation of PC). This type of column would be useful for fine separation of proteins.

4. Conclusion

Six anion-exchange-type monolithic silica columns were prepared by an on-column polymerization of acrylates and methacrylates possessing tertiary amino and quaternary ammonium functionalities on the silica surface modified with (3-methacrylamidopropyl)silyl groups. Among these columns, the 200H-HMPMA column showed good selectivity of five inorganic anions and 12 nucleotides. These monolithic silica columns provided much higher separation efficiency in comparison with a commercially available particle-packed AEX column in terms of separation impedance, based on 4.3–5.0 times higher permeability than a particle-packed column and small *H* (7–8 μm at optimal flow rate). The highly porous monolithic column realized a fast separation of inorganic ions in 2 min even using a column of 410 mm. The 200H-HMPMA could separate 12 nucleotides with a high efficiency, and the separation could be finished within 16 min under gradient elution conditions. The same column showed good recovery (95.9%) of trypsin inhibitor, and provided better resolution of proteins.

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