Talanta 85 (2011) 1298–1303

Contents lists available at ScienceDirect

Talanta

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

Surface chemical analysis and chromatographic characterization of polyethylenimine-coated hydroxyapatite with various amount of polyethylenimine

Yukiko Murakami [a,*], Ken Sugo [a], Masahiro Hirano [a], Tsuneo Okuyama [b]

[a] Research and Development Department, PENTAX New Ceramics Division, HOYA Corporation, 1-1-110, Tsutsujigaoka, Akishima-shi, Tokyo, Japan
[b] Protein Technos Institute, 99-7 Shimokawa-iri, Astugi-shi, Kanagawa, Japan

ARTICLE INFO

Article history:
Received 11 April 2011
Received in revised form 18 May 2011
Accepted 1 June 2011
Available online 12 June 2011

Keywords:
Chromatography
Hydroxyapatite
Polyethylenimine
Surface functionalization
Phosphorylated protein

ABSTRACT

Polyethylenimine (PEI) has been widely used as a coating material to produce stationary phase for ion-exchange chromatography of biomolecules. However, a precise study of the PEI coating fraction has been lacking, despite such quantification being very important for fundamental research as well as identifying further industrial applications.

In this study, we produced four types of PEI-coated hydroxyapatite (PEI-HAp) with various fractions of PEI (0.16%, 0.5%, 1.0%, 1.5%) using a spray-drying system to evaluate correlations between coating fractions and the thermochemical or chromatographic behaviors of theses products. The thermal analyses of these matrices showed two exothermic peaks when the PEI coating fraction exceeded 1.0%. The one peak indicated a PEI decomposition peak and the other would indicate bond dissociation of PEI layers formed over the HAp surface as the PEI concentration increased. Furthermore, the chromatographic analysis for the surface chemical characteristics showed the correlation between coating fraction and the retention time of protein or nucleotide. Acidic or phosphorylated proteins were more strongly adsorbed as the PEI coating fraction increased when the initial coating fraction was low, but at fraction exceeding 0.5%, constant retention was observed. The retention time of nucleotides increased in proportion to the fraction of PEI added. The good selectivity of PEI-HAp may be attributable to multifunctional interactions of electrostatic and bare Ca sites on HAp, not just the amino sites of PEI. These precise studies of PEI coating fraction are our original novel contributions, which could be achieved by quantitative consideration using thermal analysis and chromatography.

1. Introduction

Hydroxyapatite (HAp) liquid chromatography for protein purification was first introduced by Tiselius et al. in 1956 [1]. The binding mechanism bases on dual ion-exchange sites; positively charged calcium and negatively charged phosphate groups. The ion-exchange chromatography is one of the major methods in the biomolecules separation such as proteins, e.g. enzymes and antibodies, and also poly-nucleotides [2–4]. Polyethylenimine (PEI) has been widely used as a coating material to produce stationary phases for chromatography of biomolecules [5–10]. In our previous report, we described a basic method for preparing PEI-coated hydroxyapatite (PEI-HAp) and discussed its characteristics [11]. However, as with other materials (PEI-silica, PEI-cellulose and so on), no correlation was demonstrated between the quantities of PEI used to coat the matrix and properties such as the thermochemical or chromatographic characteristics of these materials [5–10]. Because conventional PEI coating methods require a decantation process for removal of excess PEI, the quantity of PEI coating material is not constant, i.e., there is instability. Therefore a precise study of the PEI coating fraction has been lacking, despite such quantification being very important for fundamental chromatographic research as well as identifying further industrial applications. Herein, we produced four types of PEI-HAp with different PEI fractions (0.16%, 0.5%, 1.0%, 1.5%) using an improved preparation method not requiring a decantation process which destabilized the amount of the coating material. Furthermore, we analyze the surface chemical condition by using thermal analysis and chromatography, and correlations between PEI coating fractions and the adsorption behaviors of proteins and nucleotides.

Abbreviations: HAp, hydroxyapatite; PEI, polyethylenimine; PEI-HAp, polyethylenimine-coated Hydroxyapatite; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; CV, column volume.

* Corresponding author. Tel.: +81 42 500 5849; fax: +81 42 500 5861.
E-mail addresses: yukiko.murakami@pc.hoya.co.jp, m-yukiko@cf6.so-net.ne.jp (Y. Murakami).

0039-9140/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2011.06.004

Table 1
Weight percent fraction of coated PEI and the quantities of reactants used in this preparation.

PEI/HAp % (w/w)	PEI (mL)	PEI-water solution (mL)	HAp (g)
1.5	50.0	9000	1000
1.0	33.3	9000	1000
0.5	16.6	9000	1000
0.16	5.3	9000	1000

2. Materials and methods

2.1. Materials

The ceramic HAp stationary phase (CHT, Type II, 40 μm particle size) was obtained from Bio-Rad (Hercules, CA, USA). This material is standardized and yields reproducible chromatographic results [12–14]. PEI (30% aq., P-1000 solution: average molecular weight = 70,000, specific gravity = 1.0) was purchased from NIPPON SHOKUBAI (Osaka, Japan). Therefore, approximately 1654 basic skeletons were included in this PEI. Proteins (ovalbumin (chicken egg white), myoglobin (equine heart), α-chymotrypsinogen A (bovine pancreas), cytochrome *c* (equine heart), α-lactalbumin (bovine milk), and trypsin inhibitor (soybean)) were supplied by Sigma Aldrich (St. Louis, MO, USA). Nucleotides (adenosine, AMP, ADP, ATP; abbreviations given in section of "Sample preparation procedure for proteins and nucleotides in solution") were purchased from Oriental Yeast (Tokyo, Japan).

2.2. Preparation of PEI-HAp

In this study, four types of PEI-HAp with different coating fractions, (PEI)/(HAp) × 100 wt%, were produced. The preparations of these fractions are summarized in Table 1: 50.0 mL, 33.3 mL, 16.6 mL and 5.3 mL of PEI (30% aq.) were individually diluted to 9000 mL with Milli-Q water. Then, 1000 g of HAp particles were added to each diluted PEI solution in a 20 L polypropylene reaction tank. The respective suspensions corresponded to 1.5, 1.0, 0.5, and 0.16% final PEI fractions. Next, each suspension was stirred for 24 h, and the particles were dried employing a spray-dryer (Ohkawara Kakohki, Kanagawa, Japan) with a centrifugal wheel atomizer at 220 °C. The varying PEI-HAp fractions produced were observed with a scanning electron microscope (SEM) (S-4300, Hitachi, Tokyo, Japan).

2.3. Thermal analysis

The thermal behavior of PEI-HAp was evaluated using a thermogravimetric-differential thermal analyzer (TG-DTA) (SHIMADZU, Kyoto, Japan). Approximately 10.0 mg of PEI-HAp were used for TG-DTA analysis under heated conditions, with temperatures rising from 35 °C to 600 °C at a rate of 20 °C/min, in a nitrogen atmosphere (using α-alumina as the standard material).

2.4. Sample preparation procedure for proteins and nucleotides in solution

Protein and nucleotide mixtures were used to evaluate chromatographic-separation behavior. For preparation of the standard protein mixture solution, ovalbumin (10 mg), myoglobin (5 mg), α-chymotrypsinogen A (5 mg) and cytochrome *c* (5 mg) were mixed with 1 mL of 1 mM sodium phosphate buffer, at pH 6.8. Furthermore, each α-lactalbumin (10 mg) and soybean trypsin inhibitor (5 mg), one of the acidic proteins, was mixed with 1 mL of 1 mM sodium phosphate buffer, at pH 6.8, for the extensive discussion of the chromatographic behaviors. For preparation of the standard nucleotide mixture solution, adenosine (1 mg), adenosine monophosphate (AMP) (1 mg), adenosine diphosphate (ADP) (1 mg) and adenosine triphosphate (ATP) (1 mg) were mixed with 1 mL of 1 mM sodium phosphate buffer, at pH 6.8.

2.5. Chromatographic procedure with phosphate buffer solution

The PEI-HAp was packed into a 4.0 mm × 100 mm stainless steel column (Sugiyama Shoji, Kanagawa, Japan). As a control, the HAp column was prepared as above. These chromatographic procedures were performed with a Biologic Duo Flow (Bio-Rad). The initial buffer A was a 10 mM sodium phosphate buffer (pH 6.8). The final buffer B was a 400 mM sodium phosphate buffer (pH 6.8). These columns were washed with 8 column volumes (CV) (10 mL) of 100% buffer B and equilibrated with 16 CV (20 mL) of 100% buffer A. Finally, 50 μL of the protein mixture and other acidic proteins or 30 μL of the nucleotide mixture were loaded onto the column. The chromatographic separation was performed according to the following protocol: 100% A (0.8 CV), linear gradient 0–75% B (12 CV), 100% B (4 CV), except for α-lactalbumin and soybean trypsin inhibitor. For α-lactalbumin and soybean trypsin inhibitor, following protocol was accomplished: 100% A (1.6 CV), linear gradient 0–75% B (12 CV), 100% B (4 CV). Chromatography was performed at a flow rate of 1 mL/min, at room temperature.

2.6. Influence of different base buffers

To evaluate the adsorption site interacting with an acidic material, the chromatographic elution pattern of the nucleotide solution was examined with a NaCl gradient based on different buffers using 0%, 0.5% and 1.5% PEI-HAp. The column washing process described in section of "Chromatographic procedure with phosphate buffer solution" was used.

2.6.1. Elution with water-based sodium chloride gradient

The initial buffer (D1) was Milli-Q water. The elution buffer (E1) was a 1 M NaCl solution and the final buffer (F) was a 400 mM sodium phosphate buffer (pH 6.8). These columns were equilibrated with 16 CV (20 mL) of 100% D1. In total, 30 μL of the nucleotide mixture was loaded onto the column. Chromatographic separation was carried out according to the following protocol: 100% (D1) (0.8 CV), linear gradient 0–100% (E1) (16 CV), 100% (E1) (4 CV), 100% F (12 CV). Chromatography was performed at a flow rate of 1 mL/min, at room temperature.

2.6.2. Elution with sodium phosphate buffer-based sodium chloride gradient

The initial buffer (D2) was a 10 mM sodium phosphate buffer (pH 6.8). The elution buffer (E2) was a 10 mM sodium phosphate buffer, pH 6.8, including a 1 M NaCl. The final buffer (F) was a 400 mM sodium phosphate buffer (pH 6.8). The chromatographic separation was carried out according to the following protocol: 100% (D2) (0.8 CV), linear gradient 0–100% (E2) (16 CV), 100% (E2) (4 CV), 100% F (12 CV). Chromatography was performed at a flow rate of 1 mL/min, at room temperature.

2.7. Durability of PEI-HAp

Multiple-cycle chromatographic durability was examined using 1.5% PEI-HAp. Measurements were made to assess the separation behaviors of protein mixtures during chromatography. The durability of repeat-use experiments was assessed with an Ez Chrom Elite (Hitachi). A 50 μL protein mixture was injected onto the column and eluted in the same manner as described in section of "Chromatographic procedure with phosphate buffer solution". The

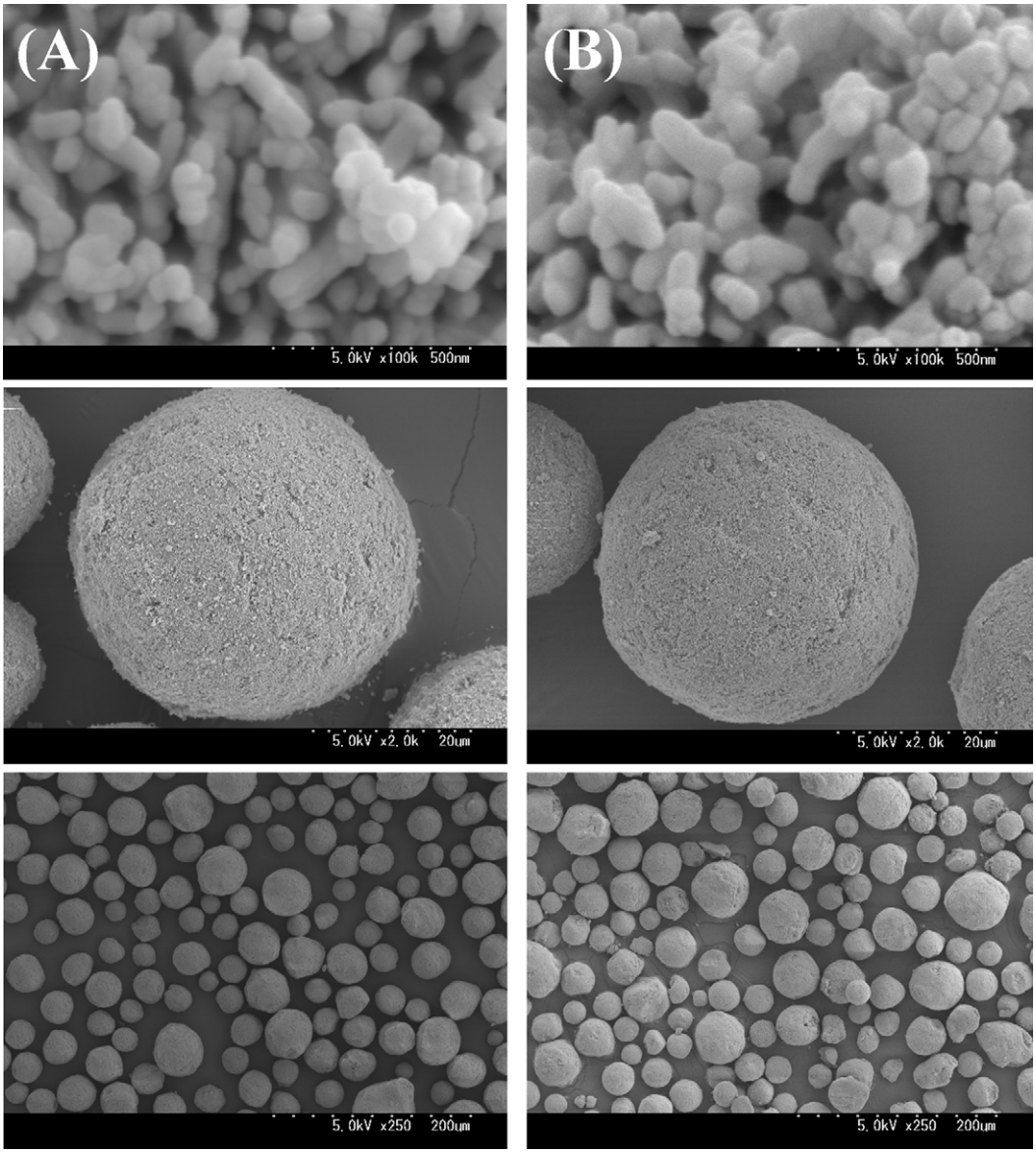

Fig. 1. Scanning electron micrograph of PEI-HAp powder prepared with different PEI fraction: (A) 0% (HAp), and (B) 1.5%.

PEI-HAp column was equilibrated with 8 CV of 10 mM sodium phosphate buffer, pH 6.8, at a flow rate of 1.0 mL/min for each protein mixture injection. This measurement procedure was repeated for 100 cycles.

3. Results and discussion

3.1. Evaluation of PEI-HAp

3.1.1. SEM images

Fig. 1 shows the SEM images of (A) HAp, without PEI coating, and (B) PEI-HAp with PEI coating of 1.5% fraction. Because there is no significant difference between the SEM images of the other PEI fractions, one typical picture of PEI-HAp was showed. Both images indicate no significant differences between HAp and 1.5% PEI-HAp in primary crystals. Furthermore, none of the PEI-HAp particles broke, and each maintained its spherical form throughout the spray-drying processing. These results show the surface structure, especially the primary HAp crystal, to be unaffected by the PEI coating. The coating process did not alter the crystal structures of the HAp particles, nor were these particles broken during the spray-drying process. Therefore, the improved coating method employed herein has the potential to allow a precise analysis of the PEI coating fraction and large-scale preparation of PEI-HAp particles which would be useful as chromatographic matrices.

3.1.2. Thermal analysis

Fig. 2 shows the DTA curves of PEI-HAp with various PEI fractions. The DTA curves had two exothermic peaks at around 260 °C and 320 °C in the 1.0% and 1.5% PEI-HAp groups, while one exothermic peak was detected at around 320 °C in the 0.16% and 0.5% PEI-HAp groups. Fig. 3(A) and (B) shows the TG-DTA profiles of products with 0.5 and 1.5% PEI, respectively. The TG curves show a two-stage weight decrease between 35 °C and 600 °C in both groups. The first weight decrease, between 35 °C and 150 °C, would be attributable to desorption of adsorbed water [15]. Fig. 3(A) shows that the second weight decrease, between 210 °C and 600 °C, was accompanied one exothermic peak on the DTA curve, indicating this weight loss to be attributable to decomposition of PEI [16]. In constant, in Fig. 3(B), the first peak of the DTA curve began to appear in the approximately 150 °C–210 °C range without an accompanying weight decrease (arrow).

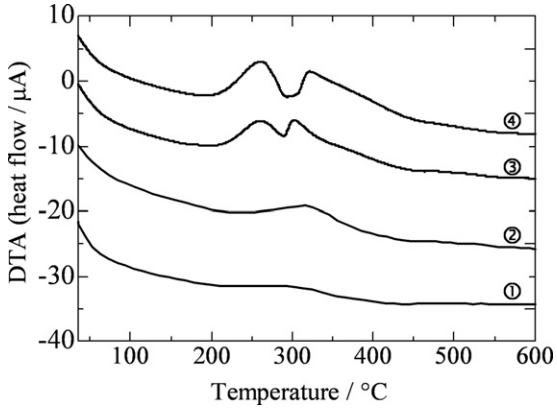

Fig. 2. DTA curves of PEI-HAp with different coating fractions: (①) 0.16%, (②) 0.5%, (③) 1.0%, and (④) 1.5%. The heating condition: heating range 35–600 °C, the heating flow rate 20 °C/min, in the atmosphere.

Based on these data, the exothermic peak at approximately 320 °C reflects PEI decomposition, while that at approximately 260 °C can be considered to represent bond dissociation of PEI layers, i.e., a strong and stable PEI "shell" formed over the HAp surface as the PEI concentration increased.

3.1.3. Chromatography of proteins with phosphate buffer solution

Protein chromatograms and the correlations between the PEI coating fraction and retention time of each protein are shown in Fig. 4(A) and Fig. 5. The separation patterns varied with PEI coating fractions. Thus, the retention time of ovalbumin (thick arrow) using PEI-HAp (0.16%, 0.5%, 1.0%, 1.5%) was longer than that using HAp (0%). At PEI coating fractions from 0% to 0.5%, the retention time of ovalbumin increased as the PEI coating fraction increased, but then remained constant above 0.5%. In contrast, the retention times of myoglobin, α-chymotrypsinogen A and cytochrome *c* decreased as the PEI coating fraction increased. Furthermore, with a PEI coating fraction of 1.5% or more, PEI-HAp retained only acidic or phosphorylated proteins (unpublished observations). Like ovalbumin, the same tendencies were observed using α-lactalbunin and soybean trypsin inhibitor which are one of the acidic proteins, as shown in Fig. 4(B) and (C), respectively. Especially, soybean trypsin inhibitor includes two major subcomponents [17]. PEI-HAp could retain and separate both of them, however, the attribution is complicated because adsorption state depends on the surface condition of protein such as electric charge. Further detailed analysis, now in progress, will be discussed in another paper.

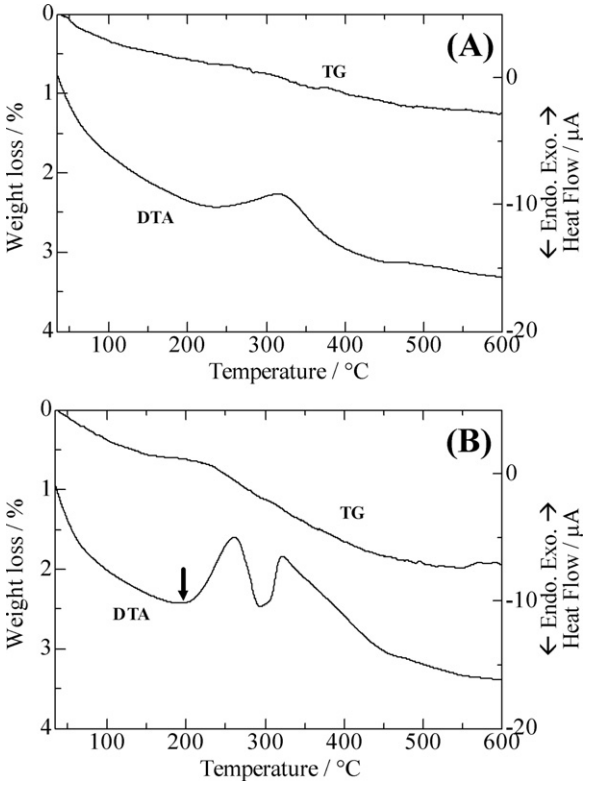

Fig. 3. TG-DTA profiles of the productions: (A) 0.5% PEI-HAp and (B) 1.5% PEI-HAp.

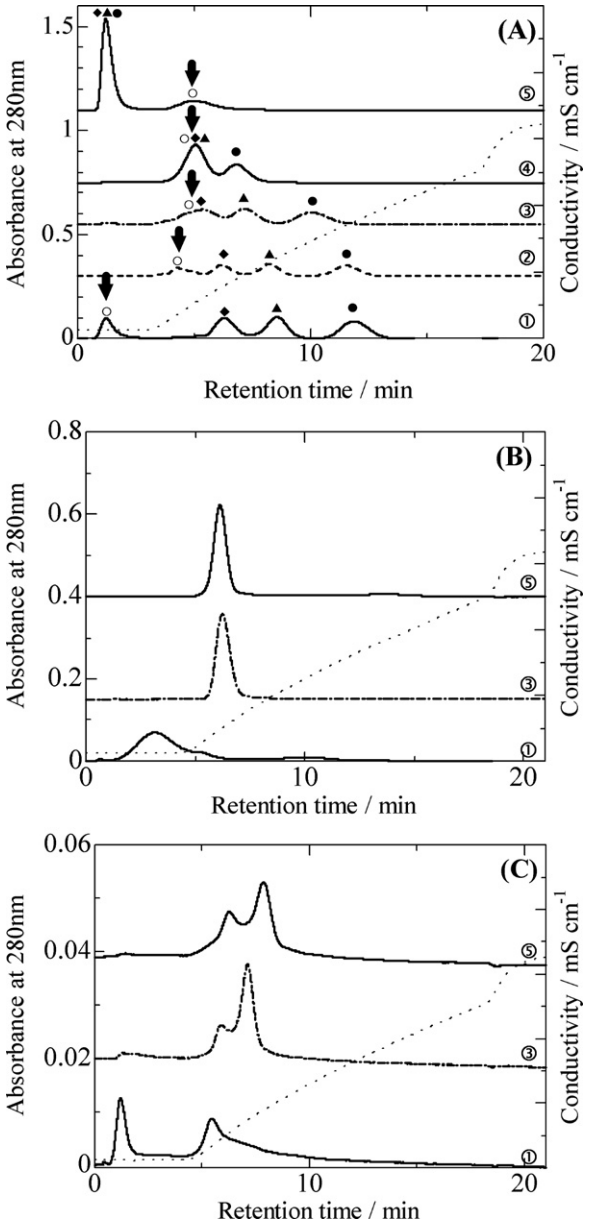

Fig. 4. Chromatogram of proteins mixture solution (ovalbumin, myoglobin, α-chymotrypsinogen A, and cytochrome *c*) and other acidic proteins (α-lactalbumin and soybean trypsin inhibitor): (A) proteins mixture solution, (B) α-lactalbumin, and (C) soybean trypsin inhibitor. (①) HAp column, (②) 0.16% PEI-HAp column, (③) 0.5% PEI-HAp column, (④) 1.0% PEI-HAp column, and (⑤) 1.5% PEI-HAp column. (○) ovalbumin, (♦) myoglobin, (▲) α-chymotrypsinogen A, and (●) cytochrome *c*. The thick arrow indicates the ovalbumin peak. Individual components were used to determine the order and position of elution.

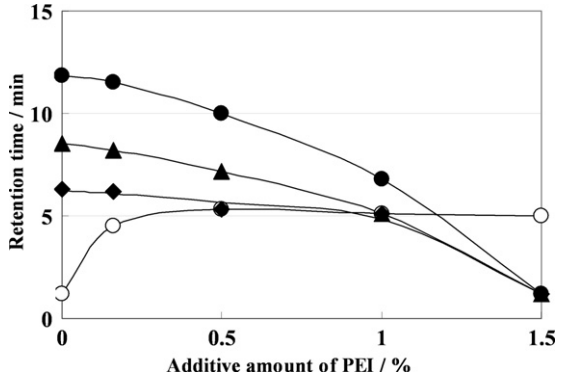

Fig. 5. Plots of correlations between the PEI coating fraction and retention time of each protein: (○) ovalbumin, (♦) myoglobin, (▲) α-chymotrypsinogen A, and (●) cytochrome *c*. This data was extracted from Fig. 4(A).

These data indicate the necessity for appropriate positive charges to prevent retention of neutral or basic proteins. Acidic or phosphorylated proteins are adsorbed more strongly as the PEI coating fraction increases, when the PEI coating fraction is low (0–0.5%). In contrast, when PEI coating fractions exceeded 0.5%, acidic or phosphorylated proteins can be considered to be constantly retained by the interaction between negatively and positively charged proteins.

3.1.4. Chromatography of nucleotides with phosphate buffer solution

Nucleotide chromatograms and the correlations between the PEI coating fraction and the retention time of each nucleotide are shown in Figs. 6 and 7. PEI-HAp, for fractions examined (0.5%, 1.0%, 1.5%), had an ability to retain even AMP, though the 0.16% PEI-HAp and HAp (0%) did not retain AMP. Clear elution of nucleotides could be obtained depending on the number of phosphate residues. The retention times of AMP, ADP and ATP increased in proportion to the fraction of PEI added. Therefore, the adsorbability of the PEI-HAp surface for AMP, ADP and ATP, which have only positive charges, increased in accordance with the PEI coating fraction on HAp. Furthermore, at a PEI coating fraction of 1.5% or more, PEI-HAp retained more of these nucleotides (unpublished observations).

3.1.5. Chromatography with sodium chloride gradient

Employing the water-based 1 M NaCl gradient, only adenosine and AMP were eluted from the PEI-HAp (Fig. 8). With the 10 mM

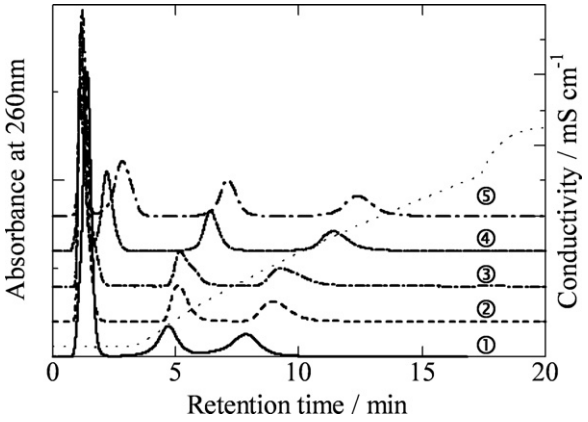

Fig. 6. Chromatogram of nucleotides mixture solution (adenosine, AMP, ADP, and ATP). (①) HAp column, (②) 0.16% PEI-HAp column, (③) 0.5% PEI-HAp column, (④) 1.0% PEI-HAp column and (⑤) 1.5% PEI-HAp column. Clear elution of nucleotides was obtained in order of adenosine, AMP, ADP, and ATP. Individual components were used to determine the order and position of elution.

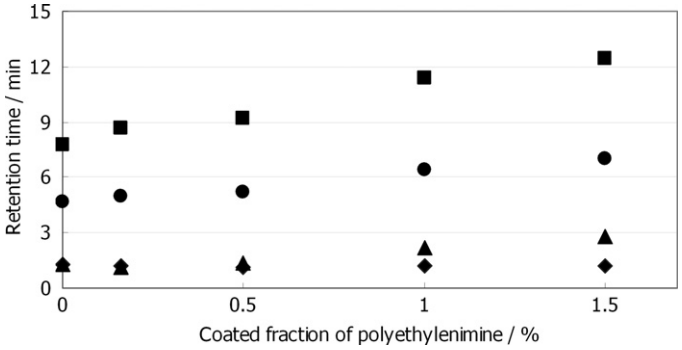

Fig. 7. Plots of correlations between the PEI coating fraction and retention time of each nucleotide: (♦) adenosine, (▲) AMP, (●) ADP, and (■) ATP. This data was extracted from Fig. 6.

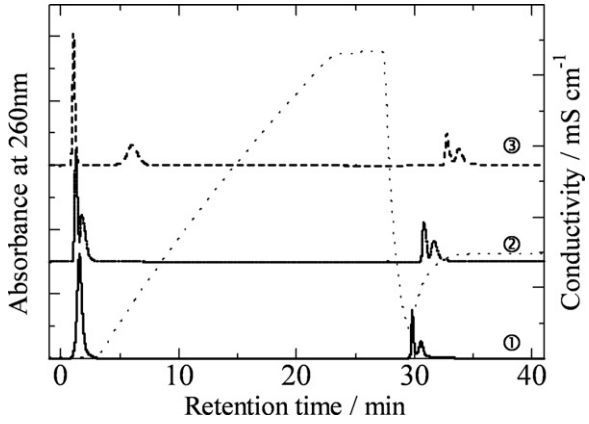

Fig. 8. Chromatogram of nucleotides mixture solution (adenosine, AMP, ADP, and ATP) using the water-based 1 M NaCl gradient. (①) HAp column, (②) 0.5% PEI-HAp column, and (③) 1.5% PEI-HAp column.

sodium phosphate buffer-based 1 M NaCl gradient, adenosine, AMP and even ADP were eluted from the PEI-HAp (Fig. 9). Each retention time increased with the additive fraction of PEI. These chromatographic patterns differed from those of conventional PEI-silica, suggesting the surface condition of PEI-HAp to be different from that of PEI-silica [5]. These data indicate sodium phosphate buffer to have a higher elution ability for acidic materials than NaCl solution. Thus, this PEI-HAp presumably has a bare calcium (Ca) site on its surface, because sodium phosphate buffer generally shows a

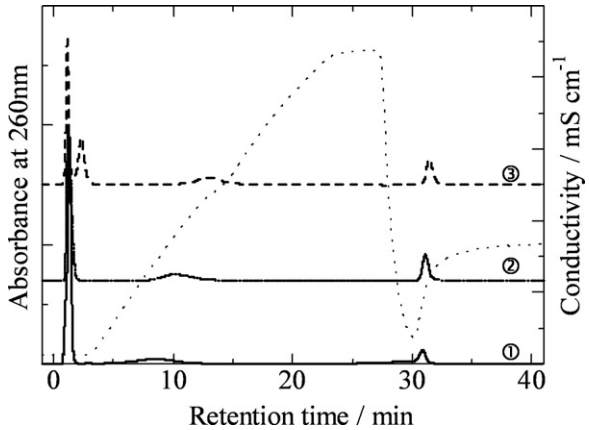

Fig. 9. Chromatogram of nucleotides mixture solution (adenosine, AMP, ADP, and ATP) using the 10 mM sodium phosphate buffer-based 1 M NaCl gradient. (①) HAp column, (②) 0.5% PEI-HAp column, and (③) 1.5% PEI-HAp column.

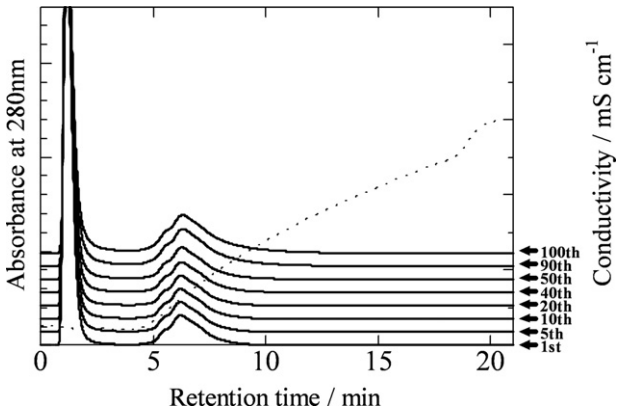

Fig. 10. Repetitive chromatograms obtained from the proteins mixture separation. Sample: 50 μL of proteins (ovalbumin, myoglobin, α-chymotrypsinogen A, and cytochrome *c*). The right side number indicates the repeating number.

stronger interaction with Ca sites than does NaCl solution. Therefore, the good selectivity of PEI-HAp may be attributable to the multifunctional interaction of the electrostatic and the bare Ca sites on HAp, not just the PEI amino sites.

3.1.6. Durability of PEI-HAp

The above results (sections of "SEM images", "Thermal analysis", "Chromatography of proteins with phosphate buffer solution" and "Chromatography of nucleotides with phosphate buffer solution") indicate that PEI coating fractions of at least 1.5% are needed to meet the performance requirements for an ion-exchange matrix. Therefore, 1.5% PEI-HAp was used as a standard material. The chromatograms are shown in Fig. 10. When these tests were repeated, to assess the reproducibility of results, the chromatographic peaks were unchanged, showing essentially the same pattern as in Fig. 4(A). Thus, PEI-HAp is a stable material even when used for up to 100 cycles.

4. Conclusion

The surface chemical condition of PEI-HAp was analyzed by using thermal analysis and chromatography, after the HAp surface was coated with various fractions of PEI using the spray-drying method.

The thermal analysis showed two exothermic peaks when the PEI coating fraction exceeded 1.0%. The one peak was attributable to decomposition of PEI and the other could be considered to represent bond dissociation of PEI layers formed over the HAp surface as the PEI concentration increased. Therefore, such a PEI-HAp has a stable PEI "shell".

In chromatography, acidic or phosphorylated proteins, like ovalbumin, were more strongly adsorbed as the PEI coating fraction increased when the initial coating fraction was low, but at fractions exceeding 0.5%, constant retention was observed. In the nucleotide mixture, the retention times of AMP, ADP and ATP increased in proportion to the fraction of PEI added. Thus, the adsorbability of PEI-HAp surfaces for molecules such as AMP, ADP and ATP, which have only positive charges, was dependent upon the PEI coating fraction added to HAp. Furthermore, it was suggested that the PEI-HAp had a bare Ca site on its own surface. The good selectivity of PEI-HAp may thus be attributable to multifunctional interactions of electrostatic and bare Ca sites on HAp, not just the amino sites of PEI.

Among the coating fractions examined herein, 1.5% PEI-HAp showed performance and properties suitable for a stable chromatographic material. Such a precise analysis of PEI coating fractions, which is our original contribution, is very important for fundamental chromatographic research as well as developing new industrial applications. It has been lacking, because the conventional preparation method requires a decantation process and thus yields an unstable coating amount material. Our novel method is anticipated to allow preparation of an optimal ion-exchange matrix, scaling-up for large-scale chromatographic processes such as antibody purification, and to offer a viable alternative to the diethyl aminoethyl (DEAE) matrix. These improvements and advances are made possible by the good selectivity and stability of PEI-HAp, which became identified in this study.

References

[1] A. Tiselius, S. Hjertén, Ö. Levin, Arch. Biochem. Biophys. 65 (1956) 132–155.
[2] C.S. Rao, Process Biochem. 37 (2001) 247–256.
[3] Y.-C. Shih, J.M. Prausnitz, H.W. Blanch, Biotechnol. Bioeng. 40 (1992) 1155–1164.
[4] R. Torres, B.C.C. Pessela, M. Fuentes, C. Mateo, R. Munilla, R. Fernandez-Lafuente, J.M. Guisán, Enzyme Microb. Technol. 39 (2006) 711–716.
[5] T.G. Lawson, F.E. Regnier, H.L. Weith, Anal. Biochem. 133 (1983) 85–93.
[6] J.D. Pearson, F.E. Regnier, J. Chromatogr. 255 (1983) 137–149.
[7] R.M. Chicz, Z. Shi, F.E. Regnier, J. Chromatogr. 359 (1986) 121–130.
[8] C. McNeff, P.W. Carrr, Anal. Chem. 67 (1995) 2350–2353.
[9] R.P. Magnusso, A.R. Portis Jr., R.E. McCarty, Anal. Biochem. 72 (1976) 653–657.
[10] K. Watanabe, W.–S. Chow, G.P. Royer, Anal. Biochem. 127 (1982) 155–158.
[11] Y. Murakami, S. Rikimura, K. Sugo, K. Kawamura, T. Ogawa, M. Hirano, T. Okuyama, J. Liq. Chromatogr. 32 (2009) 407–417.
[12] T. Kadoya, T. Isobe, M. Ebihara, T. Ogawa, M. Sumita, H. Kuwahara, A. Kobayashi, T. Ishikawa, T. Okuyama, J. Liq. Chromatogr. 9 (1986) 3543–3557.
[13] P. Lundahl, Y. Watanabe, T. Takagi, J. Chromatogr. 604 (1992) 95–102.
[14] T. Kadoya, T. Ogawa, H. Kuwahara, T. Okuyama, J. Liq. Chromatogr. 11 (1988) 2951–2967.
[15] R.Z. LeGeros, G. Bonel, R. Legros, Calcif. Tissue Res. 26 (1978) 111–118.
[16] Product manual of polyethylenimine, NIPPON SHOKUBAI Co., LTD.
[17] H. Kobayashi, M. Suzuki, Soy Protein Res. 7 (2004) 137–144 (in Japanese).