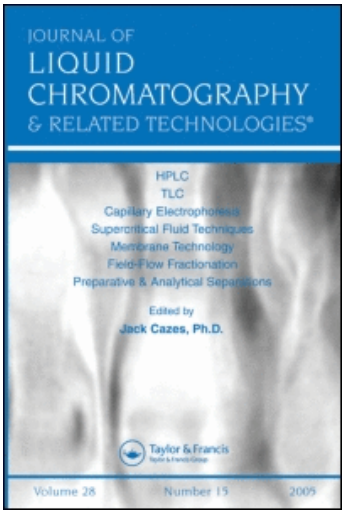

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Preparation of Polyethylenimine-Hydroxyapatite and its Chromatographic Use

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Abstract: Hydroxyapatite (HAp-CHT TypeII 40 μm) was coated with a stable layer of polyethylenimine (PEI). The resulting material was useful in column chromatography of acidic materials. A mixture of AMP, ADP, ATP, and adenosine could be resolved readily. The convenient separation of ovalbumin (one of the phosphoproteins) from other standard protein mixtures was observed.

Keywords: Chromatography, Hydroxyapatite, Nucleotide, Phosphorylated protein, Polyethylenimine (PEI), Purification

INTRODUCTION

Polyethylenimine (PEI) is regularly produced by the acid catalyzed polymerization of aziridine. Branched PEI is synthesized using this method according to Thomas et al.[1] The produced polyethylenimine has various molecular weights, and they always have three types of amino groups.[2] The various polymer levels of these materials have been commercialized. The branched structure can be illustrated schematically (Figure 1). The three types of amines are distributed as the primary (25%), secondary

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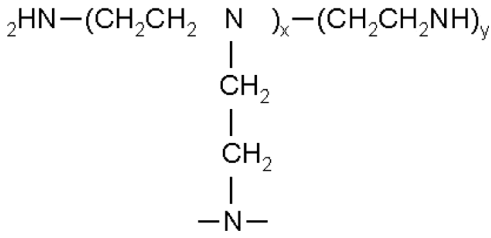

Figure 1. The PEI structure.

(50%), and tertiary amine (25%). PEI has a maximum ion exchange capacity of 23 meq/g. Figure 2 shows the one of the model drawn on the basis of the abundance rate of the three types of amines. Previously, the polymer had been adsorbed to cellulose and was commercialize as the PEI cellulose with a capacity of 1 μeq/g.[3]

But this material had a draw back as a chromatography column, since it showed the volume change due to the solvent change. While PEI-silica has better rigidity, but this material was not suitable for general use due to the large relative solubility in the neutral and basic range. Silica also sometimes showed very strong binding capacity against proteins and some more dendrimers; therefore, we tried to produce PEI-Hap, which has molder binding and more stability in the basic pH range. The covalent attachment of PEI to chromatographic grade HAp should give a promising rigid material with improved ion exchange capacity for the chromatography of bio-materials.

EXPERIMENTAL

Materials

HAp was CHT type II (particle size 40 μm) of Bio-Rad.[4–8] PEI (30% Polyetylenimine P70 solution: Av.Mw, 70,000 Mr) was purchased from Wako

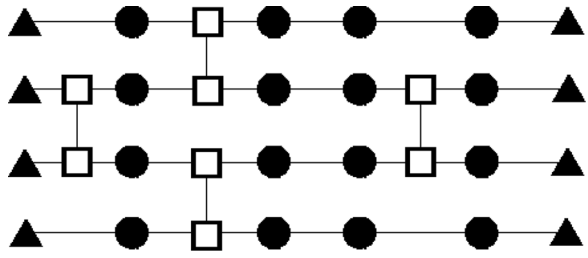

Figure 2. The PEI structure (▲)primary amine group, (●)secondary amine group, (□)tertiary amine group.

Pure Chemical Industries, Ltd. About 1654 basic skeletons were included in this PEI. Nucleotides were purchased from Oriental Yeast Co., Ltd. Proteins were purchased from WAKO Pure Chemical Industries, Ltd.

Preparation of PEI-HAp

PEI-HAp

PEI-methanol solution was prepared as follows; 5 mL of PEI was mixed with methanol, refluxed, and then the volume was adjusted to 50 mL with methanol. Five milliliters of this PEI-methanol solution was mixed with 10 g HAp in 100 mL of methanol with the boiling stone. The mixture was stirred with air bubble flow and heated on a boiling water bath for 15 min with gently shaking every 5 min. The mixture was cooled gradually at room temperature for 30 min. Then, the materials were repeatedly washed with decantation and resuspension with water 5 times. After this procedure, the wash was checked with ninhydrin reaction to negative. The product was then dried in a vacuum desiccator for 60 hours. The synthesized PEI-HAp was evaluated by a ninhydrin test. The color change after the ninhydrin test was also evaluated by the absorption spectroscopy. The PEI-HAp (0.05 g) and HAp (0.05 g) was suspended in 2 mL of water, respectively, and 3 drops of 0.5% ninhydrin solution was added to each group.

Preparation of Nucleotides Sample Solution or Proteins

The Nucleotide Solution

Each 1 mg of nucleotide (AMP, ADP, and ATP) was dissolved in 1 mL of sodium phosphate buffer (1 mM, pH 6.8).

The Nucleotide Mixture Solution

AMP (1 mg), ADP (1 mg), and ATP (1 mg) were mixed with 1 mL of sodium phosphate buffer (1 mM, pH 6.8). After prolonged storage, as indicated in results, AMP contained a small amount of adenosine and ADP contained a small amount of AMP (indicated with arrow mark in Figure 7a).

The Protein Solution

Each protein, Ovalbumin (10 mg), Myoglobin (5 mg), α-chymotrypsinogen A (5 mg), and cytochrome *c* (5 mg), was dissolved in 1 mL of sodium phosphate buffer (1 mM, pH 6.8).

The 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 sodium phosphate buffer (1 mM, pH 6.8).

Chromatography Procedure

The PEI-HAp was packed into a 3.9×100 mm stainless column (Sugiyama Shoji). As the control column, the HAp column was prepared as above.[9] All chromatographic experiments were performed with a Biologic Duo Flow (Bio-Rad). Of the nucleotide mixture (AMP, ADP, and ATP), 30 μL, or the single nucleotide solution was injected onto the PEI-HAp and HAp columns, and eluted with the liner gradient from 10 to 400 mM sodium phosphate buffer (NaPB) (pH 6.8) for 15 min. at a flow rate of 1.0 mL/min. All separations were monitored at 260 nm.

In another experiment, 30 μL of protein mixture, or respective single protein solution, was injected onto the PEI-HAp and HAp column and eluted in the same manner as above. The effluents were monitored at 280 nm.

The stability of this PEI-HAp was also examined by using chromatography. In this experiment, 30 μL of protein mixture was injected onto the PEI-HAp column and eluted with the liner gradient from 1 to 400 mM NaPB (pH 7) for 15 min at a flow rate of 0.6 mL/min. This measurement was repeated 3 times, and all separations were monitored at 280 nm.

RESULTS AND DISCUSSION

Evaluation of PEI-HAp

The synthesized PEI-HAp was evaluated by a ninhydrin test to determine the PEI derived amino group.[10] HAp without PEI coating was also used as a control materials. The color change was evaluated by visual inspection. The results were indicated in Figure 3. HAp did not show any color in the supernatant and the precipitate. On the other hand, PEI-HAp showed a very remarkable purple color in the precipitate and the very low coloration in the supernatant. This indicates that the PEI is located firmly on the solid material and the dose scarcely leaks out from the produced PEI-HAp.

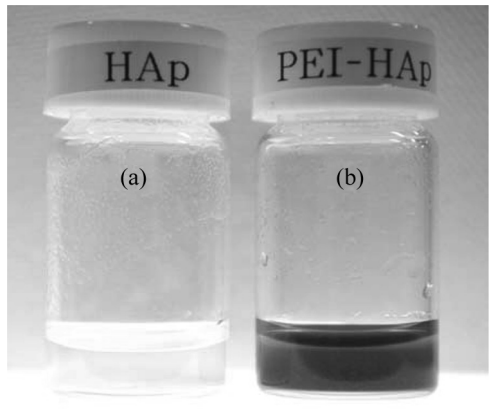

Figure 3. The result of the ninhydrine test. (a) HAp, (b) PEI-HAp.

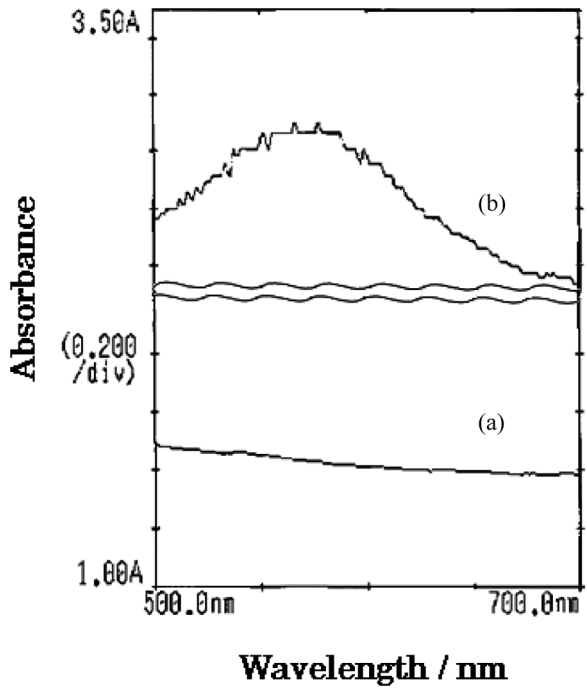

Figure 4. The absorption spectrum of (a) HAp, (b) PEI-HAp.

Absorption Spectrum

The synthesized PEI-HAp was evaluated by the ninhydrin test to determine the PEI derived amino group. The color change was evaluated by visual inspection. Subsequently, the precipitates were suspended in the 80% sucrose solution to hold the suspension rather stable and then the absorption spectra could be measured with spectrophotometer (Shimazu).

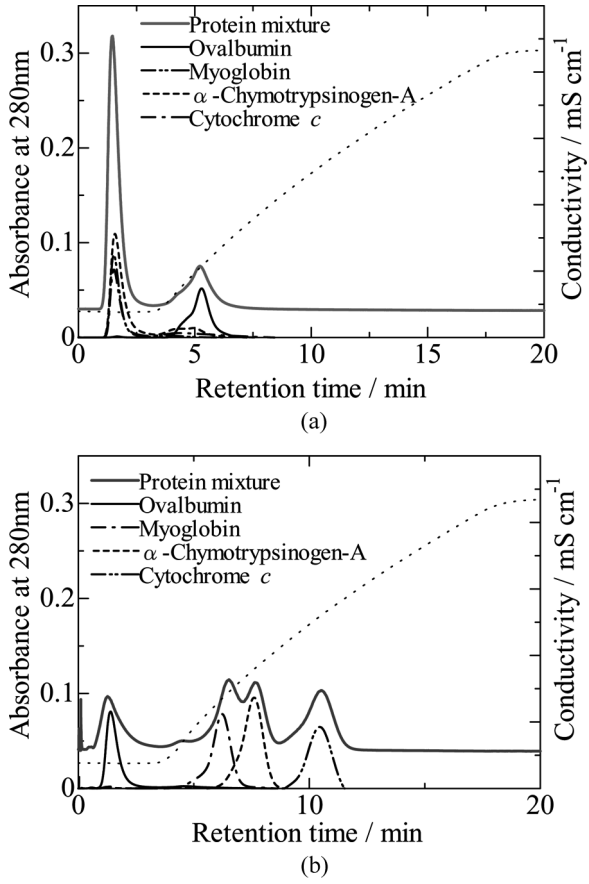

Figure 5. (a) Chromatography of protein mixture (ovalbumin, myoglobin, α-chymotrypsinogen-A, and cytochrome *c*) on PEI-HAp column (3.9 × 100 mm). The ovalbumin was separated with a linear gradient of 10–400 mM NaPB (pH6.8) at a flow-rate of 1 mL/min. (b) Chromatography of a protein mixture (ovalbumin, myoglobin, α-chymotrypsinogen-A, and cytochrome *c*) on HAp column (3.9 × 100 mm). The ovalbumin could be separated with a linear gradient of 10–400 mM NaPB (pH6.8) at a flow-rate of 1 mL/min.

The absorption maximum wave length was detected at about 570 nm (Figure 4a and b).[10] The peak was attributable to Ruhemann's purple, and it indicated the existence of amine derived on the PEI-HAp product. This result revealed that the PEI was fixed on the HAp.

Chromatography of Proteins

The protein solutions was loaded on the columns and eluted with a linear gradient. The chromatograms are shown in Figure 5a and Figure 5b.

The ovalbumin were retained and the cleared shoulder was indicated when the PEI-HAp was used, although the ovalbumin was not retained on the HAp. It is known that ovalbumin contains phosphate groups and its approximate content has been determined with 2D electrophoresis, so that the separation might be due to a phosphate group on ovalbumin.

Figure 6 also shows the relation of the retention time between HAp and PEI-HAp. The dotted lines on Figure 6 correspond to the retention time of non-retained component. These results indicated that the retention time of ovalbumin using PEI-HAp was longer than that of ovalbumin using HAp. Therefore, the acidic or phosphorylated protein like ovalbumin would be mildly separated using the PEI-HAp.

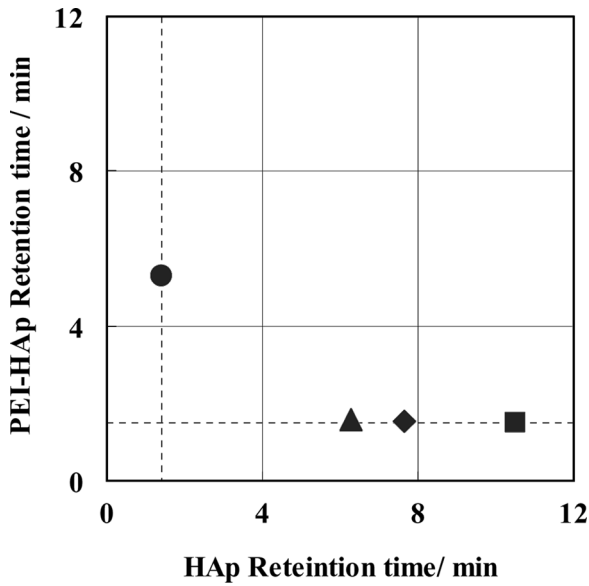

Figure 6. Correlation of retention time on the HAp with retention time on PEI-HAp. (●) Ovalbumin, (▲) Myogrobin, (◆) α-Chymotripusinogen-A and (■) Cytochrome-c.

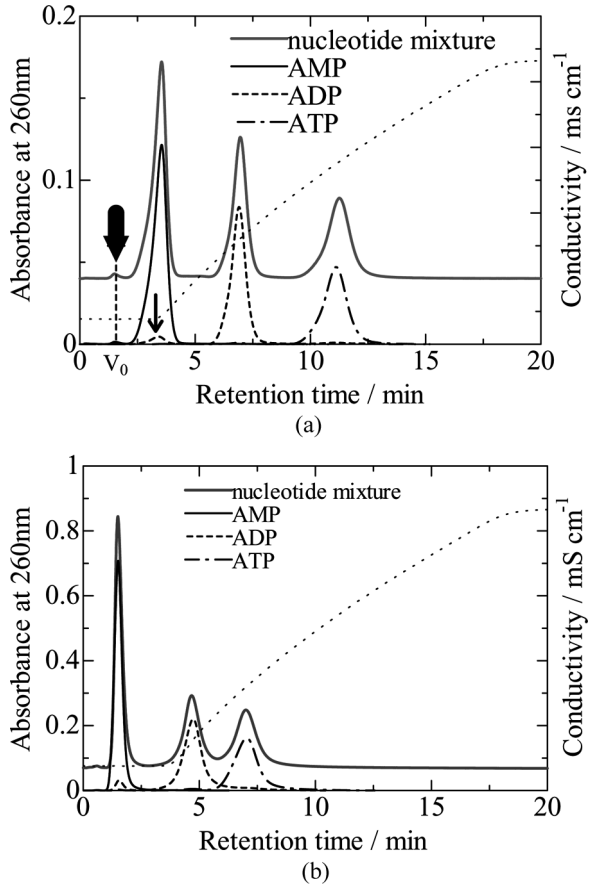

Figure 7. (a) Separation of AMP, ADP, and ATP on PEI-HAp. Chromatogram from the PEI-HAp column (3.9×100 mm) at a flow rate of 1 mL/min with linear gradient of 10–400 mM NaPB (pH 6.8) at room temperature. The fat arrow indicates the adenosine peak which is included in AMP peak and is one of the degradation products of AMP. (b) Separation of AMP, ADP, and ATP on HAp. Chromatogram from the HAp column (3.9×100 mm) at a flow rate of 1 mL/min with linear gradient of 10–400 mM NaPB (pH6.8) at room temperature.

Chromatography of Nucleotides

The nucleotide solutions and the nucleotide mixture solution was loaded on the columns and eluted in the regular manner Figure 7a,b show the chromatogram of the separation of nucleotides. The PEI-HAp could retain even AMP, although the HAp could not.

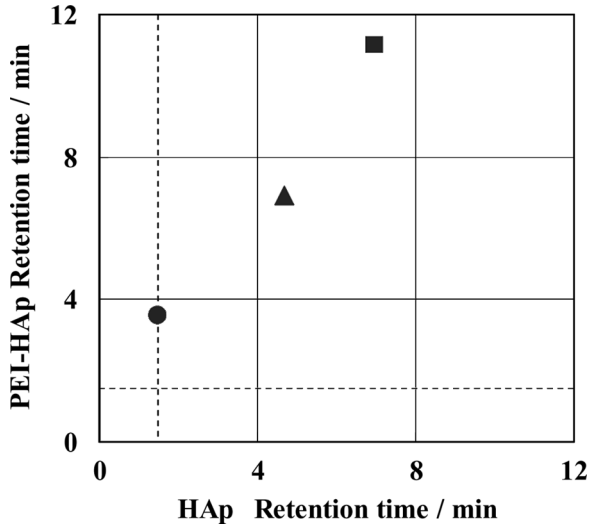

Figure 8. Correlation of retention time on the HAp with the retention time on PEI-HAp. (●) AMP, (▲) ADP and (■) ATP.

On the V_0 site you can notice the adenosine peak (indicated with a fat arrow mark) so that the nucleotides could be eluted more clearly depending on the number of the phosphate groups. Figure 8 shows the correlation of the retention time between HAp and PEI-HAp. The dotted lines in Figure 8 correspond to the retention time of the non-retention component. It was obvious that PEI-HAp is the useful material for separation of nucleotides.

Stability of PEI-HAp

The protein solution was loaded on the column and eluted with a liner gradient. The chromatograms are shown in Figure 9. In the repeating test, these chromatographic peaks didn't change and are almost the same pattern as the Figure 5a. This result shows that this PEI-HAp is the stable material.

In conclusion, the newly produced PEI-HAp showed approximately a similar chromatographic mode as PEI-silica[2] or PEI-cellulose[3] and polyallylamine carbon black.[11] It showed some more valuable results for the purification of various biological materials. First, it is stable under alkaline conditions, which are different from PEI-silica, and secondly, it is mechanically stronger and more rigid than PEI-cellulose, so that this column is useful for maybe many months. Thirdly, this is comparatively heat stable.

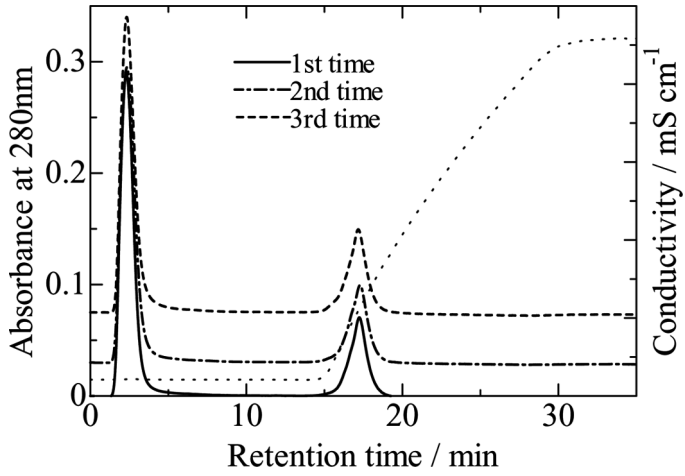

Figure 9. Repeating chromatography of a protein mixture (ovalbumin, myoglobin, α-chymotrypsinogen-A, and cytochrome *c*) on PEI-HAp column (3.9×100 mm). The ovalbumin peak is observed with a linear gradient of 1–400 mM NaPB (pH7) at a flow-rate of 0.6 mL/min.

Fourth it is rather low cost, and is suitable for the large column processing. The advantages of the matrix include low cost excellent dimensional and chemical stability and good handling properties. Also, the matrix is not susceptible to attack by microorganisms and can be reused in the same column over many months. The low degree of hydrophobicity makes the matrix an ideal choice for applications, which require the presence of detergents (neutral and positively charged). These features suggest applicability as an enzyme support.

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