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ION-EXCHANGE CHROMATOGRAPHY OF PROTEINS ON A POLYETHYLENEIMINE-GRAFTED HYDROPHILIC POLYMER FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A new, macroporous, synthetic polymer, Hydrophase™ HP-PEI, having highly hydrophilic surface characteristics enhanced by evenly distributed polyethyleneimine groups on its surface, was evaluated as an high-performance ion-exchange chromatographic packing material for the separation of proteins and nucleotides. A steel column (100 mm × 7.8 mm I.D.) packed with this material produces more than 33000 plates per m. This base polymer included large proteins, such as thyroglobulin and apoferritin, in a size-exclusion mode. With various proteins it gave high recovery, high resolution and high capacity.

INTRODUCTION

The usefulness of polyethyleneimine (PEI) in the preparation of high-performance ion-exchange chromatographic packing materials for separating proteins and nucleotides has been reported. Randerath and Randerath[1,2] first prepared such materials by adsorption of PEI and used it on cellulose for thin-layer chromatography to separate mononucleotides. Alpert and Regnier[3] demonstrated the usefulness of PEI by preparing a monolayer of PEI on a silica surface, which was subsequently cross-linked with a multifunctional oxirane and used for high-performance liquid chromatographic (HPLC) packings to separate proteins and nucleotides. Columns of PEI–silica resolved proteins quickly and effectively with excellent recoveries of enzyme activity. Vanecek and Regnier[4,5] used a similar technique with macroporous silica materials. PEI-coated silica packing material was also applied to the rapid and effective separation of oligonucleotides by Lawson *et al.*[6]. Flashner *et al.*[7] prepared a macroporous silica–PEI HPLC packing material for the separation of four major proteins and nucleotides. The technique was later applied to large-scale protein separation by Berkowitz *et al.*[8]. Schmuck *et al.*[9] also applied silica–PEI packing materials to preparative ion-exchange chromatography. They reported that 30- to 50-μm materials enabled the resolution of ovalbumin samples comparable to that of 6-μm materials.

Chicz *et al.*[10] have extended the PEI coating technique to alumina, magnesia,

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titania and zirconia-coated silica to improve pH stability. They successfully separated proteins on these columns and concluded that these non-silica supports with adsorbed stationary phases are potentially useful materials for operation in alkaline media. Gooding and Schmuck[11] compared the chromatographic characteristics of PEI-modified silica and the quaternized PEI–silica packing materials for protein separations. Kennedy *et al.*[12] reported that a cross-linked PEI–silica stationary phase could be used for multi-modal liquid chromatography columns. They collected fractions of proteins by ion exchange; these were then rechromatographed on the same column in a hydrophobic interaction mode.

Proteins, even though they are macromolecules, seem to be further distinguished from other high polymers. The long chain of each protein is folded in an unique configuration which it maintains so long as it evidences biological activity. Consequently, the interaction sites within protein molecules are arranged in more than one dimension. A good stationary phase must deal with this unique situation without disrupting the protein configuration. In other words, the interaction sites on the stationary phase must be very mobile so as not to change the protein configuration. Biologicaly active macromolecules prefer so-called dynamic surface arrangements where the ionic groups on the stationary phase are placed with flexibel spacer arms which can change the ionic interaction sites according to the shape of the proteins or the positions of their ionic sites. These requirements are directly the result of the "hydrophilicity" of the materials. From the behavior of PEI molecules in aqueous solutions, it is not difficult to speculate that PEI molecules create these dynamic surface arrangements. Because of PEI groups on the surface, the solid (polymer)/liquid (water) interface does not have a clear boundary. Thus, this region creates hydrophilicity. In other words, the interface has a very low free energy. It can be called a diffuse surface to distinguish it from conventional surfaces having a distinctive interface. The polymer surface is characterized by having polymer chains (PEI groups in this case) that "dissolve out" into a water phase when in contact with water and move freely on the surface. A portion of each PEI chain is, of course, tightly immobilized on the material surface (Hydrophase base polymer) so that it cannot dissolve away. These materials have a smooth and hard surface in the air, but the boundary of the interface becomes vague and diffuse when in contact with water. Thus, the surface treats biomolecules very gently without disturbing their natural properties. In this sense, it is safe to say that PEI-modified stationary phases are highly bio-compatible. It has been suggested that PEI-containing matrices perform better than others, such as DEAE-modified matrices, in protein separations due to their higher capacities and improved selectivities. The truth is that PEI provides an higher "active capacity". That is, the ion-exchange sites actively interact with approaching biomolecules because of the flexibility of the long PEI chain. Better selectivities are thereby obtained.

The use of PEI has been extended to synthetic polymer packing materials. Burke *et al.*[13] successfully applied PEI to non-porous polymethacrylate beads and Stevens *et al.*[14] applied such columns to protein separations. However, a major disadvantage of such pellicular materials is their small surface area, resulting in very low capacity. The material is useful only when the quantity of solute is small. Furthermore it is easily overloaded and definitely not satisfactory for preparative work.

There are two major types of materials used for chromatographic separation

media, namely silica-based and organic polymer-based materials Two major types of morphology are used to describe their different physical characteristics according to their performances, namely, non-porous and macroporous. For silica with PEI, both non-porous and macroporous materials are available, but for organic polymers with PEI, only non-porous is available.

In this paper, a new hydrophilic, macroporous, synthetic polymer, covalently coupled with PEI at the surface, is shown to be a suitable packing material for protein and nucleotide separations. Because of the nature of the macroporous polymer and the versatile PEI ligands, the material exhibits high capacity and excellent resolution of both small and large molecules.

EXPERIMENTAL

HPLC

Hydrophase HP-PEI polymer, which exhibits a chloride binding capacity of approximately 700 μequiv./g wet weight, and steel columns, 250 mm × 4.6 mm, 100mm × 6.5 mm, 100 mm × 7.8 mm and 300 mm × 7.8 mm, were obtained from Interaction Chemicals (Mountain View, CA, U.S.A.). An high-pressure eluent-delivery system (EM Science, Cherry Hill, NJ, U.S.A.) was used. It included two 655A-12 micro pumps, a D-2000 integrator, an L-5000 LC controller, a dynamic mixer (Hitachi, San Jose, CA, U.S.A.) and a 7161 sample injector (Rheodyne, Cotati, CA, U.S.A.). A Spectroflow 773 variable-wavelength UV detector (Kratos, Ramsey, NJ, U.S.A.) was integrated with the system. A Spectronic 1201 UV spectrophotometer (Milton Roy, Rochester, NY, U.S.A.) was used for the determination of protein concentrations.

Chemicals

Deionized, glass-distilled water was used for the preparation of buffers and protein solutions. All reagents were of analytical grade. Sodium chloride (Curtin Matheson, Florence, KY, U.S.A.) and hydrochloric acid (Fisher Scientific, Santa Clara, CA, U.S.A.) were used. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Aldrich (Milwalkee, WI, U.S.A.), cytochrome *c* (horse heart), hemoglobin (Hb) (bovine blood), transferrin (bovine), ovalbumin (chicken egg), thyroglobulin (bovine), apoferritin (horse spleen), nucleotides (from yeast), LDH-Isotrol, hypoxanthine, xanthine, orotic acid and uric acid from Sigma (St. Louis, MO, U.S.A.). HbA/F, HbF, HbA_2, HbA/C, HbA/F and HbAFSC were obtained from Isolab (Akron, OH, U.S.A.). Sodium azide, 0.25 g/l, was added to prevent bacterial growth.

All mobile phases were degassed under vacuum and filtered through 0.45-μm membranes prior to use Protein solutions (1–250 mg/ml) were freshly prepared and stored at 4°C when not in use. All experiments were performed at room temperature.

Columns, 100 mm × 4.6 mm, 250 mm × 4.6 mm, 100 mm × 66.5 mm, 7.8 mm × 7.8 mm, 100 mm × 7.8 mm and 300 mm × 7.8 mm, were prepared by means of a Shandon Packer using water at 6000 p.s.i.

RESULTS

Physical properties of the packing material

A 300 mm × 7.8 mm column packed with Hydrophase polymer was examined

in size exclusion mode to approximate the maximum pore size of the material. The packing material included proteins such as thyroglobulin (MW = *ca.* 669000) and apoferritin (MW = *ca.* 443000) and the eluent was 50 m*M* Tris buffer with 0.1 *M* sodium chloride at pH 8.1. The packing material had an internal pore volume of approximately 4% of the packed volume. The surface area of the packing material according to a gas absorption method (B.E.T.) performed by Micromeritics (Norcross, GA, U.S.A.) was 4.07 m^2/g with 30% nitrogen and 70% helium. The shrinkage/selling ratio of the material was less than 0.9% in aqueous buffers such as from 10 to 50 m*M* Tris buffer and from 10 to 20 m*M* phosphate buffer with pH between 2 and 10 and from 0 to 0.5 *M* sodium chloride. At flow-rates greater than 4 ml/min, the pressure–flow-rate correlation began to deviate from linearity, which may be an indication that the column should be used at less than 4 ml/min.

Protein recovery and protein binding capacity

Data on the recovery of various proteins from 100 mm × 7.8 mm column were obtained by loading approximately 10–20 mg of protein on the column in 20 m*M* Tris buffer at pH 8.0, eluting with the same buffer containing 0.5 *M* sodium chloride and collecting all of the material which absorbed at 280 nm. An equivalent protein sample was diluted to a known volume, and the absorbance of each solution at 280 nm was determined on a Milton Roy Spectronic 1201 UV spectrophotometer. The recovery represents the ratio of these two values. The results are shown in Table I.

The hemoglobin binding capacity in a 100 mm × 7.8 mm column, packed with Hydrophase HP-PEI, was determined as follows: hemoglobin solution (in 20 m*M* Tris, pH 8.5) was injected in sequential 0.10-mg pulses until it was detected in the effluent. The capacity was determined to be *ca.* 41.9 mg hemoglobin per ml wet packing material.

Chromatographic separation of proteins

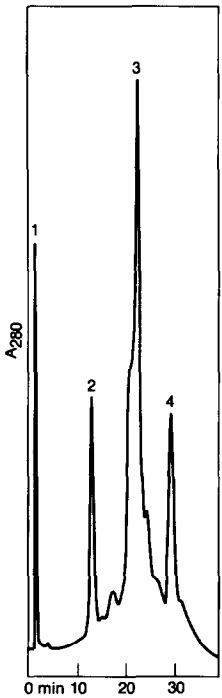
Figs. 1 and 2 show chromatograms of four proteins (cytochrome *c*, hemoglobin, transferrin and ovalbumin) obtained by 30-min and 10-min gradient elutions, respectively. A mixture of four hemoglobins (HbC, HbS, HbA and HbF) was chromatographed on columns of various sizes, packed with Hydrophase HP-PEI, with the same salt gradient. Results from a 100 mm × 4.6 mm column are shown in Fig.

TABLE I

PERCENT RECOVERY OF VARIOUS PROTEINS FROM HYDROPHASE™ HP-PEI

Loading: 20 m*M* Tris at pH 8.0. Eluent: 20 m*M* Tris with 0.5 *M* sodium chloride at pH 8.0. Sample: 10–20 mg in 10-μl loop.

Protein	*Recovery (%)*
Myoglobin	96.7
Hemoglobin	87.8
Conalbumin	89.6
α-Lactglobulin	100.0
Insulin	96.5



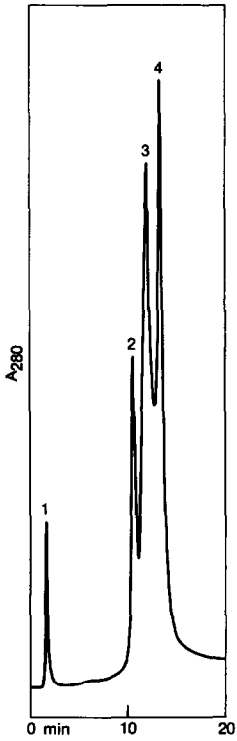


Fig. 1. Anion-exchange chromatography of proteins. Column: Hydrophase HP-PEI, 100 mm × 7.8 mm. Sample: 1 = cytochrome *c*; 2 = hemoglobin; 3 = transferrin; 4 = ovalbumin. Temperature: ambient. Flow-rate: 1.0 ml/min. Detection: UV 280. Eluents: A = 10 m*M* Tris–HCl (pH 6.02); B = A + 0.5 *M* sodium chloride; linear gradient, 0 to 100% B in 30 min.

Fig. 2. Anion-exchange chromatography of proteins. As in Fig. 1 except 10-min gradient.

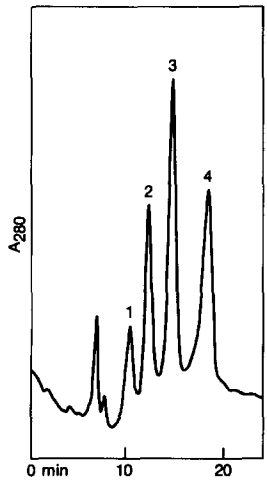
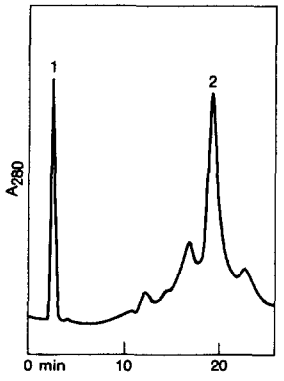


Fig. 3. Anion-exchange chromatography of hemoglobin. Column: Hydrophase HP-PEI, 100 mm × 4.6 mm. Sample: 1 = HbC; 2 = HbS; 3 = HbA; 4 = HbF. Eluents: A = 10 m*M* Tris–HCl (pH 8.06); B = A + 0.5 *M* sodium chloride: linear gradient. 0 to 100% B in 30 min.



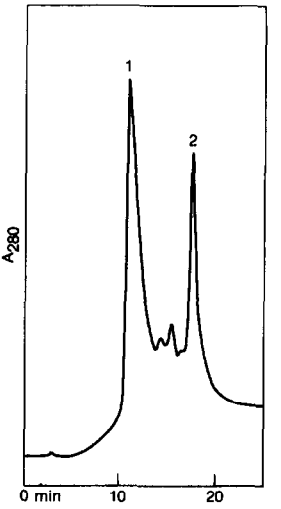


Fig. 4. Anion-exchange chromatography of proteins. Column: Hydrophase HP-PEI, 250 mm × 4.6 mm. Sample, 1 = cytochrome *c*; 2 = ovalbumin. Temparature: ambient. Flow-rate: 1.0 ml/min. Detection: UV 280. Eluents: A = 10 m*M* Tris · HCl (pH 7.80); B = A + 0.5 *M* sodium chloride linear gradient, 0 to 100% B in 20 min.

Fig. 5. Anion-exchange chromatography of proteins. Column: Hydrophse HP-PEI, 150 mm × 4.6 mm. Sample: 1 = IgG; 2 = ovalbumin. Temperature: ambient. Flow-rate: 1.0 ml/min. Detection: UV 280. Eluents: A = 10 m*M* Tris · HCl (pH 7.91); B = A + 0.5 *M* sodium chloride; linear gradient, 0 to 100% B in 15 min.

3. Fig. 4 shows the results of separating cytochrome *c* and ovalbumin, and Fig. 5 of IgG and ovalbumin.

Nucleotide separation

Adenosine 5'-monophosphate, -diphosphate and -triphosphate were separated using a salt gradient in 10 m*M* Tris buffer at pH 7.5 (Fig. 6).

Isocratic separation

The urines hypoxanthine, xanthine, orotic acid and uric acid were separated on a Hydrophase HP-PEI (100 mm × 7.8 mm) column by isocratic elution with 10 m*M* Tris buffer and 0.1 *M* sodium chloride at pH 6.02 (Fig. 7).

Preparative separation

A total of 24 mg of four proteins, cytochrome *c* (5 mg), hemoglobin (4 mg), transferring (5 mg) and ovalbumin (10 mg), were chromatographed using a 100 mm × 7.8 mm column packed with Hydrophase HP-PEI. The results are shown in Fig. 8. A total of 26.3 mg of cytochrome *c* (11 mg) and hemoglobin (16.3 mg) were also chromatographed. The separation is shown in Fig. 9.

Plate numbr calculations

A plate number of 29 500 per m with an asymmetry factor of 1.11 (10% of the peak height) was recorded with uric acid, using 10 m*M* Tris buffer with 0.15 *M*

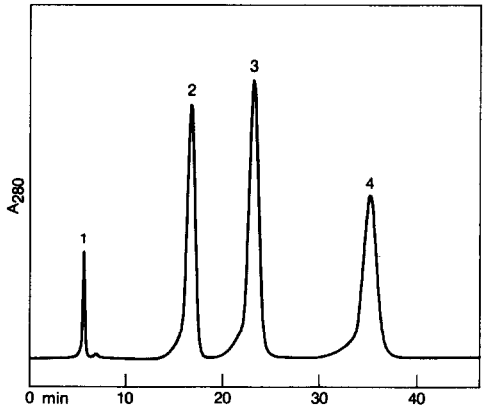


Fig. 6. Anion-exchange chromatography of nucleotides. Column: Hydrophase HP-PEI, 100 mm × 6.5 mm. Sample: 1 = AMP; 2 = ADP; 3 = ATP. Temperature: ambient. Flow-rate: 1.0 ml/min. Detection: UV 280. Eluents: A = 10 m*M* Tris · HCl (pH 7.5); B = A + 0.5 *M* sodium chloride; linear gradient from A to B in 10 min.

Fig. 7. Isocratic separation of nucleic acid derivatives. Column: Hydrophase HP-PEI, 100 mm × 7.8 mm. Sample: 1 = hypoxanthine, 2 = xanthine; 3 = orotic acid; 4 = uric acid. Temperature: ambient. Flow-rate: 1.0 ml/min. Detection: UV 280. Eluent: 10 m*M* Tris–HCl with 0.1 *M* sodium chloride (pH 6.02).

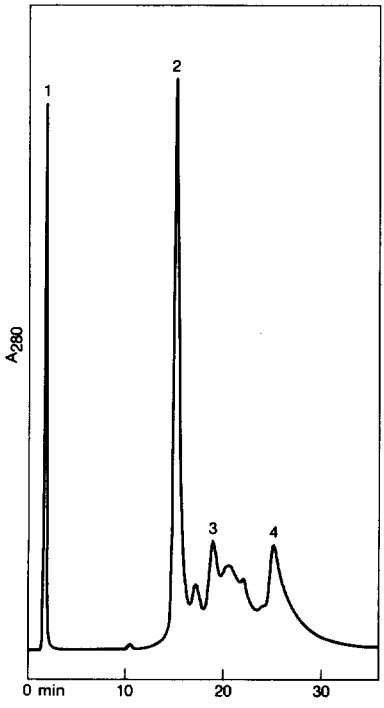


Fig. 8. Preparative separation of proteins. Column: Hydrophase HP-PEI, 100 mm × 7.8 mm. Sample: total 24 mg of cytochrome *c* (1), hemoglobin (2), transferrin (3) and ovalbumin (4). Temperature: ambient. Flow-rate: 1 ml/min. Detection: UV 280. Eluents: A = 10 m*M* Tris–HCl (pH 8.03); B = A + 0.5 *M* sodium chloride; linear gradient from A to B in 30 min.

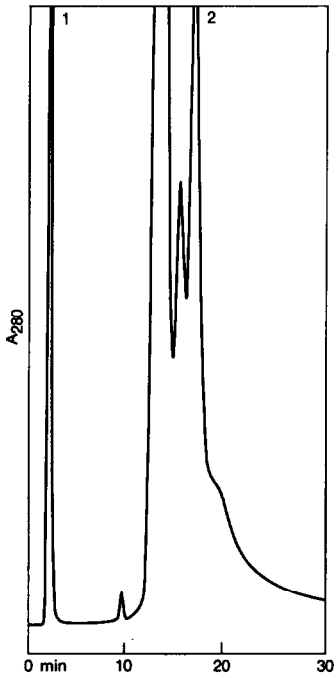


Fig. 9. Preparative separation of proteins. Sample: total of 26.3 mg of cytochrome *c* (1) and hemoglobin (2). Other details as in Fig. 8.

sodium chloride (pH 6.02) at a flow-rate of 1 ml/min. A plate numbr of 33 100 per m with an asymmetry factor of 1.24 was also recorded using 10 m*M* Tris buffer with 75 m*M* sodium chloride (pH 6.02) at a flow-rate of 0.5 ml/min.

DISCUSSION AND CONCLUSIONS

A 100 mm × 7.8 mm column packed with Hydrophase HP-PEI can be used from the microgram of analytical separations to the sub-gram range in semi-preparative aplications. The support is rigid in aqueous buffers and organic modifiers normally employed in ion-exchange chromatography of biomolecules. Separations can be routinely performed in the range pH 2–12. An efficiency of more than 33 000 plates per m has been obtained. Because of its macroporosity, Hydrophase HP-PEI showed a very high ion-exchange capacity and high sample capacity. These characteristics are important, for example, in clinical analyses, where it is often necessary to use large sample loads to detect trace constituents in mixtures. In addition, the pH stability of the material exceeds that of silica-based materials. The loading capacity of the material seems to surpass that of pellicular materials. The results from these experiments indicate that this material will make a significant contribution toward solving problems in both analytical and semi-preparative biomolecule separations.

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