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Ion-exchange high-performance liquid chromatography of nucleotides and polypeptides on new types of ion-exchange sorbents, based on polystyrene-coated silicas

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ABSTRACT

A novel type of ion exchanger was prepared by multipoint covalent binding of polystyrene chains onto the surface of porous silica followed by polymer-analogous modification of the bonded layer. Both anion and cation exchangers were synthesized and examined in the separation of nucleotides and proteins. Rapid and efficient separation of basic polypeptides on strong anion exchangers and that of acidic polypeptides on strong cation exchangers could be achieved. For the separation of complete mixtures of polypeptides the application of zwitter-ionic ion exchangers can be recommended.

INTRODUCTION

Ion-exchange high-performance liquid chromatography (HPLC) proved to be a powerful chromatographic method for separating ionic inorganic and organic compounds. This method was found to be especially useful for the separation of biologically active materials and several review papers on this topic have been published [1,2]. In this kind of separation, various types of polymer-based ion exchangers are commonly used as adsorbents, whereas the application of silica-based exchangers is limited by the hydrolytic stability of the latter. Many attempts have been made to overcome this disadvantage of the silica packing. One of the most promising approaches was introduced by Alpert and Regnier [3], who first adsorbed polyethylene imine on the surface of macroporous silicas and then cross-linked the polymer by various cross-linking agents. In this manner, a fully insoluble and hydrolytically stable polymeric layer was formed on the surface of the silica. Another type of silica-based ion exchanger with a polymeric modifying layer was introduced by Schomburg and co-workers [4,5]. Using chemical modification of polybutadiene which was cross-linked on the surface of silica they succeeded in preparing both anion and cation exchangers. In this article, we report on the synthesis and use of new ion exchangers

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which are based on polystyrene-coated silica. We were the first to describe the chemical binding of polystyrene chains onto the surface of silica as the initial step in the preparation of chiral polymeric bonded ligand-exchanging phases [6]. Other types of chemical transformations of the bonded polystyrene-coated silica easily produce cation or anion exchanging phases which are of excellent chromatographic performance and enhanced hydrolytic stability when compared to monomeric phases.

EXPERIMENTAL

Materials

Base silicas, Zorbax PSM-500 and PSM-1000, both of particle diameter (d_p) = 5 μm, were purchased from Du Pont de Nemours (Wilmington, DE, USA). All solvents used in the chromatographic experiments were of HPLC grade and were a gift of E. Merck (Darmstadt, Germany). Other materials were laboratory grade and were used as purchased.

The proteins used in the chromatographic experiments included ribonuclease A from bovine pancrease, myoglobin from horse skeletal muscle, hen egg albumin, chicken egg white lysozyme and human transferrin, (all from Serva, Heidelberg, Germany), trypsin inhibitor from soy-beans, conalbumim, ferritin, and cytochrome *c* (all from Boehringer, Mannheim, Germany). Nucleotides were purchased from Sigma Chemie, Deisenhofen, Germany.

Equipment

The chromatograph employed consisted of two LKB 2150 pumps, the LKB controller 2152, the variable-wavelength detector BT 3030 (Biotronik, Maintal, Germany) operated at 220 nm and a potentiometic recorder 2210 (LKB, Bromma, Sweden)[a]. Columns were 125 × 4.6 mm I.D. (Hyperchrom, Bischoff, Leonberg, Germany).

Preparation of ion exchangers

The coating procedure for polystyrene–vinylsilane copolymers onto silica was described elsewhere in detail [7]. The characteristic data of the polymer-modified silicas were as follows: carbon content: PSM-500 2.4% (w/w), PSM-1000 1.4% (w/w). On the basis of the specific surface area of the native silica (PSM-500 25 m^2/g, PSM-1000 15 m^2/g), the ligand density of the average polymer unit was estimated to be 9.4 $\mu mol/m^2$ for PSM-500 and 9.8 $\mu mol/m^2$ for PSM-1000. Such high values of ligand density are rather common for polymer-modified macroporous silicas [7].

The subsequent chemical modification of polystyrene-coated silicas was performed by a method quite similar to that known for pure polystyrene. An aliquot of the modified PSM-500 was sulphonated by using chlorosulphonic acid in dichloroethane at room temperature yielding a cation exchanger with a capacity of 0.21 mmol SO_3H groups per gram based on the sulphur content. Another aliquot of polymer-coated PSM-500 was chloromethylated by monochloromethyl ether in dichloromethane in the presence of $SnCl_4$ and then aminated by the action of an alco-

[a] The sensivity of the detector was 0.1 a.u.f.s.

holic solution of trimethylamine thus producing an anion exchanger with a capacity of 0.17 mmol $N(CH_3)_3$ groups per gram.

On the bases of polymer-coated PSM-1000, a zwitter-ionic exchanger was synthesized. For this purpose, the chloromethylated product was sulphonated and then aminated. Because of the low surface area of this silica, it was not possible to determine analytical capacities. The content of sulphur and nitrogen were $< 0.2\%$ and thus below the limit of determination. However, the chromatographic experiments clearly demonstrated their anion and cation exchange capabilities.

RESULTS AND DISCUSSION

The method for the synthesis of anion exchangers suggested by Alpert and Regnier [3] appeared to be very attractive because of its chemical background: The adsorption of the polymer (polyethylene imine) on the surface of silica. This kind of polymer–surface interaction results from the electrostatic attraction between the deprotonated silanol groups of silica and the protonated positively charged amino groups of the poly-ethylene amine. This prevents the formation of a thick, polymolecular adsorption layer and, at the same time, allows a dense coating of the surface. It is difficult to achieve this type of interaction for a broader series of polymers. In the method postulated by Schomburg [5], the nature of the polymer is not that critical, because it is placed on the silica surface by means of the evaporation of the polymer solution. In this particular case, however, problems arise with regard to the homogeneity of the polymer layer on the surface, which may affect the pore structure of the silica and the reproducibilty of the coating process.

In our procedure, the adsorption of the polymer is the result of the chemical reaction of silanol groups at the silica surface with the ethoxysilane units of the styrene–vinylsilane copolymer. Vinylsilane copolymers can be synthesized with different vinylic monomers. Styrene is the most attractive one since styrene-based packings are very common in classical ion-exchange chromatography and many kinds of well-known ion exchangers can currently be produced on polystyrene-coated silicas. Since the aim of this work was to separate biological substances, macroporous silicas with average pore diameters of 50 and 100 nm were chosen as base silicas. The specific surface area of the macroporous silicas was low, corresponding to the large pore size. However, the polymeric coating approximately exhibited a doubled ligand density, calculated on the basis of monomer units, as is the usual observation for silicas with bonded monomeric silanes. This meant that the polymeric-bonded ion exchangers exhibited a higher capacity than those obtained from the modification of monomeric bonded silicas. The polymeric layer did not impair the mass-transfer kinetics and hence the performance of the ion exchanger and also provided an improved shielding of the silica surface. As demonstrated in Fig. 1 a fast and complete separation of 11 nucleotides on an anion exchanger could be achieved in 10 min in a neutral phosphate buffer with a linear salt gradient. Two nucelotides co-elute in peak 9. Anion-exchange chromatography of proteins of the same exchanger is shown in Fig. 2. In this case, a double gradient is used: a linear salt gradient and an exponential proton gradient. Again, an excellent peak shape is observed for the proteins resolved under these conditions.

Fig. 3a shows a separation of a more complex mixture, and, as it often happens

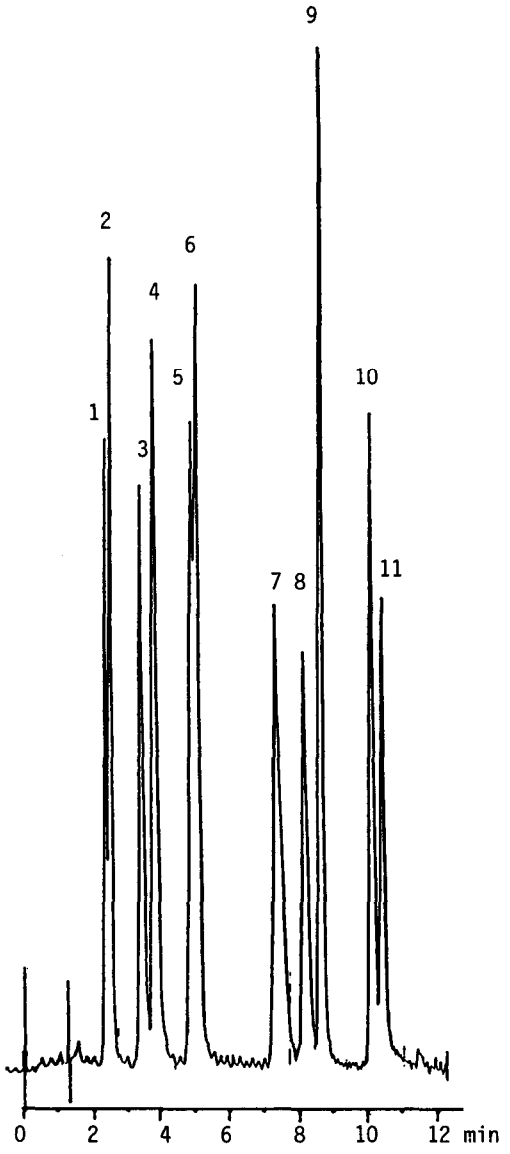

Fig. 1. Separation of a synthetic mixture of nucleotides on a strong anion exchanger made from polystyrene-coated PSM-500. Column: 125 × 4.6 mm; flow-rate 1 ml/min; gradient elution: from 0 to 14% B in 1 min, from 14 to 20% B in 5 min, from 20 to 50% B in 5 min A: 10 m*M* phosphate buffer, pH 7.0; B: 10 m*M* phosphate buffer, pH 7.0, 0.1 *M* KCl. Components: 1 = uridine monophosphate, 2 = cytidine monophosphate, 3 = adenosine monophosphate, 4 = guanosine monophosphate, 5 = uridine disphosphate, 6 = citidine diphosphate, 7 = adenosine diphosphate, 8 = guanosine diphosphate, 9 = uridine triphosphate + cytidine triphosphate, 10 = adenosine triphosphate, 11 = guanosine triphosphate.

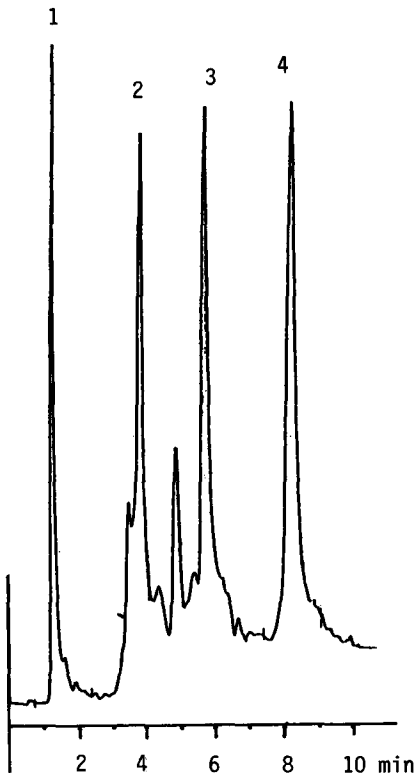

Fig. 2. Separation of a protein mixture on a strong anion exchanger made from polystyrene-coated Zorbax PSM-500. Column: 125 × 4.6 mm; flow-rate: 1 ml/min; gradient elution: from 0 to 50% B in 10 min. A: 10 m*M* phosphate buffer, pH 8.5; B: 10 m*M* phosphate buffer, pH 5.5, 1 m*M* KCl. Components: 1 = myoglobin, 2 = transferrin, 3 = ovalbumin, 4 = trypsin inhibitor.

in the separation of proteins, the general shape of the chromatogram depends on the gradient time. At a gradient time of 40 min (Fig. 3b) the peak of ferritin splits into three, one of which co-elutes with the conalbumin. The changes are well-reproducible, and on a second run of the gradient in 10 min; the shape of the chromatogram fully recovers (compare Fig. 3a and c).

The cation-exchange chromatography of proteins of the synthesized cation exchanger is illustrated in Fig. 4. As above mentioned, a double gradient was applied to achieve a better separation and to improve the peak shape. As is quite common with ion-exchange chromatography, only the appropriate species of a sample mixture can be resolved on a given type of exchanger: acidic proteins on cation exchanger and basic proteins on anion exchangers. For example, strongly basic proteins such as lysozyme elute with the void volume from the anion-exchange column, but are extremely strongly retained on the cation-exchange column. This unfavourable situation initiated the synthesis of a zwitter-ionic packing which has both sulphonic and quarternary ammonium groups. The silica packing was PSM-1000. The results shown

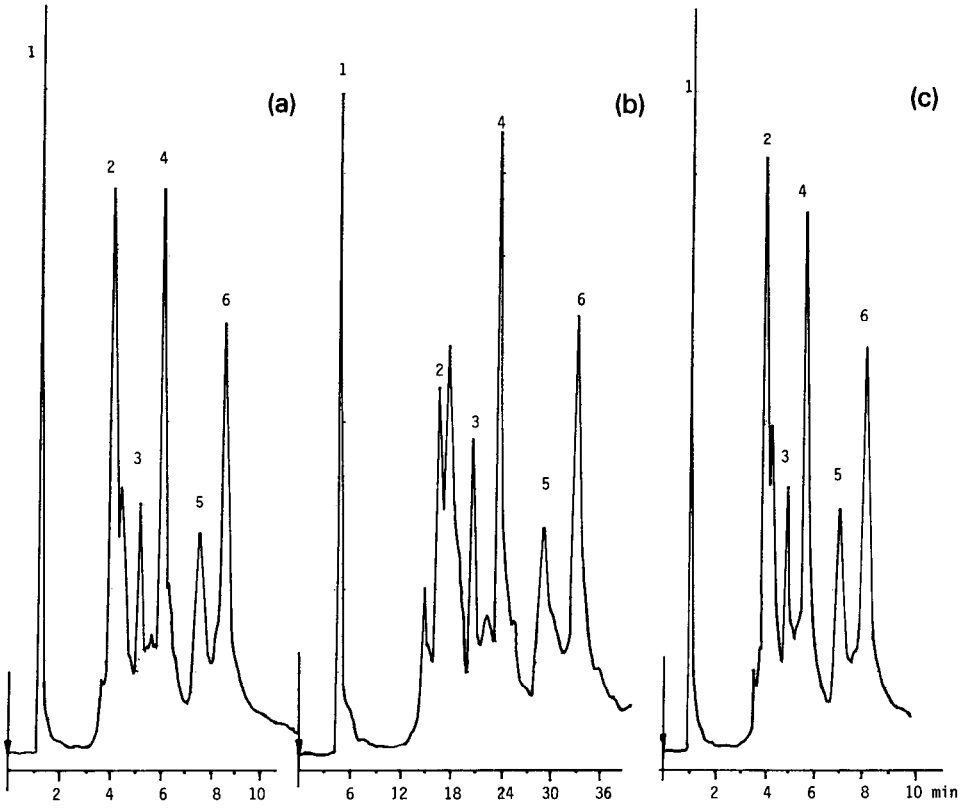

Fig. 3. Separation of a protein mixture on a strong anion exchanger made from polystyrene-coated Zorbax PSM-500. Column: 125 × 4.6 mm; gradient elution: from 5 to 50% B. Eluents A and B are described in the caption to Fig. 2. Gradient time and flow-rate: (a) 10 min, 1 ml/min; (b) 40 min, 0.25 ml/min; (c) 10 min, 1 ml/min. Components: 1 = myoglobin, 2 = ferritin, 3 = conalbumin, 4 = ovalbumin, 5 = albumin, 6 = trypsin inhibitor.

in Fig. 5 clearly show the ion-exchange properties of the packing. Myoglobin is seen to be eluted later than ovalbumin. Myoglobin is not retained on anion exchangers, ovalbumin is not retained on cation exchangers. The peak of lysozyme (last eluting peak) is relatively broad, even at the high flow-rate used for elution. It seems that this broadening is due to the mixed-mode interactions between lysozyme and the exchanger.

The results also reveal that the ion exchanger contains cationic groups in excess of anionic groups. In spite of this fact, the zwitter-ionic exchanger is well-suited to resolve different types of peptides and proteins and even lysozyme residues in about 10 min.

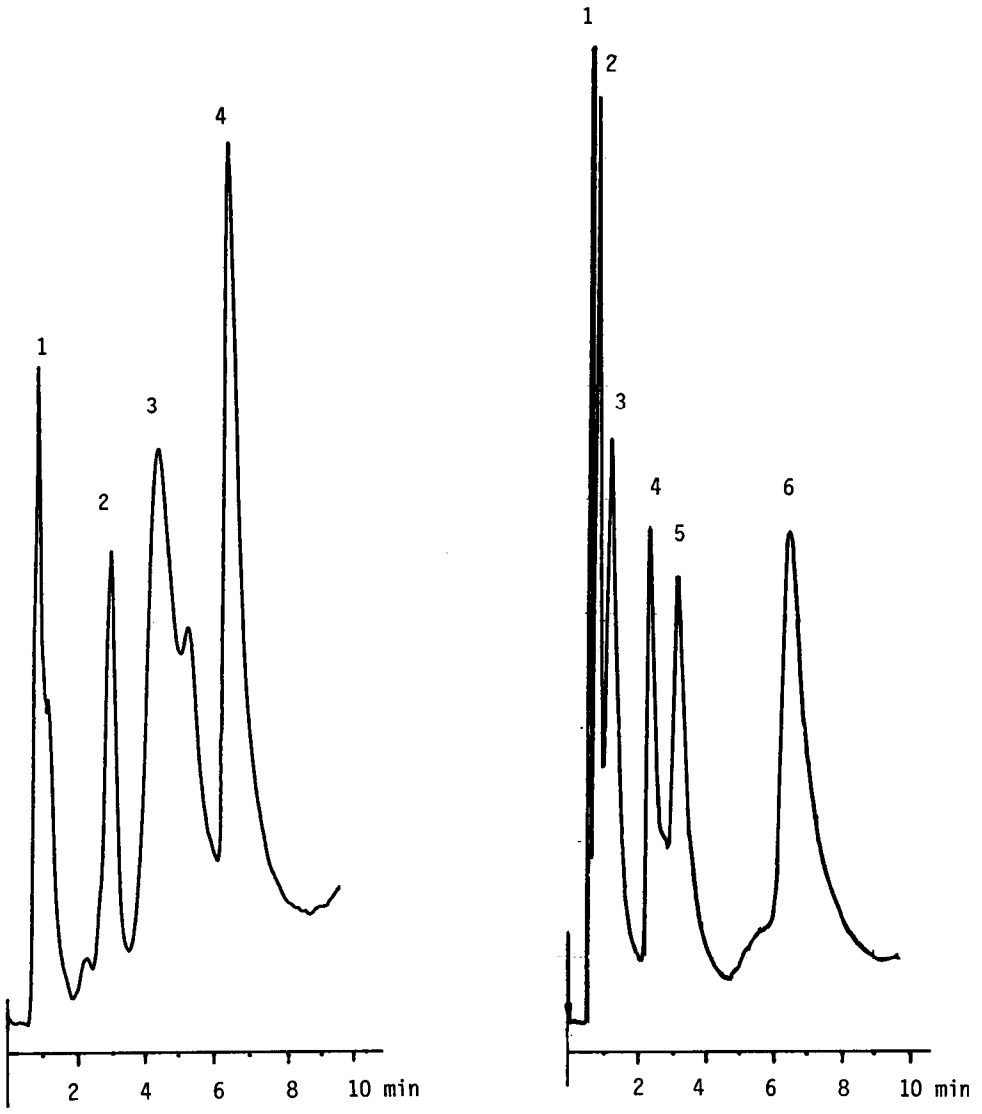

Fig. 4. Separation of a protein mixture on a strong cation exchanger made from polystyrene-coated Zorbax PSM-500. Column: 125 × 4.6 mm; flow-rate: 1 ml/min; gradient elution: from 30 to 100% B in 10 min. A: 10 m*M* phosphate buffer, pH 5.0; B: 10 m*M* phosphate buffer, pH 8.5, 1 *M* KCl. Components: 1 = ovalbumin, 2 = ribonuclease, 3 = conalbumin, 4 = cytochrome *c*.

Fig. 5. Separation of a protein mixture on a zwitter-ionic exchanger made from Zorbax PSM-1000. Column: 125 × 4.6 mm; gradient elution: from 16 to 50% B in 4 min at 2 ml/min, from 50 to 100% B in 1 min at 4 ml/min. A: 10 m*M* phosphate buffer, pH 5.0; B: 10 m*M* phosphate buffer, pH 8.0, 1 *M* KCl. Components: 1 = ovalbumin, 2 = myoglobin, 3 = transferrin, 4 = ribonuclease, 5 = cytochrome *c*, 6 = lysozyme.

CONCLUSIONS

A simple method for the preparation of both cation and anion exchangers as well as zwitter-ionic bonded phases was developed for ion-exchange HPLC of proteins and nucleotides. The synthesis is based on polymer-analogous transformations of polystyrene-coated macroporous silicas. The polymer coating permits the attainment of a dense shielding of the silica surface and a higher capacity compared to ion exchangers modified with monomeric silanes. The ion exchanger columns showed excellent performance for proteins and nucleotides. Especially interesting was the application of the zwitter-ionic exchanger which allowed us to resolve both acidic and basic proteins in one run with one buffered mobile phase.

REFERENCES

1 B. S. Welinder and S. Linde in W. S. Hancock (Editor), *Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, Vol. II, CRC Press, Boca Raton, FL, 1985, p. 357.
2 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979.
3 A. J. Alpert and F. E. Regnier, *J. Chromatogr.*, 185 (1974) 375.
4 G. Schomburg, A. Deege, U. Bien-Vogelsang and J. Köhler, *J. Chromatogr.*, 282 (1983) 27.
5 G. Schomburg, *LC · GC Int.*, (1988) 34.
6 A. A. Kurganov, A. B. Tevlin and V. A. Davankov, *J. Chromatogr.*, 261 (1983) 223.
7 A. A. Kurganov, O. Kuzmenko, V. A. Davankov, B. Eray, K. K. Unger and U. Trüdinger, *J. Chromatogr.*, 506 (1990) 391.
8 V. A. Davankov, A. A. Kurganov and K. K. Unger, *J. Chromatogr.*, 500 (1990) 519.