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Phosphorylated polystyrene resins in high-performance ion-exchange chromatography

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ABSTRACT

Non-porous polystyrene beads were highly substituted with phosphate groups, grafted via spacers on hydroxylated polystyrene. The phosphorylated polystyrene resins exhibit ion-exchange properties that are different to those of the usual stationary phases containing carboxymethyl or sulphopropyl groups. Moreover, the substituted polymers possess high mechanical and chemical stability, hydrophilicity and ion-exchange capacity. Various proteins were fractionated with these phosphorylated resins, packed in high-performance liquid chromatographic columns. The chromatographic separation occurred according to an ion-exchange mechanism; the adsorption of biomolecules was controlled by the pH and ionic strength of the eluent.

INTRODUCTION

The stationary phases used in ion-exchange chromatography (IEC) for protein separations are generally functional polymers substituted with carboxymethyl or sulphopropyl groups in the case of cationic proteins and aminoethyl or diethyl aminoethyl groups in the case of anionic proteins. However, these stationary phases, generally based on polysaccharides, usually have poor mechanical properties which prevent their use in high-performance liquid chromatography (HPLC) and limit scaling up of the separation.

Therefore, great effort [1–3] has been devoted to the development of new phases based on silica or cross-linked synthetic polymers with better mechanical properties, which would allow their use in HPLC. Cross-linked polystyrene (PS) has recently been shown to perform well at high pressure when substituted with L-arginyl methyl ester [4] or when substituted with sulphonyl groups followed by coating with polyethyleneimine [5].

Little attention has been paid to phosphorylated stationary phases despite their potential interest for both IEC and affinity chromatography, and no phosphorylated PS resins have been described. Therefore, we decided [6] to synthesize phosphorylated PS derivatives via a multi-step procedure.

This paper describes the synthesis of these new phases and their use as stationary phases in the chromatography of proteins.

EXPERIMENTAL

Preparation of phosphorylated polystyrene resins

Polystyrene beads cross-linked with 2% divinylbenzene were obtained from Fluka (Buchs, Switzerland). These are nearly spherical and are about 50 μm in diameter (200–400 mesh). The substitution of PS was performed in a multi-step reaction with analytical-reagent grade reagents.

In the first step, PS was chloromethylated in more than 90% yield as described previously [7,8]. Starting from this material poly(hydroxypropylstyrene) was then prepared [7] (Fig. 1): 4 g of chloromethylated PS were heated at 80°C for 24 h with 30 ml of diethyl malonate, 10 g of potassium carbonate and 1 g of tetrabutylammonium hydrogensulphate. After filtration, the polymer was successively suspended for 12 h in 200 ml of 6 *M* sodium hydroxide,

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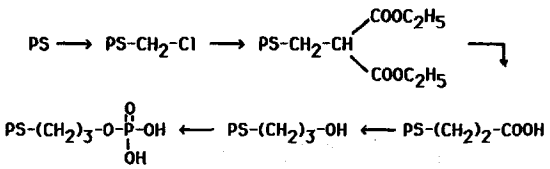

Fig. 1. Synthesis of insoluble phosphorylated polystyrene (PS) derivatives.

then in a mixture of 200 ml of water and 200 ml of 6 *M* hydrochloric acid for 24 h at 110°C. The resulting poly(carboxyethylstyrene) was resuspended in 45 ml of di(2-methoxyethyl) ether, 75 ml of 1 *M* sodium borohydride and 18 ml of boron trifluoride etherate for 12 h at room temperature, leading to poly(hydroxypropylstyrene). Finally, 4.1 g of poly-(hydroxypropyl)styrene in 25 ml of trimethyl phosphate were reacted with 3 ml of phosphorus oxychloride at 60°C for 12 h; the phosphorylated resin was stirred for 2 h in 250 ml of water, then washed with water, dioxane, methanol and dichloromethane and dried at 80°C under vacuum.

Chemical characterization

Acid–base titrations of phosphoester derivatives of polystyrene were performed [7] under a flow of nitrogen with an Tacussel TT100 + TT300 automatic titrator in freshly distilled water containing 0.2 *M* NaCl; small amounts of titration solution (10^{-3} ml of 0.1 *M* NaOH) were added with automatic monitoring which allowed the thermodynamic equilibrium to be approached; thus, the rate of addition was about 0.5 ml/day.

Infrared spectra were recorded with a Perkin-Elmer Model 580 spectrophotometer, the polymeric samples being dispersed in KBr pellets.

Elemental microanalyses were carried out by the Service Central d'Analyse (CNRS, Vernaison, France).

Chromatographic experiments

The chromatographic system consisted of a three-headed (120°) pump (Merck LC219), connected to a Rheodyne Model 7126 injection valve (sample loop 200 μl), a 25 x 0.4 cm I.D. stainless-steel column (Merck–Clevenot) in which about 2 g of phosphorylated polystyrene beads were packed by a slurry method, a variable-wavelength UV–visible detector (Merck LC313) and the gradient system. The system was controlled by an Epson QX-10 computer. Experiments were performed at room temperature.

The eluents were prepared with freshly distilled water and were filtered (0.22-μm Millipore GS membrane) and degassed. Analytical-reagent grade reagents were used in these experiments.

Samples

The proteins examined were myoglobin, bovine serum albumin (BSA), transferrin and horse cytochrome *c* purchased from Sigma (La Verpillière, France). Buffered protein solutions (2 mg/ml) in the starting mobile phase were stored at 4°C before use.

RESULTS AND DISCUSSION

Synthesis and characterization

The synthesis described under Experimental results in a three-carbon spacer between the aromatic styrene nucleus and a phosphomonoester group (Fig. 1). Elemental analysis (Table I) indicates a substitution ratio of 2.6 mequiv. of phosphate groups per gram of dry resin. Thus, on the macromolecular chains, 62% of the monomer units bear phosphate groups, randomly distributed along the polymer chains [6,9,10]. The other substituents are mainly hydroxyl groups. Less than 10% of the styrene units are unsubstituted.

As shown in Fig. 2, the acid–base titration curve

TABLE I

CHARACTERISTICS OF THE STATIONARY PHASE

Elemental analysis of the phosphorylated polystyrene

C (%)	H (%)	O (%)	P (%)	Phosphate groups
61.7	6.7	19.2	8.0	2.6 mequiv./g

Elemental analysis of the precursors

C (%)	H (%)	O (%)	Cl (%)	Precursors
79.1	8.1	10.2	–	PS–$(CH_2)_3OH$
74.3	6.8	16.2	–	PS–$(CH_2)_2COOH$
72.3	7.1	17.2	<0.2	PS–$CH_2CH(COOC_2H_5)_2$
72.0	6.1	–	20.8	PS–CH_2Cl
90.4	7.6	0.2	0.09	PS

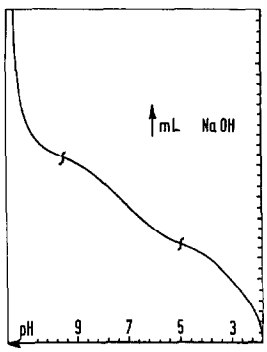

Fig. 2. Titration curve of phosphorylated polystyrene resin with 0.1 *M* sodium hydroxide.

showed evidence of phosphomonoester groups characterized by their acidic properties. Fig. 2 exhibits two inflection points, one strong and one weak acid, with apparent pK_a values of *ca.* 4 and 8.

The chemical and physical characteristics of the resin remained unchanged after treatment for several weeks at a temperature of 160°C, with aqueous solutions with pH values ranging between 1 and 14 and at pressures as high as 200 bar. Moreover, the swelling ratio of the phosphorylated polymer in aqeuous solutions was about 200% compared with the dry resin, indicating the high hydrophilicity of this material. From these results, it can be concluded that the syntheses are easily reproducible and well characterized and that the chemical and physical stabilities of the hydrophilic resin are excellent even under extreme temperature, pressure and acidic and basic conditions. Moreover, depending on the $POCl_3$ volume of phosphorus oxychloride added, the range of substitution ratios was from 0 to 80% of phosphate groups [7,11].

Chromatographic experiments

We assessed the applicability of phosphorylated PS resins in the ion exchange of proteins by performing HPLC experiments with solutions of albumin, myoglobin and cytochrome *c* and mixtures of these proteins, the molecular weights (MW) and isoelectric points (p*I*) [12] of which are reported in Table II.

Hydrophilic behaviour. Albumin is a highly hydrophobic protein [13] and near the top of the hydrophobic chromatographic scale, and would be expected to adsorb on the hydrophobic stationary phases. To demonstrate the hydrophilic behaviour of phosphorylated PS resins, isocratic elution of BSA was performed at pH 7.4 in 5 m*M* phosphate buffer–0.1 *M* NaCl, or in 5 m*M* Tris–HCl buffer–0.1 *M* NaCl, and at pH 7 in 5 m*M* Tris–HCl buffer–0.01 *M* NaCl, as shown in Fig. 3. Even at low salt concentrations (at physiological pH), BSA was eluted in the void volume of the column whatever the buffer used. This result confirmed the hydrophilic character of these stationary phases be-

TABLE II

ANION-EXCHANGE CHROMATOGRAPHY OF PROTEINS AT pH 7.6 ON A PHOSPHORYLATED POLYSTYRENE COLUMN (250 × 4 mm I.D.)

Conditions: 3.5-ml linear A–B gradient following 4.2-ml isocratic elution with buffer A, where buffer A is 5 m*M* Tris–HCl (pH 7.6) and buffer B is buffer A + 2 *M* NaCl; flow-rate, 0.1 ml/min; injection volume, 50 µl (2 mg/ml).

Protein	MW	p*I*	Elution volume (ml)
Cytochrome *c*	12 400	9.2	6.84
Myoglobin	17 400	7.1	1.42
Bovine serum albumin	66 000	5	1.44
Transferrin	80 000	5–7	1.45

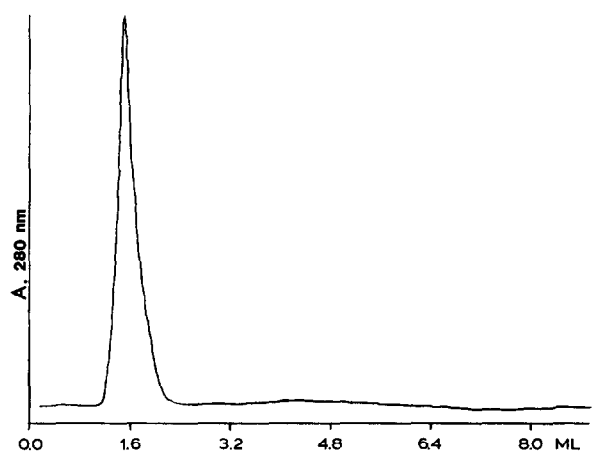

Fig. 3. Isocratic elution of BSA on a phosphorylated PS column (250 × 4 mm I.D.). Sample, 35 µl at 2 mg/ml; flow-rate, 0.1 ml/min; eluent, 50 m*M* phosphate buffer (pH 7.4)–0.1 *M* sodium chloride.

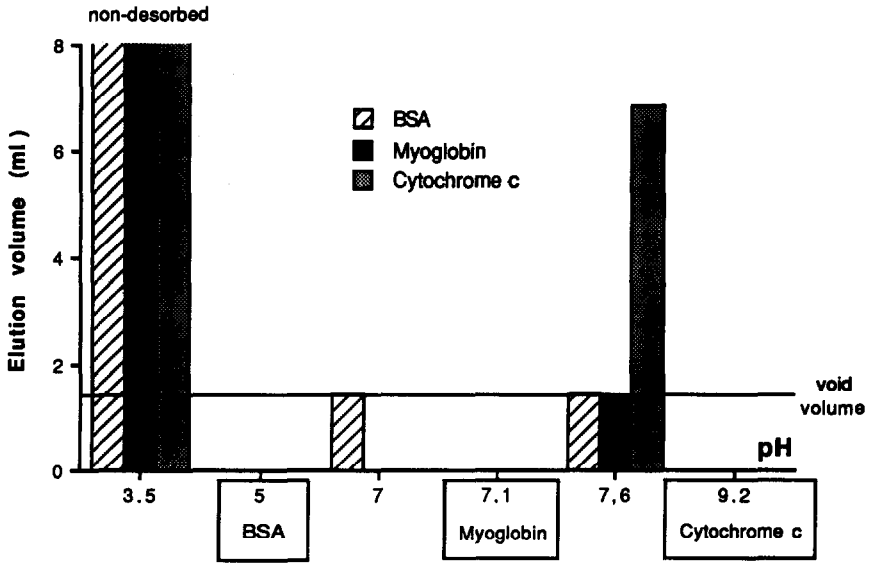

Fig. 4. Anion-exchange chromatography of proteins on a phosphorylated PS column (250 × 4 mm I.D.) at various pH values. Mobile phase, 3.5 ml linear A–B gradient following 4.2-ml isocratic elution with buffer A, where buffer A is 0.16 *M* citrate–sodium phosphate at pH 3.5, 0.05 *M* Tris–HCl–0.01 *M* NaCl at pH 7.0 and 0.05 *M* Tris–HCl at pH 7.6 and buffer B is buffer A plus 2 *M* sodium chloride; flow-rate, 0.1 ml/min; temperature, 20°C; injection volume, 50 μl at 2 mg/ml.

cause BSA is well known [13,14] to adsorb strongly on hydrophobic surfaces.

Influence of pH. Elutions of proteins were performed at various pH values in order to check whether the resins behaved as ion-exchange stationary phases. At pH 3.5 (0.16 *M* citrate–sodium phosphate buffer), *i.e.*, below the p*I* of the proteins under study, the proteins were strongly adsorbed by the resins and could not be eluted even with the use of 2 *M* sodium chloride as eluent. At pH 7, BSA (p*I* = 5.0) was eluted without retention on the stationary phase, as mentioned above. At pH 7.6 in 5 m*M* Tris–HCl without NaCl, elution of myoglobin, BSA and transferrin also occurred in the void volume, as reported in Table II. All these proteins with a p*I* lower than 7.6 did not interact, as expected, with the negatively charged phosphorylated resin, whereas cytochrome *c* (p*I* = 9.2) was desorbed at an NaCl concentration of 1.8 *M*.

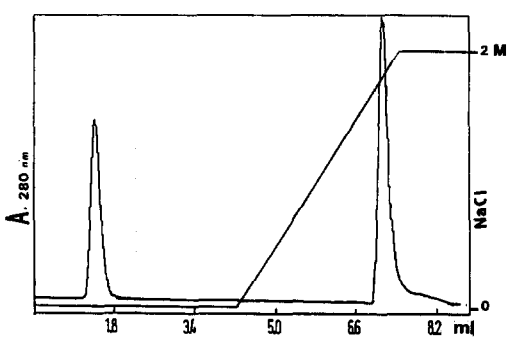

Fig. 5. Separation of myoglobin and cytochrome *c* mixture on a non-porous phosphorylated PS column (250 × 4 mm I.D.). Sample in 150 μl injection volume, 100 μg of myoglobin and 200 μg of cytochrome *c*; linear A–B gradient (100 m*M* salt/ml following 4.2-ml isocratic elution with buffer A), where buffer A is 5 m*M* Tris–HCl (pH 7.6) and buffer B is buffer A plus 2 *M* sodium chloride; flow-rate, 0.1 ml/min; temperature, 20°C.

The results of the studies are summarized in Fig. 4: at pH below the p*I* of the proteins, they are adsorbed on the anionic stationary phase, whereas at pH above the p*I* the proteins are eluted in the void volume. When a protein is retained on the stationary phase, elution occurs at high ionic strength. This implies that the "hydrophobic" contribution to the chromatographic mechanism is minimal, and interaction is primarily though an ion-exchange mechanism. Moreover, the molecular weight does not affect the elution of proteins (Table II), which

shows that the chromatographic mechanism is not one of size exclusion. In all instances, the recovery of the proteins after elution was quantitative.

Fractionation of protein mixtures

The above conclusions are supported by the results of fractionation of a myoglobin and cytochrome *c* mixture. HPLC experiments at pH 7.6 indicate a complete resolution (Fig. 5) of the two proteins when a linear salt gradient is used: myoglobin was first eluted at the initial ionic strength and cytochrome *c* was only eluted with 1.8 *M* NaCl in the eluent.

CONCLUSIONS

Phosphorylated derivatives of cross-linked PS were synthesized and characterized. They are fairly reproducible and of high ion-exchange capacity. These polymeric materials, in the form of beads 50 μm in diameter, were demonstrated to be essentially hydrophilic for proteins such as albumin.

As stationary phases in HPLC they exhibited excellent mechanical properties. Phosphorylated resins are stable in strongly acidic or alkaline solutions. They can also be autoclaved. Experiments performed under HPLC conditions indicated that the elution of the biomolecules is controlled by the pH and ionic strength of the eluent, involving an ion-exchange mechanism. These new synthetic ionized stationary phases allowed the ion-exchange chromatography of protein mixtures.

In ion-exchange chromatography, phosphorylated polystyrene permitted polycationic proteins to be adsorbed at relatively low pH. Moreover, phosphoric acid is an important tool for affinity chromatography because phosphate esters dominate the living world. Thus, phosphorylated resins can be used for the purification of proteins or enzymes interacting with the phosphate groups of biological molecules, including nucleic acids.

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