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SYNTHESIS OF A NON-POROUS, POLYSTYRENE-BASED STRONG ANION-EXCHANGE PACKING MATERIAL AND ITS APPLICATION TO FAST HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

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SUMMARY

Adsorbed coating technology has been used to produce a strong anion-exchange stationary phase on 3-μm non-porous poly(styrene–divinylbenzene) particles. In order to take full advantage of the excellent kinetic properties of the resultant packing material, small columns of 5 mm × 6 mm I.D. were used. These columns were pressure- and pH-stable and allowed protein separations to be made in less than 1 min at ambient temperature.

INTRODUCTION

There is increasing interest in the use of non-porous microparticulate packing materials for fast high-performance liquid chromatography (HPLC) of proteins. The main advantage of such packings is that stagnant mobile-phase mass transfer, which leads to band-broadening and a consequent loss of efficiency and resolution has been eliminated[1]. This concept originated with Horváth, in 1967, when he made pellicular column packings by coating solid glass beads with anion-exchange stationary phases[2]. These materials were pressure- and temperature-stable and efficient at much higher flow velocities than traditional column packings. The principal limitation of these supports was low sample capacity due to the large particle size (≥50 μm) of the glass beads used. Consequently, they were superseded by microparticulate porous media (mostly silica with bonded stationary phases) which offered both high efficiency and high load capacity. Only recently have non-porous packings become available in very small particle sizes.

Microparticulate (1.5 μm) non-porous silica was introduced for improved affinity chromatography packings in 1984[3]. Since then, 1.5- and 2.0-μm monodisperse silicas have been used to achieve rapid separations in the reversed-phase and hydrophobic-interaction modes[4–6]. Covalently derivatized polymeric media, *e.g.*, 7-μm polymethacrylate particles, are also currently in use as weak anion- and cation-exchangers[7–9]. Organic resins, of course, provide greater pH stability than silica.

Among the synthetic polymers, poly(styrene–divinylbenzene) possesses particularly good physical and chemical properties. Recent work has shown that macroporous, microparticulate poly(styrene–divinylbenzene) can be converted to a du-

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rable strong anion-exchange packing material via adsorbed-coating technology[10]. The availability of small, 3-μm, non-porous poly(styrene–divinylbenzene) led us to use similar techniques to make a pellicular polystyrene-based strong anion-exchange sorbent. (Compared to weak anion-exchangers, strong anion-exchange columns provide superior resolving power and reduce the variable of stationary-phase ionization[11]. Details of this synthesis and application of the resultant packing materials to the rapid separation of proteins by HPLC are discussed on the following pages.

MATERIALS AND METHODS

Polystyrene

Sulfonated non-porous poly(styrene–divinylbenzene) was provided by Polymer Labs. (Church Stretton, U.K.). The surface area of the 3-μm solid particles was estimated to be 2 m^2/g.

Reagents

Polyethyleneimine-6 [PEI-6; molecular weight (MW) *ca.* 600] and PEI-18 (MW *ca.* 1800) were purchased from Polysciences (Warrington, PA, U.S.A.). Polyethyleneimine-3 (MW *ca.* 300) was from Dow Chemical (Midland, MI, U.S.A.) but is no longer commercially available. Linear PEI · HCl (MW *ca.* 1000–2000) was synthesized from 2-ethyl-2-oxazoline, as described by Lewis *et al.*[12]. Aldrich (Milwaukee, WI, U.S.A.) supplied 1,4-butanediol diglycidyl ether, methyl iodide, 1,2,2,6,6-pentamethylpiperidine, 2-ethyl-2-oxazoline and tetraethylenepentamine. Diethylene glycol ("technical grade") (2,2′-oxydiethanol) was purchased from Sargent Welch (Skokie, IL, U.S.A.). Other solvents and inorganic reagents were of analytical-reagent grade or comparable quality.

Samples

Proteins employed were horse heart myoglobin (MYO), chicken egg white conalbumin (CON) and ovalbumin (OVA), bovine serum albumin (BSA), and soybean trypsin inhibitor (STI). All were purchased from Sigma (St. Louis, MO, U.S.A.) except OVA which was obtained from Calbiochem (La Jolla, CA, U.S.A.). Ascites fluid was provided by Dr. Jane Babin of the Purdue University Chemistry Department and was dialyzed against 0.01 *M* Tris–HCl prior to use.

Synthesis of packing material

Preliminary steps. Sulfonated non-porous polystyrene was stirred in a 10-fold excess (v:w) of 0.1 *M* sodium hydroxide for *ca.* 30 min. The base-washed resin was isolated on Whatman No. 50 paper in a small buchner funnel, rinsed briefly with water and methanol, and dried in a vacuum dessicator. Meanwhile, PEI-6 was converted to the hydrochloride as follows. A 5-ml volume of an aqueous solution of PEI-6 (15 mg/ml)* was transferred to a small beaker and titrated to pH 7.0, using 0.1 *M* hydrochloric acid. (Approximately 0.9 ml of acid was required.)

* The quantity of PEI needed to just coat a given weight of sulfonated resin was previously determined by means of an adsorption isotherm (mg PEI · HCl adsorbed *vs.* solution concentration). This insures that all polyamine will be removed from solution prior to direct addition of crosslinking reagent to the same reaction vessel.

Adsorption and crosslinking. A 5-ml volume of aqueous PEI-6 · HCl (above) was measured into a 50-ml round-bottom flask containing a small magnetic stir bar. To this stirred solution was added 1 g of dry, base-washed, sulfonated non-porous poly(styrene–divinylbenzene). After initial mixing, the flask was sonicated briefly to insure complete suspension of the resin, then stirred for 1 h. At the end of the adsorption period, 5 ml of a solution of 10% (v/v) 1,4-butanediol diglycidyl ether in diethylene glycol was added, dropwise, and stirring of this mixture was continued for an additional 12 h (at room temperature). The flask was then immersed in a boiling water-bath for 10 min, to complete crosslinking and hydrolyze residual epoxides. The resulting weak anion-exchange packing material was isolated in a 30 ml fine sintered-glass funnel and washed with methanol. After drying in the funnel, under suction, for 30 min, this material was subjected to a second wash with 0.1 *M* sodium hydroxide (as described above) to insure deprotonation of amines prior to methylation.

Quaternization. The PEI-coated polystyrene was methylated to produce a strong anion-exchange packing as previously described[10]. Although actual percent derivatization was not calculated, approximately constant chromatographic retention of OVA and STI at pH 6–10 indicated that the stationary phase was quaternized[10,13].

Chromatographic evaluation

Comparison of stationary phases. Static ion-pairing and protein-binding capacity assays have long been used to characterize column materials[13,14]. Unfortunately, these assays are not applicable to the non-porous polystyrene-based packings due to low surface area (2 m^2/g), low ligand density, and interaction between assay reagents and the polystyrene matrix[10]. Similarly, the low nitrogen content (<0.5%) precludes accurate elemental analysis. Consequently, the sorbents (after quaternization) were packed into small columns, as described in the following section, and evaluated chromatographically, using a mixture of MYO, CON, OVA, and STI. Resolution of CON and OVA (denoted by arrows in Fig. 1) was particularly sensitive to changes in the coating composition, since these two proteins are eluted closely together with the short gradients used.

Column hardware and packing. A guard cartridge system manufactured by Upchurch Scientific (Oak Harbor, WA, U.S.A.) was utilized to make the small columns. After insertion of one fritted disk (0.5-μm porosity stainless steel with Kel-F collar, Upchurch Scientific), the cartridge was slurry-packed and a second fritted disk was pressed into position. Final inner dimensions of the "column", excluding the area occupied by the disks, were 5 mm × 6 mm I.D. This cartridge had a volume of 140 μl and held *ca.* 150 mg of non-porous resin.

Initially, columns were packed by using a vacuum pump to pull coated resin (suspended in 0.01 *M* Tris–HCl buffer, pH 8.0) down into the cartridge. Later, an accessory was designed (custom-machined by Purdue University Central Machine Shop, W. Lafayette, IN, U.S.A.) which allowed several cartridges to be packed simultaneously by using an HPLC packing pump (Shandon Southern Instruments, Sewickley, PA, U.S.A.). In this case, the medium was suspended in 2-propanol and packed (downward) at 2500 p.s.i. The two methods did not produce significant differences in the amount of packing material in the cartridge or its chromatographic performance. The packing density of the 3-μm non-porous polystyrene (as deter-

mined from a 5 × 0.41 cm column, packed from 2-propanol at 3000 p.s.i.) was 0.8 g/ml.

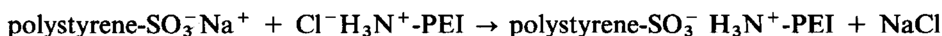
HPLC instrumentation. Chromatography was performed with a HP 1090 liquid chromatograph (Hewlett-Packard, Waldbronn, F.R.G.) fitted with a Model 7125 manual injector (Rheodyne, Cotati, CA, U.S.A.) with a 3-μl sample loop. Absorbance at 280 nm was monitored with a Kratos 757 Spectroflow detector with a 0.5-μl flow-cell (kindly lent by Kratos Division, Applied Biosystems, Ramsey, NJ, U.S.A.). Dead volume of all connecting tubing was kept at a minimum. All samples and chromatographic eluents were filtered through 0.45-μm porosity filters.

RESULTS AND DISCUSSION

Preparation of packing material

The present work was undertaken to provide a non-porous strong anion-exchange sorbent which would be pH-stable, pressure-stable and suitable for high-speed analyses. Parameters which were most important to the development of a successful coating procedure for the solid particles are discussed below.

Maximizing electrostatic interaction. Preliminary washing of the sulfonated resin with dilute sodium hydroxide and conversion of PEI to its hydrochloride prior to coating insures maximum electrostatic interaction:



$$\text{polystyrene-SO}_3^-\text{Na}^+ + \text{Cl}^-\text{H}_3\text{N}^+\text{-PEI} \rightarrow \text{polystyrene-SO}_3^-\ \text{H}_3\text{N}^+\text{-PEI} + \text{NaCl}$$

This step is particularly important in the case of small non-porous particles. Not only is there less surface, *i.e.*, fewer sites for interaction, but also a completely external polyamine layer is more susceptible to desorption prior to crosslinking. Thus, initial attempts to coat and crosslink the solid sulfonated poly(styrene–divinylbenzene) from methanolic solutions[10] were not successful. Neither was dioxane[14] satisfactory for crosslinking, because the isolated[10] polyamine-coated resin could not be uniformly resuspended. Apparently, some aggregation of the externally coated particles occurred during the isolation step, and slight solubility of PEI in the crosslinking solution was needed to dissociate them. Although diethylene glycol was found to be a satisfactory crosslinking solvent, it was concurrently recognized that the problem could be avoided by omitting the isolation of coated material prior to crosslinking. (Horváth *et al.*[2] used methylene bromide vapor to crosslink PEI adsorbed to solid glass beads.) These realizations, along with an attempt to increase ionization by coating the support from an aqueous solution, led to the "one-pot" procedure described in Materials and methods.

An important requirement of this continuous coating and crosslinking procedure is that the amount of PEI just sufficient to coat a given quantity of resin completely must be determined by means of a preliminary adsorption isotherm (mg PEI · HCl adsorbed *vs.* solution concentration; data not shown). This insures that all polyamine is removed from solution prior to addition of crosslinking reagent so that the reaction of amine and epoxide does not take place in solution. Consequently, the "one-pot" procedure results in a relatively thin adsorbed coating.

Minimizing layer thickness. Heavy PEI coatings have been found useful for increasing anion-exchange capacity[15], improving resolution[13], and masking undesir-

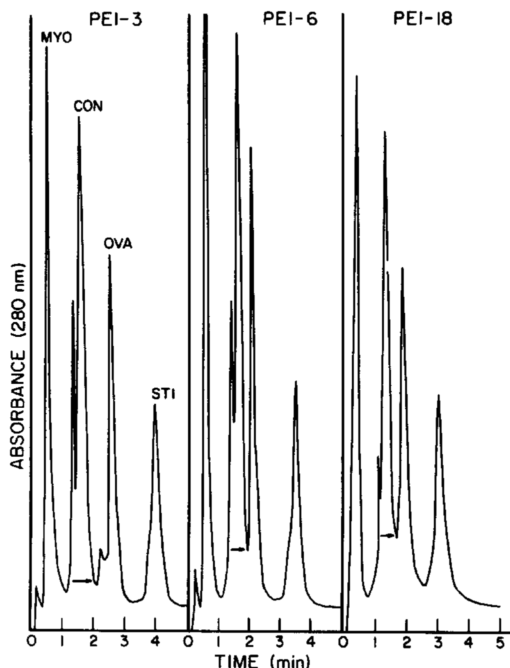


Fig. 1. Influence of the molecular weight of polyamine on chromatographic performance of non-porous polystyrene coated with branched PEIs. Sulfonated poly(styrene–divinylbenzene) was coated with PEI of MW 300 (PEI-3), 600 (PEI-6), or 1800 (PEI-18) as described in the text. Chromatographic evaluation was performed at pH 8.0, using a 5-min linear gradient from 0.01 *M* Tris–HCl to 0.35 *M* sodium chloride in 0.01 *M* Tris–HCl at a flow-rate of 1.5 ml/min. A mixture of MYO (1.5 μg), CON (3 μg), OVA (3 μg) and STI (3 μg) served as the sample.

able properties[10] of porous matrices. However, for non-porous particles, a thin, uniform coating appears to be optimal. In this study, the molecular weight of the polyamine used was found to affect chromatographic performance. Trials of branched PEIs of MW 300, 600 and 1800 (coated as the free amine) clearly showed that the smallest branched PEI gave the best chromatographic resolution (Fig. 1). Polystyrene coated with linear PEI, MW 1000–2000, and tetraethylenepentamine, MW 189, performed similarly to PEI-18 and PEI-3 packings, respectively (data not shown). Since high-molecular-weight branched PEIs are known to be "bushlike" in structure[16], it was concluded that smaller polyamines can more efficiently cover the surface of small, non-porous particles. When PEI-6 was converted to its hydrochloride prior to coating the base-washed resin, chromatographic resolution comparable to that from the PEI-3 (free amine) stationary phase was achieved. (Simply reducing the concentration of PEI-6 in the coating solution had no effect.) Presumably, increased electrostatic interaction pulls positively charged polyamine molecules closer to the negatively charged particle. This may result in a smoother surface which is more favorable to rapid protein adsorption/desorption. Alternatively, as the thickness of the stationary phase layer is decreased, adsorbed proteins are in closer proximity to the polystyrene matrix. The fact that OVA and STI retention times increased

as the polyamine size decreased suggests mixed ionic/hydrophobic contributions to retention and selectivity[13].

Chromatographic evaluation

Column format. As particle size decreases, both separation efficiency and resistance to flow, *i.e.* operating pressure, increase. In order to maximize the former and minimize the latter, small, non-porous particles are packed into short columns, *e.g.*, 30–36 mm[4–9]. Column length is relatively unimportant in the surface-mediated separation of macromolecules due to multiple-site binding[17]. Ion-exchange columns 5 cm in length have 75% of the resolving power of 25 cm columns[18]. Pearson[19] has recently shown that reversed-phase columns only 4 mm long (packed with macroporous, 3-μm silica) could fractionate proteins with a resolution comparable to that of a 5-cm column. In addition to the obvious advantages of decreased elution time and reduced back pressure, small analytical columns concentrate the eluent so that detection can be achieved with smaller volumes. The columns are also easier to pack reproducibly and are less expensive in terms of both packing material and solvent consumption. Consequently, a novel column format was employed for the 3-μm non-porous strong anion-exchange packing material which had been developed. A commercially manufactured cartridge system was adapted to provide a small, "disposable" column, 5 mm × 6 mm I.D. All data presented in the following sections were obtained with columns of these dimensions, packed with PEI-6 · HCl-coated (quaternized) media, as described in Materials and methods. In the future, the feasibility of using even smaller cartridges will be examined.

Pressure and pH stability. Previous work with macroporous poly(styrene–divinylbenzene) indicated good pressure stability[10]. A plot of back pressure *vs.* mobile phase velocity (Fig. 2) for a 5 mm × 6 mm I.D. cartridge of the 3-μm non-porous packing material showed good linearity up to a flow-rate of 5 ml/min, the upper limit of the pumping system used.

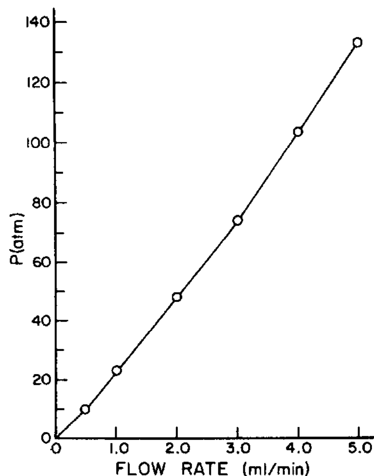


Fig. 2. Pressure *vs.* flow-rate for non-porous polystyrene-based packing in a 5 mm × 6 mm I.D. column. Data from two cartridges, packed simultaneously with a Shandon HPLC packing pump (see Materials and methods) were averaged.

Like its macroporous polystyrene-based predecessor[10], PEI-coated non-porous poly(styrene–divinylbenzene) appears to posess good resistance to aqueous acid and base. Fifty column volumes of (filtered) 0.5 *M* sodium hydroxide were pumped through a cartridge with no noticeable effect on the chromatogram of a four-component protein mixture (same sample as in Fig. 1).

Capacity. As previously stated, the main goal of this work was to develop a durable, non-porous, strong anion-exchange packing which would be suitable for high-speed analyses. The kinetic properties of the small non-porous particles were maintained by the use of a thin, highly crosslinked, adsorbed PEI coating. Thus, chromatographic efficiency was maximized at the expense of capacity. A study of resolution *vs.* load, in which a 1:1 mixture of BSA and OVA and a 1-min gradient at a flow-rate of 3.0 ml/min (conditions described for Fig. 3) was used indicated that 5 μg of each protein was the upper limit for resolution under these conditions (data not shown). Evaluations in which a mixture of MYO, CON, OVA and STI was used with a longer gradient and slower flow-rate (sample and elution conditions described for Fig. 1), showed that from 10 to 50 μg of total protein could be loaded onto a 5 mm × 6 mm I.D. cartridge (140-μl volume) with no significant change in the chromatogram (not shown).

Similar studies have been described for considerably larger columns of polymeric non-porous weak anion-exchange media. With a 35 mm × 4.6 mm I.D. column of 2.5-μm particles, a plot of peak width *vs.* load for CON, OVA, and STI, chromatographed individually, showed that the peak-width remained constant at loads up to 5 μg, and increased rapidly at loads exceeding 10 μg of pure protein[9]. Columns 30 mm × 4.6 mm I.D. (500-μl volume) of polymethacrylate-based, non-porous (7-μm particles) weak anion-exchanger can reportedly separate up to a 300-μg load of a 1:1 mixture of OVA and BSA[7]. Presumably, this higher capacity is the result of a "fuzzy" PEI stationary phase[15]. However, if columns of non-porous packing are used to analyze small or diluted samples, such a high protein load capacity is not necessary.

Speed of separation. Factors that influence the efficiency of HPLC columns include eddy diffusion, mobile phase mass transfer between and within particles, and stationary phase mass transfer. The column efficiency is greatly improved by using pellicular or non-porous particles, since intraparticulate diffusion, *i.e.* stagnant mobile phase mass transfer, is eliminated[1]. Consequently, separations on non-porous packings are characterized by very narrow bandwidths, and their efficiency is retained over a broader range of flow-rates. Comparisons of a 7-μm non-porous polymeric weak anion-exchanger with three porous supports showed that, in this case, the non-porous packing provided superior resolution when higher flow-rates and shorter gradient times were used[7,20]. Thus, when speed of analysis is essential, non-porous packing materials are superior.

In the present work, very short columns, good kinetic properties of the matrix and high selectivity of the adsorbed PEI stationary phase were combined to achieve ion-exchange separations in less than 1 min at ambient temperature. Fig. 3 shows a 32-s fractionation of four proteins with the use of a 1-min gradient and a flow-rate of 3.0 ml/min (cycle time was 1.2 min). Although shorter separation times have been achieved using reversed-phase chromatography at elevated temperature (80°C)[5], the chromatogram in Fig. 3 was obtained at *ca.* 21°C. Rate laws for the ion-exchange

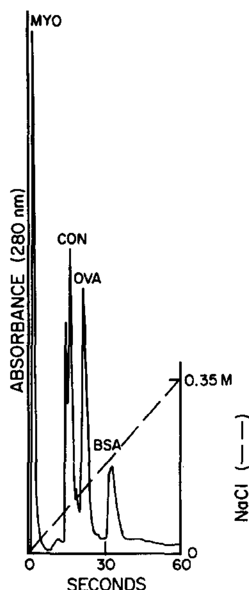


Fig. 3. High-speed separation of a protein mixture on non-porous strong anion-exchange packing. This 32-s fractionation of MYO (1.1 μg), CON (1.5 μg), OVA (1.9 μg), and BSA (1.5 μg) was obtained at ambient temperature, using a 5 mm × 6 mm I.D. cartridge of PEI-6 · HCl-coated polystyrene. A 1-min linear gradient from 0.01 *M* Tris–HCl to 0.35 *M* sodium chloride in 0.01 *M* Tris–HCl (pH 8.0) at a flow-rate of 3.0 ml/min was used.

process involve diffusion coefficients; thus, increased temperature should bring about a considerable rate increase[21]. Raising the temperature from 22 to 37°C has been found to increase resolution on an ion-exchange column by 25%[11]. The contact time of a macromolecule on 5-mm long columns of non-porous packing is obviously very short. Thus, it may be possible to use slightly elevated temperatures to increase resolution and decrease separation time even further without protein denaturation.

Column lifetime. Since the small cartridges described in this paper were initially intended to be disposable, it was of interest to determine their usable "lifetime". To this end, 200 injections of a 10-μg protein load (MYO, CON, OVA, and STI) were made, using the elution conditions described for Fig. 4. Two series of 100 injections, at *ca.* 2-min intervals, were performed on consecutive days. Nothing was done to clean the column during this time except that a blank gradient was run prior to injection 101. The chromatograms in Fig. 4 show that there was no significant difference in retention times or resolution from the beginning to the end of this study. (The disparity between chromatograms 100 and 101 is due to the use of different sample stocks.) It is believed that short contact times between protein and packing material, as well as the use of an adsorbed PEI coating, minimize fouling of the stationary phase[22]. Other investigators have reported less fouling of non-porous silica-based anion-exchangers than of macroporous supports[23].

Applications. Small, disposable cartridges, packed with non-porous media, offer obvious advantages in situations where many similar samples must be analyzed rapidly; *e.g.*, in quality control or clinical analyses. It is anticipated that they will be

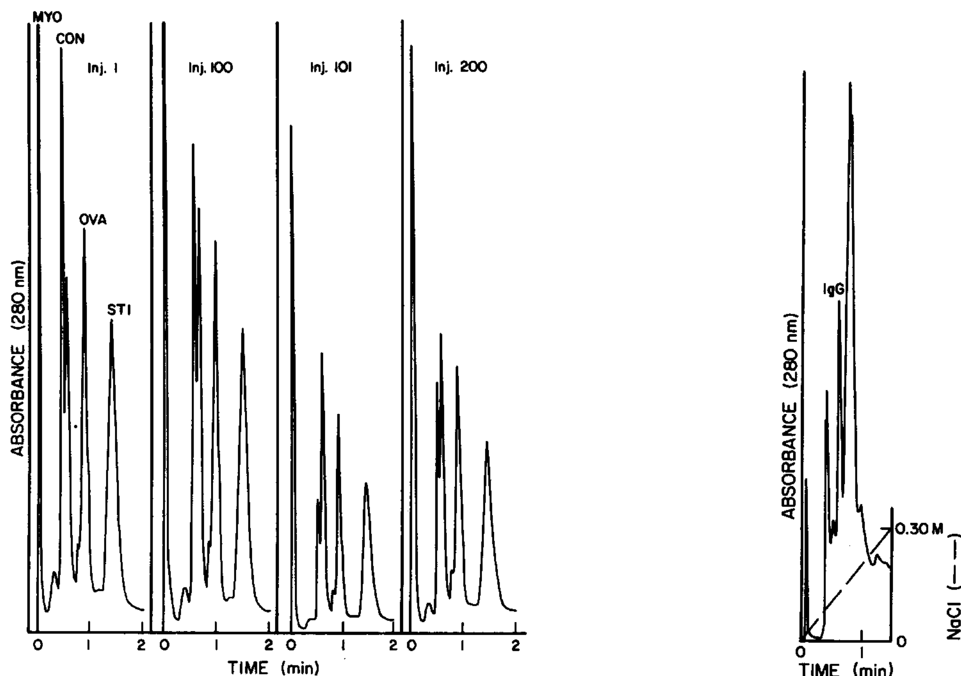


Fig. 4. Column lifetime study. Two hundred successive injections of a 10-μg load of MYO, CON, OVA, and STI were made on a 5 mm × 6 mm I.D. cartridge of the non-porous strong anion-exchange packing material. Elution conditions were a 2-min linear gradient from 0.01 *M* Tris–HCl to 0.35 *M* sodium chloride in 0.01 *M* Tris–KCl (pH 8.0) at a flow-rate of 2.0 ml/min. (Different sample stocks were used for injections 1–100 *vs.* 101–200.)

Fig. 5. Analysis (1 min) of ascites fluid. Dialyzed mouse ascites fluid was chromatographed on a 5 mm × 6 mm I.D. cartridge of the new packing material. A 1.5-min linear gradient from 0.02 *M* Tris–HCl to 0.30 *M* sodium chloride in 0.02 *M* Tris–HCl (pH 8.5) at a flow-rate of 2.0 ml/min was used.

applied to on-line monitoring of large-scale protein purifications[24]. Fig. 5 shows a 1-min fractionation of mouse ascites fluid, within a 2-min cycle, at pH 8.5. The combination of a strong anion-exchange stationary phase and poly(styrene–divinylbenzene) matrix makes this packing material applicable to separations where maximum resolution is achieved at high pH[25].

CONCLUSIONS

A procedure was developed for coating a sulfonated 3-μm non-porous poly(styrene–divinylbenzene) resin with PEI. Crosslinking with a diepoxide, followed by quaternization[10] produced a strong anion-exchange stationary phase. The application of an electrostatically adsorbed coating to solid particles is considerably more sensitive to procedural variables than is the coating of porous matrices. Parameters that were most important to the success of this procedure were: (1) maximizing electrostatic interaction and (2) minimizing thickness of the adsorbed layer. The latter was influenced by molecular weight, (*i.e.* size) of the polyamine used for coating.

Small, branched PEIs gave optimal results, presumably because they can most efficiently cover the surface of small, non-porous particles. The use of a thin, relatively smooth coating may interfere less with the adsorption and desorption of proteins from solid media. As the layer thickness decreases, however, the possibility of hydrophobic contributions to retention from the poly(styrene–divinylbenzene) matrix increases. Such a mixed-mode character may actually increase the selectivity of the stationary phase[13].

In order to take full advantage of the kinetic properties of the non-porous packing material described herein, a novel column format was employed. Columns of only 5 mm × 6 mm I.D. were obtained by adapting a commercial cartridge system. Pressure stability and high efficiency across a broad range of flow velocities allowed the use of high flow-rates. When used with a pumping system and detector designed to minimize extra-column effects, these small columns of non-porous, strong anion-exchange packing gave excellent resolution and truly fast separations. For example, a four-protein mixture was fractionated in 32 s at ambient temperature.

In the present work, chromatographic efficiency was maximized by the use of a thin, highly-crosslinked stationary phase. Consequently, the load capacity is rather low, but this does not present a problem in analytical use. Low ligand density and short residence times of macromolecules on the non-porous packing appear to decrease fouling and prolong column lifetime. Two hundred consecutive injections of a four-protein mixture (10-μg load) on a 5 mm × 6 mm I.D. cartridge of the non-porous packing caused no noticeable changes in chromatographic retention or resolution. Moreover, short equilibration times and good resistance to aqueous base allow rapid flushing of fouled cartridges, if needed.

It is anticipated that very small columns with non-porous packings will be used for on-line monitoring of large-scale protein purifications. In addition to the possibility of manipulating retention and selectivity via mobile-phase pH, ionic strength, and type of displacing ion, a strong anion-exchange column offers the additional advantages of superior resolution and constant charge density[11]. The combination of a strong anion-exchange stationary phase and a durable, pH-stable poly(styrene–divinylbenzene) matrix makes this packing material applicable to separations where maximum resolution is achieved at high pH. In the future, the feasibility of above-ambient temperature and/or additional reduction of colum dimensions (as a means of further decreasing analysis times) will be studied. Other chromatographic modes will also be examined using the small column format.

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