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POLY(STYRENE–DIVINYLBENZENE)-BASED STRONG ANION-EXCHANGE PACKING MATERIAL FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

M. A. ROUNDS, W. D. ROUNDS* and F. E. REGNIER*
Department of Biochemistry, Purdue University, West Lafayette, IN 47907 (U.S.A.)

SUMMARY

An adsorbed polyethyleneimine coating was applied to sulfonated macroporous, microparticulate poly(styrene–divinylbenzene). The chemical, physical and chromatographic properties of the resulting strong anion-exchange packing material were thoroughly characterized. The dynamic load capacity of the experimental packing was comparable to that of large-pore diameter silica. Good recoveries of protein mass and enzyme activity were achieved. The new column withstood a variety of cleaning procedures and prolonged exposure to aqueous base. The retention times on the polystyrene-based column were similar to those on a silica and a commercial, polymeric, strong anion-exchange column. Chromatographic resolution of the new packing material was equal or superior to that provided by the other two packings.

INTRODUCTION

Liquid chromatography of polypeptides often requires harsh mobile phases and extremes in pH for both elution and cleaning of denatured proteins from columns. This makes packing materials based on poly(styrene–divinylbenzene) very attractive, inasmuch as these resins possess excellent physical and chemical properties[1]. Modern technology has also made it possible to produce cross-linked organic resins with sufficient mechanical strength and porosity to be used in high-performance systems[2].

Polymeric column materials were first used in protein separations more than three decades ago[3,4]. However, as noted by Boardman and Partridge[4], the inherent hydrophobic character of hydrocarbon-based media[1] is a serious limitation. Hydrophobic interactions between resins and proteins are sufficiently strong that the proteins may be denatured either upon adsorption or during elution. Thus, masking or eliminating the hydrophobic character of the poly(styrene–divinylbenzene) matrix is essential for the chromatographic separation of proteins in all retention modes except reversed-phase.

* Present address: Department of Chemistry, Dartmouth College, Hanover, NH 03755, U.S.A.

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The problem of protein denaturation by interaction between biopolymers and matrix functional groups has also been encountered with inorganic packings. In the case of glass and silica, surface silanol and siloxane groups have been shown to have adverse effects on proteins[5]. A solution to this problem is to cover the surface of the support material with a thin, adsorbed layer of organic polyamine, such as polyethyleneimine (PEI)[6]. Adsorption of the polyamine layer occurs through ion-pairing between silanols and amine groups in the coating. Subsequent cross-linking of the polyamine with a multifunctional oxirane permanently immobilizes the coating. This thin polymeric layer simultaneously sequesters silanol groups by ion-pair formation, presents a physical barrier to contact between macromolecules and the column matrix, and serves as a chromatographic stationary phase.

Studies by Kopaciewicz[7] indicated that the same coating process could be applied to negatively functionalized organic resins. Adsorption of low-molecular-weight polyamines onto macroporous polystyrene sulfonate and polymethacrylic acid resins of large particle diameter (followed by cross-linking, as described above) produced anion-exchange sorbents which bound and released hemoglobin. This paper describes the application of an electrostatically adsorbed PEI coating to sulfonated macroporous, microparticulate poly(styrene–divinylbenzene). Questions addressed include the effects of synthetic variables on chromatographic performance and whether this technique is suitable for masking inherent hydrophobicity of the organic matrix.

MATERIALS AND METHODS

Polystyrene

The macroporous poly(styrene–divinylbenzene) resin (PLRP-S 1000 Å, 8 μm) used in this work was a generous gift from Polymer Labs. (Shropshire, U.K.). Elemental analysis data suggested a repeat unit of $C_{18}H_{18}$ (see Fig. 1); consequently, a molecular weight of 234 g/mol or equivalent weight of 117 was assumed for synthetic purposes.

Reagents

Polyethyleneimine (PEI-18, molecular weight *ca.* 1800, and PEI-6, molecular weight *ca.* 600) was purchased from Polysciences (Warrington, PA, U.S.A.). Ladd LX-112 resin* was obtained from Ladd Research Industries (Burlington, VT, U.S.A.). Aldrich (Milwaukee, WI, U.S.A.) supplied chlorosulfonic acid, methyl iodide, 1,2,2,6,6-pentamethylpiperidine, and 1,4-butanediol diglycidyl ether. Solvents and inorganic reagents were of analytical-reagent grade or comparable quality.

Proteins

Proteins employed, (their abbreviation, molecular weight and isoelectric point)[8,9] are given in Table I. Bovine Type II crude Hb was used for static Hb-binding assays. All proteins were purchased from Sigma (St. Louis, MO, U.S.A.),

* Ladd LX-112 resin is a mixture of di- and triglycidylglycerols. Chromatographic comparisons have shown it to be a satisfactory substitute for diglycidylglycerol, which is no longer commercially available.

TABLE I

PROTEINS EMPLOYED IN THIS STUDY

Protein	*Abbreviation*	*Molecular weight*	*Isoelectric point*
Horse heart myoglobin	MYO	17 500	6.9, 7.3
Egg white ovalbumin	OVA	43 500	4.7
Soybean trypsin inhibitor	STI	20 100	4.5
Chicken egg-white conalbumin	CON	77 000	6.0, 6.3, 6.6
Bovine serum albumin	BSA	69 000	*ca.* 5.0
Bovine milk β-lacto-globulin A	β-LAC A	35 000	5.1
Bacterial α-amylase	α-AMY	55 000	*ca.* 6
Hen egg-white lysozyme	LYS	13 930	11
Bovine catalase	CAT	234 000	*ca.* 6
Bovine hemoglobin	Hb	64 500	6.8

except the OVA used for retention mapping, which was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Commercial STI was further purified chromatographically by P. A. Tice and G. Robinson from this laboratory[10].

Columns

Strong anion-exchange columns used for comparative evaluation were a Mono Q HR 5/5[11], purchased from Pharmacia, and quaternized, PEI-coated silica, synthesized by the following procedure. Vydac 101TPB (5.5 μm) silica (a generous gift from The Separations Group, Hesperia, CA, U.S.A.) was coated using a 1% (w/v) methanolic solution of PEI-6 and cross-linked using a solution of 10% (v/v) 1,4-butanediol diglycidyl ether in methanol[12]. This stationary phase was quaternized as previously described[13].

Instrumentation

Chromatography was performed using an LDC Constametric I and III G pumping system with a Gradient Master controller (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Absorbance was monitored by a Micromeritics (Norcross, GA, U.S.A.) 786 variable-wavelength detector. A Varian (Walnut Creek, CA, U.S.A.) 634 Series UV–VIS spectrophotometer was used to measure concentrations in static binding capacity assays and to quantitate protein mass recovery. A Model 1052B digital conductivity meter (Amber Science, San Diego, CA, U.S.A.) was used to monitor specific conductance during ionic capacity measurements.

Synthesis of packing material

Sulfonation. Macroporous poly(styrene–divinylbenzene) (PLRP-S 1000 Å) (10 mmol, 1170 mg) was added to 100 ml of dichloromethane in a 500-ml two-neck round-bottom flask. After brief sonication, the flask was evacuated for 2 min to remove air from interstitial spaces of the resin. This suspension was then mechanically stirred for 1 h (at room temperature) prior to the addition of acid. Chlorosulfonic

acid (8 mmol, 532 μl), was added to 100 ml of dichloromethane in a 125-ml addition funnel. This dilute acidic solution was added, dropwise, to the stirring suspension of resin over a period of *ca.* 1 h. An ice bath was placed under the flask during this step. After stirring for an additional 8 h at room temperature, the sulfonated polystyrene was isolated in a sintered-glass funnel, rinsed thoroughly with dichloromethane and dried overnight in a vacuum dessicator. Elemental analyses, performed by the Purdue University Chemistry Department Microanalysis Laboratory, showed more than 60% sulfonation with 10% or less as the sulfonyl chloride.

Coating and cross-linking. The adsorbed coating method of Alpert and Regnier[6], as modified by Kopaciewicz *et al.*[12], was the basis for these procedures. An amount of 1 g of the above sulfonated poly(styrene–divinylbenzene) was weighed into a tared 50-ml round-bottom flask. To this was added 10 ml of 10% (w/v) PEI-18 in methanol. The flask was sonicated and degassed briefly, kept at room temperature for 3 h with frequent swirling, and then heated over low steam for 15–30 min. The coated support material was isolated in a sintered-glass funnel and dried under vacuum for 30 min. Cross-linking was achieved by suspending the coated resin in 10 ml of a solution of 10% (v/v) Ladd LX-112 resin in methanol. The flask was sonicated and evacuated briefly, left at room temperature 12 h (without agitation), and then heated over low steam for 30 min. The product was isolated in a sintered-glass funnel, washed with methanol, 0.1 *M* sodium hydroxide, water, and methanol, and finally dried under vacuum.

Quaternization. The PEI-coated polystyrene was methylated to produce a strong anion-exchange stationary phase. Reagent quantities were based on total amine content of the weak anion-exchange packing material, as determined by elemental analysis. For example, to a solution of 350 μl of 1,2,2,6,6-pentamethylpiperidine in 6 ml dry dimethylformamide was added 700 mg of sodium hydroxide-washed media. After brief sonication, 1 ml of methyl iodide was added and the suspension was heated in an oil bath at 60°C overnight. Isolation and workup were as previously described[13].

Static analyses

Hemoglobin-binding capacity. Assays were performed at pH 8.0 as previously described[12], except that the ionic desorption solution contained 1.0 *M* sodium chloride (instead of 0.5 *M*). At this pH, Hb is negatively charged; hence, the anionic Hb-binding capacity is measured. Ionically desorbed hemoglobin (Hb_{aec}) and non-ionically desorbed hemoglobin (Hb_{iso}) were quantitated spectrophotometrically at 410 nm. An absorbance of 1.0 corresponded to 0.158 mg/ml of Hb.

Cationic Hb binding (Hb_{cec}) was determined by performing the above assay at pH 5.5 so that Hb was positively charged[14].

Equilibrium BSA-binding capacity. A known weight of packing material (*ca.* 100 mg) was washed once with ionic desorption solution, then twice with adsorption buffer as in the Hb-binding assay. After removing excess buffer by aspiration, 1.0 ml of a 50-mg/ml solution of BSA (in 0.01 *M* Tris–HCl, pH 8.0) was added to the test tube. The tube was vortex-mixed and the suspension was then gently agitated for 18 h at 21°C. A "control", consisting of the same volume of BSA solution but no packing material, was treated in the same manner. After 18 h, the sample tube was centrifuged and the protein content of the supernatant (as well as that of the "control"

solution) was determined by the Bradford method[15]. The difference between the control and sample was assumed to be the amount of BSA bound to the packing material.

Chromatographic evaluation

The coated polystyrene was slurry-packed into 5 × 0.41 cm I.D. stainless-steel columns at 3000 ± 20 p.s.i. (packing density, 0.4 g/ml). Either 1.0 *M* sodium sulfate or 2-propanol was used as the solvent, with no discernible difference in column performance.

Gradient elution. Unless otherwise stated, analytical chromatography was performed using a 20-min linear gradient from 0.01 *M* buffer (A) to 0.35 *M* sodium chloride in 0.01 *M* buffer (B) at a flow-rate of 1 ml/min. Retention mapping was carried out as previously described[16]. (Samples were prepared at pH 7.0.) A mixture of MYO, CON, OVA, and STI (3, 5, 4 and 7 mg/ml, respectively) and a sample of β-LAC A (10 mg/ml) served as probes. Resolution was calculated for the OVA and STI major peaks in the four-component sample by $R_s = 2(t_{R2} - t_{R1})/(\Delta t_{R1} + \Delta t_{R2})$.

Dynamic load capacity. The dynamic load capacity of the new packing material was examined via frontal analysis[17]. For example, BSA-binding capacity was determined by pumping a dilute solution of BSA (2.5 mg/ml in 0.01 *M* Tris–HCl, pH 8.0) through a 5 × 0.41 cm I.D. column at 1 ml/min. The volume of protein solution needed to saturate the column was measured by dropping a perpendicular line from the front boundary at 20% of maximum absorbance[18]. This method takes column hold-up volume into account.

Ionic capacity of the strong anion-exchange packing was determined in an analogous manner, by pumping a 2.5% solution of sodium nitrate through a column (5 × 0.41 cm) at 0.5 ml/min.

Protein (mass) recovery. Protein (5 mg, in 0.01 *M* Tris–HCl, pH 8.0) was loaded onto the column in buffer A, then eluted with eluent B, described above. Controls went directly from injector to detector, *i.e.* no column was used. Equal volumes of sample and control were collected and their protein content was measured spectrophotometrically at 280 nm. The recovery (%) was calculated from $(A_{sample}/A_{control}) \cdot 100$, where each absorbance (A) value was the average of two replicates. The five proteins examined (BSA, OVA, STI, CON and CAT) represented a range of MW, p*I*, and hydrophobicity[16].

Enzymatic activity recovery. Samples were eluted using a 20-min linear gradient from 0.01 *M* Tris–HCl (pH 8.0) to 0.5 *M* sodium chloride in 0.01 *M* Tris–HCl (pH 8.0) at a flow-rate of 1 ml/min. The controls were treated as above. α-Amylase was assayed with starch and 3,5-dinitrosalicyclic acid as the color reagent[19].

Column durability. The resistance of a column to aqueous base under dynamic conditions was evaluated as follows. A solution of 0.5 *M* sodium hydroxide was pumped through a 5 × 0.41 cm I.D. column in 100-ml increments (at 1–1.5 ml/min). After washing the column with water and buffer (pH 8.0), analytical injections of an OVA–STI sample and LYS were made to monitor packing material performance. Since LYS is positively charged at pH 8.0, retention of this protein would indicate loss of the polyamine stationary phase from the sulfonated polystyrene matrix.

RESULTS AND DISCUSSION

Preparation of packing material

The poly(styrene–divinylbenzene)resin (PLRP-S) used in this study has thus far been employed as a packing for reversed-phase high-performance liquid chromatography[20,21] (HPLC). Conversion of this resin to a strong anion-exchange sorbent was achieved via an electrostatically adsorbed coating[6]. The complete synthetic route to the strong anion-exchange packing material is shown schematically in Fig. 1. Functionalization of the unsubstituted resin was carried out by addition of a dilute

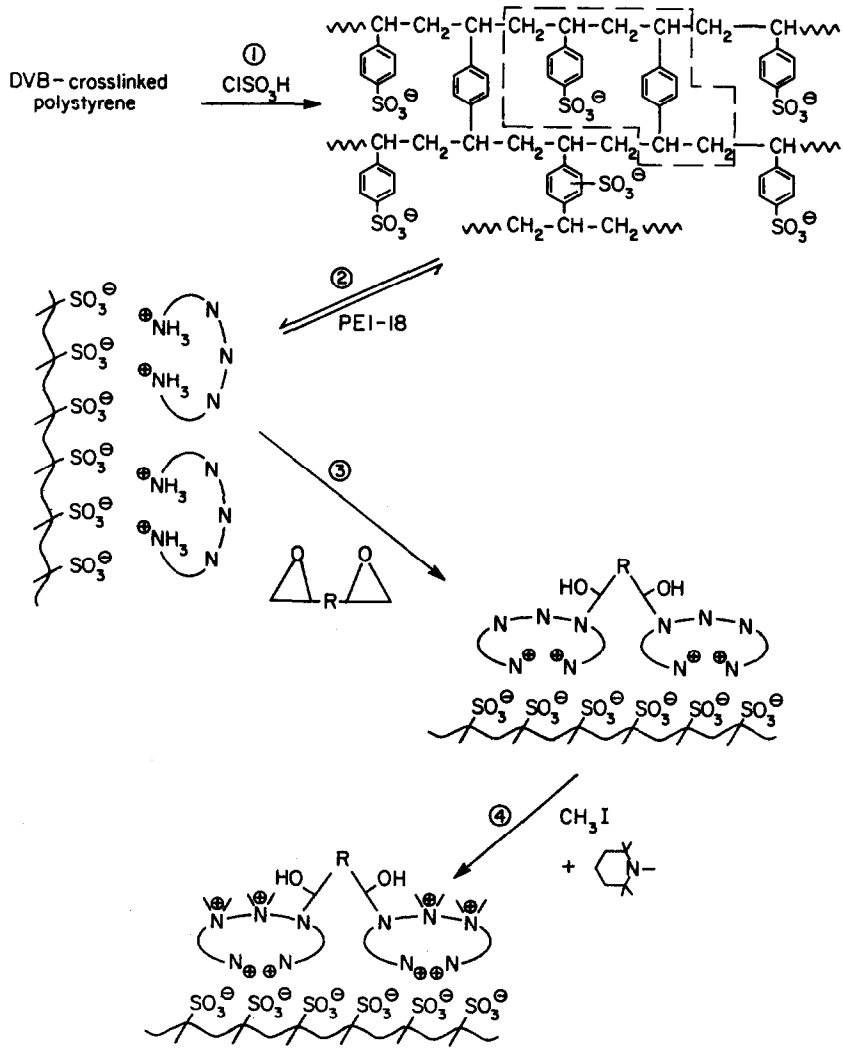

Fig. 1. Synthetic route to an adsorbed polyethyleneimine (PEI) stationary phase on divinylbenzene (DVB)-cross-linked polystyrene. A possible repeat unit of the base polymer is denoted by dashed lines. Structures have been oversimplified for illustrative purposes. "R" (step 3) denotes the variable portion of the diepoxide cross-linking reagent (*cf.* ref. 12).

solution of chlorosulfonic acid to a suspension of the resin in organic solvent. Since rate and extent of this reaction depend on diffusion of reagent through the copolymer mass[22,23], one function of the organic solvent was to swell the resin and make it more accessible to the chlorosulfonic acid[22]. It should be remembered, however, that the newer, highly cross-linked polystyrene packings used for HPLC swell relatively little compared to conventional resins[24]. Thorough degassing of the suspension was also essential for reproducible sulfonation. This removes air from the pores and facilitates penetration of the sulfonating reagent. In this work, reaction conditions were selected to maximize sulfonic acid formation (and minimize sulfones) relative to the sulfonyl chloride[22]. However, this is not necessarily a problem, since the latter is easily hydrolyzed to the acid.

The pore stability of poly(styrene–divinylbenzene) ion-exchange resins is greatest when the material is in its lowest state of hydration[25]. Consequently, highly sulfonated resins may be more susceptible to changes in pore diameter, due to the ease of solvating sulfonic acid groups. (Large pores and a high degree of crosslinking favor the conservation of porosity.) Preliminary data (not shown) indicated that more highly functionalized media, as judged by titration with sodium hydroxide, required a heavier PEI coating to mask acidic moieties on the matrix. However, application of a heavy (*e.g.* 10% PEI in the coating solution) coating reduces the pore diameter, pore volume, and surface area of the base material[13,26]. Consequently, physical characteristics of the matrix as well as variables in the sulfonation and coating procedures must all be considered. The optimization of these conditions requires further investigation. To date, 65–80% sulfonation of 1000 Å PLRP-S prior to coating, as described in this paper, produced packings of very similar Hb-binding capacity and chromatographic retention.

Experimental materials were systematically compared with each other and with silica-based packings by means of static anionic Hb-binding capacity assays[13] at pH 8.0. Assays conducted at pH 5.5 (cationic Hb) assessed binding to residual negative functionalities on the poly(styrene–divinylbenzene) resin. The ratio of these values was used to judge whether a particular coating had masked sulfonic acid moieties on the matrix. This was later verified chromatographically at pH 8.0 by the non-retention of LYS (p*I* 11).

Conversion of the weak anion-exchange medium to a strong anion-exchange stationary phase was achieved by methylation of the PEI-coated polystyrene, as recently described for silica packings[13]. However, a preliminary wash with 0.1 *M* sodium hydroxide was needed to deprotonate all amines, owing to the acidity of underlying sulfonic acid moieties. Without this step, methylation was incomplete. It was not possible to calculate the actual percent derivatization of the weak anion-exchange packing, since the picric acid ion-pairing capacity assay[6] cannot be used for polystyrene-based stationary phases. (Interaction with phenyl groups of the polymeric support[27] precludes accurate desorption of bound picric acid.) However, approximately constant chromatographic retention of OVA and STI[12] from pH 6–10 indicated that the stationary phase was quaternized.

Characterization of packing material

Composition. Elemental analyses of the strong anion-exchange packing material showed the composition of various batches to be very similar (Table II). [Static

TABLE II

ELEMENTAL ANALYSIS DATA FOR POLYSTYRENE-BASED STRONG ANION-EXCHANGE PACKING MATERIAL

The packing material was Polymer Labs, PLRP-S 1000 Å poly(styrene–divinylbenzene), sulfonated, coated, and quaternized by two independent preparations.

Batch	*% H*	*% S*	*% C (μmol C/g)*	*% N (μmol N/g)*	*C/N*
1	5.75	6.14	60.37 (50 300)	3.09 (2200)	23
2	7.19	6.45	61.08 (50 900)	2.99 (2100)	24

Hb-binding capacity and chromatographic performance of these two independently prepared lots was also quite reproducible (data not shown).] The ligand density of 2100–2200 μmol amine per gram packing material is comparable to a coating containing 20 μmol of nitrogen per m^2 of surface area on macroporous silica[26]. Vanacek and Regnier[26] noted that about 40% of the total amines of such a "heavy" layer of PEI are available for ion-pairing with small molecules, *e.g.*, picric acid. Ionic capacity of the polystyrene-based packing material was 0.12 mmol/ml (300 μmol/g), as determined by frontal uptake[17] of sodium nitrate (see Materials and methods). This value is only one-seventh (14%) of the total amine content, as determined by elemental analysis. There are two possible explanations for these results. One is that strong interaction between sulfonic acid moieties on the matrix and amines within the stationary-phase layer diminishes its capacity for the nitrate anion. The second explanation is that the coating is so dense that steric limitations prevent total penetration of the probe.

Protein load capacity. On a weight basis, the intrinsic Hb-binding capacity[13] of the PLRP-S 1000 Å weak anion-exchange packing (Table III) was *ca.* 60% that of the thinly-coated Vydac 101TPB (300 Å) silica used for chromatographic comparison in this study (data not shown). Vanacek and Regnier[26] have found that a macroporous silica-based packing with an ion-exchange capacity comparable to that of small-pore, high-surface-area silica can be prepared via a heavy polyamine coating. The same should be true for other packings.

TABLE III

INTRINSIC Hb-BINDING CAPACITY OF POLYSTYRENE-BASED PACKING MATERIAL

The packing material was Polymer Labs PLRP-S 1000 Å poly(styrene–divinylbenzene) coated and cross-linked as described in Materials and methods. Assay performed at pH 8.0. All data are averages from two independently-prepared lots of packing material (Table II).

Stationary phase	*Hb-Binding capacity (mg/g packing)*			
	Hb_{aec}	Hb_{iso}	Hb_{tot}	Hb_{aec}/Hb_{iso}
Weak anion-exchange (before quaternization)	27	28	55	1.0
Strong anion-exchange (after quaternization)	45	30	75	1.5

Quaternization of the polystyrene-based weak anion-exchange stationary phase increased anionic Hb-binding capacity (Hb_{acc}) by more than 65% (Table III). Presumably, this is due to increased surface charge, in agreement with findings for silica-based packing materials[13]. Non-ionically desorbed Hb (Hb_{iso}) appeared to be but little affected by quaternization. However, the color of the coated polystyrene makes it impossible to determine whether all residual bound Hb is actually removed by the trifluoroacetic acid–water–2-propanol eluent[12].

Although useful to the development and optimization of the new packing material, static binding capacity value do not correspond to the amount of protein which can be loaded onto a column in use. Dynamic load capacity was estimated by frontal uptake, using a dilute solution of BSA[18]. This protein is similar in molecular weight to Hb but does not exhibit the same non-ionic binding[12]. For comparison, intrinsic BSA-binding capacity was determined, as described in Materials and methods. Dynamic and static (equilibrium) BSA-binding capacities were 52 and 62 mg/ml, respectively. Thus, 84% of the equilibrium load was achieved under the dynamic conditions stated. This is comparable to (weak) anion-exchange silica-based packings of analogous particle size and pore diameter[18]. Dynamic load capacities of *ca.* 60 mg/ml were obtained by frontal analysis[17] with CON and OVA. Unfortunately, equilibrium values could not be determined for these proteins, because they were denatured in the course of the 18 h static assay.

Protein mass/enzymatic activity recovery. Mass recovery was determined for STI, OVA, BSA, CON and CAT as described in Materials and methods. A recovery of protein mass of >96% was achieved for all except CAT (84%). CAT is a large, tenaciously retained protein, which has previously been found difficult to elute from silica-based anion-exchange columns[7].

Enzyme activity recovery from the new packing material was examined with α-AMY. This enzyme is moderately retained on a strong anion-exchange column at pH 8.0[16] and is relatively easy to assay[19]. Activity recovery was 100% for a 50-μg load and 94% for a 200-μg load.

Chromatographic evaluation

Gradient conditions. Since the ultimate test of any packing material is its chromatographic performance, a four-component protein sample was fractionated (at pH 8.0) on a 5 × 0.41 cm I.D. column of the PEI-coated polystyrene strong anion-exchange packing material. A commercial polymeric strong anion exchanger (Mono Q, from Pharmacia; based on a polyether resin[11]) and a quaternized, PEI-coated silica column (see Materials and methods) were operated under identical conditions for comparison.

Initial chromatography was performed with a 20-min linear gradient from 0 to 0.35 *M* sodium chloride at a flow-rate of 1 ml/min[11]. These results are shown in Fig. 2. The three columns were also evaluated (same protein sample) with a steeper gradient slope, *i.e.* a 20-min linear gradient from 0 to 0.5 *M* sodium chloride at 1 ml/min. The steeper gradient (profiles not shown) produced negligible changes on the silica column and a decrease in resolution (R_s) of OVA/STI of <10% on the polystyrene-based packing. However, R_s for OVA/STI on the commercial column was lowered noticeably (24%). It is hypothesized that the lesser dependency of the polystyrene-based column on gradient slope is due to its larger pore diameter and, possibly, a more uniform pore-size distribution within the PLRP-S matrix.

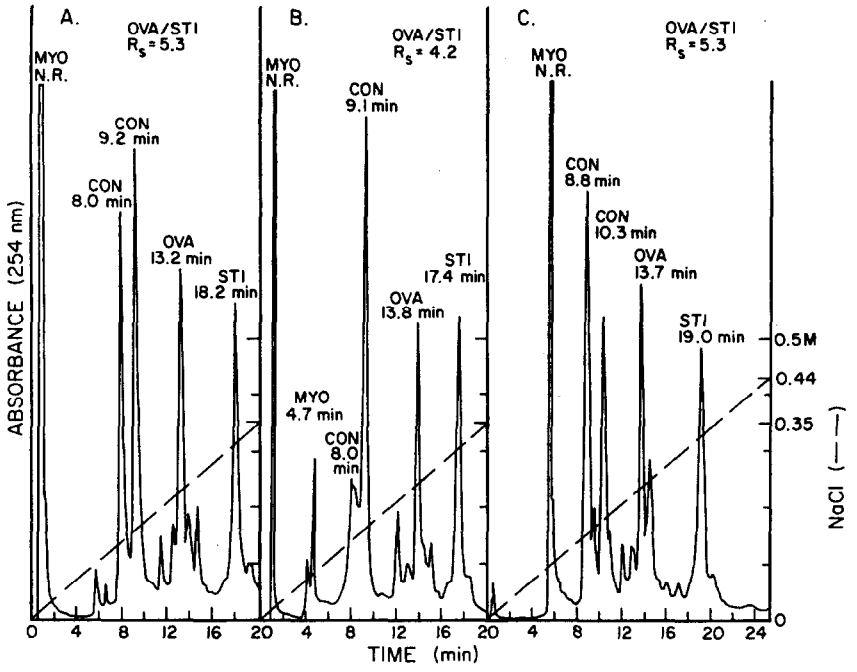

Fig. 2. Comparative protein separations. A mixture of MYO, CON, OVA, and STI (3, 5, 4, and 7 mg/ml, respectively) was chromatographed (20 μl) on (A) a quaternized PEI-coated poly(styrene–divinylbenzene) column (5 × 0.41 cm), (B) a Pharmacia Mono Q column (5 × 0.5 cm), and (C) a quaternized PEI-coated silica column (5 × 0.41 cm). A 20-min linear gradient was employed from 0 to 0.35 *M* sodium chloride in 0.01 *M* Tris–HCl buffer (pH 8.0) at a flow-rate of 1 ml/min. The gradient is indicated in the figure by the dashed line. (In the case of the silica column, this gradient was extended to insure elution of STI.)

Retention and resolution mapping. Chromatographic retention (t_R) over the range pH 4–12 was examined with five proteins of varying molecular weight and p*I*. A "retention map"[16] for the polystyrene-based strong anion-exchange packing is shown in Fig. 3, along with analogous data from the Pharmacia Mono Q column. With minor exceptions, retention of the proteins examined was virtually identical on the two polymeric columns. The retention times of OVA and STI decreased on lowering the pH from 6.0 to 5.0, compared to nearly constant retention across the range pH 5–10 on a silica-based strong anion-exchange column (data not shown; *cf.* ref. 12). In addition, retention times of OVA and STI increased at pH 11, then decreased at pH 12. Since OVA and STI have low isoelectric points, this may have been due to structural changes brought about by this extreme eluent pH.

The pH dependence of R_s between OVA and STI was compared for all three strong anion-exchange columns (silica, polystyrene, and commercial). Fig. 4 shows that R_s on the PEI-coated polystyrene packing was greater than, or equal to, that of the other two columns, except at pH 9 and 10 where, ironically, the silica-based column actually gave the best performance. (The silica column was not operated above pH 10.) High resolution on the polystyrene-based strong anion-exchange packing is attributed, in part, to good accessibility of proteins to the internal surface of the macroporous support material.

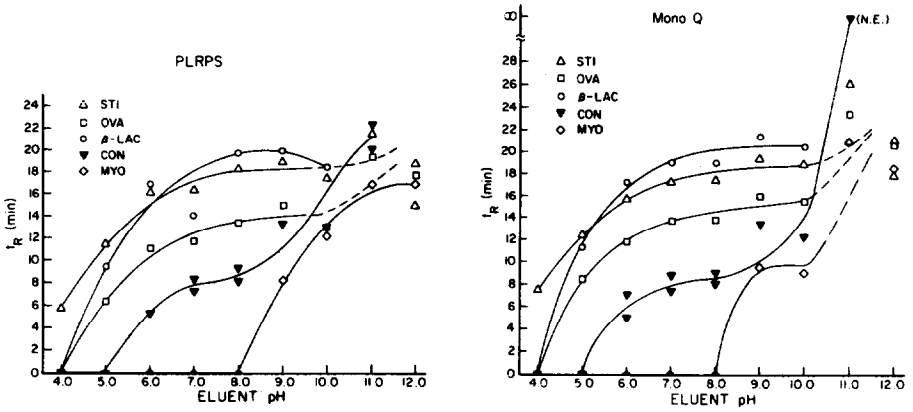

Fig. 3. Retention maps of polystyrene-based (left) and commercial polymeric (right) strong anion-exchange columns. Chromatographic retention (t_R) over the range pH 4–12 was examined for five proteins. Samples were eluted as described in Fig. 2. Two points on a curve indicate that two major peaks were observed. Conalbumin was not eluted (N.E.) from the commercial column at pH 11.0. Dashed lines denote possible protein denaturation by eluent.

Solvophobic effects. As a consequence of its organic matrix, the PLRP-S-based strong anion-exchange packing material has a C/N ratio (Table II) which is *ca.* 10 times higher than that of similarly-coated silica[26]. However, chromatographic performance did not suggest any problems (such as excessive band widths and "ghost-

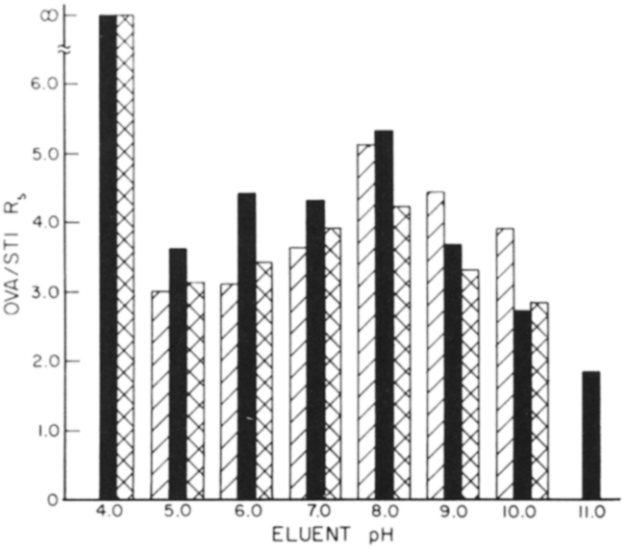

Fig. 4. Comparison of OVA/STI resolution on three strong anion-exchange columns. Chromatographic resolution (R_s) between OVA and STI was compared for silica-based (▨) and polystyrene-based (■) packings, and the Pharmacia Mono Q (▩). Elution conditions for both polymeric columns are described in Fig. 2. For the silica packing, the final sodium chloride concentration was 0.5 *M*. At pH 4.0, neither protein was retained on the silica column, and OVA was not retained on the polymeric columns; hence, infinite (∞) R_s. The silica column was not operated above pH 10.0.

ing") due to hydrophobicity. Solvophobic effects were examined more closely by repeating the separations (at pH 8) depicted in Fig. 2 with 10% acetonitrile in the eluents. (This organic modifier has previously been used to study the retention of oligonucleotides on hydrophobic anion exchangers[28].) The retentions of CON, OVA, and STI were each decreased *ca.* 10% on the polystyrene-based packing in the presence of 10% acetonitrile, compared to a 5% decrease in t_R on the Pharmacia Mono Q column. BSA, a relatively lipophilic protein[16], underwent maximum change in t_R and peak shape on the PEI-coated silica column. This suggests that the hydrophobicity of both the PLRP-S and silica-based packings is due to the methylated, heavily crosslinked polyamine stationary phase rather than to the underlying polystyrene or silica matrix. Previous work has shown that the cross-linking reagent used to immobilize an adsorbed PEI coating may contribute to stationary-phase hydrophobicity[12]. A small amount of hydrophobic character in an anion exchanger is not necessarily detrimental and may even enhance resolution via mixed-mode effects. The utility of mixed-mode column packings for the separation of proteins and oligonucleotides has been well documented[12,28,29].

Packing material durability. Throughout this study, the polystyrene-based strong anion-exchange column was subjected to a variety of cleaning procedures. Injections of 0.1% trifluoroacetic acid in 2-propanol–water (60:40, v/v) followed by aliquots of 50% hydrochloric acid (6 *M*) were used during retention mapping. Numerous injections (1 or 2 ml) of 1 *M* sodium hydroxide were also used without obvious detrimental effects. The packing material tolerated 1% acetic acid, 50% aq. formic acid, and acetone without noticeable shrinking or swelling. As a dynamic test of durability in aqueous base, *e.g.* for depyrogenation, a 5 × 0.41 cm I.D. column was subjected to 0.5 *M* sodium hydroxide as described in Materials and methods. Nearly 500 column volumes of this solution were pumped through the column with negligible change in R_s between OVA and STI. Subsequent non-retention of LYS (positively charged at pH 8) indicated that the adsorbed coating was still intact.

CONCLUSIONS

Adsorbed-coating technology[6] has been used to prepare an organic polymer-based strong anion-exchange packing for the HPLC of proteins. This packing material was prepared in three steps. First, negatively charged functional groups were imparted to small-particle (8 μm), macroporous (1000 Å) poly(styrene-divinylbenzene)[20,21] by sulfonation. Second, the sulfonated resin was coated with PEI, and this coating was subsequently cross-linked into a stable polymeric skin[6,12]. Finally, exhaustive methylation was used to achieve a quaternary amine strong anion-exchange stationary phase. Once key variables in the sulfonation, coating, and quaternization steps had been identified, it was possible to produce a packing material which was quite reproducible in chemical and chromatographic properties.

Intrinsic Hb-binding capacity assays[13] indicated that the heavy polyamine coating (along with quaternization) increased effective surface area of the macroporous polymeric matrix. The dynamic load capacity of the polystyrene-based packing material was measured by frontal uptake of BSA. A static (equilibrium) binding capacity of 84% was achieved for this protein at a flow-rate of 1 ml/min. This is comparable to (weak) anion-exchange silica-based packings of similar pore diameter

and particle size[18]. Protein mass recovery of more than 96% was obtained for four proteins of varying molecular weight, p*I*, and hydrophobicity. The recovery of enzyme activity was 94% or higher, as measured with α-AMY.

Chromatographic performance of the new packing material was evaluated relative to a quaternized, PEI-coated silica column and a commercially available, polymeric, strong anion-exchanger (Pharmacia Mono Q). Retention maps[16] of the five proteins examined, in the range pH 4–12, were virtually identical on the two polymeric columns. As measured with standard protein probes (OVA and STI[12]), resolution on the experimental packing was generally greater than, or equal to, that of the other two columns. Resolution on the PEI-coated polystyrene packing material was also less sensitive to the gradient slope employed than was the commercial column. This was attributed to the large pore diameter and, possibly, more uniform pore-size distribution within the PLRP-S matrix.

Addition of an organic solvent to the mobile phase indicated that the experimental column was less hydrophilic than the polyether-based[11] Mono Q column. However, the behavior of BSA on the PEI-coated silica column suggested that hydrophobicity was primarily due to the methylated, heavily cross-linked polyamine stationary phase rather than to the underlying matrix. Mixed ionic hydrophobic retention mechanisms[12] probably contributed to enhanced resolution on the two PEI-coated packings compared to the commercial column.

Throughout this study, the polystyrene-based material was subjected to injections of both acid and base without obvious detrimental effects. Dynamic exposure to 500 column volumes of 0.5 *M* sodium hydroxide produced negligible change in R_s between OVA and STI. Subsequent non-retention of LYS at pH 8.0 indicated that the cross-linked polyamine coating was still intact.

The versatility of adsorbed coatings[30] as well as their ability to mask undesirable properties of inorganic matrices[5] is now widely recognized[31]. In this paper, we have described the application of such a coating to a polymeric resin, originally designed for use as a reversed-phase packing[20,21]. Performance of the resultant medium in the (strong) anion-exchange mode testifies to the effectiveness of the cross-linked polyamine film in shielding proteins from the hydrophobic matrix underneath. Further work will refine this packing material and explore its utility for the separation of other biological macromolecules.

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