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PREPARATION OF A POROUS MICROPARTICULATE ANION-EXCHANGE CHROMATOGRAPHY SUPPORT FOR PROTEINS

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SUMMARY

A hydrophilic, durable anion-exchange material has been developed for high-performance liquid chromatography of proteins. Polyethyleneimine and simpler amines are adsorbed to porous, microparticulate silicas so strongly that the adsorbed coatings may be crosslinked into a stable layer by a wide variety of reagents in organic solution. Epoxy resins, alkyl bromides, and nitro alcohols are the best crosslinkers. The resulting pellicular coating is more stable in aqueous media than the underlying silica. Porous glass, alumina, and titania can also be coated in this manner. These materials are quite reproducible and of high ion-exchange capacity: up to 2.7 mequiv./g. Columns of polyethyleneimine-coated silica resolve proteins quickly and efficiently with excellent recoveries of enzyme activity. Nucleotides are also well resolved. Efficiency does not change during a column's lifetime.

INTRODUCTION

Anion-exchange chromatography has been a useful technique for resolving mixtures of biological compounds ranging from intermediates in the tricarborylic acid (TCA) cycle to nucleotides, peptides, and proteins. In the particular case of proteins, anion-exchange supports are of far more general utility than cation-exchange supports. This paper reports the preparation of a simple, durable anion-exchange support for proteins that functions in high-performance liquid chromatographic (HPLC) separations. This support is prepared by crosslinking a thin layer of polyethyleneimine (PEI) on the surface of controlled porosity inorganic supports.

The early observation by Wilfinger[1] that cellulose fibers strongly bound PEI led to the preparation of PEI-cellulose chromatography adsorbents. Using PEI-derivatized cellulose thin-layer plates, the Randeraths[2–9] were able to obtain sharp separations of nucleotides. The success of this ion-exchange thin-layer technique is due to the high ion-exchange capacity and the presence of a macroporous matrix.

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Selective desorption under milder conditions and enhanced equilibration are the desirable features of such an open network. The ion-exchange capacity of the support also has an important influence on migration[3]. Lowering the ion-exchange capacity of the support resulted in greater migration and diminished resolution. Elution of substances such as ATP from high ion-exchange capacity supports required mobile phases of low pH and salt concentrations greater than 1 *M*. Anionic materials were separated on PEI-cellulose plate by an ion-exchange mechanism while basic and neutral compounds separated by some other partition mechanism.

Ion-exchange chromatography of proteins stems from the early work on derivatized cellulosics[10,11] and synthetic resins such as Amberlite IRC-50[12–15]. The very high loading capacity of derivatized cellulosics relative to the synthetic resins and the ease of eluting proteins from the carbohydrate supports have since led to their wide acceptance. However, there are some reports from this early work that relate to the bonding of stationary phases to silica as reported in this paper. In efforts to increase the ion-exchange capacity (IEC) of synthetic supports, Boardman and others[16–19] applied ionic stationary phases to macroporous inorganic supports. Ion-exchange adsorbents were prepared by depositing either styrene–divinylbenzene or methacrylic acid–divinylbenzene polymers on the surface of diatomaceous earth. For example, polymer deposition occurred on Celite particles when they were heated with a methanolic solution of methacrylic acid and divinylbenzene in the presence of a suitable catalyst such as benzoyl peroxide[18]. The amount of polymethacrylic acid deposited on the Celite was controlled by the concentration of methacrylic acid in methanol. Polystyrene coatings were formed in much the same way by the substitution of styrene for methacrylic acid. Further derivatization of the polystyrene matrix was achieved by sulfonation to produce a strong cation exchanger[17–19]. Ion-exchange supports produced by this procedure had high IEC values and were used in the separation of insulin, cytochrome *c*, bovine serum albumin, bovine hemoglobin, lactoglobulin, and tobacco mosaic virus.

Later reports on the preparation of surface-coated inorganic ion-exchange supports for proteins all used organosilane coupling agents to covalently bond stationary phases to the surface of the support material. Eltekov *et al.*[20] bonded γ-aminopropylsilane (APS) to the surface of glass which then functioned as a weak anion-exchange support for proteins. However, non-specific and irreversible binding of proteins to APS-coated supports seriously limit their utility in protein chromatography. The nature of the APS coating appears to vary appreciably with reaction conditions[21–23]. It has been shown that protein recovery from APS bonded phase supports could be increased by simply crosslinking with butadiene dieproxide[24]. Still another technique for producing an ion exchanger is to crosslink the APS bonded phase support with carboxymethyl dextran or cellulose[24]. These supports, however, still have some of the adsorption problems of the original APS material. The use of amine-containing epoxy polymer coatings overcame the non-specific protein binding problem and produced supports of high efficiency that could be used in HPLC[25–27]. Since this coating procedure required several steps and the ion-exchange capacity was difficult to reproduce in large-scale synthesis, the possibility of circumventing the organosilane coating step was investigated.

HPLC anion-exchange supports of three types have been produced: porous inorganic materials with organosilane bonded phases such as those described above;

pellicular materials; and the rigid, porous organic gels. At present, most of the rigid organic supports are based on a derivatized polystyrene–divinylbenzene matrix that has been of limited use for the chromatography of proteins because of its hydrophobicity. However, recent reports[28] of the use of methacrylate-based Spheron supports for the ion-exchange chromatography of proteins indicate that it is possible to prepare rigid organic gels that are suitable for protein separations.

It is noteworthy that the original pellicular anion-exchange supports of Horvath *et al.*[29] did not use organosilanes to bond the coatings to the support. Nonporous glass beads were simply immersed in solutions of monomer or polymer and either polymerized in the case of polystyrene coatings or crosslinked by a gaseous agent in the case of PEI coatings. The resulting polymer pellicules were physically held in place on the non-porous beads and produced stable ion exchangers. However, it is desirable to use the higher surface area porous materials to increase the IEC of HPLC supports. The techniques reported above[29] for the coating of non-porous supports are undesirable with porous supports, since they tend to produce heavy coating that fill the support pores. A later procedure for applying PEI coatings to inorganic supports involved crosslinking the polymine in an aqueous phase during high-speed stirring. These supports were then used for enzyme immobilization[30–32]. This later technique is not suitable for HPLC supports, however, since particles are crosslinked into aggregates. This paper reports techniques for the production of pellicular coatings on totally porous supports.

THEORETICAL

The concentration of a solute at a liquid–solid interface is often greater than that in the bulk solution due to adsorption. By manipulating solvent polarity, the adsorption of solutes on an inorganic surface may be controlled. Solvents of low polarity are conducive to tight adsorption. If we designate the polarized inorganic surface as $P_s^{\delta-}$ and the polar solvent component as $P_m^{\delta+}$, then adsorption of the polar solvent to the surface may be described as follows:

$$P_s^{\delta-} + P_m^{\delta+} = P_s^{\delta-}P_m^{\delta+}$$

while the adsorption of solute $S^{\delta+}$ to the surface is represented by the equation

$$P_s^{\delta-} + S^{\delta+} = P_s^{\delta-}S^{\delta+}.$$

Both $P_m^{\delta+}$ and $S^{\delta+}$ are obviously competing for the same surface sites. The ultimate composition on a surface depends on the relative affinities of the different components for the surface and their concentrations.

When the affinity of an organic solute (R) is sufficiently large, the surface will become saturated with a layer of R ranging from one to a few molecules thick. This technique provides a convenient method for organizing molecular films on a surface that are thin and uniform. Accumulation of organic phase on the surface will be self-limiting; when all active sites on the surface are covered, adsorption ceases. Such a thin, reproducible film would be highly desirable in both the preparation and operation of a chromatography support.

It is apparent however, that adsorbed organic layers might not have sufficient long range stability to withstand elution with thousands of volumes of mobile phase in a chromatographic column. A gradual leeching of even the most tenaciously adsorbed solutes would occur.

The central concept of this paper is to use adsorption to establish films on inorganic surfaces that are stabilized by crosslinking adjacent molecules into a continuous surface layer. By crosslinking, the solubility of the organic phase is substantially decreased and the number of adsorption sites at which desorption must occur simultaneously to elute the coating is greatly increased.

This paper reports studies of the adsorption onto and crosslinking of PEI on inorganic supports. The utility of these composite supports in a chromatographic system is examined.

MATERIALS AND METHODS

Apparatus

HPLC was performed on a Constametric I and IIG system (Laboratory Data Control, Riviera Beach, Fla., U.S.A.). Samples were applied with a Rheodyne 7120 Sample Injector (Anspec, Ann Arbor, Mich., U.S.A.) equipped with a 100-μl loop. Proteins were monitored at 280 nm with a Perkin-Elmer LC-55 variable-wavelength detector (Perkin-Elmer, Norwalk, Conn., U.S.A.). Enzyme activities were monitored with a continuous-flow post-column reactor[33,34] and an Aminco Fluoro-Monitor (American Instrument, Silver Springs, Md., U.S.A.). Nucleotides were monitored at 254 nm with an Altex UV detector, Model 153 (Anspec). Columns were of precision-bore stainless steel with stainless steel frits at the ends. The inlet frit had 10-μm pores and the outlet frit (with a plastic rim) had 2-μm pores. Columns were packed with a Micromeritics Column Packer, Model 705 (Micromeritics, Norcross, Ga., U.S.A.).

Reagents

LiChrosorb and LiChrospher were purchased from EM Labs. (Elmsford, N.Y., U.S.A.). Partisil 10 was purchased from Whatman (Clifton, N.J., U.S.A.). Vydac TP, 10 μm, was a gift of The Separations Group (Hesperia, Calif., U.S.A.) while Johns-Manville (Denver, Colo., U.S.A.) furnished a sample of Chromosorb LC-6 (10 μm). Controlled-pore glass and porous titania were gifts of Corning Biological Products Dept. (Medfield, Mass., U.S.A.). Spherisorb alumina was a gift of Phase Separations (Queensferry, Great Britain). Alumina was also purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). PEI 6 (average mol. wt. = 600), Epon 812, Epon 826, ethylene glycol diglycidyl ether, and glyceryl diglycidyl ether were purchased from Polysciences (Warrington, Pa., U.S.A.). PEI 12, 18, and 200 were gifts of Dow Chemical (Midland, Mich., U.S.A.). Dithiobis(succinimidyl propionate) and dimethyladipimidate dihydrochloride were from Pierce (Rockford, Ill., U.S.A.). All other reagents were commercially obtained, reagent grade if available or the purest grade obtainable if not.

Preparation of pellicular anion-exchange coatings with various crosslinkers

Epoxy resins. 3 g of porous silica were weighed into a large test tube and

covered with 20–30 ml of a 10% (w/v) solution of an amine in methanol. The mixture was agigated on a vortex mixer. The tube was then put under vacuum (via a one-holed stopper connected to a water pump) for about 20 sec to remove air from the pores of the silica. A second agitation followed, and the coated silica was collected on a medium-pore sintered-glass funnel. Reduced pressure was used to filter off the amine solution, and was continued for at least 50 min to evaporate the methanol from the solution inside the pores.

The resulting silica (with an uncrosslinked coating of amine) was transferred to a large test tube and covered with 25 ml of a 5–10% (w/v) solution of an epoxy resin in dioxane. The mixture was swirled for a few seconds and the silica degassed *in vacuo* as before. The mixture was left overnight at room temperature, then heated on a steambath for 40 min with vortex mixing every 10 min. The product was collected on a sintered-glass funnel and washed well with acetone, water, and acetone, then dried under reduced pressure on the funnel.

The coating develops a light yellow color at the onset of the crosslinking reaction. This color is not observed when crosslinker is omitted from the system.

1,3-Dibromopropane. 300 mg of silica, with a coating of PEI 6 (see above), were vortexed and degassed in 5 ml of dioxane containing 404 mg of 1,3-dibromopropane. The mixture was left 24 h at room temperature, then heated 30 min on a steambath with occasional vortexing. The product was collected and washed as above.

2-Methyl-2-nitro-1,3-propanediol. The same procedure was followed as with *1,3-Dibromopropane*, save that the crosslinker was 270 mg of 2-methyl-2-nitro-1,3-propanediol and the mixture was left at room temperature for 3 days before heating.

Dithiobis(succinimidyl propionate). The same procedure was followed as with *1,3-Dibromopropane.* The crosslinker was 809 mg of dithiobis(succinimidyl propionate).

Cyanuric chloride. 300 mg of PEI 6-coated silica were vortexed and degassed in 5 ml of dioxane containing 369 mg of cyanuric chloride. The mixture was left 24 h at room temperature, then filtered out and washed with methanol, water, diethylamine, water, methanol, and acetone, then dried.

Dimethyl adipimidate dihydrochloride. 300 mg of PEI 6-coated silica were vortexed and degassed in 5 ml of 0.01 *M* sodium borate containing 490 mg of dimethyl adipimidate dihydrochloride. The mixture was left 24 h at room temperature, then collected by filtration and washed with water, diethylamine, water, and acetone.

Control (no crosslinker). 300 mg of PEI 6-coated silica were vortexed and degassed in 5 ml of dioxane. The mixture was heated 30 min on a steambath with occasional vortexing, then collected by filtration and washed well with water and acetone.

Preparation of APS-coated silica

3.0 g of LiChrosorb Si 100 (10 μm) were degassed in 3 *M* nitric acid and heated 45 min on a steambath with occasional swirling. The LiChrosorb was collected in a sintered-glass funnel and washed with water and acetone.

6 g of 3-aminopropyltriethoxysilane were dissolved in 60 ml of water and the pH adjusted to 3–4 with 6 *M* HCl. The LiChrosorb was degassed in this solution

and heated 2 h on a steambath, with occasional swirling. The product was collected in a funnel and washed well with water and acetone.

Assay of picric acid ion-pairing capacity (IPC)

An assay used for solid-phase peptides[35] was modified for use with silica.

100 mg of the silica to be assayed is placed in a small test tube. It is agitated by vortex with 4 ml of methylene chloride and sedimented by centrifugation. The supernatant is decanted with an aspirator and replaced by 4 ml of 0.2 *M* picric acid in methylene chloride. The mixing, sedimentation, and decanting are repeated. Non-bonded picric acid is washed out by repeating the procedure with four 4-ml washes of methylene chloride. Bound picric acid is then released with 5% (v/v) triethylamine in methylene chloride; the silica is agitated in this solution and sedimented, and the supernatant transferred by pipet to a volumetric flask. The washing with amine solution is repeated as long as the supernatant is yellow (about 4 washings). The combined washings are diluted and the triethylamine picrate assayed spectrophotometrically. The molar extinction coefficient of triethylamine picrate is 14,500 at 358 nm. Duplicate assays differ by 2% or less.

Assay of protein anion-exchange capacity

A published assay[36] based on hemoglobin was used to measure this property.

Assay of recovered enzyme activities

Lactate dehydrogenase (LD) was assayed by a Worthington procedure[37]. Trypsin was assayed with BAPA[38]. DAHP synthetase was assayed with a published method[39].

Tissue preparation for hexokinase isoenzyme profiling

A male Wistar rat, 10 weeks old, was killed by cervical dislocation and the liver removed rapidly. It was rinsed and homogenized in one volume of the following cold buffer: 150 m*M* KCl, 10 m*M* K_2HPO_4, 10 m*M* glucose, 5 m*M* EDTA disodium salt, and 3 m*M* dithiothreitol, pH 7.0. The homogenate was centrifuged 1 h at 105,000 *g*. The supernatant below the lipid (top) layer was decanted and analyzed.

HPLC column packing

Anion-exchange silica is slurried and degassed in water or isopropanol. The minimal amount of silica should be used; about 2.2 g for a 25 × 0.41 cm column. The silica is then packed into the column with the appropriate solvent. The flow-rate is increased from 2 to 10 ml/min over 25 min and is maintained at that rate for an additional 20 min (note: pressure should not be allowed to exceed 3000 p.s.i. when LiChrospher Si 500 is packed).

Column durability test

A 25 × 0.41 cm column is eluted with 0.1 *M* potassium phosphate, pH 8.5, at a flow-rate of 1.0 ml/min while it is heated on a steambath. The elution is continued until the column fails, as indicated by a rise in the inlet pressure above 4000 p.s.i.

Production of a tightly-adsorbed, non-crosslinked coating

1–3 g of porous silica were agitated thoroughly and degassed in a 10% (w/v) solution of PEI 6 in methanol, then collected on a medium-pore sintered glass funnel. The solution was filtered through and suction continued until the methanol in the pores had evaporated. 100 mg of the product was removed for IPC assay; the remainder was wetted and slurried briefly with a jet of methanol from a wash bottle, then dried again with suction. The washing was repeated several times, with 100-mg samples removed for assay after each washing. In some cases, additional 150-mg samples were removed after washings and crosslinked with an epoxy resin before assay.

Titration of anion-exchange silica

Deionized water was freshly distilled before use to eliminate CO_2; reagents were prepared with this water.

0.2–0.8 g of the silica to be titrated was placed in a 20-ml beaker and suspended with stirring, in 10 ml of either water or 1 *M* NaCl. The suspension was titrated with 0.1 *M* HCl. The pH reached equilibrium within 30 sec after each addition and was then measured with a Radiometer pH meter 36 equipped with a Markson 1885 electrode.

Elemental analysis

The elemental composition of coatings was determined by the Purdue Chemistry Department or by Hercules (Wilmington, Del., U.S.A.).

Synthesis of pentaerythritol tetraglycidyl ether (PETE)

Pentaerythritol was treated by the method of Smith *et al.*[40] to give a product which contained 90% pentaerythritol tetraallyl ether and 10% triallyl ether, as determined by gas chromatographic (GC) analysis. The tetraallyl ether was distilled at 122° (0.8 torr); lit.[41]: 125° (1.0 torr). A small amount of boric acid, added to the distillation pot, esterified the triallyl ether[42] and prevented its codistillation with the tetraallyl ether.

30.0 g (0.101 mol) of the tetraallyl ether was dissolved in 850 ml of methylene chloride. To this was added 90.9 g (0.527 mol, a 1.3-fold excess) of *m*-chloroperbenzoic acid (99%; prepared by washing 85% acid with 0.02 *M* sodium phosphate, pH 7.5). The resulting solution was stirred for 4 days at room temperature. The precipitated *m*-chlorobenzoic acid was filtered out and washed twice with methylene chloride; the filtrate and washings were combined. Excess peracid was then destroyed by addition, with stirring, of 20% sodium sulfite until the suspension did not darken moist starch–iodide paper. Precipitated *m*-chlorobenzoic acid was again filtered out and washed once with methylene chloride. The filtrate and washing were combined in a separatory funnel and washed 3× with 200 ml of 5% sodium bicarbonate and 3× with 200 ml of water. The organic layer was dried ($MgSO_4$), filtered through Celite 545, and the solvent evaporated. Upon cooling, the resulting fluid deposited additional crystals of *m*-chlorobenzoic acid, which were filtered out. The product was a clear, colorless fluid, slightly viscous, which was stable for over a year at 4°. Yield: 32.4 g (0.090 mol; 89%). The epoxide content was found to be 98% of theoretical in a titration assay[43]. Attempts at distillation were unsuccessful, the material distilling at 260° (0.02 torr) with decomposition.

RESULTS

Adsorbed amine coatings

A variety of amines were readily adsorbed to the surface of porous silicas from an organic solvent such as methanol. Removal of the remaining solution prevented packing of the pores. The adsorbed coatings were then crosslinked into pellicles by a suitable agent in dioxane. Since polyfunctional epoxy resins are reported to yield more durable materials than bifunctional resins[40], the tetrafunctional resin PETE was used as the crosslinking agent. The resulting coatings could be compared quantitatively through elemental analyses or the use of an ion-pairing agent (picric acid) to determine the number of amine residues available in organic solution. Table I lists the IPC of adsorbed coatings made with several different amines. The highest IPC was obtained with PEI 6, while those obtained with simple amines were slightly lower. An exceptionally poor coating was obtaining with N,N-diethylethylenediamine. Possibly the primary amine is preferentially adsorbed to the surface. This would leave the end with the tertiary amine, with no active hydrogens, projecting into solution. The resulting coating could not be crosslinked, and washes off.

TABLE I

LiCHROSORB Si 100 (10 μm) COATED IN A 10% SOLUTION OF AMINE

The coatings were crosslinked with PETE.

Amine	*Picric acid IPC (mmol/g support)*
N,N-Diethylethylenediamine	0.31
Ethylenediamine	1.45
Tetraethylenepentamine	1.47
1,3-Diamino-2-hydroxypropane	1.53
Polyethyleneimine 6	2.10

PEI 6, 12, 18, and 200 gave coatings with almost identical IPC and hemoglobin IEC values. However, columns packed with PEI 12-coated material were found to give poorer resolutions of proteins than columns of PEI 6-coated material. Accordingly, further study was confined to the properties of PEI 6 coatings.

Polyethyleneimine adsorption

PEI 6 is readily adsorbed to a variety of porous silicas from methanol solution. It is possible that multiple layers are adsorbed, the top layers being weakly retained. A series of washings with methanol in fact rapidly removed all but a tightly-bound layer of PEI as seen in Fig. 1. The amount of amine retained in this layer was unique for each silica, consistently giving the same quantitative analysis. Table II shows the IPC for the silica supports after six methanol washes. The 100-Å pore diameter supports lost at least 35–45% of their initial coating while the 500-Å material lost at least 65%. However, the specific IPC (IPC/m²) of the 500-Å support is still greater than that of the small pore diameter supports. The methanol apparently had limited access to the narrow pores of Partisil. Since little PEI could be washed out, a characteristic tightly-retained layer was not obtained.

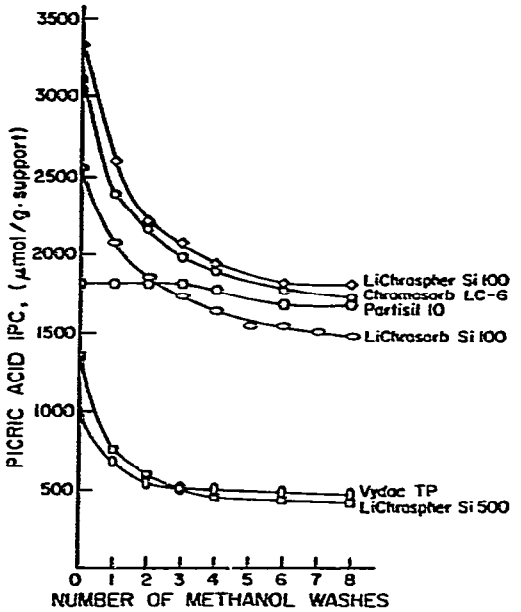

Fig. 1. Retention of adsorbed PEI 6 by HPLC silicas (10 μm) after washing with methanol.

TABLE II

IPC OF SILICAS WITH TIGHTLY BOUND COATINGS

Samples were coated with PEI 6 and washed 6 times with methanol. Manufacturers' stated dimensions are given.

Silica	*Pore diameter (Å)*	*Surface area (m^2/g)*	*Picric acid IPC (mmol/g support)*	*IPC ($\mu mol/m^2$)*
LiChrospher Si 100 (10 μm)	100	370	1.8	4.9
Chromosorb LC-6 (10 μm)	120	400	1.8	4.5
LiChrosorb Si 100 (10 μm)	100	300	1.6	5.3
Vydac TP (10 μm)	330	100	0.50	5.0
LiChrospher Si 500 (10 μm)	500	50	0.45	9.0
Partisil 10 (10 μm)	50	400	—	—

It is important to note here that methylene chloride did not remove PEI adsorbed to silica. This permitted the use of the picric acid assay with non-crosslinked coatings. Its accuracy was evaluated by comparison with elemental analysis. Fig. 2 shows that the picric acid assay detects only 55% of the nitrogen residues in the initial adsorbed coating but over 90% when the coating is thinned by 6 methanol washes. The IPC assay was used throughout these studies because it is equivalent to IEC measurements.

The concentration of silanol groups on the surface of silica is 8–9 $\mu mol/m^2$ (ref. 45). Only 1 mol/m^2 will react covalently, but a larger portion adsorbs molecules from solution[46]. The specific IPC values in Table II may reflect the concentration of adsorptive sites in the silicas listed.

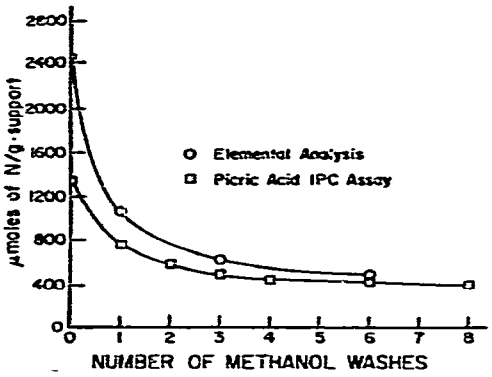

Fig. 2. Nitrogen content of methanol-thinned coatings of PEI 6 on LiChrospher Si 500 (10 μm).

Crosslinked coating

A variety of reagents were used to crosslink adsorbed PEI 6 coatings, as described in Materials and methods. Table III lists the picric acid IPC and the hemoglobin IEC of the products. Epoxy resins gave coatings with similar IPC. Coatings prepared with other reagents varied considerably in their IPC and IEC. 1,3-Dibromopropane produced a good coating but was not used in further work, since alkyl dibromides are mutagenic and may also generate a coating with quaternary amine residues. Since no other crosslinker was appreciably better than PETE for our purposes, it was retained as the crosslinker for the rest of this study. However, other crosslinkers may be more suitable for applications which require a particular coating matrix. The properties of the matrix may be modified with ease, since the crosslinker may contain any functional group which does not interfere with the crosslinking process. For example, the use of Epon 826 (Bisphenol A diglycidyl ether) incorporates aromatic groups into the coating. We have noted, however, that

TABLE III

CROSSLINKING AGENTS USED TO PREPARE PELLICULAR COATINGS

The coating of PEI 6 on (A) LiChrosorb Si 100 (30 μm) or (B) LiChrospher Si 500 (10 μm) was crosslinked by the indicated reagent.

Crosslinker	*Support*	*Picric acid IPC (mmol/g support)*	*Hemoglobin IEC (mg/g support)*
Pentaerythritol tetraglycidyl ether	A	1.42	—
Epon 826	A	1.42	—
Glyceryl diglycidyl ether	A	1.47	—
Epon 812	A	1.47	—
Ethylene glycol diglycidyl ether	A	1.51	—
1,3-Dibromopropane	B	1.85	58
Ethylene glycol diglycidyl ether	B	1.58	55
2-Methyl-2-nitro-1,3-propanediol	B	1.03	61
Epichlorohydrin	B	0.86	52
Dithiobis(succinimidyl propionate)	B	0.62	30
Dimethyl adipimidate dihydrochloride	B	0.43	79
Cyanuric chloride	B	0.36	26
None	B	0.016	16

these crosslinking agents do not all produce supports with comparable selectivity for small molecules. Matrix selectivity will be the subject of a future paper.

Part of the IPC is lost during the crosslinking process. In a typical case with LiChrosorb Si 100, the IPC of a PEI 6 coating decreased 30% when crosslinked with PETE in dioxane. However, when the PETE was omitted from the reaction mixture and the coated support simply heated in dioxane, the IPC decreased 22%. This leads to the conclusion that the reaction solvent is desorbing part of the PEI during the crosslinking reaction.

A variety of solvents and curing conditions were examined. In general, the optimum conditions for crosslinking and adsorbed PEI coating were to expose the coated support to a 10% solution of a multifunctional epoxy resin in dioxane for at least 14 h at room temperature followed by a 40-min treatment at 85°. The extended treatment at room temperature gave a product with 30% higher IPC than found in supports directly treated at 85° for 40 min. The PEI is probably crosslinked extensively at room temperature under conditions which do not desorb weakly-adsorbed PEI.

Support materials

The PEI coating may be applied to a variety of HPLC silicas, as well as to other inorganic materials. Table IV lists the picric acid IPC of various coated materials. Alumina and titania are denser than silica and the coated products have lower IPC when compared on the basis of mass. However, the specific IPC are similar. Coated aluminas and titanias could be useful for applications which require prolonged exposure to elevated pH.

TABLE IV

INORGANIC SUPPORT MATERIALS USED TO PREPARE PELLICULAR ANION-EXCHANGE PACKINGS

Coatings were PEI 6 crosslinked with PETE. Manufacturers' stated dimensions are given.

Support coated	*Pore diameter (Å)*	*Surface area (m^2/g)*	*Picric acid IPC (mmol/g support)*	*IPC ($\mu mol/m^2$)*
LiChrospher Si 100 (10 μm)	100	370	2.6	7.0
LiChrosorb Si 100 (10 μm)	100	300	2.1 ± 0.1	7.0
Chromosorb LC-6 (10 μm)	120	400	2.1	5.2
Controlled-pore glass (5–10 μm)	100	170	1.6	9.4
LiChrosorb Si 60 (5 μm)	60	500	1.3	2.6
LiChrospher Si 500 (100 μm)	500	50	1.1 ± 0.1	22
Partisil 10 (10 μm)	50	400	0.86	2.1
Vydac TP (10 μm)	330	100	0.85	8.5
Spherisorb alumina (10 μm)	100	95	0.78	8.2
Alumina (basic), activity I (40 μm)	—	—	0.66	—
Alumina (acidic), activity I (40 μm)	—	—	0.62	—
Titania (40–60 mesh)	400	11	0.29	26

Materials with pellicular PEI coatings compare favorably with other ion-exchange materials on the basis of IEC per unit volume. Coated LiChrosorb Si 100 (10 μm) has an IEC of approximately 1.2 mmol/ml of support, based on the titration data given below. Porous polystyrene-based gels possess IEC up to 1.7 mmol/ml[47].

The specific IPC in Table IV decrease with pore diameter. This suggests that the coating is of sufficient thickness to introduce steric hindrance which is prohibitive in 50- and 60-Å pore supports. This observation led to the exclusion of all materials with pore diameters less than 100 Å in future studies.

Coating characterization

Elemental analysis of the crosslinked PEI coatings on two different preparations with LiChrospher Si 500 are given in Table V. The C/N molar ratio in sample 1 is 2.89. This value may be used to determine the extent of crosslinking by making two assumptions: the average molecule of PEI 6 contains 14 nitrogen residues [represented by the formula $(C_2H_5N)_{14}$]; and none of the oxiranes are hydrolyzed to diols during the crosslinking reaction. The observed C/N ratio is then best explained by a model in which each PEI molecule has three of its nitrogen atoms crosslinked to adjacent PEI molecules. Since PETE has the formula $C_{17}H_{28}O_8$, the coating may be represented by $C_{39.3}H_{88.7}N_{14.0}O_{5.3}$, with a molar ratio of C/N = 2.81. A similar calculation for sample 2 suggests that an average of four amine residues per molecule are crosslinked. The picric acid assay detected about 75% of the nitrogen residues in the coatings, the efficiency of detection being slightly lower with the more highly crosslinked coating.

TABLE V

ANALYSIS OF COATINGS OF PEI 6 CROSSLINKED WITH PETE ON LiCHROSPHER Si 500 (10 μm)

Sample	*%C*	*%H*	*%N*	*mmol N/g support*		*% N Detected by picric acid assay*
				By elemental analysis	*By picric acid assay*	
1	5.59	1.29	2.26	1.61	1.24	77
2	6.39	1.12	2.30	1.64	1.11	68

The pH range of a PEI-coated silicas operation was determined with a titration curve, since ionizable groups on silica particles in suspension can be titrated[48]. Silica and APS-bonded silica were also examined, as were PEI 6 and 3-aminopropyltriethoxysilane in solution. The materials were either suspended or dissolved in 1 *M* NaCl, which reportedly permits freer access of a titrating agent to an insoluble electrolyte[49].

The coated silicas exhibit a broad range of ionization as seen in Fig. 3. This continuum of charge has been reported for PEI in solution[50], but is not characteristic of free 3-aminopropyltriethoxysilane, as indicated in Fig. 3. This suggests that the protonation of the cationic coatings is affected by the ionization state of neighboring groups and/or by interaction with the silica surface. The picric acid IPC of the PEI support is comparable to the IEC determined by titration in water. In both assays, insoluble polyelectrolyte is probably closely associated with the silica surface. When this association is broken with 1 *M* NaCl, the IEC is increased about 20% above the IPC and approaches the value for the number of nitrogen residues in the coating (Table V).

Columns of PEI-coated LiChrosorb Si 100 had lifetimes in excess of 400 h.

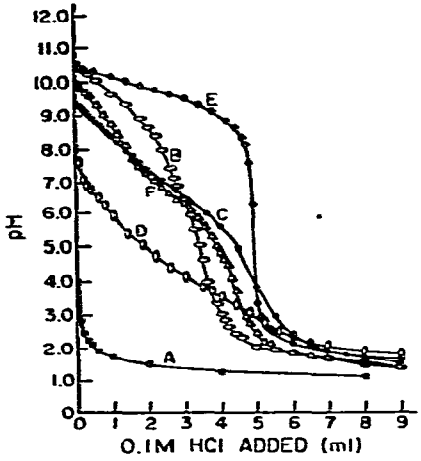

Fig. 3. Titration of amines and anion-exchange silicas. (A) LiChrosorb Si 100, 250 mg in 10 ml 1 *M* NaCl. (B) PEI 6, 21.6 mg in 10 ml 1 *M* NaCl. (C) PEI 6–LiChrosorb Si 100, 250 mg (IPC = 495 μmol) in 10 ml 1 *M* NaCl. (D) PEI 6–LiChrosorb Si 100, 250 mg (IPC = 495 μmol) in 10 ml water. (E) 3-Aminopropyltriethoxysilane, 111 mg (IPC = 500 μmol) in 10 ml 1 *M* NaCl. (F) APS–silica, 800 mg (IPC = 472 μmol) in 10 ml 1 *M* NaCl.

Columns were operated in the pH range 2–9.2 with no change in efficiency throughout the lifetime of the column. Failure was characterized by a rapid increase in head pressure of several thousand p.s.i. Accelerated column aging tests were carried out as described in Methods. Columns of PEI-coated LiChrosorb Si 100 failed in 6 h, while columns of the uncoated material failed in 2 h. It may be concluded that the coating protects the silica from solvent erosion. Elemental analysis of the coated support both before and after failure testing indicated that the percentages of total organic coating, carbon, and nitrogen had changed from 25.8, 14.35, and 5.24 to 27.3, 14.61, and 6.29, respectively. The increase in relative percentage of organic coating indicates that the coating is slightly more stable than the underlying silica. This could also account for the unchanging capacity and separation factors during aging. The total amount of amine on the column remains constant, even though the underlying silica is dissolving.

Macromolecular ion-exchange capacities of PEI-coated 10-μm supports were determined using hemoglobin, as described previously. Typical IEC for LiChrospher Si 100, Vydac TP, and LiChrospher Si 500 are 36, 33, and 65 mg hemoglobin/g of coated support. The higher value obtained with the Si 500 reflects the fact that hemoglobin can more fully penetrate the 500-Å diameter pores than it can the narrower pores of the other supports. Thus, a greater fraction of the support's surface is available for ion-exchange of the protein.

LiChrospher Si 500 supports with thin PEI coatings were prepared washing the adsorbed coatings with methanol before crosslinking. Fig. 4 shows that hemoglobin IEC of the resulting materials increases linearly with picric acid IPC until the IPC reaches 450 μmol/g support. Above that level, hemoglobin IEC increases much more slowly. This IPC is the same value which is characteristic of a tightly-retained PEI layer on LiChrospher Si 500 (Table II). There is no explanation as to

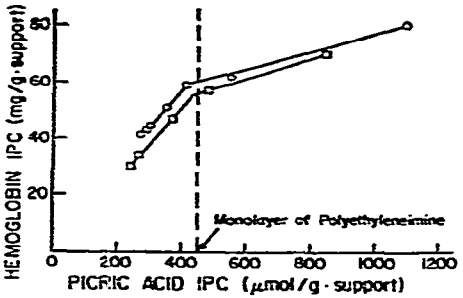

Fig. 4. Hemoglobin IEC as a function of picric acid IPC. Support: LiChrospher Si 500 (10 μm) with PETE-crosslinked coatings containing varying amounts of PEI 6.

why the presence of such a layer has such a marked effect on the macromolecular ion-exchange characteristics of the crosslinked coating.

Applications

Protein mixtures may be resolved on these WAX supports by means of a gradient. The high IEC of the supports makes desalting of serum samples unnecessary. Fig. 5 shows the A_{280} profile of serum. The constituents elute approximately in the order obtained with electrophoresis. Through the use of a post-column reactor, specific isoenzyme profiles were obtained from a crude mixture. Fig. 6 is the lactate dehydrogenase (LD) isoenzyme profile obtained with a rat kidney homogenate, and Fig. 7 shows the hexokinase isoenzyme profile from a rat liver homogenate. The use of this support material in the analysis of rat tissue proteins[33]* and commercial proteins has been described.

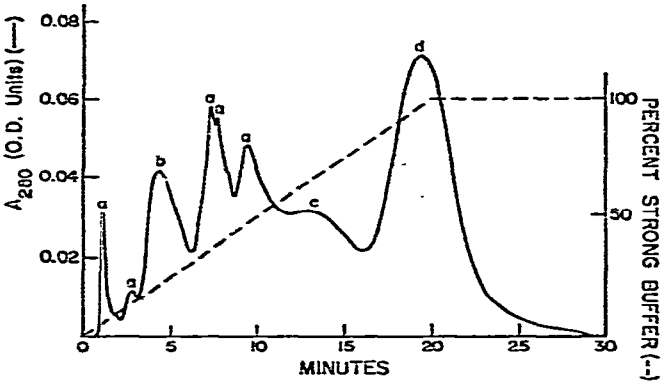

Fig. 5. Anion-exchange chromatography of human serum. Column: PEI 6–LiChrospher Si 500 (10 μm) crosslinked with PETE, 25 × 0.41 cm. Sample: 100 μl of serum, dil. 33%; temperature: 25°; flow-rate: 2.0 ml/min; inlet pressure: 1400 p.s.i.; detection: A_{280}, 0.195 a.u.f.s. Weak buffer: 0.02 *M* Tris acetate, pH 8.0; strong buffer: 0.5 *M* sodium acetate in weak buffer. Peaks: a = γ-globulin; b = unidentified; c = β-globulin; d = albumin.

* A PEI 6–LiChrospher Si 500 column, 25 × 0.41 cm, is described in ref. 18 as analytical column IV.

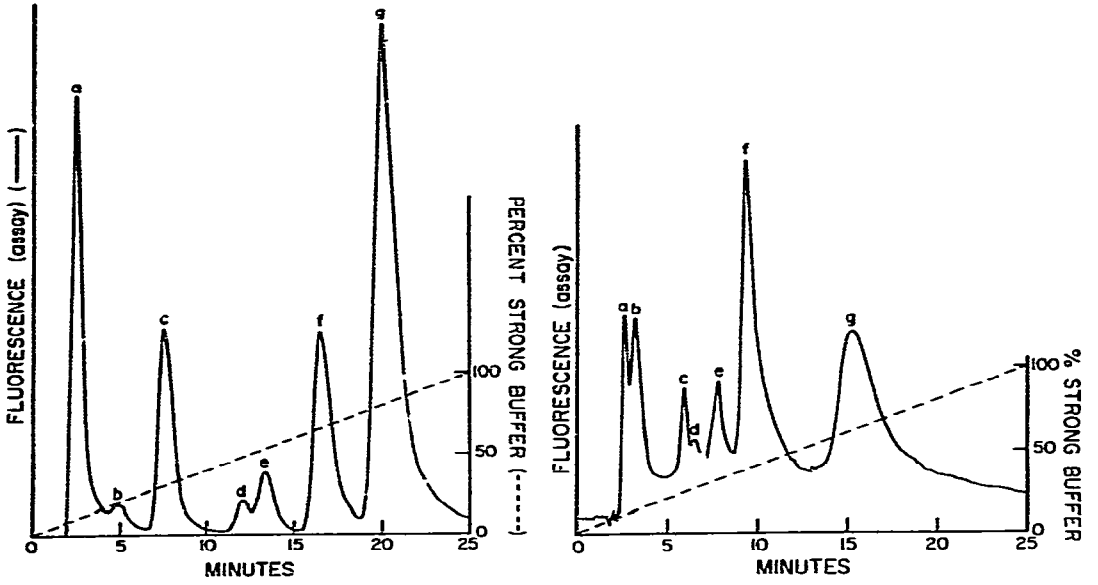

Fig. 6. Anion-exchange chromatography of rat kidney LD isoenzymes. Column: PEI 6–LiChrospher Si 500 (10 μm) crosslinked with PETE, 25 × 0.41 cm. Sample and conditions: see ref. 33. Weak buffer: 0.02 *M* Tris acetate, pH 7.9; strong buffer: 0.5 *M* sodium acetate in weak buffer. Peaks: a = LD_5; b = LD_4'; c = LD_4; d = LD_3'; e = LD_3; f = LD_2; g = LD_1.

Fig. 7. Anion-exchange chromatography of rat liver hexokinase isoenzymes. Column: PEI 6–LiChrospher Si 500 (10 μm) crosslinked with PETE. Sample: 100 μl of 105,000 *g* supernatant; temperature: 25°; flow-rate: 1.5 ml/min; inlet pressure: 1300 p.s.i.; detection: post-column reaction at 37° with immobilized glucose-6-phosphate dehydrogenase[18]; NADH monitored by fluorescence. Post-column reaction solution: 100 m*M* Tris-Cl, 60 m*M* glucose, 6 m*M* $MgCl_2$, 3 m*M* ATP, 1.5 m*M* NAD, and 1 m*M* dithioerythritol, pH 7.5. Weak buffer: 10 m*M* KH_2PO_4, 10 m*M* glucose, 5 m*M* $MgCl_2$, 0.5 m*M* dithioerythritol, pH 7.2; strong buffer: 0.6 *M* KCl in weak buffer. Peaks: a = background; b = hexokinase I; c, d = hexokinase IIA, IIB; e = hexokinase III; f = glucokinase; g = background.

Enzyme activity recoveries were measured by applying known amounts of enzymes to a column and eluting them with buffers similar to those used for elution from DEAE-cellulose. The eluate was collected and assayed for enzymatic activity. Activity recoveries were 100% for LD-1, 95% for LD-5, 100% for trypsin, and 89% for DAHP synthetase. The last two enzymes are quite sensitive to uncoated glass[51]. These recoveries are equal or superior to those obtained from DEAE-cellulose.

Although a detailed comparison of the PEI supports with DEAE ion exchangers has not been made, they have the same general chromatographic characteristics. In general, mobile phases that function well with DEAE supports will also function with the PEI supports.

It is noteworthy that the resolution of these columns for protein mixtures improved during the first few hours of column operation. This phenomenon is caused by exposure to buffers of high ionic strength. We can as yet offer no explanation for this behavior. However, we do suggest that a new column be eluted for 2 h with a strong buffer before its first use.

Some small peptides were retained on a PEI column, but polypeptides with over 20 residues were retained weakly or not at all. In general, cation exchange is more suitable for peptide analysis than anion exchange. PEI columns may be useful for the analysis of anionic peptides.

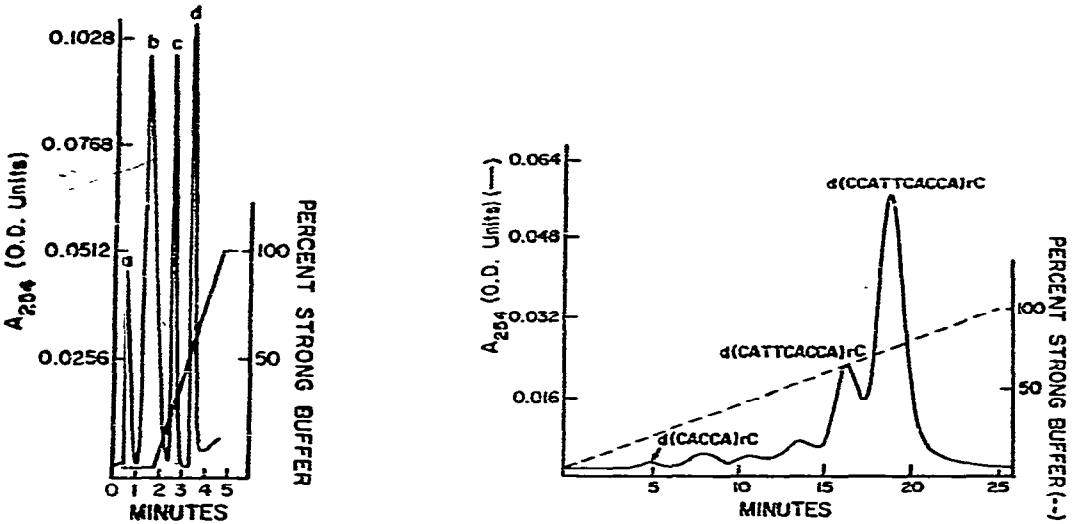

Fig. 8. Resolution of 5′-mononucleotides: Column: PEI 6–LiChrosorb Si 100 (10 μm) crosslinked with PETE, 6.2 × 0.46 cm. Sample: 5′-mononucleotides, 0.07 mg each/100 μl; temperature: 25°; flow-rate: 3.0 ml/min; pressure: 250 p.s.i.; detection: A_{254}. Weak buffer: 0.01 *M* KH_2PO_4, pH 3.0; strong buffer: 1.0 *M* KH_2PO_4, pH 2.0. Peaks: a = CMP, b = AMP; c = UMP; d = GMP.

Fig. 9. Resolution of oligonucleotides. Column: see Fig. 7. Sample: deoxyribonucleotides, 6–11 bases long; 0.1 O.D. unit/100 μl; temperature: 25°; flow-rate: 2 ml/min; pressure: 180 p.s.i.; detection: A_{254}. Weak buffer: 0.05 *M* K_2HPO_4, pH 7.0, with 30% methanol; strong buffer: 1.0 *M* NH_4Cl in weak buffer.

Nucleotides could be resolved on a 6.2-cm column by gradient elution. Resolution of 5′-mononucleotides and oligonucleotides is shown in Figs. 8 and 9, respectively.

The use of pellicular PEI materials for the resolutions of organic acids and phenols will be the subject of a separate paper.

DISCUSSION

Adsorbed coatings on inorganic supports may be crosslinked to produce viable chromatography support materials. The materials have been used in the anion-exchange resolution of compounds, including proteins, up to several hundred thousand daltons. These pellicular stationary phase supports have several advantages over supports with covalently-bonded organic phases. First, the number of functional groups in the coating is not limited by the Van der Waals radius of a silane monomer or by the number of reactive silanols on the silica surface. This permits the preparation of silica-based anion-exchange materials with several times the functional groups/m^2 that are currently obtainable on any covalently-bonded phase. Secondly,

the stability of the coating is independent of the stability of the underlying support, so a column's capacity factors and resolution do not change during its life if it is well packed.

Perhaps the most notable advantage of this coating technique is that coatings are self-assembling. Every step of the coating procedure is surface-directed, which prevents reaction in the solvent medium and cementing of the particles. First, a surface adsorbs a thin, reproducible layer of amine from solution. Adsorption terminates when the surface is covered. The layer is then crosslinked under conditions that retain the amine on the surface and permit the crosslinker to react only at the surface. The resulting coating is quite reproducible. By contrast, the monomeric silanes used to make covalent coatings can react with each other as well as with surface silanols. This leads to polymerization of silane monomers to a degree that depends upon the amount of water on the silica surface[52], the water content of the solvent[52], and the structure of the organosilane. The resulting coatings vary both qualitatively and quantitatively.

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