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CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON POLY(ASPARTIC ACID)-SILICA

ANDREW J. ALPERT*
Department of Pediatrics, Baylor College of** Medicine, **Texas** Medical **Center, Houston, TX 77030 (U.S.A.)

SUMMARY

A simple cation-exchange material for high-performance liquid chromatography of proteins was developed. Poly(succinimide) reacted rapidly with aminopropyl-silica and the product was hydrolyzed to poly(aspartic acid)-silica. Reaction conditions were optimized to yield a material with an ion-exchange capacity of 430 mg hemoglobin/g material. High-performance liquid chromatographic columns of the material featured excellent performance in terms of capacity, selectivity, recovery of enzyme activity. peak shape and durability. Protein standards and clinical hemoglobin samples were well resolved in minutes. Poly(succinimide)-silica was readily derivatized to give products other than poly(aspartic acid)-silica, and several such materials were prepared. Such materials could be useful for affinity chromatography or enzyme immobilization.

INTRODUCTION

Ion-exchange chromatography is one of the most useful techniques available for resolving mixtures of proteins. Most proteins have isoelectric points below 7 (refs. 1 and 2), and therefore anion exchange is more generally useful than cation exchange at neutral pH values. However, there are still a large number of protein mixtures which are best resolved by cation exchange.

The most widely used materials for protein cation exchange have been cellulose or dextrans containing carboxymethyl (CM) groups. The success of these materials is due to their high capacity and hydrophilicity, which is necessary for general protein chromatography. Recent advances in chromatography involve the use of finer column packings with high surface areas, a technique known as high-performance liquid chromatography (HPLC). Until recently, packings were not available for ion-exchange HPLC of proteins. The carbohydrate-based materials cannot withstand the high pressures involved. Hydrophilic organic supports have recently been developed for the purpose[3,**]. However, the pressure limit of these packings is still appreciably

* Present address: Department of Biochemistry, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030, U.S.A.
** Mono S, from Pharmacia (Uppsala, Sweden).

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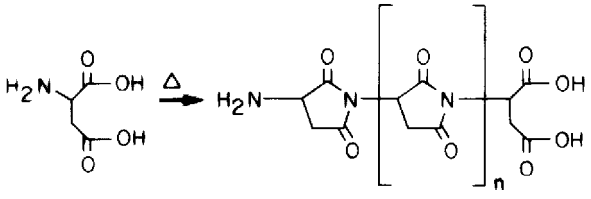
lower than that for inorganic-based materials. and limits their application. Inorganic materials are also available in a wider range of pore and particle diameters.

An inorganic support which is to be used for protein HPLC must be microparticulate, hydrophilic and macroporous (to give proteins free access to the entire surface area). An anion-exchange material is available which meets these requirements[4–6], but until recently no such cation exchanger has been available. Glass is naturally anionic and porous glass beads have been used for cation exchange of proteins'.'). However, glass binds many proteins irreversibly, and the cation exchange is best carried out through anionic groups in a hydrophilic, organic coating on the surface of the inorganic support. Chang *et al.*[10] developed a material of this type which required several steps to prepare and was difficult to reproduce. In the past year, Regnier and co-workers"." have developed a more promising coating, and two materials have become commercially available*.

Several studies have shown the advantages of using short polymers to prepare coatings for inorganic chromatography supports[4,13–15]. If the polymer reacts readily with the support surface and not with itself, then the coating is self-assembling. Such coatings are reproducible and cover the inorganic surface well; they also tend not to fill in pores or cement particles together. This report describes a self-assembling coating which converts an inorganic support into a cation-exchange material suitable for protein chromatography. The reactive polymer is anhydropoly(aspartic acid), or poly(succinimide). It is formed in almost quantitative yield by heating aspartic acid under conditions which cause it to condense (Scheme 1)[16–20]. Subsequent hydrolysis produces poly-α,β-D,L-aspartic acid[17,18].



Aspartic Acid Poly(Succinimide)

Scheme 1.

This polymer offers a promising route to a cation-exchange coating. Poly(succinimide) could be immobilized on a surface through reaction with surface amino groups. Subsequent hydrolysis would generate a cation-exchange group as an integral part of the polymer and so no additional reaction would be needed to add one. This would promote reproducibility. Such a coating would be useful for protein chromatography since it would contain many carboxylic acid groups in a hydrophilic, polypeptide matrix. This report details the preparation of poly(aspartic acid)–silica and its use for HPLC of proteins.

Poly(succinimide) could be used to prepare a variety of chromatographic media in addition to the cation exchanger, since amines add readily in N,N-

* SynChropak CM300, from SynChrom (Linden, IN, U.S.A.); and TSK-GEL IEX-530CM SIL and TSK-GEL IEX-535CM SIL from Toyo-Soda Manufacturing Co. (Tokyo, Japan).

dimethylformamide (DMF) solution to yield poly-α,β-D,L-aspartamides[18,19,21–25]. Several coatings of this type are examined in this study as well.

MATERIALS AND METHODS

Apparatus

HPLC was performed on a Varian 5000 LC system (Varian, Palo Alto, CA, U.S.A.). Samples were applied with a Valco valve (Valco Instruments, Houston, TX, U.S.A.) equipped with a 25-μl loop. Hemoglobins were monitored at 415 nm with a Vari-Chrom detector (Varian). Other proteins were monitored at 220 nm. Poly(aspartic acid)–silica was slurry-packed into stainless-steel columns by Custom LC (Houston, TX, U.S.A.). The columns were 20 x 0.46 cm with Valco end fittings. Such columns are available from Custom LC under the name PolyCAT A.

Reagents

Vydac 101 TP silica gel, with a pore diameter of 330 A, was purchased from The Separations Group (Hesperia, CA. U.S.A.). The particle diameter was 8.0 ± 0.2 (nominally 10) μm. Vydac with a particle diameter of 4.9 ± 0.1 (nominally 5) μm or 6.7 ± 0.2 (nominally 7) μm was also used as noted. The following were from Sigma (St. Louis. MO. U.S.A.): D,L-aspartic acid, ovalbumin (Grade V), bacitracin, myoglobin (type II, from sperm whale skeletal muscle), chymotrypsinogen A (type II, from bovine pancreas). ribonuclease A (type I-A, from bovine pancreas), cytochrome *c* (type VI, from horse heart), and lysozyme (Grade I). Hemoglobin AFSC Control (cat, No. 5331) was obtained from Helena Labs. (Beaumont, TX, U.S.A.). Bis-tris [2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; Gold Label] and 3-aminopropyltriethoxysilane were from Aldrich (Milwaukee, WI, U.S.A.) Polyethyleneimine 6 (PEI 6) [average molecular weight (mol.wt.) 600] was purchased from Polysciences (Warrington. PA, U.S.A.), All other reagents were reagent grade if available or the purest grade obtainable if not.

Clinical hemoglobin samples were prepared from heparinized blood. Erythrocytes were sedimented and washed once with saline solution. Packed cells were lysed by mixing them with an equal volume of water. Cellular debris was removed by centrifugation for 5 min at 1500 *g*.

Preparation of poly(succinimide)

D,L-Aspartic acid (50 g, 0.38 mol) was placed in a thin layer in a crystallization dish and heated in an oven at 190° for 50 h. The resulting light tan powder weighed 37.9 g (indicating virtually quantitative dehydration, assuming a unit mol. wt. of 97 for the product). The powder dissolved with heating in 150 ml of DMF except for a small amount of white material. This was removed by centrifugation, leaving a brown solution. Poly(succinimide) could be collected by pouring the DMF solution into 4 volumes of water or diethyl ether. with rapid stirring, and collecting the precipitated product by centrifugation. The precipitate was freed of DMF by shaking it several times with the precipitating solvent followed by resedimentation. The precipitate was then lyophilized to yield a light tan powder, completely soluble in DMF. The mol.wt. of the product is reported'" to be about 13,000. The use of either water or ether gives a product acceptable for the purpose here. The precipitate sediments more rapidly

from ether, and its use also precludes the possible hydrolysis of some of the succinimide rings.

***Preparation** of aminopropyl–silica*

Vydac silica (4.0 g) was weighed into a large test tube and covered with 30 ml of 5 % (w/v) of 3-aminopropyltriethoxysilane in toluene. The mixture was swirled with a vortex generator and then kept under vacuum (with a one-holed stopper connected to a small pump) for 30 sec to remove air from the pores of the silica. The mixture was heated for 2 h in a boiling water-bath with occasional swirling. The product was collected in a medium-pore sintered-glass funnel and washed well with toluene and acetone, then dried by continued suction.

***Preparation** of poly(succinimide)–silica*

Aminopropyl-silica (4.0 g) was swirled and degassed as above in 20 ml of 5 % (w/v) of poly(succinimide) in DMF. The mixture was left for 24 h at room temperature with occasional swirling. The tan-colored product was collected in a funnel and washed well with DMF and acetone.

Preparation** of poly **(aspartic acid)-silica

Poly(succinimide)-silica (4.0 g) was swirled and degassed as above in a solution containing 15 ml of DMF, 10 ml of water, 0.825 g (9.3 mmol) of β-alanine, and 0.625 ml (4.5 mmol) of triethylamine. The mixture was left for 24 h at room temperature with occasional swirling (this removes much of the tan color). The light-tan product was collected in a funnel and washed well with water, 0.05 *M* hydrochloric acid, water, and acetone, then dried by continued suction.

***Preparation of** poly(2-aminoethyl aspartamide)–silica*

The procedure was the same as for poly(aspartic acid)-silica except that the solution consisted of 0.25 ml (3.7 mmol) of ethylenediamine, 0.50 g (3.7 mmol) of ethylenediamine dihydrochloride. 20 ml of DMF. and 4 ml of water. The product was washed with a saturated aqueous solution of triethylamine instead of 0.05 *M* hydrochloric acid.

***Preparation** of poly **(PEI** aspartamide)–silica*

The procedure was the same as for poly(aspartic acid)-silica except that a 5 % (w/v) solution of PEI 6 in DMF–water (1:1) was allowed to react for 46 h and the product was washed with a saturated aqueous solution of triethylamine instead of 0.05 *M* hydrochloric acid.

***Preparation of** silicas coated with derivatized poly(succinimide)*

Solutions were prepared containing 2.5 ml of a 10% solution of poly(succinimide). The additives ethanolamine or tris(hydroxymethyl)aminomethane (Tris) were dissolved in 2.5 ml of DMF to produce solutions containing 0, 5, 10 or 20 % of the molar equivalent of succinimide residues. Both the poly(succinimide) and additive solutions were cooled to 5°C. The additive solutions were then introduced dropwise into the swirling poly(succinimide) solutions and left for 3 h at room

temperature. The solutions darkened almost immediately in proportion to the amount of additive present. The solution without additive received 2.5 ml of DMF alone.

Samples of aminopropyl-Vydac (each 140 mg) were each degassed and swirled in one of the solutions of derivatized poly(succinimide). They were left for 24 h at room temperature, with swirling after 12 h. Finally, they were recovered by filtration, washed well with DMF and acetone, and then dried by continued suction. Residual succinimide residues were hydrolyzed with β-alanine and triethylamine solution as with poly(aspartic acid)–silica.

Assay of picric acid ion-pairing capacity

This assay has already been described[4]. It permits the quantitation of accessible amine groups on a solid surface through the reversible formation of ion-pairs with picric acid.

Assay of protein ion-exchange capacity

This quantity was measured by a published method, based on hemoglobin". The amount of silica assayed was 100 mg instead of 1 ml, and the concentration of the original hemoglobin solution was doubled. Undissolved sediment was centrifuged from the hemoglobin solution before its use in the assay.

Assay of recovered enzyme activities

Published methods were used to assay adenylosuccinate synthetase[26], thiolase I (porcine)*' and β-hydroxyacyl-CoA dehydrogenase (porcine)".

Titration of silica and poly(aspartic acid)–silica

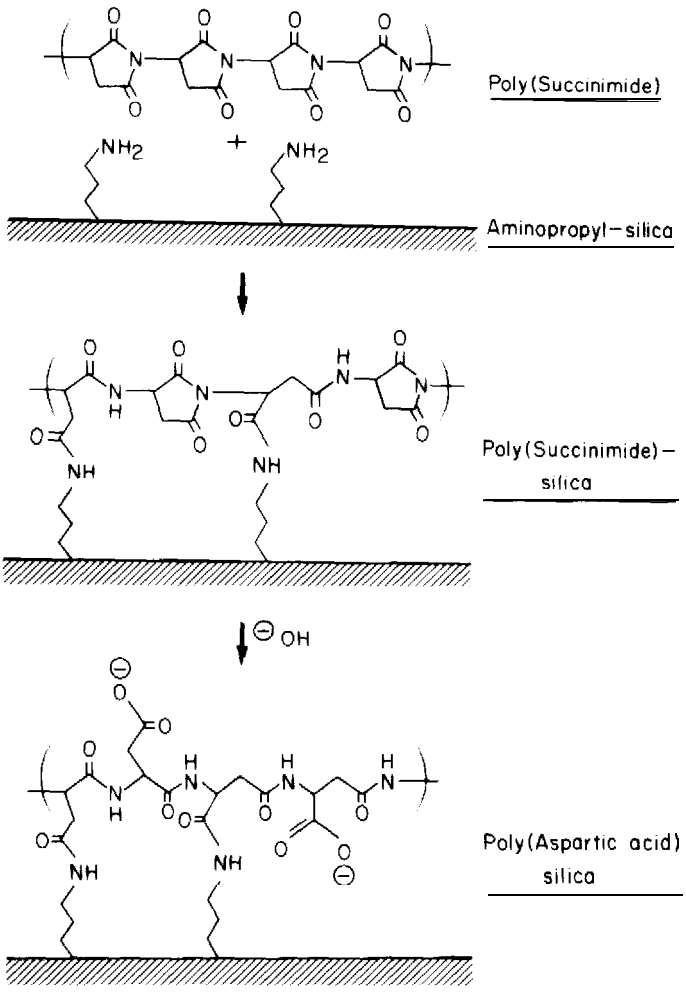
Reagents were prepared with water which had been freshly distilled to eliminate carbon dioxide.

A 3.5-g portion of the silica to be titrated was placed in a 30-ml beaker with a magnetic stirring bar. The sample was suspended, with stirring, in 15 ml of 1 *M* sodium chloride containing sufficient added 0.1 *M* hydrochloric acid to adjust the pH of the suspension to 3.0. The suspension was titrated with 0.1 *M* sodium hydroxide. The pH reached equilibrium within 1 min after each addition and was then measured with a Radiometer pH Meter 26 equipped with a Radiometer electrode.

RESULTS

Preparation of poly faspartic acid)-silica

Scheme 2 illustrates the method used to prepare poly(aspartic acid)-silica. A microparticulate silica gel was selected having pores wide enough to give most proteins free access to the pore interior. The silica was given a covalently bonded coating of aminopropyl groups, Poly(succinimide) was allowed to react with the amino groups. This produced a poly(succinimide)-silica in which the polymer was immobilized through amide bonds to the surface. Subsequent treatment with base catalyzed the hydrolysis of unreacted succinimide rings, producing the poly(aspartic acid) coating.



Reaction of aminopropyl–silica with poly (succinimide)

The reaction of poly(succinimide) with surface aminopropyl groups causes free amino groups to disappear. The rate of disappearance could be measured with the picric acid assay. Samples of aminopropyl-silica were immersed in a 5 % solution of poly(succinimide) and worked up for the assay at various times. Fig. 1 shows that the reaction is 85 % complete within 0.5 h. Thorough coating of the surface could be achieved by allowing the reaction to proceed for 24 h.

The concentration of the poly(succinimide) solution was also optimized. Solutions of several concentrations were used to prepare poly(succinimide)-silica in 24-h reactions and the products were hydrolyzed to poly(aspartic acid)-silica as described in Materials and methods. The ion-exchange capacity for hemoglobin was used to relate the properties of the products to the quality of the poly(succinimide) coatings, Fig. 2 shows that there is an appreciable difference in the coatings when the poly(succinimide) concentration is raised from 1 to 2 %, but little difference when the

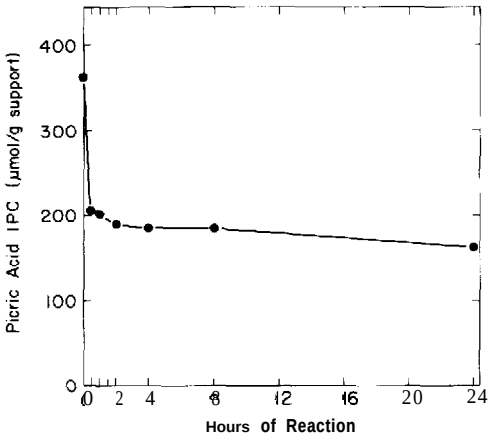


Fig. I. Reaction of aminipropyl-silica with poly(succinimide). IPC = Ion-pairing capacity.

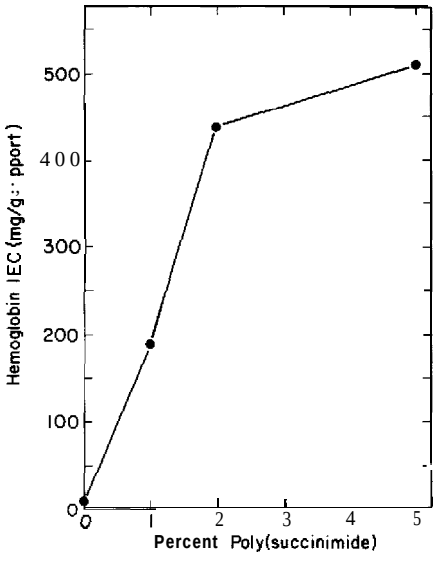


Fig. 2. Effect on poly(aspartic acid)–silica of varying the concentration of the poly(succinimide) solution. IEC = Ion-exchange capacity.

concentration is raised from 2 to 5 %. Thus, a 5 % solution is suitable for producing a reproducible coating rapidly.

Hydrolysis of poly(succinimide)–silica

It has been reported[28] that β-alanine reacts with poly(succinimide) to give poly(2-carboxyethyl aspartamide). The subsequent hydrolysis of unreacted succinimide residues is catalyzed with the base triethylamine. This reaction was investigated as a possible route to a cation-exchange material. Table I shows the hemoglobin ion-exchange capacity of several such products prepared from poly(succinimide)–silica. Reaction of β-alanine with succinimide residues is far slower than their hydrolysis in the presence of triethylamine; thus, the coatings prepared here are primarily poly(aspartic acid) and not poly(2-carboxyethyl aspartamide). The inclusion of β-alanine in the triethylamine solution gives a product with a slightly higher ion-exchange capacity, and so β-alanine is routinely added to the hydrolysis solution. It is evident from Table I that there is slow hydrolysis of the coating in the absence of a catalyst; this hydrolysis is slower in DMF-water than in water alone. However, catalyzed hydrolysis is faster in DMF-water solutions.

Untreated poly(succinimide)-silica has a modest hemoglobin ion-exchange capacity. This may reflect the presence of aspartyl groups in the middle of the polymer as well as the C-terminus. A study of one poly(succinimide) sample found it to contain 14 % aspartyl-residues[29], although other estimates were lower[22,30].

Quantitation of carboxyl groups in poly(aspartic acid)-silica

Hydrolysis of a poly(succinimide) coating to a poly(aspartic acid) coating generates numerous carboxylic acid groups. It would be helpful to know the concentra-

TABLE I

EFFECT OF ADDITIVES ON CATION-EXCHANGE MATERIALS PRODUCED FROM POLY(SUCCINIMIDE)-SILICA

Portions (each 120 mg) of poly(succinimide) Vydac were swirled and degassed in solvent (4.4 ml). The solvent was water or a mixture of DMF (2.6 ml) and water (1.8 ml). β-Alanine (147 mg) and/or triethylamine (111 mg) were present in the solvent as noted. Mixtures were left for 15 h at room temperature with occasional swirling. The products were filtered off and washed as with poly(aspartic acid)–silica (see Materials and methods).

Solvent	*β-Alanine*	*Triethylamine*	*Hemoglobin ion-exchange capacity (mg/g support)*
DMF-water	+	+	382
	+		95
	–	+	356
	–	–	84
Water	+	+	370
	+	–	158
	–	+	305
	–	–	170

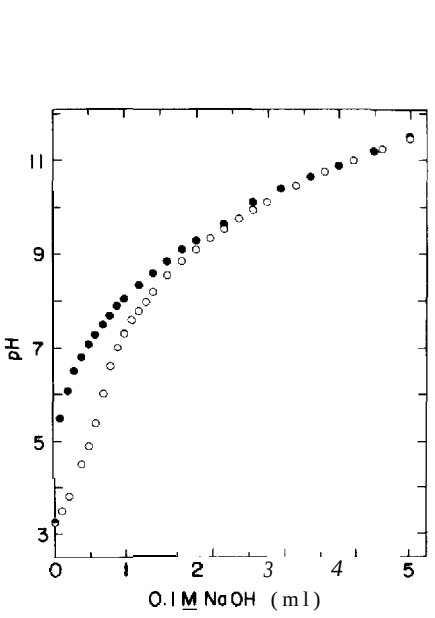


Fig. 3. Titration of 3.5 g of Vydac TP silica (●) and poly(aspartic acid))Vydac (0).

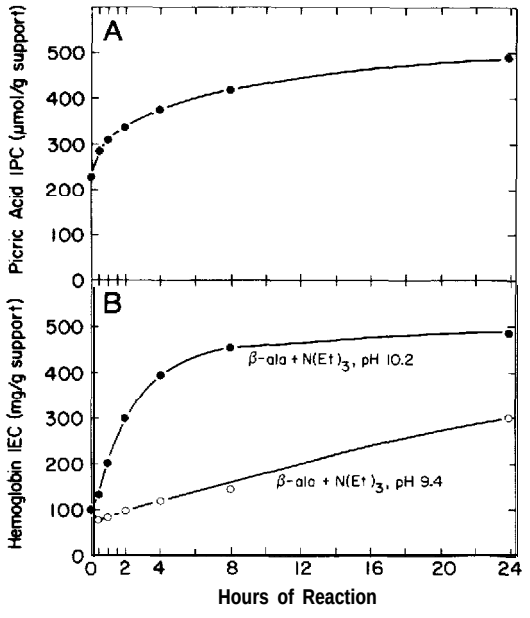


Fig. 4. (A) Reaction of poly(succinimide)-silica with ethylenediamine monohydrochloride. The pH was 9.3. (B) Hydrolysis of poly(succinimide)-silica to poly(aspartic acid)–silica. Et = Ethyl; IEC = ion-exchange capacity; IPC = ion-pairing capacity.

tion of these groups in order to determine the time needed for hydrolysis of the coating. Unfortunately, there is no assay which can quantitate carboxyl groups conveniently in the manner that the picric acid assay quantitates amine groups. The native silanol groups of silica interfere with their quantitation by aqueous titration, as can be seen from the titration curves in Fig. 3. The titration curve of the silica resembles one already published[31]. Poly(aspartic acidjsilica possesses a modest buffering capacity in the pH range 3.5-5.5 which silica lacks.

The concentration of carboxyl groups in the coating can be estimated by indirect methods. The reaction of poly(succinimide)-silica with ethylenediamine produces a coating containing aminoethyl groups. These can be quantitated with the picric acid assay. The monohydrochloride of ethylenediamine was used to promote reaction with only one amine residue and not both (which would simply cross-link the coating through amide bonds and leave the picric acid ion-pairing capacity unchanged). Fig. 4A shows the time course of reaction. Reaction is almost complete after 24 h, with 0.26 mmol of ethylenediamine added per g of poly(succinimide)–silica. This constitutes a lower limit for the concentration of hydrolyzable succinimide residues, since this method would not measure the following types of residues: (a) residues involved in a cross-linking reaction with ethylenediamine and not end-on addition; (bj aspartyl groups present before the reaction; (c) residues the hydrolysis of which to aspartyl groups was catalyzed by the basic ethylenediamine. The product, poly(2-aminoethyl aspartamide)-silica, had almost no hemoglobin ion-exchange capacity (see Table II). This indicates that there are enough carboxyl groups in the coating to give the product a neutral, zwitterion character. If the number of carboxyl groups equals the number of amine groups, then the concentration of carboxyl groups in poly(aspartic acidjsilica would be twice the concentration of the amine groups added here, or 0.5 mmol/g material.

The rate of coating hydrolysis can be followed by a second indirect method: the increase in the hemoglobin ion-exchange capacity as hydrolysis proceeds. Fig. 4B shows the time course of hydrolysis with two different concentrations of triethylamine in the hydrolysis solution. The rates differ by a factor of 8, and are directly proportional to the difference in the concentration of hydroxyl groups in solution. At the higher pH, hydrolysis seems to be complete by 24 h. However, a study with an anion-exchange silica showed that the hemoglobin ion-exchange capacity leveled off once

TABLE II

DERIVATIVES OF POLY(SUCCINIMIDE)–SILICA

Products were prepared as described in Materials and methods.

Derivative	*Hemoglobin cation-exchange capacity (mg/g support)*	*Hemoglobin anion-exchange capacity (mg/g support)*	*Picric acid ion-pairing capacity (mmol/g support)*
Poly(2-hydroxyethyl aspartamide)-Vydac	150	1	0.23
Poly(2-aminoethyl aspartamide)–Vydac	8	3	0.44
Poly(PEI aspartamide)–Vydac	3	19	0.72

the surface ion-exchange sites reached a certain concentration and was little affected by any additional increase[4]. Thus, the rate of hydrolysis could be slower than indicated in Fig. 4B. On the other hand, prolonged exposure to base could cause dissolution of the silica. Therefore, hydrolysis for 24 h seems to be a reasonable compromise between the need to complete the reaction and to preserve the support integrity.

Ion-exchange properties of poly(aspartic acid)–silica

Poly(aspartic acid)-silica prepared under optimal conditions has a hemoglobin ion-exchange capacity which is remarkably high: 430 ± 80 mg hemoglobin/g support based on 10-μm Vydac TP. The capacity was lower for coated 5- and 7-μm Vydac TP: 310 ± 60 mg hemoglobin/g. The lower capacity is probably due to the lower surface areas of these materials when compared with 10-μm Vydac (manufacturer's communication).

In the hemoglobin ion-exchange capacity assay, most chromatographic media release some bound hemoglobin during the initial washes. Also, an appreciable amount of hemoglobin is frequently retained by some process other than ion exchange, and is not released during the high-salt washes. By contrast, poly(aspartic acid)–silica retained bound hemoglobin strongly during the initial washes and released the hemoglobin quite cleanly during the high-salt washes. This strong binding and ready release of proteins may account for the sharp peaks and absence of tailing in protein chromatography on packed columns of poly(aspartic acid)-silica (see *Applications).*

Derivatives of poly(succinimide)–silica

The reactivity of poly(succinimide)silica permits a variety of functional groups to be incorporated into the coating. Table II shows the ion-exchange properties of the products of reaction with ethanolamine (prepared as with ethylenediamine hydrochloride), ethylenediamine hydrochloride, and PEI 6. The poly(PEI aspartamide)–silica is an anion exchanger for both small molecules and proteins, although the ion-exchange capacity for hemoglobin is only 58 % of the ion-exchange capacity of the anion-exchange silica with a pellicular coating of PEI[32]. This may reflect the presence of carboxyl groups in the coating. Similarly, poly(2-hydroxyethyl aspartamide)–silica, which should be neutral, has an appreciable hemoglobin cation-exchange capacity. Poly(2-aminoethyl aspartamide)-silica seems to have enough amino groups to balance the carboxyl groups and yield a neutral, zwitterion coating, as discussed earlier.

TABLE III

PRODUCTS MADE WITH POLY(SUCCINIMIDE) DERIVATIVES

	Additive					
	None	*Ethanolamine*		*Tris*		
% Additive	0	5	10	5	10	20
Hemoglobin cation-exchange capacity (mg/g support)	487	349	345	351	325	325

An alternative means of adding functional groups is to incorporate them into poly(succinimide) prior to its immobilization. This may yield products which are more reproducible than when incorporation follows immobilization. Pre-derivatization must not cross-link the polymer and must leave enough free succinimide residues to permit immobilization. This method was examined for the incorporation of ethanolamine or Tris into poly(succinimide). The resulting polymers would contain some neutral, hydrophilic residues generated at the expense of potential anionic residues. Table III shows the ion-exchange capacity of poly(aspartic acid)-silica made with these derivatized polymers. As would be expected, the additives decreased the hemoglobin ion-exchange capacity, although the effect was less pronounced above 5 % additive.

Applications

Protein mixtures are well resolved on poly(aspartic acid)-silica columns using gradients similar to those employed with CM-type materials. The columns display high capacity and selectivity. Fig. 5 shows the separation of several standard proteins with isoelectric points ranging from 4.7 to over 11. Peaks are sharp and show minimal tailing. Reasons for this favorable performance were discussed earlier. The gradient was formed with sodium chloride in order to permit the effluent to be monitored at 220 nm. If longer wavelengths can be used, then the gradient can be formed with sodium acetate in order to preclude possible corrosion of stainless steel in the HPLC system.

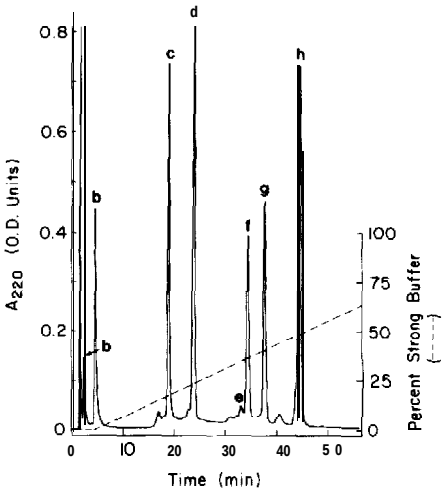


Fig. 5. Cation-exchange chromatography of protein standards. Column: poly(aspartic acid)–Vydac (10-μm), 20 x 0.46 cm. Sample: 25 μl containing 12.5 μg of ovalbumin and 25 μg each of the other proteins in the weak buffer. Flow-rate: 1 ml/min. Weak buffer: 0.05 *M* potassium phosphate, pH 6.0. Strong buffer: same + 0.6 *M* sodium chloride. Elution: 80-min linear gradient, O-100 % strong buffer. Detection: A_{220} = 1.0 a.u.f.s. Peaks: a = ovalbumin; b = bacitracin (two major peaks); c = myoglobin; d = chymotrypsinogen A; e = cytochrome *c* (reduced); f = ribonuclease A; g = cytochrome *c* (oxidized); h = lysozyme. The cytochrome *c* peaks were identified by oxidation with potassium ferricyanide and reduction with sodium dithionite.

Clinical hemoglobin samples are frequently analyzed with cation-exchange columns[33]. Poly(aspartic acid)-silica functions especially well in this application, Fig. 6 is the profile of a standard containing hemoglobins A, F, S and C. Fig. 7 is a normal hemoglobin profile, generated using a shallower gradient than for Fig. 6. Figs. 8 and 9 are the hemoglobin profiles of individuals who are heterozygous and homozygous, respectively, for sickle-cell trait. Fig. 10 is the profile of an individual heterozygous for both sickle-cell trait and β-thalassemia. Excellent selectivity is observed in the region of hemoglobins A_{1}, A_{2}, and **S,** and even better resolution is obtained if a column of 5-μm packing is used[34]. This is important for the resolution of some mutant hemoglobin forms of clinical significance. For example, hemoglobins E and A, have not been resolved on other ion-exchange materials[35,36], but are resolved on poly(aspartic acid)–silica[34].

Column operation

The poly(aspartic acid) coating is quite stable. When eluted with the buffers specified under ***Applications,*** columns last for hundreds of hours of use without decrease in efficiency or capacity. In some cases, their protein retention increased by up to 5% during the first 100 h of operation. This could reflect **ongoing** hydrolysis **of** residual succinimide residues. Alternatively, there could be a reorientation of branch ends toward the outside of the coating, increasing its surface area. This process is hastened by eluting the column with a strong buffer solution for several hours, Be-

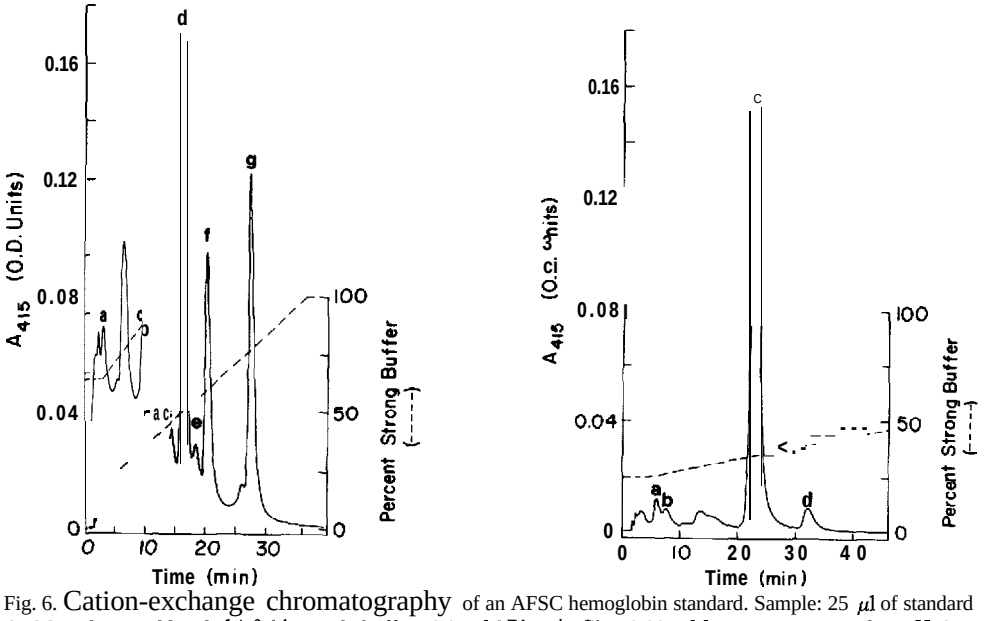


Fig. 6. Cation-exchange chromatography of an AFSC hemoglobin standard. Sample: 25 μl of standard AFSC solution diluted 14-fold. Weak buffer: 0.04 *M* Bis-tris-Cl + 0.004 *M* potassium cyanide, pH 6.5. Strong buffer: 0.04 *M* Bis-tris-Cl + 0.004 *M* potassium cyanide + 0.2 *M* sodium chloride, pH 6.8. Elution: 32-min linear gradient, 22–100% strong buffer. Detection: $A_{415} \simeq 0.2$ a.u.f.s. Other conditions as in Fig. 5. Peaks: a = Hb A_{1}, (?); b = Hb F; c = unknown; d = Hb A_{2}; e = Hb A_{2}; f = Hb S; g = Hb C.

Fig. 7. Normal hemoglobin profile. Sample: 25 μl of hemoglobin solution diluted 37-fold. Elution: 140-min linear gradient, 25-100 % strong buffer. Other conditions as in Fig. 6. Peaks: a, b = Hb F; c = Hb A_{2}; d = Hb A_{1}.

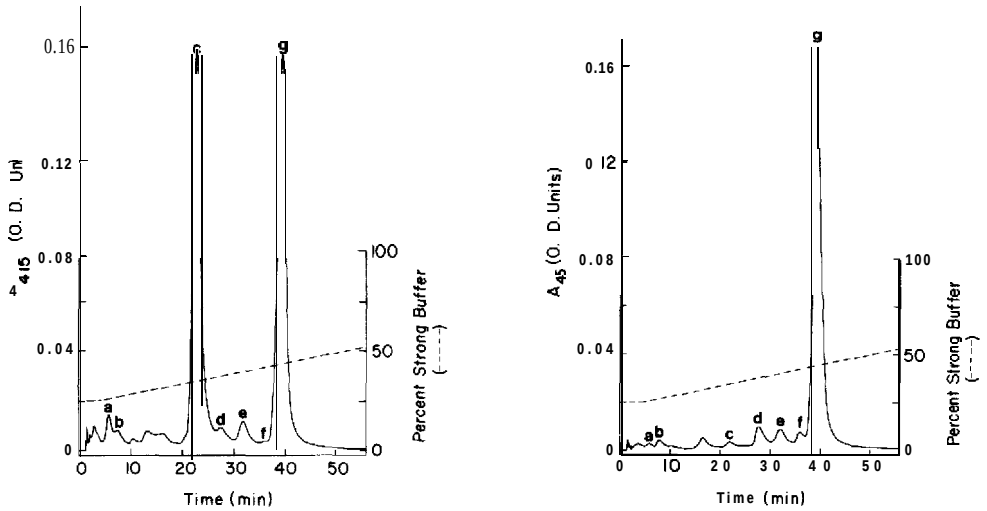


Fig. 8. Hemoglobin profile from an individual heterozygous for sickle-cell trait. Sample: 25 μl of hemoglobin solution diluted 23-fold. Conditions as in Fig. 7. Peaks: a, b = Hb F; c = Hb A_0; d. f = minor Hb S forms; e = Hb A,; g = Hb S.

Fig. 9. Hemoglobin profile from an individual heterozygous for sickle-cell trait. Sample: 25 μl of hemoglobin solution diluted 34-fold. Conditions as in Fig. 7. Peaks: a, b = Hb F; c, d, f = minor Hb S forms; e = Hb A_2; g = Hb S.

cause the process is reversed by organic solvents, these columns should be stored in water and not in methanol when not in use.

Recovery of enzyme activity

Several enzymes were applied to a poly(aspartic acid&silica column and eluted with gradients similar to those used with CM-cellulose. Adenylosuccinate synthetase,

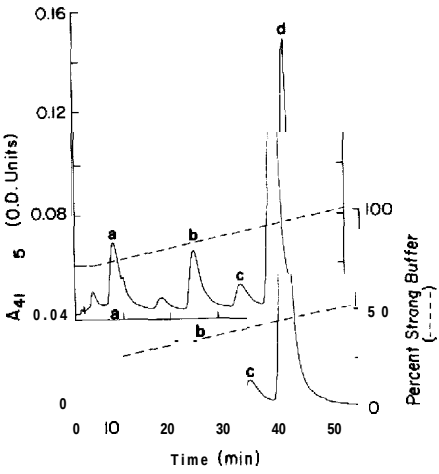


Fig. 10. Hemoglobin profile from an individual heterozygous for both sickle-cell trait and β-thalassemia. Sample: 25 μl of hemoglobin solution diluted 20-fold. Conditions as in Fig. 7. Peaks: a = Hb F; b = Hb A,,; c = Hb A,; d = Hb S.

thiolase I, and β-hydroxyacyl-CoA dehydrogenase were eluted with 100 % recovery of applied activity. The high recovery may reflect the polypeptide nature of the coating, which would make it suitable for interaction with enzymes without causing denaturation.

DISCUSSION

Poly(aspartic acid)–silica shows great potential as a cation-exchange material for HPLC of proteins. The coating is simple and is easy to prepare reproducibly from inexpensive materials. Columns packed with this exchanger feature excellent performance in terms of capacity, selectivity, recovery of enzyme activity and peak shape.

The poly(aspartic acid) molecule is probably attached to the silica surface at several points. This would form an "island"-type coating since the individual polymer molecules are not cross-linked into a continuous network (a pellicular coating). Nonetheless, the surface seems to be well covered and the coating is quite durable. Even a moderate amount of cross-linking is sufficient to stabilize a silane-based coating greatly[37]. The high ion-exchange capacity of the coating and its ready release of adsorbed proteins may be ascribed to its hydrophilic, polypeptide nature, and to the location of the ionized groups on branch ends removed from the solid surface. Such "fuzzy" coatings have a high surface area and function well in the chromatography of proteins. Their hemoglobin ion-exchange capacity is several times higher than that of the CM-polyamide material developed by Regnier and co-workers[11,12]. This could he attributed to the long threads of poly(aspartic acid) projecting from the surface well into the center of the pores, a structure which would possess a much higher surface area than the other, more surface-oriented coating.

It is also possible to generate the poly(aspartic acid) coating *in situ* by circulating a solution of poly(succinimide) through a column of aminopropyl-silica and then circulating the usual hydrolyzing solution through the column. However, columns prepared in this manner have exhibited slightly lower capacity and resolution than columns packed with material prepared batchwise.

The reactivity of poly(succinimide)--silica offers a convenient route to derivatives other than poly(aspartic acid)-silica. The neutral, zwitterion poly(2-aminoethyl aspartamide)–silica may be useful for steric exclusion chromatography, Various ligands could be added to the poly(succinimide) coating for use in affinity chromatography. The polypeptide nature of the coating would favor good performance in this application. Amine-containing compounds could be reacted with the succinimide residues either before or after immobilization. In the case of small molecules, reaction before immobilization would probably yield more reproducible products. Reaction after immobilization would afford a means of immobilizing enzymes on a polypeptide matrix. One problem with this material is the seemingly unavoidable presence of some aspartyl groups in the coating. It may be possible to neutralize the effect of these groups by adding ethylenediamine to the coating along with the affinity ligand or enzyme.

Incorporation of sulfonate or phosphonate residues into the poly(succinimide) coating would produce strong cation-exchange materials, while incorporation of other groups would give materials which may be useful for chromatofocusing. Synthesis and application of these materials will be the object of future research.

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