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SYNTHESIS OF CATION-EXCHANGE STATIONARY PHASES USING AN ADSORBED POLYMERIC COATING*

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SUMMARY

We have prepared several silica-based cation-exchange materials that were suitable for the high-performance liquid chromatography of basic proteins. Two synthetic routes were examined. Central to both procedures was the adsorption of a low molecular weight polyamine. One method crosslinks the adsorbed polyamine with a multifunctional oxirane, which is then extensively derivatized with a monomeric cyclic anhydride. The second involves an adsorbed uncrosslinked polyethyleneimine layer which is reacted with polyacrylic anhydride, thereby crosslinking and imparting anionic character simultaneously. The resulting media prepared by either of these methods bound more than 40 mg of hemoglobin per gram of support depending on the reaction conditions. These cation-exchange stationary phases also exhibited good chromatographic performance, successfully resolving (horse heart) cytochrome *c* and lysozyme. Two of the more promising support materials were effectively used to isolate cytochrome c_{553} from a crude extract of cyanobacteria.

INTRODUCTION

High-performance anion- and cation-exchange chromatography have become powerful tools for the analysis and isolation of biological molecules. Typical separations can be performed in less than 1 h with good resolution and recovery of biological activity[1,2]. Of the two ion-exchange techniques, anion-exchange chromatography appears to be more popular. Such preference probably results from the fact that 70% of the proteins reported in literature are acidic, rather than superiority of this method[3]. However, for those instances where cation-exchange chromatography is applicable, it is sometimes the more satisfactory technique.

Cation-exchange coatings for high-performance media have been synthesized by several routes. The simplest is the silylation of the silica surface with an anionic organosilane. However, such reactions leave residual silanols which can bind protein irreversibly[4]. Although Chang *et al.*[5] circumvented this problem by bonding a hydrophilic organic polymer layer over the silica surface, the procedure did not give the

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required reproducibility. More recently, Gupta *et al.*[6] and Alpert[7] described cation-exchange coatings which were fairly simple to synthesize, stable and of high binding capacity. Both procedures require three steps, the first of which utilizes an organosilane to place a reactive moiety on the silica surface. A preformed polymer is then joined to the functionalized silica providing a covalently bonded "patched" polymeric stationary phase. The last step further modifies the anchored polymer so that it is anionic. These methods for preparing stationary phases are indeed useful, but lengthy.

Using the existing technology pioneered by Alpert and Regnier[8] for the synthesis of adsorbed polymeric anion-exchange media, several cation-exchange coatings were prepared from inexpensive reagents. Owing to their self-assembling nature, adsorbed polymeric coatings are inherently simple to make. Previous work with the adsorbed polyethyleneimine (PEI) stationary phases has also shown them to be extremely versatile for the synthesis of a wide range of anion-exchange media, varying in ligand density and hydrophobicity[9]. This paper further demonstrates the versatility of this coating method by showing that the ionic character of the stationary phase can be altered from cationic to anionic without noticeably influencing its stability. Standard evaluation procedures will show these cation-exchange materials to exhibit high loading capacity and excellent chromatographic characteristics. In addition, two of the stationary phases prepared by this method proved effective in the purification of cytochrome c_{553} from a crude cyanobacteria (*Aphanizomenon flos-aquae*) extract.

MATERIALS AND METHODS

Silica

Vydac 101TPB 5.5 μm (spherical, 330 Å) silica used in this study was a gift from The Separations Group (Hesperia, CA, U.S.A.).

Reagents

The following were purchased from Aldrich (Milwaukee, WI, U.S.A.): succinic anhydride, diglycolic anhydride, glutaric anhydride, tetrahydrofuran-2,3,4,5-tetracarboxylic dianhydride, polyacrylic acid (2000 MW) and diisopropylethylamine. Polyethyleneimine-18 and diglycidylglycerol were obtained from Polysciences (Warrington, PA, U.S.A.). Buffers and solvents were of AR grade or comparable quality.

Proteins

Cytochrome *c* (horse heart, pI = 9.2) and lysozyme (egg white, pI = 11) were used as standard chromatographic probes. Hemoglobin (bovine crude, type II) was used for static binding assays. All the above were purchased from Sigma (St. Louis, MO, U.S.A.). A crude extract of *A. flos-aquae* containing cytochrome c_{553} ($CYTc_{553}$) was a gift from Dr. David Krogmann (Purdue University, West Lafayette, IN, U.S.A.).

Instrumentation

Chromatography was performed using an LDC Constametric I and IIIG system with Gradient Master (Laboratory Data Control, Riviera Beach, FL, U.S.A.). Absorbance at 254 nm (during the initial evaluations) was monitored by a Model 153 Altex UV detector (Anspec, Ann Arbor, MI, U.S.A.).

A Perkin-Elmer Model 55 spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) was used to measure picric acid and Hb concentrations.

Dual wavelength monitoring (at 260 and 410 nm) during the *A. flos-aquae* fractionation was performed with an HP 1040 A Detection system (Hewlett-Packard, Corvallis, OR, U.S.A.).

Static analysis

The picric acid ion-pairing capacity assay was performed as previously described[8] to estimate stationary phase amines.

Cationic hemoglobin binding (Hb_{cec}) was determined using a modification of a previously reported procedure[9]. The assay was performed at pH 5.5 so that the protein was positively charged.

Chromatographic evaluation

Portions of the coated silicas were packed into 5 × 0.41 cm I.D. columns for evaluation[10]. Chromatography was performed using a 20-min linear gradient from 0.01 *M* sodium acetate (pH 5.5) to 0.5 *M* sodium chloride in 0.01 *M* sodium acetate (pH 5.5) at a 1 ml/min flow-rate (unless otherwise noted). The analytical test sample (20 μl) consisted of 3 mg/ml cytochrome *c* (CYTc) and 5 mg/ml lysozyme (LYS) along with a trace of ascorbic acid to inhibit oxidation. Retention mapping was performed as previously described[11]. Resolution (R_s) between CYTc and LYS was calculated according to the equation:

$$R_s = 2(t_{R_2} - t_{R_1})/(\Delta t_{R_1} + \Delta t_{R_2})$$

The symbols t_{R_1} and t_{R_2} are the retention times of each peak, and Δt_{R_1} and Δt_{R_2} are the peak widths.

Synthesis of polyacrylic anhydride

Polyacrylic acid (5 g; 2000 MW, chain length of 28) was weighed into a 100-ml round-bottomed flask and placed in an oil bath at 180°C. The flask was then connected to a vacuum pump and evacuated for 3 h. The resulting yellow solid was chipped from the flask and stored in a desiccator. Analysis by NMR showed 79% of the carboxyls to be dehydrated corresponding to *ca.* eleven anhydride functions per polymer molecule.

Synthesis of the basic PEI coating

The synthesis was a modification of the method of Alpert[8]. Silica (1 g) was suspended in 10 ml of a methanolic 1% (w/v) PEI-18 solution. The adsorbed coating was then crosslinked using 10 ml of a 5% (v/v) methanolic diglycidolglycerol solution[9].

Synthesis of the monomeric anhydride cation-exchange stationary phase

Silica (0.7 g), previously coated and crosslinked, was placed in an oven at 110°C for 30 min. The dry silica was then suspended in a solution consisting of 4 ml of dry dimethylformamide, 250 μl (dry, redistilled) of diisopropylethylamine (DIEA) and 200 mg of the appropriate anhydride. The reaction was allowed to proceed

overnight at 60°C. The product was then isolated on a sintered glass funnel and successively washed with methanol, water, triethylamine and methanol. After drying under vacuum, this material was stored in a dessicator.

Synthesis of polyacrylic anhydride cation-exchange stationary phase

Silica (0.7 g) was suspended in 10 ml of a 1% (w/v) methanolic PEI-18 solution and allowed to stand at room temperature for 30 min. The silica was reisolated on a sintered glass funnel and placed in an oven at 110°C for 30 min. The dry media was then transferred to a 50-ml round-bottomed flask containing 4 ml of dry dimethylformamide, 250 μl of dry redistilled DIEA and 50, 100 or 200 mg of polyacrylic anhydride. The reaction was allowed to proceed overnight at 60°C and worked up as in the previous synthesis.

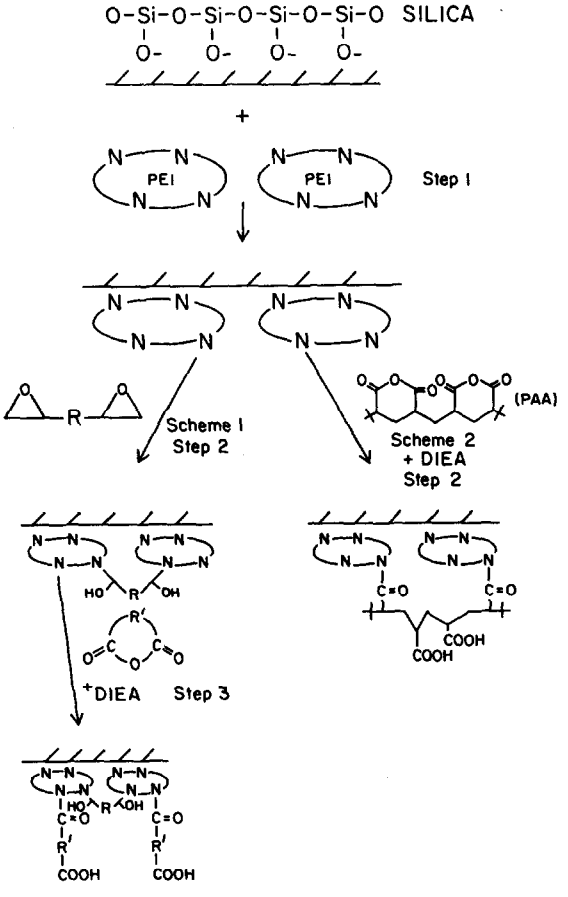

Fig. 1. Two synthetic routes for adsorbed cation-exchange coatings. Scheme 1 depicts the "monomeric" anhydride chemistry. The symbols R and R′ denote variable portions of the crosslinking reagent and anhydride, respectively. Scheme 2 outlines the polyacrylic anhydride synthetic route. Both approaches use DIEA as a proton scavenger. Structures have been abbreviated for illustrative purposes.

RESULTS AND DISCUSSION

Synthesis and evaluation

Two synthetic routes were examined. The first method was a two-step procedure in which an adsorbed crosslinked PEI layer is first placed on the silica and then reacted with a monomeric cyclic anhydride (Fig. 1, Scheme 1). The acylation reaction simultaneously derivatizes surface amines and generates a carboxylic acid. Since an acid which could titrate adjacent amines (rendering them unreactive) is produced during the reaction, DIEA was added as a proton scavenger. The reaction was monitored by picric acid assay. Picric acid ion-pairs with accessible (nonionized) amines, but not with amides. Therefore, the amount of acylation can be determined as the percent loss of ion-pairable amines after derivatization.

In general, 70% of the surface amines could be acylated (Table I). Slight deviations from this number (depending on the anhydride) resulted from either inaccuracies in measurement (± 5%) or variations in reactivity. Since picric acid did not pair with *ca.* 30% of the stationary phase amines, there are amines interspersed with carboxyl moieties in this packing. However, these chromatographic materials did not bind hemoglobin at pH 8, indicating that these amine ligands are not accessible.

The thickness of a crosslinked 1% PEI layer has been estimated as *ca.* 20 Å[10]. In addition, Vanecek and Regnier[10] and Alpert and Regnier[8] have shown that only a portion of the total amines in this coating is detectable with picric acid. The remaining ligands are buried within the layer and/or ion-paired to acidic silanols. Although not shown, residual amines may be involved in ion-pair formation with surface silanols. It should be noted that amides can also adsorb to silica. Thus, both

TABLE I

EVALUATION OF ADSORBED CATION-EXCHANGE STATIONARY PHASES SYNTHESIZED FROM MONOMERIC CYCLIC ANHYDRIDES

*Anhydride**	*% SUB***	*Hb_{cec}(mg/g)****	*t_R(min)*§		*$R_s \frac{CYTc}{LYS}$*
			CYTc	*LYS*	
Succinic	80	42	6.6	15.2	11.4
Glutaric	69	39	10.3	15.2	6.4
Diglycolic	86	51	9.0	12.4	5.2
Tetrahydrofuran-2,3,4,5-tetra-carboxylic dianhydride	74	41	11.6	21.0	4.5

* The anhydride was reacted with the stationary phase amines of a 1% PEI-18, 5% diglycidylglycerol coating (see Materials and methods).

** Percent substitution determined from picric acid ion-pairing capacity assay before and after derivatization: $\frac{[\mu\text{mole amine/g before} - \mu\text{mole amine/g after}]}{\mu\text{mole amine/g before}} \times 100 = \%\ \text{SUB}$

*** Hemoglobin cation-exchange binding capacity.

§ Retention during a 20-min linear gradient from 0.01 *M* (pH 5.5) to 0.01 *M* sodium acetate plus 0.5 *M* sodium chloride (pH 5.5) using a 1 ml/min flow-rate. Ascorbic acid was added as an antioxidant.

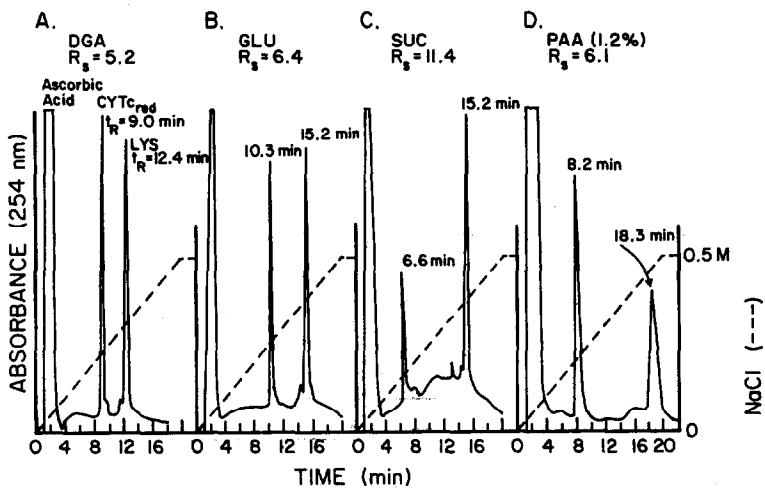

Fig. 2. Chromatographic evaluation of four selected cation-exchange columns. A 20-μl aliquot of a CYTc (3 mg/ml)–LYS (5 mg/ml) test mixture was chromatographed on the diglycolic (A), glutaric (B), succinic (C) and 1.2% polyacrylic (D) anhydride columns (5 × 0.41 cm I.D.) see Materials and methods for chromatographic conditions.

amide and amine groups are probably involved in adherence of these coatings to the silica surface.

Cation-exchange packing materials synthesized with glutaric anhydride (GLU), succinic anhydride (SUC), or tetrahydrofuran-2,3,4,5-tetracarboxylic dianhydride (TETRA) all bound *ca.* 40 mg Hb/g support (determined at pH 5.5, see Table I). This value is equivalent to the anion-exchange capacity (determined at pH 8) of the underivatized PEI coating (data not shown). The diglycolic anhydride (DGA) stationary phase bound slightly more hemoglobin, possibly owing to increased derivatization.

These materials were evaluated chromatographically by their ability to separate reduced CYTc and lysozyme (see Table I; Fig. 2, A, B and C). The TETRA column bound test proteins with the greatest tenacity, perhaps because of the presence of vicinal carboxyls. The DGA column was least retentive. We could not correlate any distinguishing physical characteristic of the remaining anhydrides to chromatographic performance. The highest R_s value was obtained on the SUC column. This resulted from unique selectivity rather than reduced peak width. Although lysozyme was strongly retained, CYTc eluted early (Fig. 2C). Performance of the succinic anhydride coating was also noteworthy for economic reasons; *i.e.*, it is the least expensive of these anhydrides.

The second synthetic method utilized polyacrylic anhydride (PAA) (MW *ca.* 1700; see Fig. 1, Scheme 2) as the crosslinking reagent. Since each PAA molecule contains eleven anhydride moieties (as determined by NMR analysis), it can effectively crosslink adsorbed amines via amide linkages. Moreover, as in the first method, a carboxyl group is generated for each reacted anhydride. Polyacrylic anhydride can also be added to a polyamine coating which has been previously crosslinked (data not shown). However, this procedure defeats the synthetic advantages of PAA in circumventing the epoxide crosslinking step.

TABLE II

EVALUATION OF ADSORBED CATION-EXCHANGE STATIONARY PHASES SYNTHESIZED FROM POLYACRYLIC ANHYDRIDE

*PAA (w/v%)**	*% SUB***	Hb_{cec} *(mg/g)*	t_R *(min)****		$R_s \frac{CYTc}{LYS}$
			CYTc	*LYS*	
4.7	59	59	10.6	NE	∞
2.4	68	54	8.8	15.4	6.6
1.2	55	36	6.6	10.6	4.9§

* Vydac silica was coated with PEI-18 and crosslinked with the indicated concentration of PAA (w/v%).

** Determined as specified in Table I. A base value of 560 μmole amine/g support was obtained from the uncrosslinked layer.

*** Obtained during a 20-min linear gradient from 0.01 *M* sodium acetate (pH 5.5) to 0.01 *M* sodium acetate plus 1 *M* sodium chloride (pH 5.5) at a 1 ml/min flow-rate. (NE = not eluted.)

§ An R_s of 6.1 was obtained when the gradient slope was halved.

Three supports were synthesized using various concentrations of PAA (Table II). Interestingly, the percent acylation of these materials was less than that obtained with the monomeric anhydrides. Access of the large PAA molecule to stationary phase amines may be hindered for steric reasons. Since the columns were stable and there was no anion-exchange hemoglobin binding, the coating was assumed to be adequately crosslinked with no accessible residual positive charge.

Hemoglobin binding increased with PAA concentration (Table II). The largest value was obtained on the medium synthesized with the highest concentration (4.7% PAA). Both this material and the packing prepared with 2.4% PAA bound more hemoglobin than the DGA stationary phase discussed earlier. Since all these coatings started with a common intermediate (*i.e.* uncrosslinked PEI adsorbed to Vydac silica), increased Hb binding capacity must be related to the ratio of amide to carboxyl groups of the covalently bonded PAA. Polyacrylic anhydride is a linear polymer. Unlike the monomeric anhydrides where the carboxyl group must be within five atoms of the surface, lengths of polyacrylic acid (stems and loops) can reach out into the silica pore volume. The existence of such structures would give the surface a "serrated" topography, effectively increasing surface area. Since binding capacity is directly related to the latter, an increase results.

The packing material crosslinked with 1.2% PAA adsorbed 36 mg Hb/g which was comparable with the monomeric anhydride stationary phases discussed earlier. At lower concentrations of polyacrylic anhydride, stem and loop structures may not predominate, since there is less competition for surface amines and the PAA molecules react extensively. Stem and loop formation is concentration dependent and has been previously investigated with regard to the adsorption of linear polymers[12].

The retention of CYTc and lysozyme also increased with PAA concentration (Table II). In fact, lysozyme could not be eluted from the 4.7% PAA column with 1 *M* sodium chloride, and 0.77 *M* sodium chloride was required for desorption from the 2.4% PAA column. These values are substantially higher than those obtained from columns synthesized by Scheme I. Since the retention times of CYTc and ly-

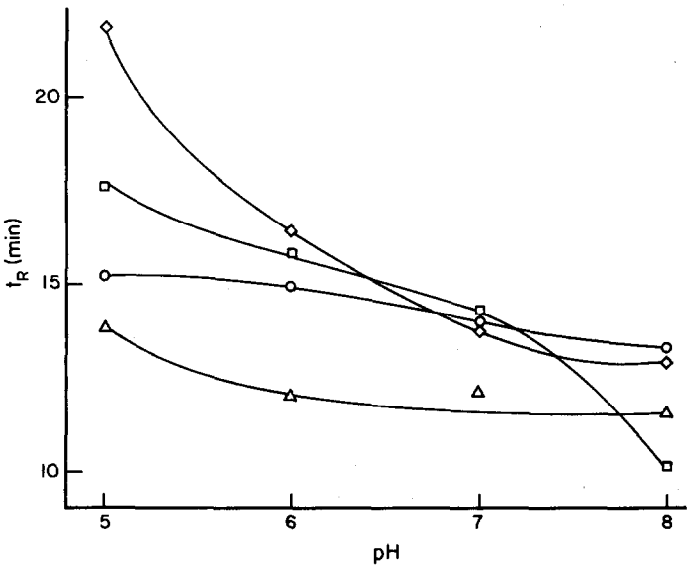

Fig. 3. Retention maps of lysozyme chromatographed on four cation-exchange columns. The retention of lysozyme was measured as a function of pH on the indicated column. The retention times were obtained during a 20-min linear gradient from 0 to 0.5 *M* sodium chloride at the specified pH (see Materials and methods). ◇ = GLU; □ = PAA (1.2%); ○ = SUC; △ = DGA.

sozyme chromatographed on the 1.2% PAA column were similar to that observed on the previously discussed media, retention is probably also related to the stem and loop surface topography. Using a 20-min linear gradient from 0 to 1 *M* sodium chloride, the resolution between CYTc and LYS varied from "infinite" (undefined) to a value of 4.9 on the 4.7% and 1.2% PAA columns, respectively. The 4.7% PAA column gave an undefined R_s, owing to the infinite retention of LYS. The 1.2% PAA column provided the lowest R_s value under the specified conditions; however, this value increased to 6.1 when the gradient slope was decreased by one-half (Fig. 2D).

Retention maps

Since the organic acids of the various anhydrides have unique pK_a(s) and the extent of stationary phase ionization can influence chromatographic performance, the effect of pH on retention was studied. A mixture of CYTc and lysozyme was chromatographed on the GLU, DGA, SUC and PAA (1.2%) columns at several eluent pH values (other conditions constant). In all cases, retention was inversely related to pH (Fig. 3). This behavior results from an increase in the protein net positive charge as eluent pH drops below the isoelectric point. For simplicity, only the retention of lysozyme has been plotted. These data are in agreement with earlier studies conducted on a Pharmacia Mono S (strong cation-exchange) column[11]. Closer examination shows the GLU column to be most pH sensitive. Since glutaric acid has the lowest $COOH:CH_2$ ratio of the anhydride reagents, strong pH dependence may result from a cooperative hydrophobic–ionic interaction. A similar phenomenon has been demonstrated for anion-exchange protein chromatography[9].

The three remaining columns were less pH sensitive. However, no correlation could be made with their respective pK_a values. This may result from the high density of carboxyl groups in the stationary phase. Alpert and Regnier[8] have shown the ionization curve to be broad for anion-exchange columns of high amine density.

Although this study failed to identify any outstanding ionization characteristic, it did define the general pH operating range of these cation-exchange packing materials. A resolution of 4 or more between CYTc and LYS was obtained from pH 5.5 to 7.5. If the limit of acceptable resolution is set at 2, the pH limits could be expanded to include pH 5 and 8. Consequently, the operational range of these media appears to span at least a two-pH-unit range from 5.5 to 7.5. These limits correlate to the loss of stationary phase and protein charge at acidic and basic pH, respectively.

Application

The succinic anhydride and polyacrylic anhydride (1.2%) columns were chosen as representative media for the fractionation of a crude protein mixture. The sample consisted of an extract from the cyanobacteria *A. flos-aquae* which contained $CYTc_{553}$. This protein is of interest for its role in the electron transport chain of photosynthesis[13]. It has an isoelectric point of 9.3, a molecular weight of 11 000 and unique spectral properties[14]. In the reduced state, the absorption spectrum exhibits maxima at 280, 410 and 553 nm. Its elution was followed by simultaneously monitoring absorbance at 410 and 260 nm with a Hewlett-Packard photodiode array detector. The 260-nm signal detects all proteins, while the 410-nm signal monitors those which contain polyporphorin rings (*e.g.* $CYTc_{553}$).

The crude cell extract was partially processed by ultrafiltration (30 000 M.W. cutoff membrane). The resulting filtrate, which contained less than 0.5 mg/ml protein, was collected and adjusted to pH 7. An 80-μl aliquot of this mixture was then loaded

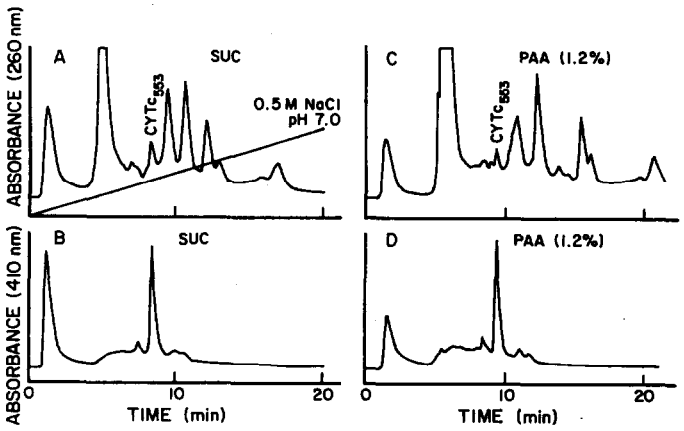

Fig. 4. Fractionation of *Aphanizomenon flos-aquae* extract containing cytochrome c_{553}. A sample was chromatographed on the SUC (A and B) and 1.2% PAA (C and D) cation-exchange columns (5 × 0.41 cm I.D.). Absorbance signals at 260 and 410 nm are indicated in quadrants A and C, and B and D, respectively. ($CYTc_{553}$ was identified by its absorbance at 410 nm). Absorbed proteins were eluted during a 20-min linear gradient from 0.01 *M* sodium acetate (pH 7.0) to 0.01 *M* sodium acetate (pH 7) plus 0.5 *M* sodium chloride at a 1 ml/min flow-rate.

on each (5 × 0.41 cm I.D.) cation-exchange column. Examination of the chromatogram obtained from the SUC column (at 260 nm) shows a substantial number of ultraviolet absorbing materials (Fig. 4A). However, the small peak eluting at 8.5 min was identified as $CYTc_{553}$ (Fig. 4B) based on its spectral properties. The 553:280 absorbance ratio at the peak apex was 0.4. Since a value of 1 is considered 90% pure, impurities are still present[15]. Nevertheless, judging from the peak area of $CYTc_{553}$ relative to the remaining peak areas, substantial purification was achieved.

Chromatography on the 1.2% PAA column under identical conditions gave similar results (Fig. 4C). In general, this support was slightly more retentive than the SUC column as shown by CYTc and LYS during the initial evaluation. The 1.2% PAA stationary phase was also slightly more selective, separating the mixture into twelve distinct peaks as compared with ten in the first instance (Fig. 4C). Again $CYTc_{553}$ was identified by its visible absorbance at a retention time of 9.4 min. Spectral analysis at the peak apex gave a 553/280 absorbance ratio of *ca.* 0.45. Although this PAA column slightly out-performed the SUC media under the stated conditions, this may not be true in general. Other samples were examined where the opposite was found.

CONCLUSIONS

Cation-exchange stationary phases can be successfully synthesized by utilizing adsorbed coating technology. Coatings made with monomeric cyclic anhydrides or polyacrylic anhydride bound *ca.* 40 mg Hb/g support and successfully separated CYTc from lysozyme. In the first method, none of the several anhydrides tested was superior to the others. The choice of anhydride is best determined by cost. Succinic anhydride, the least expensive, performed as well if not better than the other reagents.

Picric acid ion-pairing capacity analysis showed 70–80% of the surface amines to be acylated. Since these stationary phases did not bind Hb at pH 8, the residual amines must not be accessible. Moreover, these molecules appear to be buried sufficiently deep so that internal titration of carboxyls does not occur.

The stationary phases obtained using polyacrylic anhydride also proved to be simple and effective. Polyacrylic anhydride crosslinks adsorbed PEI molecules through amide linkages while simultaneously generating carboxyl groups. Loading capacity and retentiveness were directly related to PAA concentration. Hemoglobin binding capacities of supports synthesized with high concentrations of polyacrylic anhydride were greater than those determined for the monomeric anhydride stationary phases suggesting the existence of stem and loop structures. Lower concentrations of polyacrylic anhydride produced a coating which behaved similar to the "monomeric" anhydride media. In fact, of the three polyacrylic anhydride packing materials synthesized, the 1.2% PAA column was most broadly applicable. It offered good selectivity, and permitted elution of the probe proteins at salt concentrations less than 0.5 *M*. Although the strongly retentive PAA (4.7%) column may not be chromatographically practical because it is too retentive, it might be of use for protein immobilization. Cationic polypeptides, such as antibodies, could be tightly adsorbed to the matrix for immunoaffinity chromatography.

A study of retention as a function of pH conducted on the GLU, DGA, SUC and PAA (1.2%) columns showed no advantage of any one stationary phase. All

performed well between pH 5.5 and 7.5. However the GLU column was slightly more pH sensitive. Since glutaric anhydride contains an additional methylene group, a cooperative hydrophobic–ionic interaction may be responsible.

The SUC and PAA (1.2%) columns were further evaluated relative to the chromatography of an *A. flos-aquae* extract containing cytochrome c_{553}. In both cases, a substantial separation was achieved demonstrating the applicability of these media for complex biochemical mixtures. The PAA (1.2%) column retained $CYTc_{553}$ slightly longer as was seen during the initial coating evaluation. Thus, the CYTc–lysozyme test mixture appears to be an adequate probe for the general evaluation of cation-exchange chromatographic behavior.

The ability to alter the predominant charge of an adsorbed PEI stationary phase from cationic to anionic in a controlled manner testifies to the versatility of this coating chemistry. The simplicity of adsorbed coatings makes them attractive as a general method for preparing high-performance stationary phases. Future work will examine whether anionic and cationic binding sites can operate on the surface simultaneously.

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