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Fimbriated stationary phases for proteins

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ABSTRACT

This paper describes synthetic procedures for preparing fimbriated stationary phases on poly(styrene–divinylbenzene) (PS–DVB) packing materials. The synthesis consists of a five-step procedure in which the order in which the steps are carried out may be varied. These steps are (i) polymerization of monomers to form an amphiphilic copolymer or oligomer, (ii) adsorption of either monomers, or polymer onto the PS–DVB surface, (iii) solvent induction of functional group orientation at the PS–DVB–polymer interface and polymer–water interface, (iv) a cross-linking reaction that forms a hydrophilic surface layer, and (v) derivatization of the surface layer with stationary phase.

INTRODUCTION

Polar, fillamentous polymer particles such as cellulose, agarose, cross-linked dextrans, and cross-linked acrylates have long been used as supports in the chromatography of proteins [1]. The success of these filliform matrices is attributed to the fact that they are neutral, hydrophilic, easily derivatized, of high surface area, and chemically stable under use conditions. It is likely that these media were used to purify more than 90% of the proteins currently known. Although of enormous utility, these filamentous, gel-type materials also have serious limitations. One is that they imbibe large quantities of water and are easily deformed under pressure. This

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precludes their use at elevated mobile phase velocity in high-performance analytical and preparative columns. A second limitation is their resistance to mass transfer. High fillament density, molecular motion of fillament segments, and limited matrix porosity work in concert to restrict intraparticle diffusion and macromolecular mass transfer in gel-type materials.

Efforts to overcome these limitations in the mid-1970s [2–6] focused on the use of microparticulate, high-porosity silica. Through the use of small 10-μm particles to reduce diffusion path length, large 500 Å pores to increase macromolecular diffusivity in the pore matrix, and a thin layer of bonded phase on the support surface to mask silanols, columns were produced that could separate proteins more than an order of magnitude faster than soft gel columns [2]. The negative features of these high-performance silica-based sorbents were their poor stability above pH 8–9, residual silanol effects, and lower capacity.

Several of these problems were addressed with advances in organic surface coatings. Highly cross-linked epoxy surface layers were found to control residual silanol effects and increased the stability of silica-based columns [7]. Sorbents with these organic polymer coatings were used up to pH 10. The loading capacity problem remained, however.

This loading capacity problem was attacked through the use of filamentous or fimbriated polymer layers at the support surface. Incorporating stationary phases into a hair-like border of polymer filaments at the surface of a silica support increased the surface area and loading capacity 3–5 fold in most cases [8–11]. These filliform stationary phases were generally prepared by immobilizing preformed polymers onto silica supports. In other cases, polymerization was initiated at the sorbent surface to create linear "tentacles" [11].

Recent efforts to reduce mass transfer limitations in porous liquid chromatography media have been directed at the preparation of materials that allow mobile phase to flow or perfuse through the support matrix [12]. This perfusion process transports solutes to the interior of a sorbent particle much more rapidly than by diffusion, enabling separations to be achieved in a min or less. Supports used in perfusion chromatography are based on poly(styrene–divinylbenzene) (PS–DVB) with a combination of large 6000–8000 Å pore diameter, particle transecting pores and smaller 1000–1500 Å interconnecting pores. These very high porosity PS–DVB matrices are generally of lower surface area than either soft gels or high-performance silica supports.

The objective of the work reported in this paper was to develop a surface modification procedure for producing chromatography sorbents for proteins that have good chemical stability and high protein loading capacity. This paper describes the preparation of PS–DVB-based sorbents for ion-exchange and immunoaffinity chromatography of proteins in which a surface layer is established in a multi-step process consisting of polymer adsorption onto PS–DVB, cross-linking, and functionalization of the surface with stationary phase.

THEORY

Charged polymers may be concentrated from solution at a surface of opposite charge through electrostatic forces [13,14]. When all charges at the sorbent surface are ion paired with complementary groups on the polymer, adsorption ceases. The thickness of this "fuzzy" or fimbriated polymer layer is between 20 and 100 Å, depending on the solvent, polymer concentration, and polymer molecular weight [10]. Subsequent cross-linking of this adsorbed layer provides a surface coating that can only be removed by chemical degradation.

Adsorption is a general phenomenon in which any of a variety of forces could be used to create polymeric surface layers and hold them in place while they are cross-linked into a permanent film. For example, a hydrophobic polymer could be adsorbed from a poor solvent onto the hydrophobic surface of PS–DVB and cross-linked into a continuous layer. The problem is that this would only create a hydrophobic surface with properties similar to those of PS–DVB. This would not create the requisite hydrophilic surface necessary for protein separations.

It is proposed that a hydrophilic surface coating could be created by using a copolymer containing both hydrophobic and hydrophilic monomers units or segments. The copolymer can be a block polymer or an alternating polymer, or even a statistical polymer. If this copolymer were dissolved in a polar solvent and exposed to PS–DVB, hydrophobic seg-

ments of the polymer would adsorb to PS–DVB. The driving force for adsorption would be minimization of the hydrophobic contact area of both the polymer and PS–DVB sorbent with the polar solvent. In contrast, there would be no driving force for adsorption of polar segments of the copolymer. It has been demonstrated that oligomers containing oxyethylene –(CH_2CH_2O)–, glyceryl-($CH_2CHOHCH_2O$)–, or –[$CH_2CH(CH_2OH)O$], and vinylalcohol –(CH_2CHOH)– are only weakly adsorbed to hydrophobic surfaces [15]. When a hydrophilic segment containing these units is located between two adsorbed hydrophobic segments, the hydrophilic segments would loop outward away from the surface in a filament. In a given copolymer, the ratio and distribution of hydrophobic and hydrophilic units are governed by reaction conditions such as the monomer ratio and the catalyst used. The physical appearance of the polymer at the surface would be that of a loop and train arrangement [16–26] (Fig. 1). It is expected that the hydrophobic character of PS–DVB would be completely masked and that the copolymer-support composite would take on the properties of the polar oligomer filaments.

MATERIALS AND METHODS

Reagents

All proteins were obtained from Sigma (St. Louis, MO, USA). Monomers used in the synthesis of surface coatings were purchased from Poly-Science (Warrington, PA, USA). The PS–DVB support, POROS R, from PerSeptive Biosystems (Cambridge, MA, USA) was used to prepare all sorbents.

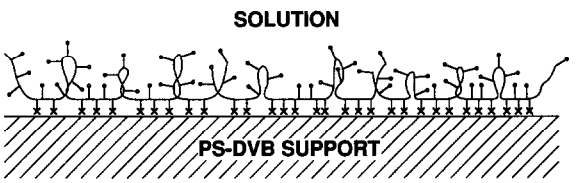

Fig. 1. The proposed physical appearance of the polymer–copolymer coating at a hydrophobic PS–DVB surface. This is referred to as a "loop and train" arrangement. × = hydrophobic functionalities; ○ = hydrophilic functionalities.

Instrumentation

Chromatographic separations were carried out on two instruments; a BioCad liquid chromatograph from PerSeptive Biosystems (Cambridge, MA, USA) and a Varian 5500 liquid chromatograph from Varian Instruments (Walnut Creek, CA, USA). Absorbance detectors set at 254 nm were used on both instruments.

Synthesis of the ether cross-linked polyglycerol coatings (IV)

Epibromohydrin (I) and glycidol (II) (2 ml in a 2:1 ratio) were dissolved in 15 ml of dichloromethane and 50 μl of boron trifluoride etherate added to initiate polymerization. The polymerization reaction was carried out at room temperature for 24 h after which the solvent was evaporated and the remaining viscous polymer (III) dissolved in a mixed solvent comprising 10 ml water, 7 ml polyethylene glycol, and 30 ml of isopropanol. The solution was filtered and 3 g of PS–DVB particles added. After sonication and gentle agitation for 14 h the suspension was filtered, washed with water and dried. The neutral, hydrophilic polyglycerol matrix (PG-OH) was prepared by treating this copolymer-coated PS–DVB matrix for 14 h at 80°C with 3 *M* KOH. This material was subsequently washed to neutral pH and dried.

The copolymer-coated PS–DVB matrix was also used to prepare a strong anion-exchange sorbent (PG-SAX) (V). These particles (1 g) were suspended in 30 ml of 10% (v/v) dimethylethanolamine in methanol and refluxed for 14 h. The particles were then washed with water, isopropanol, and acetone before drying.

Synthesis of the polyglycerol–methacrylate coating (IX)

A weak cation-exchange coating was applied to PS–DVB in this coating process. An aliquot of 6 ml of dichloromethane was transferred into a 50-ml round-bottomed flask and 1ml of glycidyl methacrylate (VI) added with gentle swirling. Boron trifluoride etherate (5 μl) was added to 2 ml of dichloromethane in a test tube and added to the glycidyl methacrylate solution by drop-wise addition. The round-bottom flask was wrapped in aluminum foil and shaken at room temperature for 24 h after which 6 ml of 50% aqueous propanol was added to

stop the reaction. Solvent was removed by vacuum evaporation and the cloudy solution of polymer (VII) suspended in 16 ml of 50% aqueous polyethylene glycol. PS–DVB particles (1 g) were added to this solution and after a 5-min sonication and degassing, the system was shaken for 24 h.

After filtering and washing three times with 50 ml of water, the polymer-coated PS-DVB (VIII) was transferred into an acrylic acid solution (20 ml, 0.1% v/v) and 0.5 ammonium persulfate added. The reaction was carried out at room temperature under nitrogen for 24 h. Tetramethylene diamine (20 μl) was then added and the reaction continued for another 30 min. The reaction was quenched by the addition of 10 mg hydroquinone and the particles washed sequentially with water (3 × 30 ml), methanol (3 × 20 ml), and acetone (50 ml) after which they were dried overnight under vacuum.

Synthesis of the sorbitol (diol) sorbent (XI)

An amount of 2 g of PS–DVB which had been coated with amphiphilic copolymer (IV) were cross-linked in 10 ml of 2 *M* KOH for 2 h at room temperature. At the end of this time 2 g of sorbitol (X) was added and the reaction continued for an additional 12 h under the same conditions. The resulting sorbitol containing sorbent was washed to neutrality with water and dried.

Cyanogen bromide activation (XII)

The sorbitol sorbent (XI) (1 g) was activated with 50 mg of cyanogen bromide at pH 10. As the reaction progressed, the reaction pH was maintained at 10 through the addition of 1 *M* KOH. After the reaction was completed the sorbent was filtered and washed with 20 ml of 0.05 *M* sodium carbonate [27,28].

Antibody immobilization on cyanogen bromide sorbent (XIII)

Cyanogen bromide-activated sorbent (XII) (1 g) was incubated with 100 mg of antibody (IgG) at pH 10 for 12 h [29]. This immunosorbent was then washed with 20 ml of Tris buffer (pH 7) and packed into a column by high-pressure slurry packing.

Preparation of the aldehyde support (XIV)

The sorbitol (XI) support (1 g) was treated with 100 mg of sodium periodate for 1 h at room temperature. The support was washed with 20 ml of 0.05 *M* sodium carbonate and used immediately to immobilize antibodies.

Antibody immobilization on the aldehyde sorbent (XVI)

The aldehyde sorbent (XIV) (1 g) was reacted at pH 10 with 100 mg of antibody (IgG) for 4 h at room temperature. At the end of this reaction the sorbent was washed with 20 column volumes of buffer (pH = 7.0) and treated with 100 mg of $NaBH_4$ in 10 ml of water for 1 h. The solution was subsequently filtered, washed with 20 ml of buffer (pH = 7.0) and packed into a column by high-pressure slurry packing.

RESULTS AND DISCUSSION

General features of the coating process

The coating process used to prepare the packing materials described below can be divided into five steps. The order in which these five steps are presented is not necessarily the order in which they must be carried out to produce a coating. They are: (i) a polymerization reaction that produces a polymer containing segments or units with hydrophobic and hydrophilic functional groups, (ii) adsorption of either polymer or monomers onto the surface of a hydrophobic material, (iii) the triggering of functional groups to orient at the PS–DVB-solvent interface on the basis of polarity, (iv) cross-linking of adjacent oligomers, polymers, or monomers to form a continuous surface layer and (v) derivatization with stationary phase. It will be shown that multiple chromatographic packing materials may be prepared using these five steps and that the sequence of these coating steps may be varied.

The letters A, B, C, D and E will be used to designate different moieties in the surface coating. The general formula for the surface coating derived from the series of steps described above is $-(A)_l-(B)_m-(C)_n-(D)_o-(E)_p-$, where (A) designates the hydrophobic moiety that orients inward toward the hydrophobic support surface and is used to adsorb the coating hydrophobically to PS–DVB, (B) designates the hydrophilic functional group (generally hydroxyl) containing moiety or moieties that orient outward away from PS–DVB toward the aqueous medium where they imbibe water and enable PS–

DVB to easily disperse in water, (C) designates the moiety or moieties in the coating that are used to cross-link adjacent chains of oligomer or polymer, (D) designates the monomeric unit that carries the chromatographic stationary phase, and (E) is any other moiety that is incorporated into the coating to convey secondary surface properties. The symbols *l*, *m*, *n*, *o* and *p* are used to designate the quantitative ratio of the various moieties in the adsorbed polymer layer.

Polyglycerol (PG) coating

The two epoxy monomers, epibromohydrin (or epichlorohydrin) (I) and glycidol (II) were polymerized (Fig. 2) with boron trifluoride in step A of the coating process to give a copolymer (III) that was relatively hydrophobic [30]. By varying the monomer ratio, the *x*/*y* ratio and concomitantly the hydrophobic character of the polymer could be altered. Hydrophobicity, as judged by hydrophobic interaction chromatography, increased with increasing content of the halide containing moiety. The copolymer (III) was dispersed in water containing polyethylene glycol and then mixed with porous PS–DVB. Step B of the coating process in Fig. 2 indicates that the copolymer (III) is adsorbed onto the hydrophobic surface of PS–DVB with specific functional group orientation. This concept is based on the fact that neither the copolymer (III) nor PS–DVB could be dispersed in water until the two were combined, after which PS–DVB particles were easily dispersed. The most likely explanation for this behavior is that the non-polar halide containing functional groups orient inward toward the PS–DVB surface and hydroxyl containing functional groups orient outward where they are solvated with water. Step C of this coating process involves cross-linking by ether bond formation in strong base according to Fig. 2. Reaction time and temperature determine the amount of residual halide. When it was the intention to prepare a hydrophilic, underivatized surface layer, reaction was continued until all halide was displaced from the matrix. This material (IV) will be referred to henceforth as PG-OH. One route for functionalization of the surface was to interrupt the reaction before complete halide displacement. Step D indicates how a strong anion-exchange sorbent (PS-SAX) was prepared by nucleophilic displacement of residual halide in the surface layer.

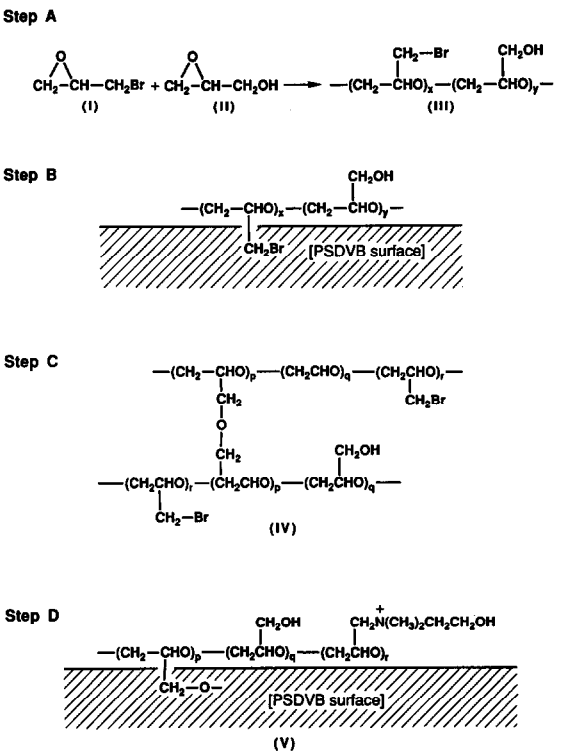

Fig. 2. Preparation of an strong anion-exchange sorbent based on the polyglycerol (PG) coating.

Because the support matrix is PS–DVB and there are no hydrolyzable functional groups in the coating, this sorbent may be operated in either strong base or acid. The PG-SAX sorbent (V in Fig. 2) was subjected to 300 wash cycles with 1 *M* KOH and 60% formic acid. No loss of the coating was observed either after this treatment nor 3000 chromatographic cycles of operation as determined by the chromatographic retention of anionic analytes.

A protein separation on the strong anion-exchange sorbent (V) is seen in Fig. 3. The wide separation of β-lactoglobulin A and B (M_r = 35 000) which vary by four amino acids indicates good selectivity and resolution with this strong anion-exchange matrix. Derivatization of the PG-OH matrix (IV) with either ethane sulfonic acid or the carboxymethyl group provide cation exchangers that were used in the resolution of a series of cationic proteins such as chymotrypsinogen, ribonuclease A, cytochrome *c*, and lysozyme (data not shown). The base stability and surface abundance of hy-

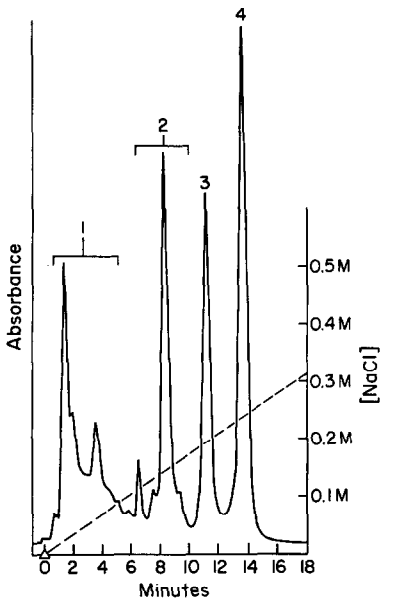

Fig. 3. Separation of proteins on the strong anion-exchange sorbent based on PG coating. Column: 5 × 0.46 cm I.D. (Poros R/H, 10 μm); mobile phase: A = 10 m*M* Tris–HCl buffer (pH 8.0), B = 0.32 *M* NaCl in A; flow-rate: 1 ml/min; gradient: 0–100% B in 20 min. Detection: UV 254 nm. Peaks: 1 = conalbumin; 2 = ovalbumin; 3 = β-lactoglobulin B; 4 = β-lactoglobulin A.

droxyl groups allow the PG-OH matrix (IV) to be derivatized in Williamson ether syntheses similar to the manner in which cellulose is functionalized with stationary phases [31].

Based on Bradford [32] assays, recovery from these ion-exchange columns was greater than 94% for the proteins examined in Fig. 3. The dynamic loading capacity of the SAX sorbent was determined to be 30 mg/ml by frontal analysis [33]. The capacity is within the range of several commercial strong cation-exchange (SCX) and SAX packing materials such as the Monobead and Neobar resins.

Polyglycerol–methacrylate (PGM) coating

Methacrylate-containing polyglycerol oligomers were used to further test the concept of using a hydrophobic support to sterically organize amphiphilic oligomers at a surface in the generation of chromatographic stationary phases. Hydrophobic adsorption of the copolymer and cross-linking in this case is due to the presence of methacryl groups instead of $-CH_2Br$ groups in the oligomer (III). The first step in the synthesis (Fig. 4) is the boron trifluoride-catalyzed polymerization of glycidyl methacrylate (VI) to form the glyceryl methacrylate polymer (VII). Mass Spectroscopy indicated that the chain length of the polymer varied from 5 to 15 residues. Following deposition of the oligomer (VII) onto PS–DVB, the coated PS–DVB support was suspended in an aqueous solution of methacrylic acid containing ammonium persulfate. The x/y ratio in VIII, *i.e.*, the ratio of methacryl groups oriented inward toward the surface as opposed to those oriented outward toward the solution, is not known. Cross-linking of methacrylate groups in the coating and polymerization of methacrylic acid in the solution was initiated simultaneously upon addition of the catalyst. During the course of polymerization, methacrylate oligomers in the solution were grafted to the surface of the sorbent through reaction with methacryl groups in the coating. The coating on this cation-exchange sorbent (IX) was stable to both organic and aqueous mobile phases from pH 2–10.

The chromatographic properties of this weak cation-exchange sorbent are seen in Fig. 5. Elution or-

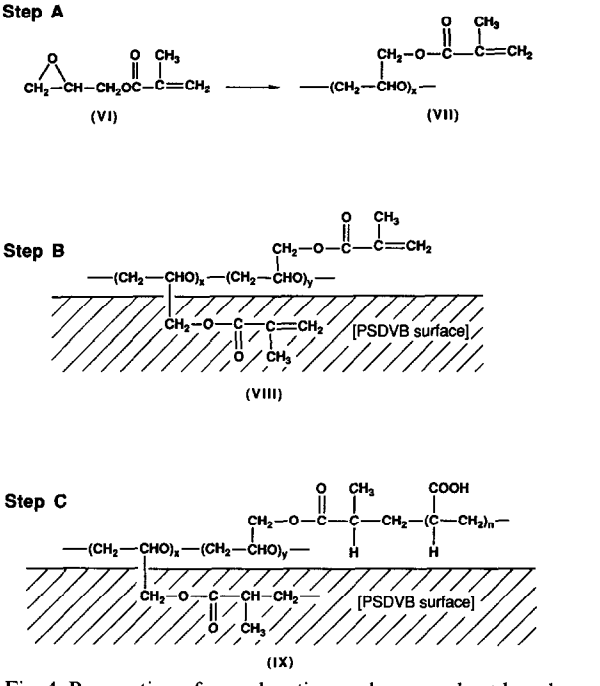

Fig. 4. Preparation of a weak cation-exchange sorbent based on the methacrylate cross-linked polyglycerol coating.

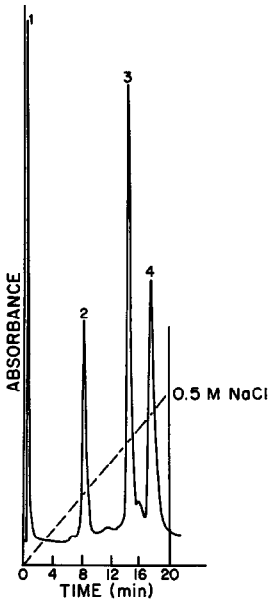

Fig. 5. Separation of protions on the weak cation-exchange column based on polymeric glycidyl methacrylate. Column: 5 × 0.41 cm I.D. (Poros, 10 μm); mobile phase: A = 50 m*M* phosphate buffer (pH = 7.0), B = 0.5 *M* NaCl in A; flow-rate: 1.0 ml/min; gradient: 0–100% B in 20 min; detection: UV 254 nm. Peaks: 1 = myoglobin; 2 = ribonuclease A; 3 = cytochrome *c*; 4 = lysozyme.

der of cationic proteins is similar to that of other high-performance ion-exchange materials. The dynamic loading capacity of this material (IX) for lysozyme was 90 mg/ml as measured by frontal chromatography [33]. By changing acrylic acid to other monomers in the coating process, a series of stationary phases can be produced. Detailed properties of these materials will be described in a future publication.

Diol phase

Carbohydrate matrices with vicinal hydroxyl groups are widely used in the preparation of affinity chromatography matrices, particularly in the case of cyanogen bromide-activated materials. A "diol" type sorbent was prepared in these studies by coupling sorbitol to a PS–DVB support that had been coated with the epibromohydrin–glycidol copolymer (III) and incompletely cross-linked. It was noted above that when cross-linking of copolymer (III) was interupted, residual halide (–CHBr) remains in the coating. Sorbitol was coupled to the PG-OH (IV) phase through an ether linkage as shown in Fig. 6. Two types of affinity matrices were prepared by the sorbitol sorbent (XI)

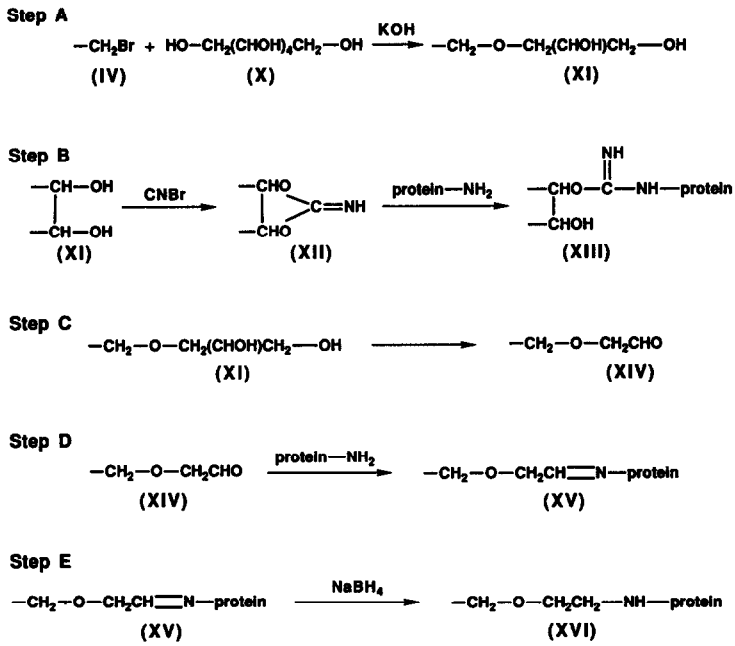

Fig. 6. Preparation of matrices for protein immobilization. Although it is indicated that sorbitol is coupled exclusively through primary hydroxyl groups, it is probable that immobilization also occurred through secondary hydroxyl groups.

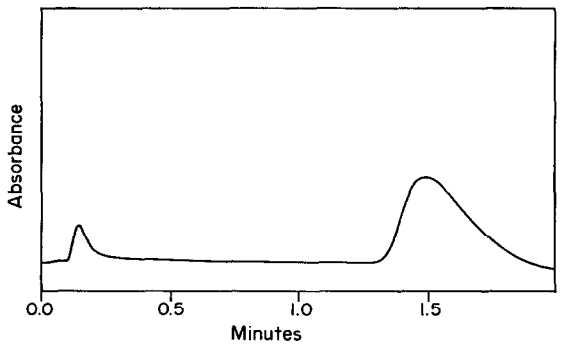

Fig. 7. Elution of Human Serum Albumin (HSA) from an anti-HSA IgG immobilized on PG-coated Poros R/H (10 μm). Column: 5 × 0.46 cm I.D.; mobile phase: binding buffer: 150 m*M* NaCl in 20 m*M* phosphate buffer (pH = 7.5), elution buffer: 0.3 *M* MgCl in 20% AcOH; flow-rate: 1.0 ml/min; detector: UV 280 nm.

(Fig. 6). Cyanogen bromide activation produced a material (XII) that immobilized proteins through an isourea linkage to amino groups on the surface of the protein [28]. Periodate oxidation produced an aldehyde material (XIV) [7] capable of immobilizing proteins by Schiff base formation. Subsequent reduction of the Schiff base with borohydride produced a stable coupling of the protein to the sorbent through a secondary amine linkage. Both of these forms of activation produced sorbents which were capable of immobilizing approximately 10 mg/ml of IgG. Application of an immunosorbent prepared from the aldehyde material (XIV) in an immunoaffinity chromatographic separations is seen in Fig. 7.

CONCLUSIONS

It may be concluded that adsorption and subsequent cross-linking of amphiphilic copolymers onto the surface of PS–DVB can produce chromatographic matrices that are both hydrophilic and stable. When these coated materials are derivatized with either ion-exchanging stationary phases or antibodies they may be used in high-performance separations of proteins.

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