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Perfusion chromatography packing materials for proteins and peptides

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ABSTRACT

    This paper describes the performance and properties of column packings for very high-speed, high-capacity and high-resolution separations of proteins and peptides by perfusion chromatography. Perfusion of mobile phase through the supports is achieved by using through-pores of 6000–8000 Å. In addition, these large through-pores are combined with smaller (800–1500 Å) pores which provide high-binding surface area. Media produced from poly(styrene–divinylbenzene) having such a pore structure plus a thin, crosslinked polymer coating allow perfusion chromatography to be performed in the reversed-phase, ion exchange, hydrophobic interaction, immobilized metal affinity and bioaffinity modes.

INTRODUCTION

    Liquid chromatography has dominated protein purification for more than three decades. It can even be claimed that new developments in liquid chromatography have been pivotal in the evolution of modern biochemistry and biotechnology. Advances in support matrices for protein separations have occurred in four distinct phases:

Soft gels

    It was recognized in the late 1950s that polysaccharides such as cellulose, agarose, and cross-linked dextrans were excellent supports for the preparation of chromatographic packings [1–3]. Because these materials were inexpensive, easily derivatizable, of high porosity, and of high surface area, they were widely adopted in laboratory scale preparative separations. The most significant problem with these soft gel supports was their poor mechanical strength. This limitation precluded the use of small particles and high flow-rates to improve resolution and decrease separation time.

Silica supports

Evolution of the soft gel chromatographic materials was followed by an era in which inorganic supports, primarily silica, came to prominence. When macroporous, microparticulate silica matrices were introduced in the early 1970s [4], it became possible to increase both the resolution and speed of chromatographic columns for the separation of proteins [5–7]. Through the use of a variety of surface coating techniques, packings were prepared that separated proteins in 10–60 min with resolution and capacity comparable or superior to that of the soft gel materials. These media have had an enormous impact on protein chemistry and biotechnology and continue to be widely used today. The problems with silica-based materials are that they often show cation exchange effects from residual silanols and are of limited stability above pH 9. The pH limitation is a particular liability when it is necessary to clean and sterilize columns at higher pH.

Rigid organic resins

During the late 1970s, a third phase of support development began with the introduction of new forms of organic resins for protein and peptide separations. Through advances in suspension polymerization technology, it became possible to prepare macroporous polymethacrylate and poly(styrene–divinylbenze) (PS–DVB) resins with orders-of-magnitude-greater mechanical strength than the soft gel resins [8–10]. In addition, these materials were stable from at least pH 2–12. As in the case of silica-based packings, these media can be prepared in particle sizes as small as a few microns and with pore diameters ranging up to 1000 Å or more. Current use of rigid, macroporous resins probably exceeds that of silica-based materials in the cases of ion-exchange chromatography (IEC), immobilized metal affinity chromatography (IMAC), and biospecific affinity chromatography (BAC).

Kinetically enhanced resins

The fourth phase of modern column development is focused on overcoming the problem of stagnant mobile mass transfer in porous chromatographic packing materials. Recent studies by Unger and co-workers have shown that small (1–3 μm) non-porous particles give very rapid separations of macromolecules [11,12]. For example, five proteins have been separated in 10 s with a non-porous reversed-phase packing [13]. Because these particles have no pores, slow diffusion within the sorbent particles is eliminated and columns may be operated at least an order of magnitude faster. There are however, limitations with non-porous particle columns. Very small particles are more difficult to pack, produce columns of much high back-pressure, and are of diminished loading capacity.

During the past year, an alternative approach has been suggested to the stagnant mobile phase mass transfer problem. It has been shown that support materials with some pores approaching a micron in diameter allow mobile phase to flow through chromatographic sorbents and transport solutes into the interior of packing materials by convection [14–16]. This "perfusion" mechanism of intraparticle solute transport is much more rapid than diffusive transport.

This paper will describe the first commercial materials (Table I) which enhance the separation of proteins and peptides by this perfusion process. These packing media are based on the POROS support matrix from PerSeptive Biosystems (Cambridge, MA, U.S.A.).

TABLE I

SURFACE CHEMISTRIES USED WITH PERFUSION CHROMATOGRAPHY

Chromatographic mode	Functional group[a]
Reversed phase	Native PS–DVB (POROS R), C_{18}
Anion exchange	Quarternized PEI (POROS Q), DEAE
Cation exchange	Sulfoethyl (POROS S), CM
Hydrophobic interaction	Phenyl butyl ether
Hydrophilic interaction	Diol
Immobilized metal	Imidodiacetate
Biospecific affinity	Protein A, immobilized antibody
Affinity activations	Aldehyde, epoxy, tresyl

[a] PEI = Polyethyleneimine; CM = carboxymethyl, DEAE = diethylaminoethyl.

CHARACTERISTICS OF PERFUSION CHROMATOGRAPHY PACKINGS

The POROS supports are synthesized by suspension polymerization processes and are available in 10 μm (H-Series) and 20 μm (M-Series) particle sizes. This matrix has several features that make packing materials based on POROS supports unique. First, the support matrix is a tightly crosslinked hydrocarbon, PS–DVB. This polymer was chosen because it is known to be of outstanding chemical and mechanical stability. Second, aqueous-compatible stationary phases are applied as a thin, crosslinked polymer film at the surface of the support. The objective of this design element is to both diminish solvent equilibration time and make it more difficult for the support to shrink or swell as columns are cycled between organic and aqueous mobile phases. And third, the support has very large transecting pores that enable mobile phase to flow (perfuse) through the sorbent particles [14–16]. The objective of designing this perfusion process into chromatographic materials is to increase the rate of intraparticle solute transport and allow columns to be operated at much higher velocity without loss of resolution or capacity.

Support stability

Chemical stability of the base POROS supports has been assessed in the reversed-phase mode with both peptides and proteins using a mobile phase gradient ranging from 0.1% trifluoroacetic acid (TFA) to 60% acetonitrile containing 0.1% TFA. No loss in protein resolution was observed after columns were operated for extended periods of time with 10–60% formic acid (6 months), 1% phosphoric acid (2 months), 1 M potassium hydroxide (1 week), and most common organic solvents (6 months). It should be noted that there may be residual double bonds in this support which can be oxidized if the matrix is exposed to strong oxidizing agents. This will change the surface characteristics of the support and may adversely affect separations. It is also not recommended that this support be used above 85°C. The sorbent is synthesized at about this temperature, and there may be some structural change if the matrix is operated above the temperature at which it was synthesized.

Mechanical strength studies indicate that 10 μm POROS R/H particles in 250 × 4.6 mm columns are stable to greater than 200 bar without bed compression. When column diameter is increased to 2 cm, bed compression begins at approximately 140
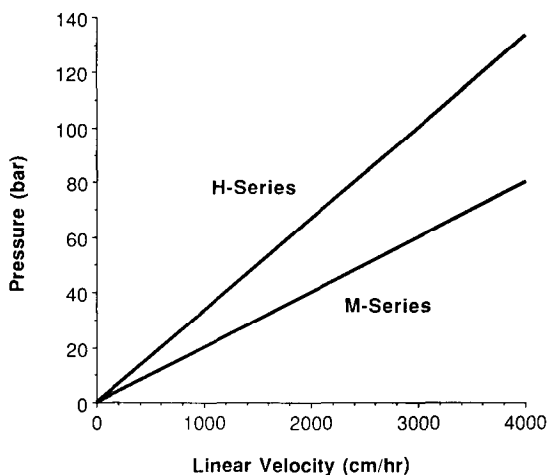
Fig. 1. Pressure-flow curves for POROS media. Data presented are for water in 50 × 4.6 mm columns for H-Series and 100 × 4.6 mm columns for M-Series.

bar. The pressure/flow behavior of the H-Series and M-Series packings is shown in Fig. 1.

Pore structure

Transmission and scanning electron microsopy show that the POROS support is a very high-porosity matrix (Fig. 2) composed of several types of pores [14–16]. One family of pores ranges from 6000–8000 Å in diameter. Electron micrographs show that these pores form a network that transects the particle, albeit not by a direct route. It is by this network of suprapores that mobile phase flows through particles and solutes are rapidly conducted by convection into the interior of sorbents. These through-pores are thought to be of relatively low surface area and therefore make a small contribution to loading capacity. [Due to the presence of micropores (< 50 Å) direct surface area distribution measurements using methods such as BET nitrogen desorption are not suitable with polymeric media. Pore size and surface area measurements are estimates based on a number of indirect methods including gel permeation chromatography (GPC).]

A second family of pores ranging from 800–1500 Å are of much larger surface area and responsible for the bulk of the loading capacity in these sorbents. The electron micrographs show that these pores generally interconnect the through-pore network and are seldom greater than 1 μm in length. Because liquid flow velocity through a porous matrix is proportional to the square of the pore radius at constant pressure, this pore system is far less important in the perfusion process. Transport in these smaller pores is more likely to occur by diffusion. It is for this reason that the length of smaller pores in the matrix should be less than (1 μm) to insure rapid transport.

Stationary phase characteristics

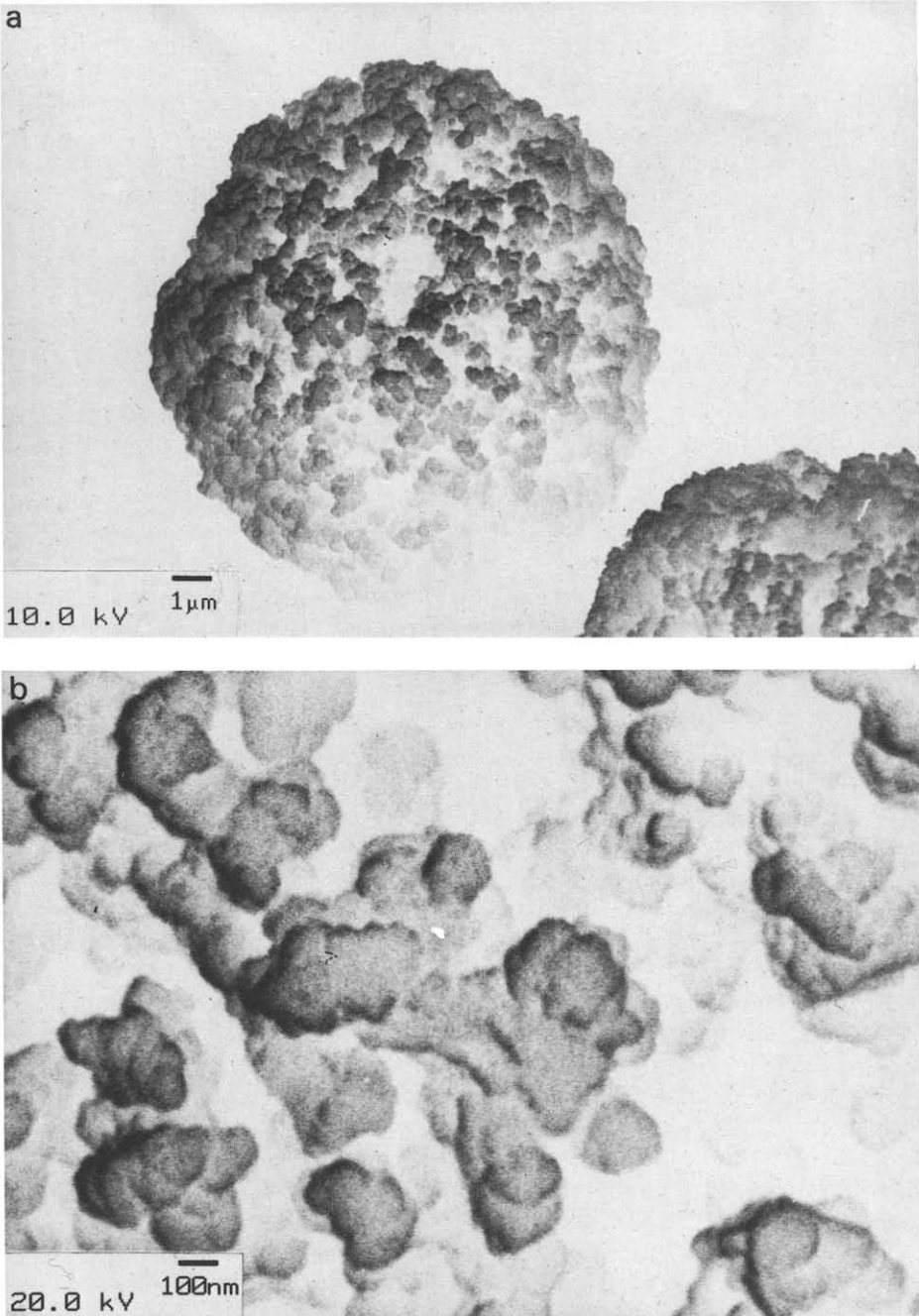The pellicular stationary phase layer in the POROS media (Table I) is com-

Fig. 2. Scanning electron micrographs of POROS R/H at two different magnifications.

posed of a cross-linked copolymer layer that is bonded to the surface of the PS-DVB support. This layer is rich in hydroxyl groups that cause the polymer to imbibe water and that render the surface readily derivatizable with conventional chemistries used on agarose and cellulose. All covalent bonds in these coatings are stable from pH 1–13. In addition, these coated packings have been cleaned repeatedly with 1 M sodium hydroxide and 60% formic acid without problems. Stationary phase ligand density is fixed by the distance between the functional groups in the coating and is readily modified to suit a particular problem [14].

The physical and chemical characteristics of the stationary phase applied to a support are major factors in determining the chromatographic properties of the sorbent. The dominant variables in stationary phase physical characteristics are coating thickness and surface rugosity. Because pores must be 300–500 Å in diameter to allow free access of large proteins [17], the coating must not occlude the support pores either at the time of application or by swelling during column operation. The polymeric stationary phases used for IEC, IMAC, hydrophobic interaction (HIC), and most of the BAC POROS packings are located in a pellicular surface layer of less than 80 Å thickness. Since the smallest pores in POROS are approximately 800 Å, there is sufficient porosity after application of the stationary phase to allow penetration of proteins with molecular weights in excess of 500 000 dalton.

Coating thickness was determined by inverse size-exclusion chromatography [18,19] in the case of the IEC and HIC materials and calculated from surface area and elemental analyses in the other cases. Through extensive cross-linking within this pellicular layer, it is possible to diminish shrinking and swelling in the stationary phase and prevent peptides and proteins from coming in contact with the hydrophobic PS–DVB support below the coating. One line of evidence for this conclusion is that when a highly cross-linked layer of stationary phase is applied to both silica and PS-DVB supports, chromatographic retention of proteins in the ion-exchange mode is independent of the support matrix [20]. A second line of evidence is that lysozyme can not be induced to adsorb to the hydroxylated POROS BAC support matrix in the presence of 2 M ammonium sulfate. It should be noted however, that these surface coatings are not totally impermeable. Small hydrophobic drugs have been observed to penetrate the surface coating in some of the BAC materials. Polar, tightly cross-linked surface layers have also been found to virtually eliminate swelling in organic solvents in the case of the IEC sorbents.

APPLICATIONS TO PROTEIN AND PEPTIDE SEPARATIONS

POROS media have been functionalized and run in the perfusion chromatography mode with most of the usual surface chemistries used for proteins and peptides (Table I). Both the nature of the support matrix–stationary phase combination and the pore structure which allows operation at very high speed have an effect on the performance in various applications. Recent findings are discussed below.

Reversed-phase chromatography (RPC)
RPC on porous PS–DVB matrices is equivalent to that on alkylsilane-derivatized silica [21] with several exceptions; (i) silanol effects may play a role in the retention of some solutes on silica based materials, (ii) π–π interactions may play a

role in the retention of some solutes in PS–DVB matrices, and (iii) differences in the nature of the hydrocarbon surface and specific surface area of any two RPC sorbents will cause phase ratio-related variations in chromatographic behaviour. A problem in RPC of polypeptides on silica-based sorbents is to overcome silanol effects, *i.e.*, electrostatic interactions between amino groups in solutes and residual silanols on the sorbent. Even in those cases where the mobile phase has been manipulated to control silanol effects, they probably still play a role in the rentention of basic polypeptides. Because PS-DVB matrices are hydrocarbons, they will be devoid of silanol effects and give different selectivity. This does not mean that either matrix is better or worse; they are just different.

Aromatic effects can be andther reason for differences in selectivity. Aromatic stationary phases can interact with hydrophobic solutes in two ways. The first, and largest, is by the normal solvophobic mechanism common to all hydrocarbon stationary phases. In this mode of retention, the surface tension of the polar mobile phase drives non-polar solute residues to minimize their contact area with the solution through hydrophobic interactions with the hydrocarbon stationary phase. With PS–DVB columns, secondary π–π interactions can then occur between aromatic residues on the sorbent surface and those in the solute. The magnitude of this effect is small unless a large number of aromatic residues is involved.

One of the advantages of doing RPC on a PS-DVB column is that it is possible to carry out separations under basic conditions. An example is shown in Fig. 3 in the separation of the angiotensins. Angiotensins II and III can not be separated at all under normal acidic conditions (0.1% TFA, Fig. 3a), but are easily separated at high pH (10 mM tribasic sodium phosphate, Fig. 3b).

The combined effects of differences in the hydrocarbon surface and specific surface area are shown in the retention maps of Figs. 4 and 5. In these maps, the log of the solute k' is plotted as a function of organic concentration [22]. Fig. 4a–c shows retention maps for angiotensin III and a two amino acid variant on POROS R, C_4 and C_{18} 300 Å silica. The slopes of the curves are all similar (-0.22 and -0.21, -0.17 and -0.15, -0.17 and -0.19 for angiotensin III and the variant on POROS
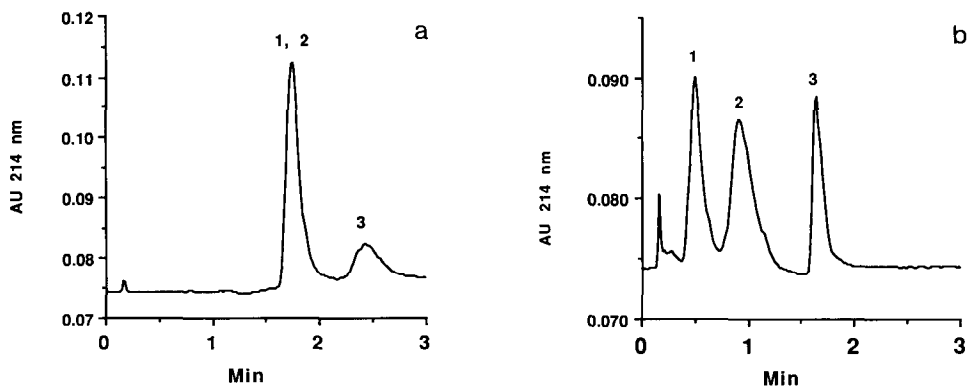

Fig. 3. Separation of angiotensins I, II and III on POROS R/H at different pH values. A 2 mg/ml concentration each of angiotension I (3), II (1) and III (2); 10-μl injection, 50 × 4.6 mm column; 5 ml/min flow-rate; detection at 220 nm. (a) 0.1% TFA–acetonitrile; 5 min gradient 12–14% acetonitrile; (b) 10 mM tribasic sodium phosphate–acetonitrile; 5 min gradient 12–14% acetonitrile.
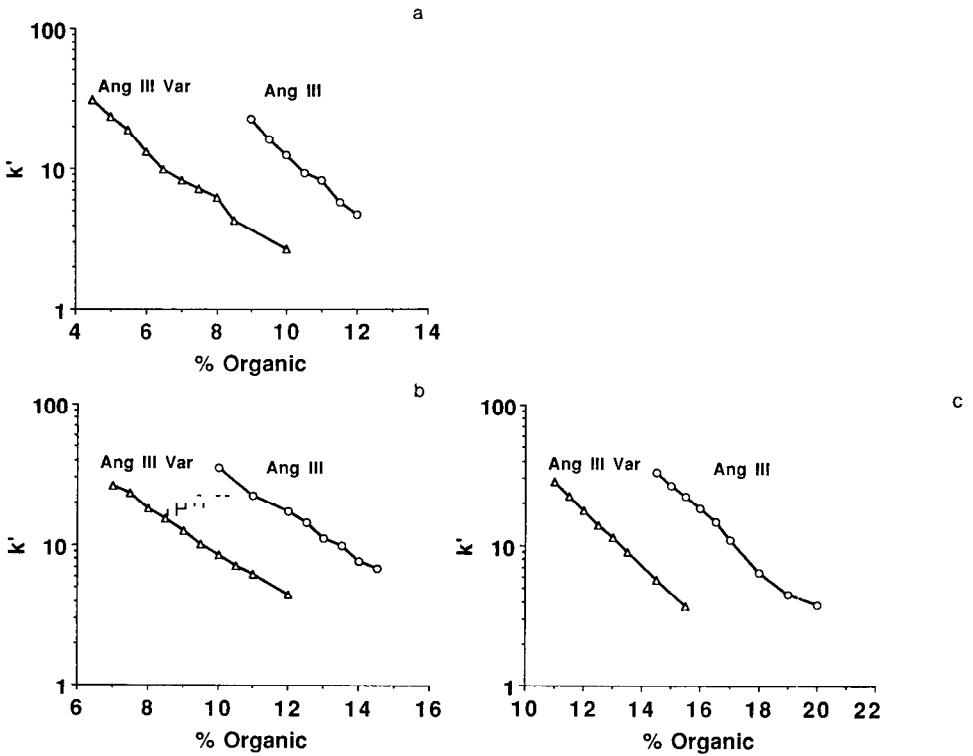
Fig. 4. Retention maps of angiotensin III variants on different reversed-phase packing media. A 10 mg/ml concentration each of angiotensin III and [Val^4, Ile^7] angiotensin III; 10-μl injection; 50 × 4.6 mm columns; 0.1% TFA–acetonitrile, acetonitrile (%) as shown run isocratically; 5 ml/min flow-rate; detection at 214 nm. (a) POROS R/H; (b) Vydac C$_4$ silica 10 μm; (c) Vydac C$_{18}$ silica 10 μm.
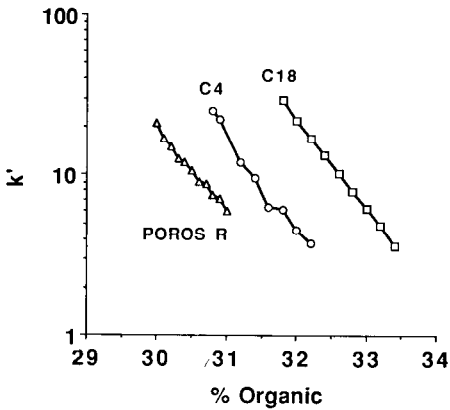

Fig. 5. Retention maps of lysozyme on different reversed-phase packing media. A 10 mg/ml concentration of lysozyme; 10-μl injection; other conditions as in Fig. 4.

R, C_4 and C_{18}, respectively), as is the difference in organic concentration for any given k' value of the two solutes. However, the organic concentration required for a given k' with either solute differs significantly among the three supports. The net result is that similar selectivity is available on all three packings, but that the gradient endpoints would have to be adjusted in moving from one to the other.

Fig. 5 shows similar maps for lysozyme, which has a 10-fold higher molecular weight. Again, the retention map slopes for the three packings are similar (-0.51, -0.59 and -0.45 for POROS R, C_4 and C_{18}, respectively). It should be noted, however, that with larger polypeptides the concentration of organic solvent required for elution tends to be higher, and the slope of the retention map is greater than for the smaller peptides such as angiotensin. The fact that large polypeptides elute from an RPC column over a narrow concentration range is related to cooperative binding at the hydrocarbon surface.

Ion-exchange chromatrography (IEC)

IEC separates polypeptides based on differences in the number and force of electrostatic interactions between the solute and IEC sorbent. Although there are a small number of charged amino acids, there can be large differences in the net charge of polypeptides and the manner in which they interact with the surface of a sorbent. This is based on factors such as (i) the fact the pK values of charged groups in amino acids are not the same, (ii) the pK of a charged residue is dependent on the surrounding environment in the polypeptide, (iii) protein confirmation can contribute to local environmental effects, and (iv) the fact that there can be large differences in charge distribution within proteins. A major problem in IEC is determining the effect of pH on the interaction of solutes with the IEC matrix.

It has been shown that this is done best with a chromatographic retention map [23]. In this particular case, the map would be a plot of the ionic strength required for elution with a particular displacing salt on cation-exchange and anion-exchange col-
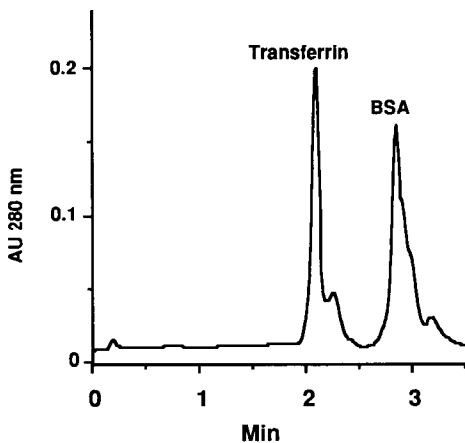

Fig. 6. Typical chromatographic run on POROS Q/H used to generate a pH retention map. A 4 mg/ml concentration of bovine serum albumin, 2 mg/ml bovine transferrin in starting buffer; 100-μl injection; 50 × 4.6 mm column; 20 mM Tris–bis-tris propane pH 6.5; gradient 0–0.5 M NaCl in 5 min; 5 ml/min flow-rate; detection at 280 nm.
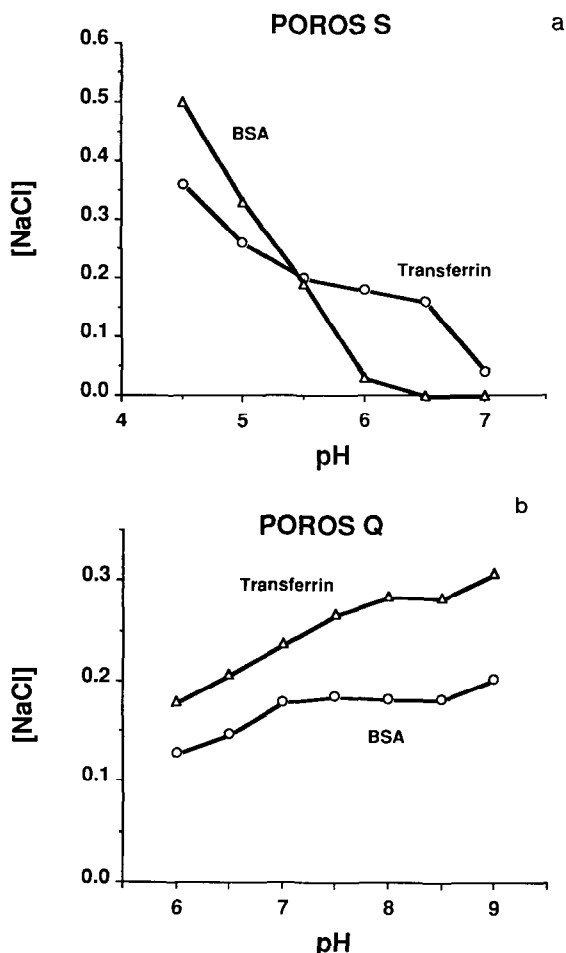
Fig. 7. pH Retention maps for (a) POROS S/H and (b) POROS Q/H for bovine serum albumin and bovine transferrin. Conditions as in Fig. 6, with buffer pH adjusted to that shown. The NaCl concentration at elution for each protein as a function of buffer pH is shown.

umns as a function of pH. Constructing such a map requires a different chromatographic run at 0.5 pH unit intervals from pH 4.5–9.5.

The advantage of high-speed columns in retention mapping can be seen in Figs. 6 and 7. It is seen in Fig. 6 that individual separations of the mixture in question can be achieved in less than 5 min. By using high-speed columns, the retention maps for both the anion- and cation-exchange columns were constructed in an hour (Fig. 7).

Although of apparent value in the analytical environment, high-speed columns may be equally useful in preparative separations. It has been demonstrated that a POROS Q strong anion-exchange column repetitively cycled every 5 min can process 5 g of serum protein/ml of column volume/day (data not shown).

Hydrophobic interaction chromatography (HIC)

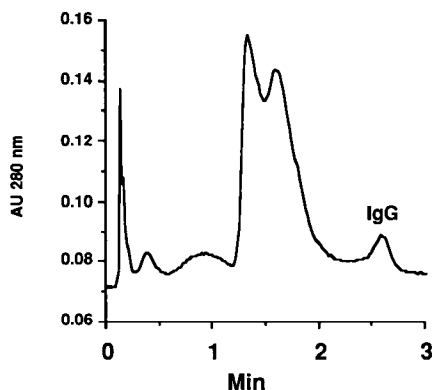The behavior of POROS HIC packings is very similar to other media used for

Fig. 8. Hydrophobic interaction separation of hybridoma cell culture supernatant on phenyl POROS M. A 20-μl injection; 100 × 4.6 mm column; 100 mM phosphate pH 7.0; gradient 2.0–0 M ammonium sulfate in 5 min; 10 ml/min flow-rate; detection at 280 nm.

this application, except of course for the run speed. Phenyl, butyl and polyether [immobilized poly(ethylene glycol)] phases have been tested. A typical high-speed separation of immunoglobulin G (IgG) from a hybridoma cell culture supernatant on a phenyl column is shown in Fig. 8.

Immobilized metal chelate (IMAC)
As with HIC, the primary difference with IMAC columns is the speed of the separation. The separation of some protein standards is shown in Fig. 9. The stationary phase in this sorbent is of the conventional imidodiacetate type in the copper form. The dynamic binding capacity in this test case was approximately 20 mg/ml.

Biospecific affinity chromatography (BAC)
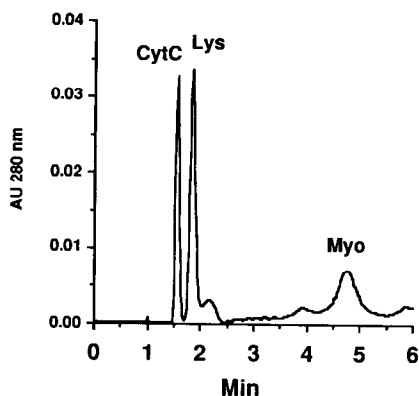The POROS family of bioaffinity supports that are produced for derivatization

Fig. 9. Immobilized metal affinity separation of standard proteins on imidodiacetate POROS M (copper form). A 5 mg/ml concentration each of cytochrome c, lysozyme and myoglobin; 20-μl injection; 100 × 4.6 mm column; 20 mM phosphate pH = 7.0, 0.5 M NaCl; gradient 0–50 mM glycine in 5 min, 50–200 mM glycine in 1 min; 5 ml/min flow-rate; detection at 280 nm.
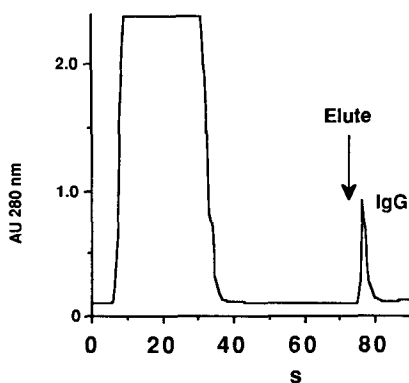
Fig. 10. Analytical separation of hybridoma cell culture supernatant on protein A POROS M. A 500-μl injection; 30 × 2.1 mm column; 10 mM phosphate pH 7.4, 0.15 M NaCl; elution with 0.3 M acetic acid (2%, v/v), 0.3 M MgCl$_2$; 2 ml/min flow-rate; detection at 280 nm.

have a diol-rich surface that may be treated as a polysaccharide both in terms of types of derivatization reactions that may be used and reaction conditions. Aldehyde, epoxy and tresyl activation reactions have all been tried successfully (data not shown). The sorbent may also be treated with periodate to generate an aldehyde-rich surface without damage to the surface coating.

Application of a POROS protein A (aldehyde-coupled) column to purification of IgG from hybridoma cell culture supernatant is seen in Fig. 10. The dynamic binding capacity of the packing is approximately 12 mg/ml human IgG. The micro-column used in the figure is particularly useful for rapid quantitation of IgG, with a linear range from 1–50 μg human IgG. Larger columns have also been used for very high through-put preparative separations.

CONCLUSIONS

Based on data presented here and in a companion paper describing perfusion chromatography [16], it may be concluded that large pores (approaching 1 μm in size) allow the flow of mobile phase through particles and that this flow enhances the mass transport performance of porous chromatographic supports. In addition, these high-speed materials may be used in both the preparative and analytical modes without compromising resolution or capacity. It has been demonstrated that all of the modes of chromatography normally used in protein purification may be carried out on surface-modified PS–DVB supports which have a pore structure suitable for perfusion chromatography.

ACKNOWLEDGEMENTS

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