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High-performance membrane chromatography of proteins, a novel method of protein separation

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ABSTRACT

Macroporous polymeric membranes, approximately 1 mm thick, were synthesized from poly(glycidyl methacrylate-co-ethylene dimethacrylate). The epoxide groups were further derivatized to add different functional groups which are commonly used as stationary phases in column chromatography. Separations of model mixtures of proteins were carried out on membranes modified with sulpho, C4 or C8 groups; the results were similar to those obtained using a column separation in the ion-exchange mode and in hydrophobic interaction chromatography. The advantage of high-performance membrane chromatography (HPMC) is that the pressure used is lower by as much as two orders of magnitude than that required to reach the same flow-rate in a packed column, although a high loading capacity can still be achieved. This makes HPMC suitable for both analytical and preparative separations.

INTRODUCTION

New methods for the separation of proteins should combine high selectivity and a high loading capacity [1]. The methods should also have the potential to be scaled up and be able to work at the low pressures required by membrane technology.

The way to obtain a membrane with the same properties as a chromatographic column is to use the Bio-Rex ion-exchange membranes (Bio-Rad) for fast separations. These consist of 90% standard chromatographic media in a bead form based on a styrene–divinylbenzene ion-exchange resin embedded in a web of poly(tetrafluoroethylene) [2]. Dominick Hunter Filters, now part of Millipore, have introduced the technique of shallow bed liquid chromatography. In this method the cartridge is filled with several sheets (up to a 10 mm layer) of modified cellulose paper supporting weak ion-exchange groups [3]. CUNO has developed a technique called Zetachromatography, which uses similar cellulose sheets consisting of a cross-linked vinyl polymer supporting ion-exchange groups [4]. Cellulose membranes with attached Protein A, Protein G or other ligands for affinity chromatography are also commercially avail-

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able (Memsep from Millipore, MAC Discs from Memtek and AbSorbent G from Genex [5]). However, membrane chromatographic techniques using hydrophobic interactions are currently not available commercially, and all the "membranes" used for the chromatographic separation of proteins are based on cellulose, which is characterized by a fibrous texture.

Calculations of the optimum particle diameter and length of a column for the separation of different molecular weight proteins suggest a bead size considerably less than 1 μm, a column $\leqslant 1$ cm in length and a pore size exceeding the Stokes diameter of the separated molecule by at least three-fold [6]. Gradient elution effects as a function of factors such as column length and gradient steepness have been investigated both theoretically [6–9] and experimentally [8–11].

The objective of this paper is to describe the use of modified macroporous polymeric flat bodies (thick membranes), prepared by a free radical bulk polymerization, for the chromatographic separation of proteins.

EXPERIMENTAL

Characterization of membranes

The membranes were synthesized by a free radical co-polymerization of glycidyl methacrylate and ethylene dimethacrylate in the presence of pores producing solvents using a procedure similar to that used for the synthesis of beads [12]. The polymerization took place in a space formed between two heated plate moulds separated by a silicone rubber gasket, the thickness of which corresponded to the required thickness of the membrane. The sheets obtained in this manner, from which membranes of the desired shape and size were cut, were washed with methanol, a methanol–water mixture (1:1, v/v) and water to remove the residue of unreacted compounds. The membranes were then stored without drying.

The permeability of the membranes to water was measured by a method described elsewhere [13] using an ultrafiltration cell (Amicon) and was calculated using Poiseuille's equation. Micro-photographs of the internal structure of the membrane were obtained with a JEOL JSM 35 scanning electron microscope. The specific surface area was calculated using the dynamic desorption of nitrogen obtained with a Quantasorb apparatus (Quantachrome).

A chemical modification of the epoxide groups of the polymer has been described elsewhere [14,15].

Sorption measurements

To determine the static sorption capacity, a 2 cm diameter, 1 mm thick membrane, with an area of 3.14 cm^2, was made from a macroporous copolymer with a specific density of 1.3 ml/g. The membrane was immersed in a 1 mg/ml protein solution. The concentration changes were monitored spectrophotometrically until equilibrium was reached. The amount adsorbed per membrane was recalculated to give the sorption per square metre of a membrane by multiplying the weight of sorbed protein by 3185. This value was obtained by dividing 1 m^2 (10 000 cm^2) by the area of the 2 cm diameter membrane (3.14 cm^2). The surface area covered by the protein was calculated as a percentage of the specific surface area of the porous polymer, divided by the sum of the known cross-section areas of the sorbed protein molecules.

The dynamic sorption data were determined on the same set of membranes with the membrane located in a chromatographic cell. A 10-ml volume of buffered protein solution (1 mg/ml) was passed through the membrane at a flow-rate of 1 ml/min, adjusted by a pressure of approximately 0.2 MPa. The effluent was collected and the overall protein content was determined photometrically. The difference between the starting and final protein concentrations gave the amount of protein sorbed on the membrane.

Chromatography[a]

Membrane chromatography of proteins was performed in a special magnetically stirred cell, 5.5 ml in volume, with a membrane at the bottom. The cell has an inlet capillary for the supply of the mobile phase and an outlet, in which the solution which has passed through the membrane is collected and then transmitted through a capillary to a UV detector (LKB, Sweden). The mobile phase is pumped into the cell using an 300 pump (Laboratory Instruments Works, Czechoslovakia). In a typical experiment the mixture of proteins dissolved in the initial buffer solution is injected into the cell and a buffer solution the same as mobile phase A is pumped into the cell. As they pass through the membrane, the proteins are sorbed onto the solid phase; the non-adsorbed protein is washed through. This process is monitored by a UV detector. After the signal has returned to baseline, the mobile phase is changed to solution B. With respect to the final volume of the cell, phase A is gradually diluted with phase B, and the change in the composition of the mobile phase entering the membrane has an exponential gradient. During the formation of the concentration gradient, the individual components of the original mixture are desorbed, depending on the interaction with the groups localized on the inner surface of the membrane, and separation is achieved.

RESULTS AND DISCUSSION

Current theories for the separation of large molecules by high-performance liquid chromatography (HPLC) favour small particles and short columns. The reduction in volume, which lowers the loading of very short columns, can be compensated by using a large diameter column, *i.e.* a flat or shallow bed column. In such a column the thin layer of polymer particles can be replaced by continuous material with properties close to those of a column packing. This continuous bed will not contain the interstitial spaces characteristic of a filled column containing spherical beads. These interstitial spaces amount at least 26% of the column volume; this volume will not contribute to the separation and causes peak broadening.

An early approach to this problem was the use of modified paper sheets to replace the modified cellulose sorbents (porous beads or microcrystalline fibres). This was widely used in biochemistry but was not established as a standard HPLC method.

Most packings for column HPLC consist of macroporous beads of both inorganic and organic origin. Each individual particle consists of mutually connected spherical entities called globules [16], the size of which is usually in the range 100–300 nm. Voids between the globules are permanent pores representing the macroporosity.

[a] The HMPC cartridges Quick Disc are now available from Säulentechnik Knauer, Berlin, Germany.

TABLE I

BASIC CHARACTERISTICS OF CHROMATOGRAPHIC MEMBRANES

Abbreviations: GMA = glycidyl methacrylate; EMDA = ethylene dimethacrylate; S_g = specific surface area; L = specific permeability; r_p = mean pore radius.

Membrane	Polymerization feed (wt. %)		S_g (m^2/g)	L (l/h m^2 MPa)	r_p (nm)
	GMA	EDMA			
G-5	5	95	250	–	5[a]
G-25	25	75	139	390	10[b]
G-40	40	60	80	1120	17[b]
G-50	50	50	60	1940	23[b]
G-60	60	40	43	–	25[a]

[a] Mean pore radius measured on beads of identical composition.
[b] Mean pore radius calculated from L.

The particles are synthesized by a suspension process which automatically yields beads from droplets of the dispersed liquid phase. The suspension polymerization is, in fact, a bulk polymerization, in which each dispersed polymerizing droplet is regarded as an individual block. This is why it is easy to move from this suspension polymerization to a polymerization of the same monomer mixture in a system not containing any continuous dispersion phase (water phase). The patented process of Švec and co-workers [17,18] yields a single piece of sorbent, which resembles a flat board polymerized in a bulk layer. The properties of this board are the same as those of a standard beaded sorbent. Macroporous membranes which match the conditions for chromatographic use are cut from this board.

Membrane properties

Characteristic data for a series of membranes prepared using different monomer mixtures are summarized in Table I. The table shows that the properties can be easily varied by changing the ratio of the monomers, *i.e.* the monovinylic glycidyl methacrylate and the divinylic cross-linking agent ethylene dimethacrylate. Earlier studies of the suspension polymerization of the same set of monomers showed that the structure and properties of a macroporous matrix are also affected by the amount and composition of the inert pores producing solvent in the organic phase, the initiator concentration, the reaction temperature and the polymerization time [12,19].

With respect to their permeability to the liquid, the membranes behave in a similar manner to the common ultrafiltration membranes. Their basic feature is a linear permeability *versus* pressure relationship (Fig. 1). The slope of this dependence is inversely proportional to the fraction of the cross-linking agent in the polymerization mixture. A high ethylene dimethacrylate concentration causes an increase in the specific surface area and moves the pore size distribution towards smaller values. To use a 2 cm diameter membrane containing 75% ethylene dimethacrylate at a flow-rate 1 ml/min, a pressure of over 0.75 MPa is required whereas only 0.1 MPa is needed for a membrane with 50% cross-linking. Any direct comparison with the permeability of a chromatographic column is incorrect. The permeability of a mem-

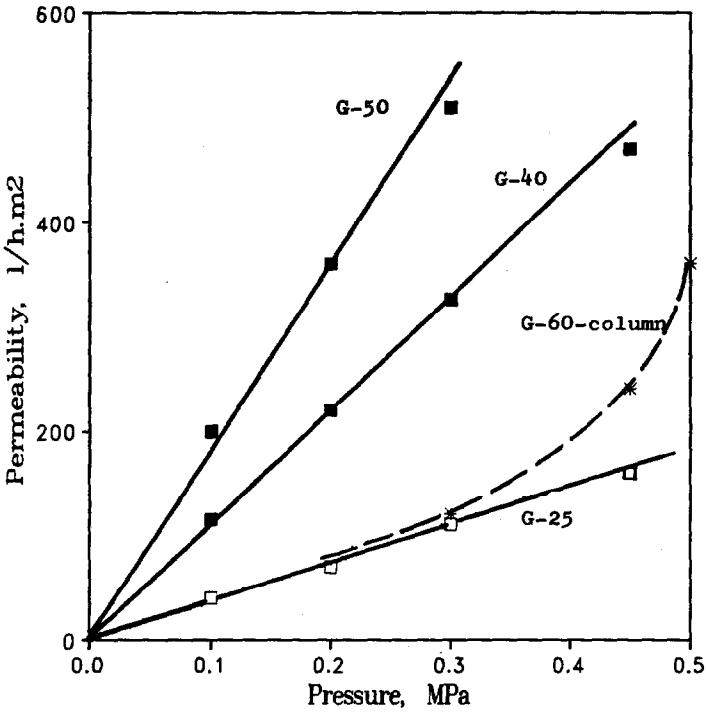


Fig. 1. Pressure dependence of flow through a 1 mm thick membrane made from poly(glycidyl methacrylate–ethylene dimethacrylate) copolymers with various glycidyl methacrylate contents. The results are compared with a 300 × 8 mm column packed with 5–7-μm beads of the copolymer containing 60% glycidyl methacrylate.

brane represents the flow through the pores, whereas the flow through a column proceeds via the interconnected interstitial voids. The pressure drop in a column depends on the particle size and particle size distribution of the sorbent, the packing efficiency, the column diameter and column length but, except in perfusion chromatography [19,20], does not depend on the porous properties of the packing material. It is possible to project the properties of a column down to the size of a membrane, but the physical properties of both are so different that any comparison is not relevant. A simplified direct comparison shows that a 1 mm thick membrane is more "permeable" than a 300 mm long column packed with 5–7 μm particles of identical composition, even when the diameter is recalculated to the same size (Fig. 1).

The mechanical stability of the membrane is equal to that of macroporous beads [21] which resist a column pressure of up to 30 MPa without damage. A detailed study of the mechanical properties of membranes has been published elsewhere [22].

A comparison between permeability and the mean pore size obtained by chromatographic measurements indicates that the pore size distribution is broad [23]. The membranes contain both relatively large transport pore (canals) and small separation pores. The former ensure a high flow-rate at low pressures, whereas the latter mainly contribute to the high specific surface area. From this point of view membrane chro-

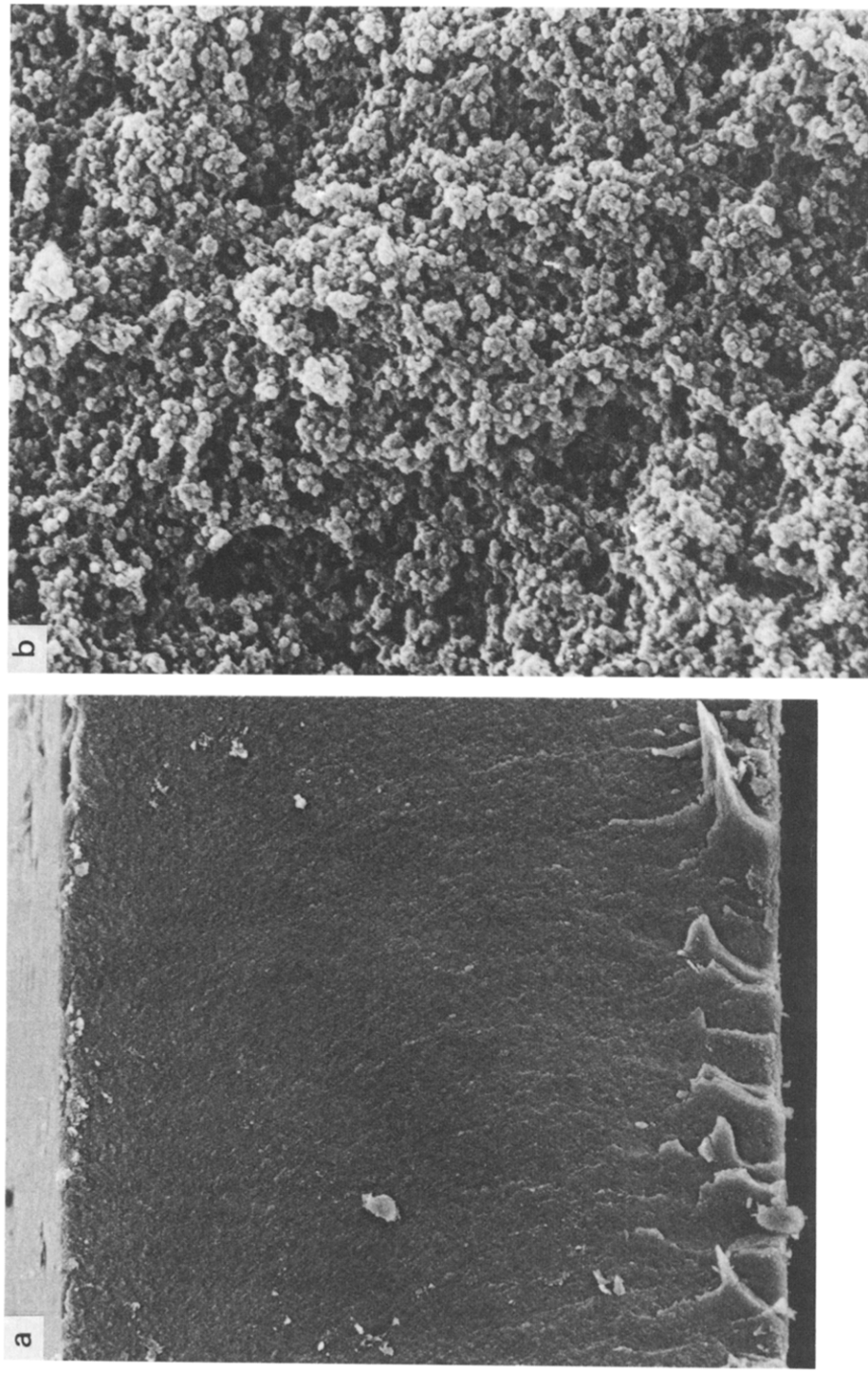
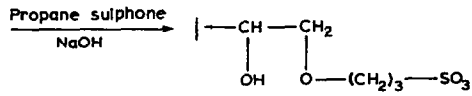


Fig. 2. Scanning electron microscopy pictures of a standard poly[glycidyl methacrylate-co-ethylene dimethacrylate] membrane. Magnification (a) 800 and (b) 5400.

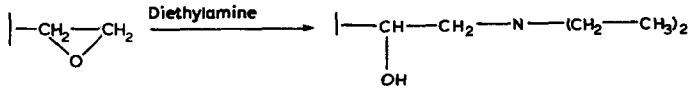
matography resembles perfusion chromatography, in which the column packing also contains two kinds of pores: through pores and diffusion pores [19,20].

Fig. 2 shows a scanning electron microscopy (SEM) picture of the membrane cross-section at two magnifications. The globular structure characteristic of macroporous polymers is distinct, especially at the higher magnification, and large voids (transport canals) are seen between agglomerates of globules.

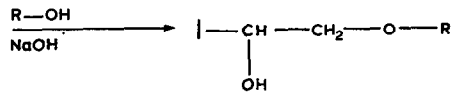
An exceptional advantage of glycidyl methacrylate copolymers is the possibility of preparing, from a single initial membrane, a whole set of chromatographic membranes containing groups commonly used in column ion-exchange chromatography or hydrophobic interaction chromatography, or, *i.e.* sulpho, diethylamino, butyl, octyl and phenyl groups, and many others:



(1)



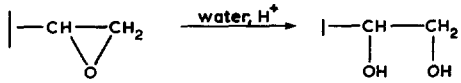
(2)



(3)

where R is an alkyl or aryl group.

The oxirane group (if any) remaining after the modification can be readily removed within 3 h by acid catalysed hydrolysis in a 0.1 mol/l sulphuric acid solution at 60–80°C. As shown schematically in eqn. 4, hydrolysis increases the hydrophility of the macroporous membrane surface and reduces the probability of non-specific interactions. At the same time, the absence of reactive oxirane groups also rules out chemical reactions in which the separated proteins could be covalently immobilized on the sorbent, particularly in alkaline medium:



(4)

Experiments with albumin, chymotrypsinogen, lysozyme and ribonuclease A adsorbed from a solution containing 0.5 mg/ml protein proved that with the hydrolysed membranes in which the fraction of the cross-linking agent (ethylene dimethacrylate) is less than 60%, almost no interactions take place between proteins and the polymer. The protein recovery exceeds 96%. Table II summarizes the data on the sorption capacity of a membrane modified with butyl groups. The adsorption was determined by frontal analysis with a high excess of protein by following the decrease in concentration photometrically. Loading of the membrane reaches 20–40 g of protein per square metre of the 1 mm thick membrane. As the volume of 1 m^2 of a 1 mm thick macroporous membrane is 100 ml, the loading is up to 40 mg/ml. This corresponds to the loading capacities of leading commercially available chromatographic packing materials [24]. However, the dynamic capacity calculated from flow-through experiments is considerably lower.

TABLE II

STATIC AND DYNAMIC CAPACITIES OF C_4 MODIFIED MEMBRANES FOR SORPTION OF PROTEINS

Protein	Amount sorbed (mg/g)		Covering of surface (%)		Specific capacity (g/m^2)	
	Static	Dynamic	Static	Dynamic	Static	Dynamic
Ribonuclease	17.6	4.4	17	4.3	20	5
Lysozyme	22.1	–	20	–	26	–
Ovalbumin	41.2	4.4	18	1.9	47	5

Chromatography

The mixture of proteins is separated using liquid chromatography equipment, but instead of the column a specially designed cell, as described under Experimental, is used.

A simple calculation assuming that the polymer has a porosity of approximately 50% [12] (the volume fraction of pores) reveals that solvent regain in a 2 cm diameter membrane 1 mm thick is only 0.15 ml. The retention time of this volume in the membrane is 9 s when the flow-rate is 1 ml/min. Hence the composition of the eluent inside the membrane is almost homogeneous and the concentration gradient between the top and bottom of the membrane is negligible. This means that the dissolution of the protein is simultaneous throughout the whole membrane, and occurs just as the capacity factor k' reaches a sufficiently low values. This results in a narrow peak. An increase in the flow-rate accelerates the changes in the composition

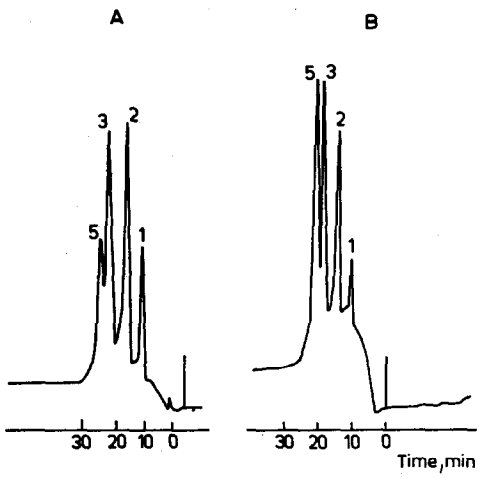


Fig. 3. Separation of a mixture of (1) myoglobin, (2) ovalbumin, (3) lysozyme and (4) chymotrypsinogen using a G-50-C8 membrane. Membrane 1 mm thick, 20 mm diameter, total loading (A) 5 mg or (B) 1.2 mg of proteins, elution rate 1 ml/min, mobile phase gradient from 2 mmol/l ammonium sulphate in 0.02 mol/l phosphate buffer solution (pH 6.8) to buffer solution only in 20 min.

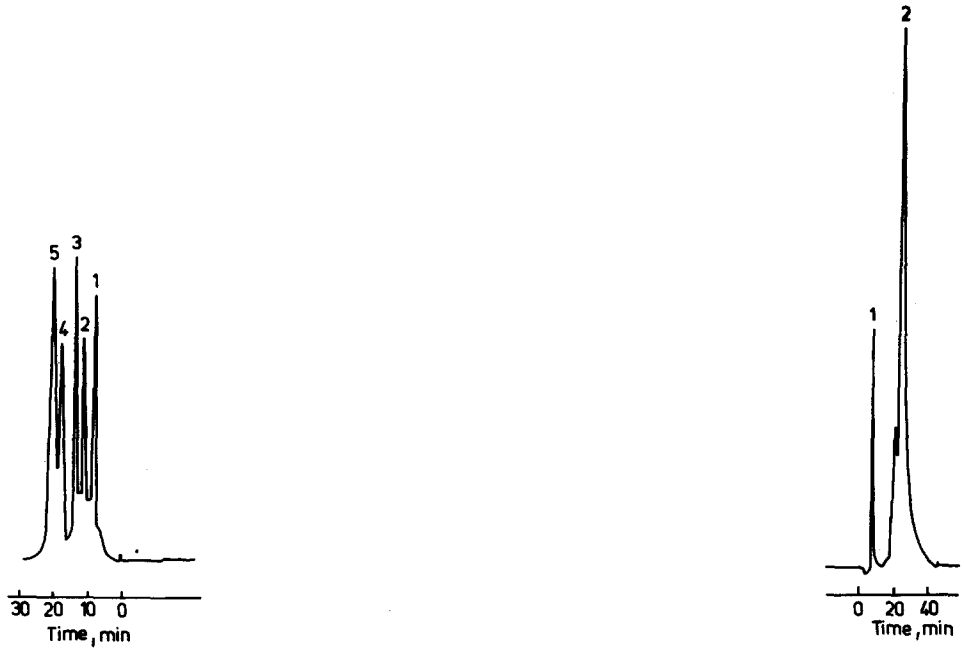


Fig. 4. Separation of a mixture of (1) myoglobin, (2) ovalbumin, (3) ribonuclease A, (4) lysozyme and (5) chymotrypsinogen using a G-50-C4 membrane. Gradient time 10 min, total loading 5 mg of proteins. Other conditions as in Fig. 3.

Fig. 5. Separation of (1) ribonuclease A and (2) lysozyme using a G-50-SP membrane. Membrane 1 mm thick, 20 mm diameter, elution rate 1 ml/min, mobile phase gradient from 0.02 phosphate buffer (pH 6.8) to 0.5 mol/l solution of sodium chloride in the buffer, loading 0.1 mg of each protein.

of the eluent, but the retention time in the membrane is shorter and the difference in concentration between the inlet and outlet does not change very much. The shape of the peaks remains almost unchanged.

The mechanism of separation does not change considerably when the thickness of the membrane is doubled or even tripled. The volume retained is still very low and represents 1–1.5% of the total eluent volume. Unless the thickness of the layer matches the length of a column, no typical chromatographic separation mechanism (repeated sorption–desorption steps) can take place and the separation proceeds by simple gradient elution.

Fig. 3 shows the separation of a model mixture of proteins on a G-50-C8 membrane with octyl groups. This can be compared with the separation of a similar mixture in a column 20 cm × 4.6 mm I.D. packed with specially treated C_{18}-silica [25]. Separations with membrane loadings of 1.5 and 5 mg are satisfactory and are fully comparable with column separation at a pressure 8 MPa. Recalculated dynamic loading gives 20 g/m^2 of proteins (20 mg/ml). The similar membrane G-50-C4 gives a fast separation of a mixture of five proteins (Fig. 4).

A chromatographic separation of proteins using a membrane modified with ionogenic sulphopropyl groups (Fig. 5) shows the feasibility of the ion-exchange mode of membrane chromatography.

CONCLUSIONS

The chromatographic data reported here demonstrate that a membrane may be used instead of a column for the separation of proteins. The separations are comparable with HPLC using standard chromatographic columns, but the membrane chromatography possesses the following advantages over traditional column methods:

(1) The membrane is synthesized directly at the final size from any combination of monomers in a mould; classical chromatographic supports prepared by suspension processes have to be size-classified.

(2) In the fabrication of beads, many are produced which are not of the desired size, which causes considerable waste. The membranes are prepared efficiently in almost the correct shape and are used after only minor mechanical treatment.

(3) The cell is simple and the membrane is easily positioned, whereas the packing of an efficient column requires substantial experience of packing procedures.

(4) The price of a quality column is relatively high, making long-term use necessary, whereas a membrane cartridge is cheaper may be economically disposed of much earlier.

(5) Flow-rates in membrane chromatography are obtained using pressures which are at least one order of magnitude lower than those used in analytical columns, thus making the use of highly efficient pump unnecessary.

(6) Scale-up in membrane chromatography is achieved by enlargement of the membrane area. This can be achieved either by enlarging the size of cartridge or by using several small cartridges connected in parallel. In preparative HPLC scale-up gives rise to specific problems of column packing and radial gradients and requires the use of heavy duty equipment.

These advantages show that membrane chromatography, although not necessarily exceeding the efficiency of HPLC, is still a competitive separation method. It has a wide range of uses in less challenging analytical separations and in preparative applications. Owing to the number of advantages and the simplicity of synthesis and use, this separation method may be appropriately called high-performance membrane chromatography.

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