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# Mixed-matrix membrane adsorbers for protein separation

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**Abstract**

The separation of two similarly sized proteins, bovine serum albumin (BSA) and bovine hemoglobin (Hb) was carried out using a new type of ion-exchange mixed-matrix adsorber membranes. The adsorber membranes were prepared by incorporation of various types of Lewatit ion-exchange resins into an ethylene–vinyl alcohol copolymer porous structure. The obtained heterogeneous matrices, composed of solid particles surrounded by the polymeric film, display high static and dynamic protein adsorption capacities. The effect of operational parameters such as filtration flow-rate, pH, and ionic strength on the protein separation performances was investigated for cation- as well as anion-exchange adsorber membranes. An average separation factor was calculated by numerical integration of the protein concentration in the permeate curve during the filtration run. High average separation factor values were obtained for BSA–Hb separation at physiological ionic strength with a filtration flow-rate up to 20 l/h per $m^2$, until the protein breakthrough point at 10% of the feed concentration.


*Keywords:* Membranes; Adsorbents; Ion exchangers; Albumin; Hemoglobins; Proteins

## 1. Introduction

The employment of solids for the removal of specific components from liquid mixtures has been widely used in the past. Nowadays, packed bed chromatography has become a useful tool for purification and separation processes with a large number of applications in the biological and biomedical fields, in biotechnology and the food industry [1,2].

In recent years, there has been considerable interest in developing membrane chromatography systems that function as a short, wide chromatographic column, in which the adsorptive packing consists of one or more microporous membranes [3–6]. The main advantages of microporous disks in comparison with packed beds are the low flow resistance due to higher porosities, decreased change in plugging by reduced bed heights, prevention of channelling, no bed compression and a rapid mass transfer especially when small particles $<10$ μm are used [4,7].

In a previous paper [8], we presented a new concept for the preparation of mixed-matrix adsorber membranes having particulate ion-exchange material entrapped in a porous matrix. A slurry of dissolved polymer and the particulate material is cast as a thin film or spun into a fiber and then solidified by a phase inversion process. The prepared mixed-matrix adsorber membranes contain Lewatit type ion-exchange particles tightly held together within an ethylene–vinyl alcohol copolymer (EVAL) polymeric matrix (25–75%, w/w, sorbent), whereby the latter does not influence the activity of the particles. The

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proposed adsorber membranes can be prepared in different shapes and can be operated either in a stack of microporous flat membranes or as a bundle of fiber membranes. The developed concept is extremely flexible and offers the possibility to easily vary the geometry, the adsorption capacity, as well as the selectivity of the adsorber membrane. The porous mixed-matrix adsorber membranes offer a wide variety of applications, depending on the particle selection. Applications include peptide and protein isolation from fermentation broths, protein fractionation, ligand immobilisation for affinity-based separations, chromatography, immobilised catalysts and enzymes for reactions, blood detoxification, product protection and drug release systems.

It was previously concluded that protein separation by membrane filtration processes could only be carried out with proteins differing in molecular mass by at least a factor of 10 [9]. Effective protein fractionation using membrane filtration for mixtures of albumin–myoglobin and albumin–immunoglobulins was reported in the literature [10,11]. Recently it has been demonstrated that using electrostatic interactions, the separation factor even for two similarly sized proteins like bovine serum albumin (BSA) and hemoglobin (Hb), can be achieved. Musale and Kulkarni [12] developed a method based on hydrophobic and electrostatic interactions for BSA–Hb separation using poly(acrylonitrile) and poly(acrylonitrile-co-acrylamide) ultrafiltration membranes. A maximum separation factor of 3.2 was obtained at low pH and the separation performance decreases with increasing pH.

Eijndhoven et al. [13] used electrostatic interactions to effectively separate BSA–Hb mixtures by membrane filtration. They obtained high initial protein separation factors (up to 70) at pH 7, ionic strength of 2.3 m*M* and a filtration velocity of 11 l/h per $m^2$. After reaching a Hb recovery of 60%, the overall selectivity dropped to 10.

The process developed by Causserand et al. [14] proposed a selective protein adsorption on montmorillonite clay particles. Once the adsorption equilibrium had been reached, the mixture of BSA–Hb–clay particle was filtered through a polyvinyldifluoride membrane with a nominal pore size of 0.1 μm. Free BSA permeates through the membrane, while the Hb adsorbed on the clay particles was retained by the membrane. The maximum obtained separation factor was 6.4 for an operational flux of 50 l/h per $m^2$, 1 m*M* ionic strength and low protein concentrations (0.1 mg/ml). Affinity assisted membrane filtration was also carried out by Yoon et al. [15]. With amino groups attached to the surface of microspheres they obtained selectivities for BSA over Hb of 13.

In this study, we provide an extensive characterisation of the new type of mixed-matrix adsorber membranes. The separation of two proteins of similar size such as BSA and bovine Hb was carried out using the newly developed adsorber membrane system at different operational parameters. The flexible concept of mixed-matrix adsorber membranes allows the incorporation of cation- as well as anion-exchange particles. Hence we hypothesise that adjustment of the adsorption conditions allows only one protein either BSA or Hb to pass the mixed-matrix adsorber membrane freely while the other is retained inside the membrane by adsorption.

## 2. Experimental

### 2.1. Materials

EVAL (a random copolymer of ethylene and vinyl alcohol) with an average ethylene content of 44 mol% was purchased from Aldrich and used as membrane material without further modification. Dimethyl sulfoxide (DMSO, Merck) was employed as solvent and 1-octanol (Fluka) as nonsolvent-additive in the casting solution. Water was used as nonsolvent in the coagulation bath. Different types of Lewatit ion-exchange resins kindly supplied by Caldic, Belgium, were incorporated into the polymeric membranes. BSA (Sigma) and bovine Hb (Sigma) were used in the adsorption/separation experiments, in freshly prepared buffer solutions in ultrapure water. All other chemicals were used as received. Ultrapure water was prepared using a Millipore Milli-Q plus purification unit.

### 2.2. Adsorbent preparation

Different types of Lewatit ion-exchange resins were incorporated into an EVAL porous matrix as a

particulate material to prepare heterogeneous mixed-matrix adsorber membranes with high protein adsorption capacity. One advantage of the Lewatit ion-exchange resins is their low price. Despite a somewhat lower protein adsorption capacity compared with other types of ion-exchange particles, the Lewatit type resins offer economic feasibility in protein separation processes [16]. A complete and detailed description for the ion-exchange resins is beyond the scope of this study, but the principal characteristics are listed in Table 1. The resin beads, with a size of 0.4–1.6 mm, were washed with demiwater in a stirred vessel until neutral pH, and then dried at 60 °C in a conventional oven. Before incorporation into the polymeric matrix, the ion-exchange resins were ground and sieved to obtain fractions with particle sizes smaller than 20 μm.

### 2.3. Membrane preparation

EVAL is a suitable candidate as polymeric material for adsorptive membrane preparation since it displays a good mechanical strength, has a high thermal stability, a good chemical and biological resistance, and is easy to sterilize using, e.g. γ-radiation. Different types of Lewatit ion-exchange particles with diameters smaller than 20 μm were added to a solution of 10%wt EVAL in DMSO to obtain membranes with protein adsorptive properties; 10% 1-octanol was added to the casting solution in order to improve the membrane morphology [17]. The mixtures were stirred overnight to break the particle agglomerates. EVAL-based mixed-matrix adsorber membranes were prepared by immersion precipitation; the polymeric solution was cast on a glass plate with a 500 μm casting knife and immediately immersed in a water coagulation bath at 40 °C. The diffusional exchange of solvent and non-solvent brings the film solution into an unstable state resulting in phase separation and formation of porous particle-loaded membranes with a thickness of about 300 μm. The preparation of the mixed-matrix adsorber membranes is described in detail in a previous paper [8].

### 2.4. Membrane characterisation

To characterise the prepared mixed-matrix adsorber membranes, the membrane morphology was investigated by scanning electron microscopy. Membrane porosity, permeation rate, and protein retention experiments as well as static and dynamic protein adsorption capacity data are included to demonstrate that the obtained structures are suitable as adsorber membranes.

For *scanning electron microscopy*, pieces of the membranes were frozen in liquid nitrogen and fractured to visualise cross-sectional areas. The fractured membranes were dried and platinum coated using a Jeol JFC-1300 Auto Fine Coater. The coated samples were examined using a Jeol JSM-5600 LV scanning electron microscope.

The *membrane porosity* was determined from the volume difference between the volume occupied by the polymer, equal to the volume of the dried membrane, and the volume of the membrane equilibrated in pure water. The polymer volume was calculated as the ratio between weight of dried membrane and the polymer density. Average values were obtained from three different samples.

The *permeation rate* through the flat adsorber membranes was determined using a nitrogen pressurised stirred dead-end filtration cell. Solutions of 50 m*M* acetate buffer at pH 4.5 and 50 m*M* phosphate buffer at pH 7 were permeated through a stack of 10 sheets of ion-exchange adsorber membrane.

The characterisation of porous adsorptive membranes with respect to their *static protein adsorption capacity* was determined by batch experiments with

Table 1
Principal characteristics of the filling ion-exchange resins

| Resin type | Functional group | Ionic form | Ion-exchange capacity (equiv./l) | p$K_a$ |
|---|---|---|---|---|
| 112 WS | Sulfonic acid | $Na^+$ | 1.75 | 2 |
| MP 500 | Quaternary amine | $Cl^-$ | 1.1 | 9–9.5 |

BSA as model protein. A known amount of membrane containing 65% of ion-exchange resins was equilibrated with a known volume of 1 mg/ml BSA solution. The protein uptake in 24 h per membrane volume was measured at 280 nm with a PU 8720 UV–Vis spectrophotometer.

The *dynamic adsorption performance* of EVAL-based mixed-matrix adsorber membranes was measured at a constant permeation rate of 10 l/h per $m^2$ using the stirred dead-end filtration cell previously described. To overcome the limited adsorption capacity of a single adsorptive membrane, 10 sheets of membrane were stacked and mounted in series in a conventional ultrafiltration device. The obtained configuration permits rapid, low-pressure adsorption of protein in either the batch or the continuous recycle mode. Furthermore, the targeted product is concentrated substantially through its adsorption into the membrane. BSA and Hb solutions were permeated through the adsorber membrane stack at a constant flow-rate. Protein concentrations of 1 mg/ml were employed in the experiments as realistic concentrations for many commercial separation processes. The permeate was collected using a LKB Frac-100 fraction collector from Pharmacia. All the filtration experiments were carried out at room temperature at a transmembrane pressure of 0.1 bar, and a stirring rate of 200 rpm.

The protein mass adsorbed per unit of membrane bed at a breakthrough concentration of 10% of the protein feed concentration was calculated by numerical integration of the protein concentration over the filtration run. For single component protein solutions, the BSA and Hb concentrations were determined in the feed and the permeate samples measuring the absorbance at 280 and 406 nm, respectively. Hb solutions exhibit maxima at two different wavelengths (280 and 406 nm), while the BSA absorbance shows a maximum at 280 nm and is negligible at 406 nm. Thus, for experiments with BSA–Hb mixtures, the Hb concentration was determined directly from the absorbance at 406 nm. The BSA concentration at 280 nm was then determined by subtracting the contribution associated with the Hb, which was evaluated directly from the Hb concentration. In the mixture, the position of the peaks for each individual protein was not affected by the presence of the other protein, indicating that no complex of the two proteins was formed.

### 2.5. Fractionation of BSA–Hb mixtures

To demonstrate and to quantify the potential of the adsorber membranes for protein fractionation, the effect of process characteristics such as pH, ionic strength, and filtration flow-rate on the BSA–Hb separation performances was studied in detail. Since the ion-exchange particles incorporated into the EVAL porous matrix exhibit different fixed ionic charges, therefore different adsorption–desorption conditions were involved in the protein separation process for each type of ion-exchange membrane.

For the *cation-exchange adsorber membrane* ($C_{MMA}$) operating at a lower pH than the protein isoelectric point, the protein is positively charged, while the ion-exchange particles possess a negative net charge. Therefore the protein is adsorbed into the mixed-matrix adsorber membranes by the ion-exchange particles. For high performances in protein fractionation, the appropriate protein solution environment and filtration conditions have to be chosen in a way that high Hb adsorption into the membrane takes place, while the BSA adsorption is minimised. The optimum pH for fractionation in the permeation step was therefore chosen to be slightly on the acid side of the hemoglobin ($6.5<pH<7.1$), since Hb was the target protein in the adsorption stage. Under this pH condition, the net charge of Hb is slightly positive and compensates the negative surface charge of the sorbent (Fig. 1). Meanwhile, BSA is negatively charged and thus a low BSA adsorption is expected. Moving the pH to either side of the optimum point would result in a less favourable adsorption owing to electrostatic repulsion [18,19].

The two proteins were mixed in equal concentrations and the competitive adsorption on the adsorber membrane was studied for different operational conditions. A solution of 50 m*M* phosphate buffer containing 1 mg/ml of each protein, was permeated through a stack of 10 membranes at a constant filtration flow-rate of 10 l/h per $m^2$ and the permeate was collected using a fraction collector. The BSA and Hb concentrations in the feed and the permeate

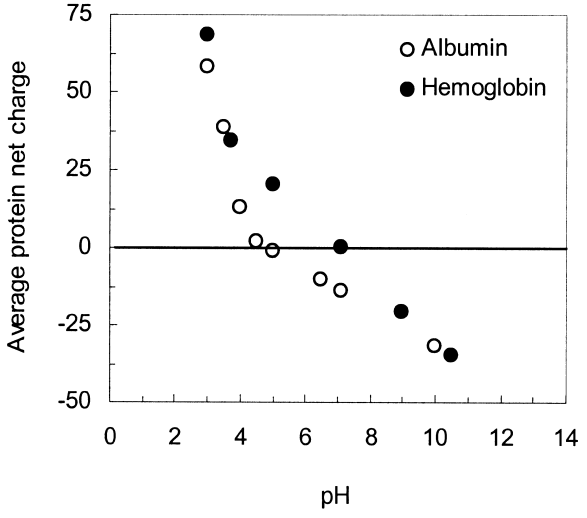


Fig. 1. Net charge of serum albumin and hemoglobin as a function of pH plotted with data from Ref. [20].

fractions were determined spectrophotometrically as mentioned before. The protein mass adsorbed per unit of membrane bed volume was calculated by numerical integration of the protein concentration over the filtration run at a breakthrough concentration 10% of the Hb feed concentration.

The protein separation efficiency was evaluated by the separation factor $S_{BSA/Hb}$, where $C_{f,BSA}$ and $C_{f,Hb}$ are the protein concentrations in the feed solution and $\overline{C}_{p,BSA}$ and $\overline{C}_{p,Hb}$ are the average protein concentrations in the collected permeate fractions during filtration, calculated by numerical integration of the permeation curve over the filtration run.

$$S_{BSA/Hb} = \frac{\overline{C}_{p,BSA}/\overline{C}_{p,Hb}}{C_{f,BSA}/C_{f,Hb}}$$

The proteins adsorbed into the mixed-matrix adsorber membrane were consecutively eluted with 50 m*M* borate buffer at pH 10 at a flow-rate of 10 l/h per $m^2$ in order to dissociate the protein-adsorber membrane complex. At a pH higher than the isoelectric point, the protein becomes negatively charged and is therefore released from the ion-exchange matrix passing freely through the porous membrane in the bulk solution. The eluent was fractionated and the BSA and Hb concentrations in the collected eluent fractions were monitored as previously described. Adsorption/desorption experiments were also conducted with individual BSA and, respectively Hb protein solutions.

For the adsorber membranes with *anion-exchange resins* incorporated ($A_{MMA}$), 50 m*M* acetate buffer at pH 5.5 was used in the permeation experiments. At this pH, the BSA is negatively charged and compensates the positive surface charge of the sorbent and thus adsorbs on the membrane. Since the net charge of Hb is positive, Hb is repelled by the anion-exchange particles and passes almost freely through the porous membrane into the permeate. The BSA and Hb concentrations were determined in the feed and the collected permeate fractions by the method previously described and the protein mass adsorbed per unit of membrane bed was calculated by numerical integration over the filtration run at a breakthrough concentration of 10% of the BSA feed concentration. The protein separation factor $S_{Hb/BSA}$ was calculated as being the inverse of $S_{BSA/Hb}$ as mentioned for protein fractionation involving the adsorber membrane $C_{MMA}$.

The desorption measurements were carried out in 50 m*M* acetate buffer pH 3.5 at a flow-rate of 10 l/h per $m^2$. Under these conditions, both proteins have the same positive charge as the membrane, being released from the complex adsorber-protein. The BSA and Hb concentrations in the eluent collected samples were monitored spectrophotometrically and the protein recoveries were calculated by numerical integration over the elution curves.

## 3. Results and discussion

This section starts with the characterisation of the prepared mixed-matrix adsorber membranes in terms of membrane morphology, porosity, transport properties and protein retention experiments. For the adsorber membranes having different particulate materials entrapped in the porous matrix, emphasis is laid on the efficiency of separation of the two similarly sized proteins (BSA and Hb) for different operational conditions.

### 3.1. Mixed matrix adsorber membrane characterisation

Different types of ion-exchange resins were employed in the preparation of the mixed-matrix adsorber membranes. The structure for the flat EVAL-based mixed-matrix adsorber membranes prepared by incorporation of 65% Lewatit 112WS ion-exchange resins with particle size diameter smaller than 20 μm ($C_{MMA}$) is presented in Fig. 2. The membranes possess an open, interconnected porous structure, without evident finger-like macrovoids across the entire cross-section (Fig. 2A,B) and pores larger than 0.1 μm on both the glass (Fig. 2C) and the air surface (Fig. 2D). The ion-exchange particles are tightly held together within the porous polymeric matrix. No significant loss of particles was observed during the membrane formation process on the glass surface. Similar morphologies were obtained by incorporation of 65% Lewatit MP500 ion-exchange resins with particle size diameters smaller than 20 μm into an EVAL porous structure ($A_{MMA}$).

Porosities in the range of 75±5% were obtained from swelling experiments, with no significant differences for various types of ion-exchangers. Due to the high pore interconnectivity and porosity of the top layer, high permeation rates through a stack of 10 adsorber membranes (≈300 l/h per $m^2$ per bar) were obtained. The continuous porous structure of the adsorber enables membrane stacking and thus avoids the well-known packing problems that arise with conventional particles in packed bed systems.

Static protein adsorption capacities were measured both for the unloaded EVAL membranes and the prepared mixed-matrix adsorber membranes with 65% Lewatit type of ion-exchange resins incorpo-

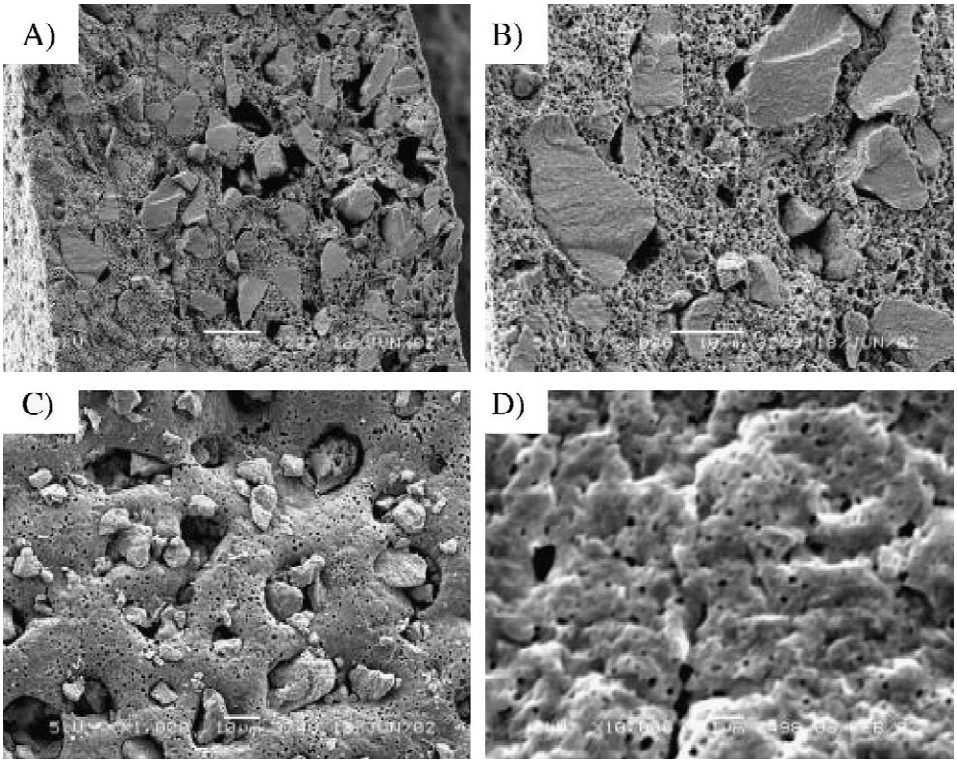


Fig. 2. SEM photomicrograph for the mixed-matrix adsorber membranes $C_{MMA}$ prepared from solutions of 10% EVAL in DMSO in the presence of 10% 1-octanol as additive, by incorporation of 65% Lewatit 112WS ion-exchange particles into the polymeric support: (A) cross-section, magnification ×750, the size bar indicates 20 μm; (B) cross-section, magnification ×2000, the size bar indicates 10 μm; (C) glass-surface magnification ×1000, the size bar indicates 10 μm; (D) air-surface, magnification ×10 000, the size bar indicates 1 μm.

rated into the porous structure. The BSA uptake within 24 h was around 100 mg BSA/g $C_{MMA}$ adsorber, equivalent to about 34 mg BSA/ml membrane. Since unloaded EVAL membranes contribute to less than 3% of the total BSA adsorption capacity, we concluded that the incorporated ion-exchange particles are responsible for the high adsorption capacity of the mixed-matrix adsorber membranes.

BSA adsorption measurements previously carried out with the same experimental conditions for both Lewatit ion-exchangers and prepared membranes, demonstrated that there was no loss of ion-exchange capacity during the particle entrapping and membrane forming process [8]. The conclusion was that the prepared adsorber membranes possess a good accessibility for proteins to the adsorptive sites, with no loss of ion-exchange groups during the membrane forming process.

Typical breakthrough and elution curves for permeation of single protein component solutions through the stack of mixed-matrix adsorber $C_{MMA}$ membranes are presented in Fig. 3. The obtained dynamic capacity (about 90 mg BSA/g adsorber membrane, equal to 30 mg BSA/ml adsorber) was slightly smaller than the corresponding static BSA adsorption capacity (34 mg BSA/ml adsorber),

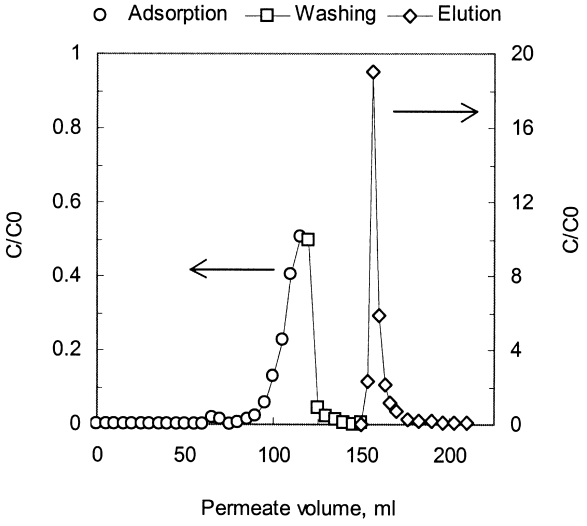


Fig. 3. Typical BSA breakthrough and elution curves through a stack of 10 $C_{MMA}$ membranes, at an ionic strength of 50 m*M* and a constant filtration flow-rate of 10 l/h per $m^2$. Loading and elution steps were carried out at pH 4.5 and pH 9, respectively.

because some adsorptive capacity remains unused mainly due to non-uniform flow distribution and resistance from mass transport rate processes. This effect was also found in packed bed chromatography where, because of slow adsorption/rearrangement/unfolding processes and axial dispersion, the dynamic adsorption is often 5–10 times lower than the equilibrium or maximum binding capacity [20]. In contrast to ideal flow conditions, a lower transport rate within the adsorptive membrane is principally caused by dispersion and diffusional resistances to mass transport and the unfolding and rearrangements of the protein at the adsorptive interface. The feed solution containing the protein molecules flows preferentially through the large pores and saturates the adsorptive sites surrounded by large pores. Meanwhile, adsorption in smaller pores and into the particles is more dominated by diffusional processes that are usually one order of magnitude slower. Full adsorption capacity can be achieved only if the flow-rate through the adsorber membrane is slow enough to allow each adsorbate molecule to diffuse to the adsorptive site and to rearrange/unfold their structure to its most favorable one before the interstitial volume continues passing through the membrane. Furthermore, it is known from packed columns that the axially directed velocity of a mobile solvent can be much faster in the center of the separation unit than near the edges of the adsorptive bed. Bypassing and uneven fluid distribution are additional sources of non-uniform flow which can lead to low dynamic capacities.

Reported capacities for several adsorptive membranes are summarised in Ref. [21] with protein adsorption capacities ranging from 3 up to 50 mg protein/ml of membrane. This indicates that the technical concept of mixed-matrix adsorber membranes shows performances equivalent to the highest reported in literature.

By using a Tris buffer solution at pH 9 for protein elution from the adsorbed protein–adsorber membrane complex (Fig. 3), the biomolecule can be rapidly concentrated up to 10 times in the desorptive buffer with a protein recovery of 90%. This offers perspectives for the adsorber membranes to function both as purifier and concentrator. In the initial stages of the purification processes of proteins and other secondary metabolites from biological sources, the

biomolecules are usually present in dilute solutions. Therefore, it is often necessary to concentrate such biological fluids in order to reduce the process liquid volume and thus to speed up the subsequent downstream processing steps. The results indicate that it is possible to concentrate a protein such as BSA using the mixed-matrix adsorber membrane with high protein recoveries in the final concentrated volume.

### 3.2. Fractionation of BSA–Hb mixtures

#### 3.2.1. $C_{MMA}$ membrane

In single component adsorption experiments at pH 4.5, close to the isoelectric point of BSA, both proteins are positively charged and therefore are adsorbed almost equally strongly onto the adsorber membrane $C_{MMA}$ (90±10 mg protein/g membrane, Fig. 4A). Fig. 4B shows the single component breakthrough curves for BSA and Hb at pH 7, close to the isoelectric point of Hb. Under these conditions, the membranes adsorb a major proportion of Hb due to the opposite protein/adsorber membrane charge (around 40±5 mg Hb/g adsorber membrane $C_{MMA}$). BSA is adsorbed to a significantly smaller extent (5±3 mg BSA/g membrane $C_{MMA}$) and passes almost unhindered into the permeate. Therefore, the operating pH for the fractionation of BSA–Hb from binary protein mixtures was chosen slightly below the isoelectric point (p$I$) of Hb.

For the BSA–Hb mixture, the permeation curve for Hb is, within experimental error, similar to that for the single component: only BSA shows a slight decrease in adsorption (Fig. 5A). Since BSA lacks competitive power with respect to Hb due to electrostatic repulsion, Hb adsorption is not affected by the other component [15]. BSA passes very quickly through the adsorber membrane and its average concentration in the permeate reaches 80% of the feed concentration while the Hb is still totally adsorbed in the matrix (Fig. 5B).

The average separation factor was calculated from the adsorption experiments and is presented in Fig. 6 as a function of permeate volume at two different pH values. At the operating pH ($pI_{BSA} < pH < pI_{Hb}$), the membrane $C_{MMA}$ adsorbs a major proportion of Hb due to opposite protein/membrane charge and allows preferential passage of BSA into the permeate. During the first part of the filtration, the hemoglobin concentration in the permeate was below the detection limits. Practically no Hb was detected in the first 20 ml collected. Hypothetical Hb concentration values of the detection limit of the spectrophotometer were used to calculate the separation factor, since infinite values of the separation factor have no meaning. With these hypothetical Hb concentrations, values of 100±10 for the separation factor of BSA–Hb were obtained within the first part of filtration (notice that other authors [13] preferred arbitrary

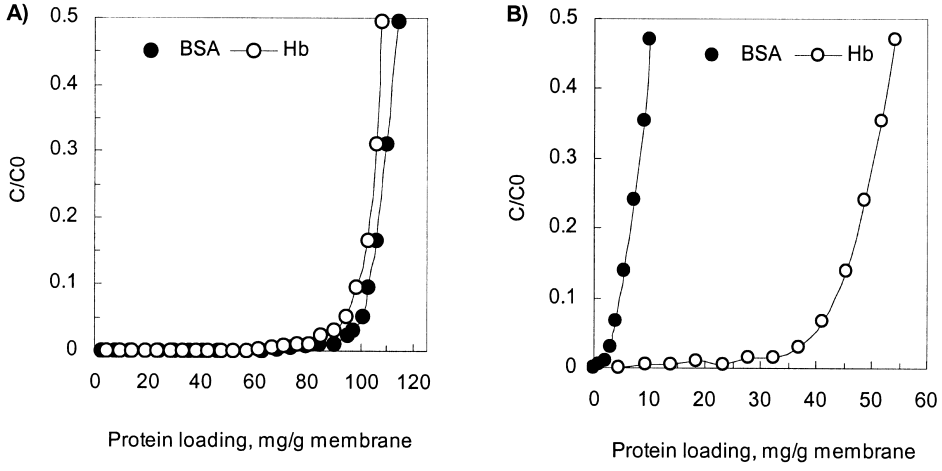


Fig. 4. BSA and Hb breakthrough curves for single components at pH 4.5 (A) and pH 7 (B). The ionic strength was set at 50 m$M$ and the filtration flow-rate through a stack of 10 $C_{MMA}$ membranes was kept constant at 10 l/h per $m^2$.

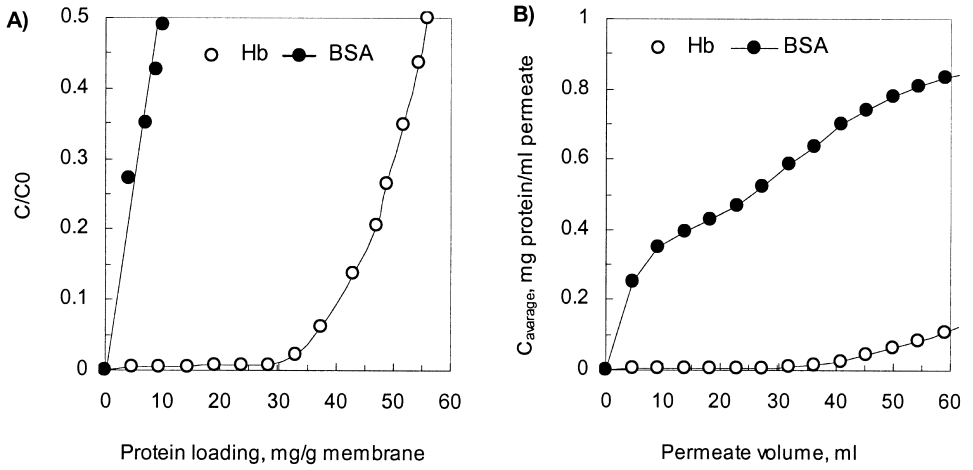


Fig. 5. BSA and Hb breakthrough curves for a BSA–Hb mixture with ratio 1:1 at pH 7. The ionic strength was set at 50 m*M* and the filtration flow-rate through a stack of 10 $C_{MMA}$ membranes was kept constant at 10 l/h per $m^2$.

values of 100 for the separation factor at the beginning of the experiments). At longer filtration times, Hb starts to appear in the permeate and therefore the separation factor values decrease with increasing permeate volume. If we compare the results obtained for the separation of BSA–Hb mixtures operated at a lower pH value, no improvement is observed in separation efficiency. Higher hemoglobin adsorption into the mixed-matrix adsorber membranes $C_{MMA}$ was obtained for an operating pH of 6.5 until the Hb breakthrough point of 10% of the feed concentration (more than 50 mg Hb/g membrane in comparison with 40–45 mg Hb/g membrane at pH 7). Unfortunately, under these conditions the BSA–Hb separation factor has lower values since the BSA adsorption into the membrane is slightly increased. This can be due to the appearance of a double layer effect. Fig. 7 presents the elution curves of single protein solutions for the $C_{MMA}$ membrane−adsorbed protein complex with a borate buffer solution at pH 10. The measured protein concentrations in the collected eluent samples are plotted as a function of eluent volume for BSA (Fig. 7A) and Hb (Fig. 7B). The protein recoveries calculated by numerical integration over the elution curves lead to high BSA desorption values (around 80%) while lower Hb recovery up to 60% was obtained. Overall hemoglobin recoveries of 60% are usually reported in the literature [12,22]. The results obtained by Kondo and Mihara [23] who used a desorption protocol consisting of increasing the pH from 7 to 10 with NaOH for 30 min, show, in the most favourable case, a Hb recovery of 63%. The denaturation of hemoglobin molecules on the adsorbent surface might increase the contact area

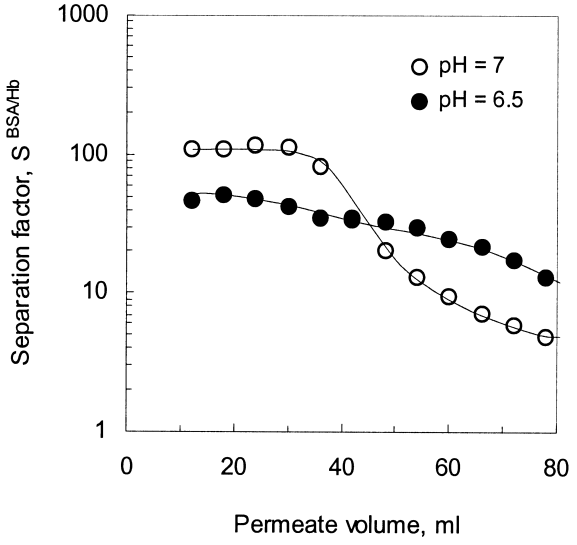


Fig. 6. Separation factor for the BSA–Hb mixture as a function of the operating pH. The filtration flow-rate through a stack of 10 $C_{MMA}$ membranes was kept constant at 10 l/h per $m^2$ at an ionic strength of 50 m*M*. The lines are included to guide the eye.

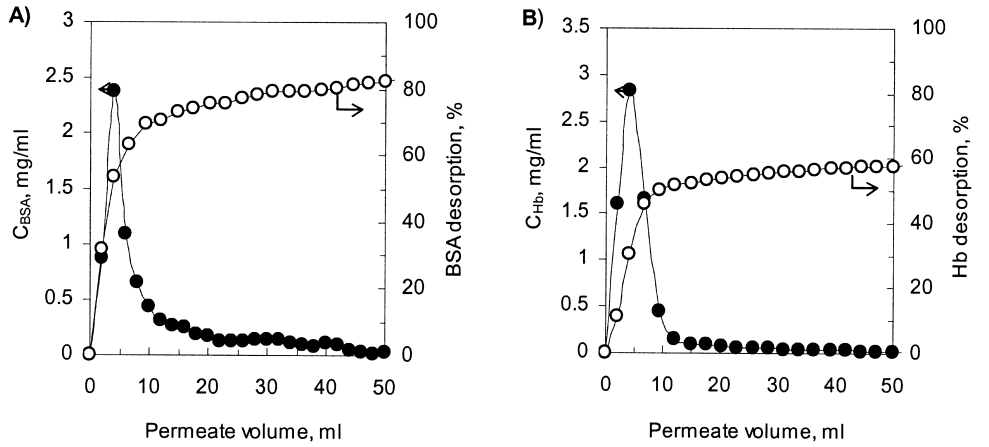


Fig. 7. Elution curves for BSA (A) and Hb (B) with 50 m*M* borate buffer at pH 10 and a constant filtration flow-rate through a stack of 10 $C_{MMA}$ membranes of 10 l/h per $m^2$.

between the protein and the surface and thus reduce the amount of protein desorbed [24]. Thus, more systematic studies using for instance a gradient elution method can reduce the protein denaturation onto the adsorber surface and might improve the percentage of Hb desorption.

### 3.2.2. $A_{MMA}$ membrane

In order to prove that the mixed-matrix adsorber membrane systems are extremely flexible and offer the possibility to easily vary the adsorption capacity, as well as the selectivity of the adsorber membranes, similar studies of protein fractionation were carried out using the anion-exchange membranes $A_{MMA}$. At pH 5.5, slightly on the basic side of BSA (Fig. 1), the $A_{MMA}$ membrane adsorbs preferentially the BSA due to the opposite protein/membrane charge; meanwhile the Hb is adsorbed to a lesser extent and passes rapidly into the permeate (Fig. 8). The Hb average concentration in the permeate reaches almost 90% of the feed concentration while the BSA is still totally adsorbed (Fig. 8B).

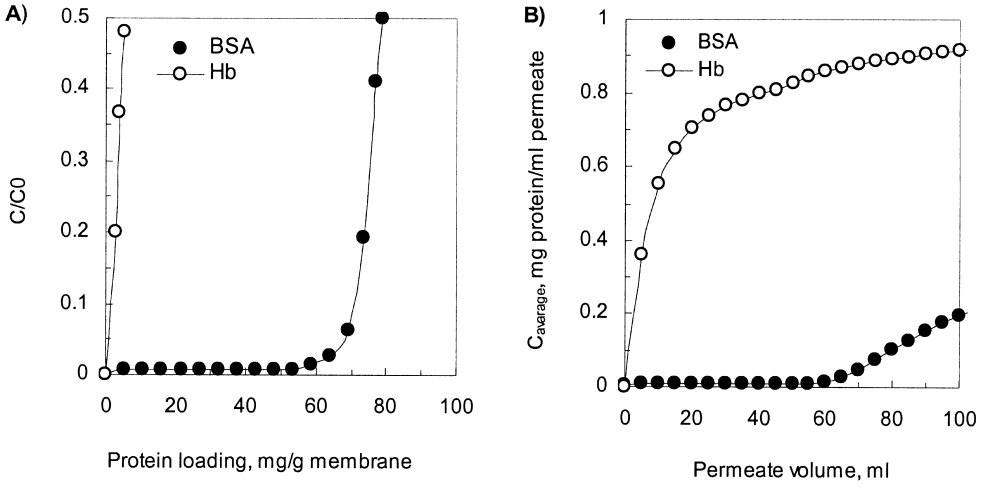


Fig. 8. BSA and Hb breakthrough curves for a BSA–Hb mixture with ratio 1:1 at pH 5.5. The ionic strength was set at 50 m*M* and the filtration flow-rate through a stack of 10 $A_{MMA}$ membranes was kept constant at 10 l/h per $m^2$.

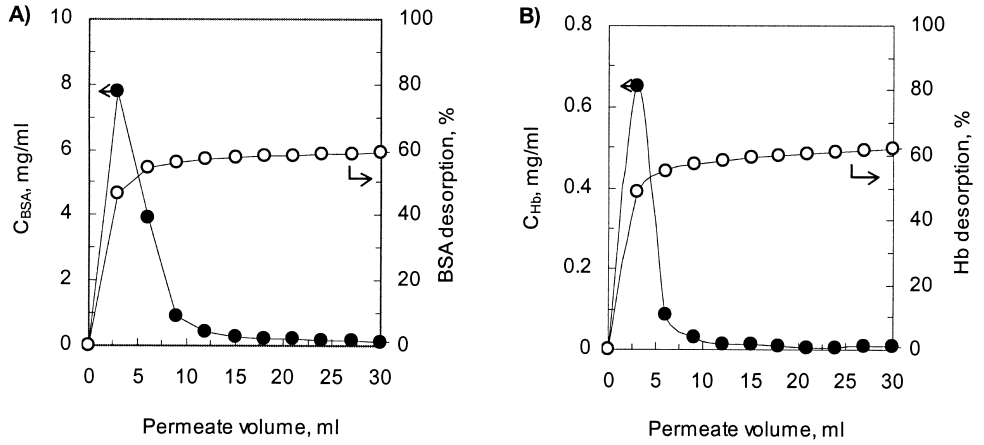


Fig. 9. Elution curves for BSA (A) and Hb (B) with 50 m*M* acetate buffer at pH 3.5, at a constant filtration flow-rate through a stack of 10 $A_{MMA}$ membranes of 10 l/h per $m^2$.

Measurements of protein desorption from the complex adsorbed protein–$A_{MMA}$ membrane carried out in acetate buffer at pH 3.5, are presented in Fig. 9. The measured BSA (Fig. 9A) and Hb (Fig. 9B) concentrations in the eluent samples were plotted as a function of eluent volume. The protein recoveries calculated by numerical integration over the elution curves lead to values of around 60% desorption for both BSA and Hb. Furthermore, BSA was concentrated fivefold into the desorptive buffer thus offering perspectives for the adsorber membranes to function as well as a concentration medium.

### *3.2.3. Flow-rate and ionic strength dependence on the separation factor*

The effect of filtration flow-rate on the separation factor for the BSA–Hb mixture is presented in Fig. 10 for both membrane systems investigated. For the $C_{MMA}$ membrane operating at pH 7 with a filtration flow-rate of 10 l/h per $m^2$, the average protein

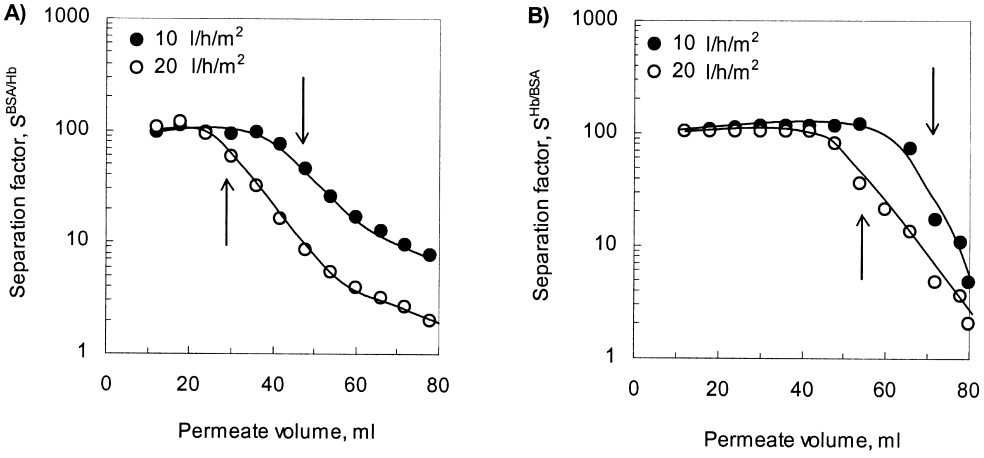


Fig. 10. Separation factor for the BSA–Hb system as a function of the filtration flow-rate for the mixed-matrix adsorber membrane $C_{MMA}$ (A) and $A_{MMA}$ (B) operated at an ionic strength of 50 m*M*. The arrow indicates the breakthrough point at 10% of the protein feed concentration. The lines are included to guide the eye.

concentrations in the permeate were 0.64 mg BSA/ml and 0.014 mg Hb/ml, at a hemoglobin breakthrough point of 10% of the feed concentration (data from Fig. 5). This results in an average separation factor higher than 45. A lower average separation factor of 32 (Fig. 10A), calculated over the filtration until the Hb breakthrough point, was obtained when the filtration flow-rate increased from 10 to 20 l/h per $m^2$, since the efficiency of adsorptive sites utilization is inversely related to the flow velocity. Full adsorption capacity can be achieved only if the flow-rate through the adsorber membrane is slow enough to allow each adsorptive site to bind the protein before the interstitial volume continues to flow through the membrane. The separation of the BSA–Hb mixture, carried out with the membranes $A_{MMA}$ at pH 5.5 and filtration flow-rate of 10 l/h per $m^2$ until the BSA breakthrough, offers a separation factor of almost 50. If the filtration flow-rate was increased up to 20 l/h per $m^2$, the separation factor decreases to a value of 35 (Fig. 10B).

One has to be aware that the arbitrary choice of the breakthrough point at 10% of the feed concentration determines the protein separation performances. In industrial applications, the filtration process can be stopped at a lower permeate concentration, e.g. 5% breakthrough and the absolute value of the average separation factor will increase up to double the value. Nevertheless, the obtained separation factors are at least 10 times higher than that obtained by the competing ultrafiltration process using the same filtration velocities [13].

The results presented in Fig. 10 were obtained for the separation of an BSA–Hb mixture in buffer solutions at ionic strength of 50 m*M*. Since real complex media often contain electrolytes at higher concentrations, the efficiency for the BSA–Hb separation using the mixed-matrix adsorber membranes was also investigated for an increased ionic strength. As previously discussed [8], the protein adsorption capacity slowly decreases with increasing ionic strength. We therefore anticipate the separation factor to follow the same trend.

Using the adsorber membranes $C_{MMA}$ under physiological ionic strength conditions (150 m*M*), the average protein concentrations in the permeate were 0.52 mg BSA/ml and 0.033 mg Hb/ml at a hemoglobin breakthrough point of 10% of the Hb feed concentration. Under these conditions, the calculated average separation factor over the filtration run until the hemoglobin breakthrough decreases to 15 (Fig. 11A). A lower average separation factor (12) was obtained for BSA–Hb fractionation using the adsorber $A_{MMA}$ operating at physiological ionic strength (Fig. 11B). Nevertheless, the values of the BSA–Hb separation factor obtained with the mixed-

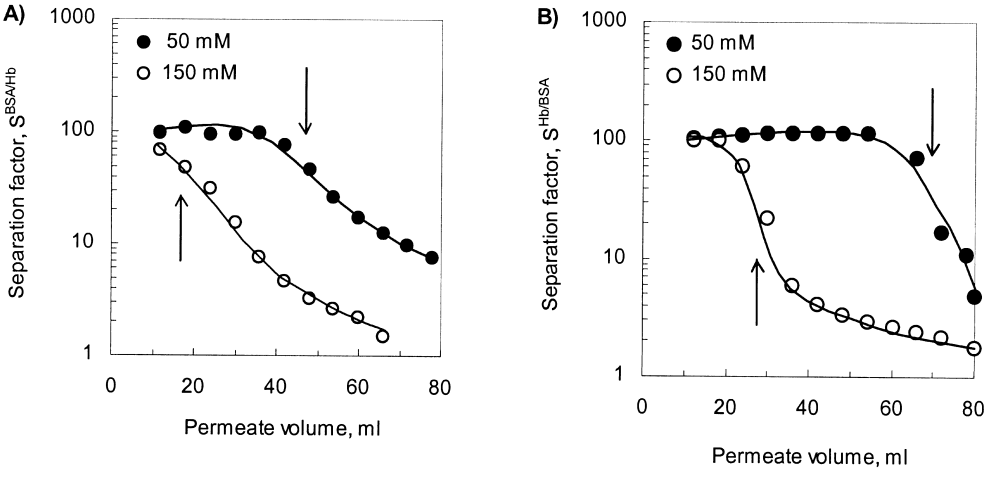


Fig. 11. Separation factor for the BSA–Hb mixture as a function of ionic strength. The filtration flow-rate through a stack of 10 membranes $C_{MMA}$ (A) and $A_{MMA}$ (B) was kept constant at 10 l/h per $m^2$. The arrow indicates the breakthrough point of 10% of the protein feed concentration. The lines are included to guide the eye.

matrix adsorber membranes are still higher than those reported in the literature for similar operating conditions [14].

## 4. Conclusion

The mixed-matrix adsorber membranes developed in our laboratory feature high protein adsorption capacities. They offer a wide variety of applications, mainly for the adsorption and/or purification of compounds from a reaction mixture. Since the protein can be concentrated up to 10-fold in the eluent, the mixed-matrix adsorber membranes can also function as a concentration medium. The developed adsorber combines the advantages of membrane technology (easy scale-up, low-pressure drop) with classical column chromatography (high binding capacity, high recovery). The advantages of this method compared with high-performance liquid chromatography consist of a lower sensitivity to fouling and plugging resulting in a less extensive pre-treatment of the feed solution and a comparable high protein binding capacity at low-pressure drop. This leads to lower protein denaturation during the separation process.

Of particular interest for the mixed-matrix adsorber membrane is the fractionation of proteins with similar molecular mass. Selective adsorption of Hb into the cation adsorber membrane $C_{MMA}$ allows separation of a BSA and Hb mixture with an average separation factor of 40, calculated over the filtration run until the hemoglobin breakthrough at 10% of the Hb feed concentration, at 50 m*M* ionic strength and 10 l/h per $m^2$ flow-rates. To prove the flexibility of the mixed-matrix adsorber membrane system, an anion adsorber membrane $A_{MMA}$ was also used for the Hb–BSA separation. High separation factors were obtained at physiological ionic strength conditions, up to 10 times higher than those reported in literature.

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