

**MEMBRANE ADSORBERS
DEVELOPMENT AND APPLICATIONS**

Maria Elena AVRAMESCU

MEMBRANE ADSORBERS – Development and applications

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DEVELOPMENT AND APPLICATIONS**

DISSERTATION

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A theory is something nobody believes, except the person who made it. An experiment is something everybody believes, except the person who made it.

-- attributed to Albert Einstein (1879-1955)

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CHAPTER 1

INTRODUCTION

1.1 Scope of the thesis

This thesis comprises a comprehensive study on novel adsorptive membranes for protein recovery processes based on either *affinity separation* or *ion exchange* processes. In order to develop the affinity membranes, the preparation and modification of hydrophilic microfiltration membranes obtained from ethylene vinyl alcohol (EVAL) by immersion precipitation has been studied in detail. Adsorptive membranes have been prepared by incorporation of various functionalized ion exchange particles into microporous EVAL membranes and investigated with respect to their protein separation and fractionalization characteristics. Coupling of BSA onto modified EVAL membrane surfaces has been investigated and applied in the separation of bilirubin from aqueous solutions.

1.2 General introduction

In biotechnology, the downstream processing of fermentation broths normally involves numerous steps for biomass removing and product purifying. In the early days of biomolecule purification, the only practical way used for protein separation from complex mixtures was based on protein precipitation by water miscible organic solvents [1]. Alteration of the solvent properties by addition of neutral salts and/or organic solvents leads to precipitation of the macromolecule due to a difference in solubility. Additionally, selective precipitation by denaturation has been achieved for certain proteins. For separation, the precipitate is filtered off and redissolved. Filtration can however, present a critical step since the filter can clog due to flocculation of proteins.

Nowadays the demands and techniques used for protein purification have changed, allowing protein recovery in mild processing conditions, which help to maintain the native conformation and hence the biological activity of the biomolecule. Alternative processes including adsorption techniques, gel filtration, liquid phase partitioning, electrophoretic methods and membrane technologies have been developed for protein purification. Adsorption techniques often result in purification steps that give the greatest increase in protein purity. Therefore, these techniques have become widely employed especially when adopted in combination with chromatographic processes.

Membrane chromatography has been recently proposed for protein recovery as an alternative method to classical bead chromatography [2-6]. Adsorptive membranes allow high velocities and very short residence times in the chromatographic support at a low transmembrane pressure drop and exhibit high binding capacities. Using stepwise elution, the biomolecules can be rapidly concentrated 10-fold and more with 85-100% recovery. The operating interactions involved in the chromatographic separation process in the microporous membranes are identical to those in the packed columns including affinity interactions, ion exchange, hydrophobic interactions and reverse phase. The functional groups immobilized on the porous membrane surface include affinity ligands e.g., Protein A/G, BSA, biomimetic dyes, immobilized metal, various hydrophobic amino-acids, ion-exchange groups such as carboxylic or sulfonic acid, tertiary or quaternary amines and hydrophobic ligands such as octyl and phenyl groups.

- **The affinity techniques** are based on a biospecific interaction that results in a change of protein properties such that the protein can be separated from other biomolecule-containing complex mixtures. The ligand molecule is immobilized on the porous surface and the mixture containing the protein of interest is passed through the adsorptive membrane. A specific interaction takes place between the ligand and the ligate and retains the desired protein, while the other components from the feed solution pass through the adsorber freely. Affinity chromatography allows the purification of proteins based on biological functions rather than individual physical or chemical properties. Isolation of a protein or a group of proteins such as γ -globulins fractions, human serum albumin, various clotting factors from body fluids was successfully achieved by using affinity membrane chromatography. Immobilized antibodies were used for example for industrial scale production of human interferon- α 2a, interleukin-2, interleukin-2 receptor. Pseudoaffinity ligands such as dyes, lysine and histidine are nowadays well established in membrane chromatography. A

unique application for affinity purification is the separation of proteins differing by a single histidine molecule in their sequence by immobilized metal affinity chromatography.

- The principle of **separation by ion exchange** is the electrostatic interaction between the charges of the proteins and the adsorber surface. The protein must displace the counter ions of the exchangers and become attached on the sorbent's surface. The amount of protein bound per unit volume of adsorptive membrane can be very high. However, for most exchangers, the binding capacity depends on the molecule size of the protein and adsorption conditions (pH, ionic strength, protein concentration). Ion exchange membranes can be produced by modification of commercially available microfiltration membranes. BSA and HSA, α -chymotrypsinogen, lysozyme, trypsin inhibitor, cytochrom c, ovalbumin, α -lactoalbumin, conalbumin, ferritin, myoglobin, chymotrypsin, have been isolated by ion exchange membrane chromatography. Furthermore, anion and cation exchange membranes allowed the purification of monoclonal antibodies and human recombinant antithrombin from cell culture supernatants and the isolation of immunofusion proteins produced extracellularly by *E. coli*.
- The protein separations based on **hydrophobic interactions and reverse phase** use the interaction between aliphatic chains on the adsorbent and corresponding hydrophobic regions on the protein surface. Typical hydrophobic adsorbents commercially available include C₄, C₆, C₈, or C₁₀ linear aliphatic chains, eventually with a terminal amino group. The main problems in hydrophobic chromatography are the slow association-dissociation process and protein-protein interactions. Similar proteins can interact with each other as well as with the adsorbent leading to a large degree of overlapping between the elution components. Hydrophobic chromatography has not been used in protein separation as intensive as ion exchange or affinity chromatography since sharp separations are not achieved.
- **Multistage chromatography** combines different types of chromatographic supports to achieve a higher degree of selectivity. A sequence of cation exchanger, dye-ligand and anion exchange membranes enabled the purification of formate dehydrogenase from *Candida boidinii*. Using a sequence of ultrafiltration, diafiltration, Cibacron Blue, anion exchanger and heparin-membrane adsorber, pure recombinant human Antithrombin III was obtained from hamster cell culture.

1.3 Outline of the thesis

The work reported in this study, focuses on the preparation of microporous membrane adsorbers for protein recovery based on affinity or ion exchange interaction modes. A detailed description of materials used as membrane substrates, preparation and modification of basic membranes, coupling of spacer arms and ligand molecules to the membrane supports as well as the main membrane chromatography applications is introduced in **Chapter 2**.

The use of EVAL copolymer to prepare the basic macrovoid-free, open cellular-type membranes, with a high internal surface area and interconnectivity that can be chemically modified in aqueous and organic media is presented in **Chapter 3**. To tailor the required membrane morphology, we investigated the ternary water/DMSO/EVAL system and quaternary systems using a series of n-alcohols (n=2-12) as non-solvents and additives in the casting solution. A model is proposed to understand the influence of the alcohol chain length and concentration on the membrane morphology.

In **Chapter 4** we investigate covalent coupling of bovine serum albumin (BSA) as model ligand onto cellular-type EVAL microfiltration membranes. EVAL membranes prepared from the ternary water/DMSO/EVAL system are because of a bad dry-wet reversibility only suitable for activation and coupling reactions in aqueous media, e.g., using glutaraldehyde and oxiran activation reactions. Preparing microfiltration membranes from the quaternary system water/1-octanol/DMSO/EVAL, using 1-octanol as nonsolvent-additive in the casting solution, enables to perform surface functionalization reactions in organic media as well as surface activation by a low-pressure glow discharge treatment. Such membranes can function as adsorptive devices for a large number of metabolic toxins such as free fatty acids, unconjugate bilirubin, endotoxins of gram-negative bacteria, mercaptans as well as medications like nortriptyline, amitriptyline, diazepam, bromazepam, which bind preferentially to the albumin fraction of the blood plasma.

Chapter 5 presents a generic technology for the preparation of adsorptive membranes by incorporation of functional entities into a porous substrate that can function as an alternative separation process to expanded bed reactors and/or classical chromatographic columns. A variety of ion exchange particles was incorporated into an EVAL porous matrix by immersion phase separation process in order to form a heterogeneous matrix composed of solid particles surrounded by a polymeric film. The developed concept is rather flexible and offers the possibility

to easily adjust the geometry, the adsorption capacity, as well as the functionality of the structure. A first series of fibers as well as flat membranes bearing an adsorptive function and high protein binding capacities were prepared. The membranes were characterised with respect to their morphology, porosity, permeability and adsorption capacity.

Applying of the prepared mixed matrix adsorber membranes for protein recovery is reported in **Chapter 6**. The obtained heterogeneous matrixes feature high static and dynamic protein adsorption capacities. In a sequential desorption step by changing the pH and/or the ionic strength of the eluent, high protein recovery was obtained. The membrane adsorbers can be reused in multiple adsorption/desorption cycles with good adsorption performances.

The separation of two proteins of similar size, bovine serum albumin (BSA) and bovine hemoglobin (Hb) carried out using a new type of ion exchange mixed matrix adsorber membranes is presented in **Chapter 7**. The effect of operational parameters such as filtration flow rate, pH, ionic strength, on the protein separation performances was investigated for cation as well as anion exchange adsorber membranes. High average separation factor values, calculated by numerical integration of the permeation curve during the filtration run, were obtained for BSA-Hb separation at physiological ionic strength with filtration flow rate up to 20 l/h/m².

The removal bilirubin by the prepared EVAL-based adsorber membranes is reported in **Chapter 8**. In fact, Chapter 8 aims to compare the concepts described in the earlier chapters. The retention of bilirubin on these adsorber membrane system was comparable with the results presented in the literature and allowed us to conclude that this system could be an alternative for the retention of other substances such as tryptophan, barbiturates or antidepressant.

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CHAPTER 2

GENERAL INTRODUCTION IN AFFINITY SEPARATION PROCESSES

2.1 Affinity based separation

Affinity based separation has become the preferred method for selective removal of proteins and other molecules from biological fluids. There are several affinity-based separation techniques: affinity precipitation, affinity chromatography and affinity-based filtration.

Affinity precipitation is a purification method especially used for enzymes and other biological molecules [1]. The first step in affinity precipitation involves mixing of a bifunctional ligand with an oligomeric enzyme. Precipitation may occur if the spacer linking the two ligand entities together is long enough to bridge the distance between the two enzyme molecules and if the binding between the ligand and the enzyme is strong enough. This phenomenon occurs because the bis-ligand interacts with two enzyme molecules. Since the enzyme is oligomeric, an elaborate network of enzymes and bifunctional molecules will form. When such a network has grown to a sufficient size, it can no longer be kept in solution and precipitates out.

Affinity chromatography using particulate materials is a highly developed method for the purification of biomolecules and presents the most important applications for affinity separation [2]. Affinity chromatography features high purification factors (50 to 100) and is commonly used as the final step in the purification of biomolecules out of crude mixtures [3].

Affinity chromatography is typically carried out in packed beds containing porous particles (or beads) onto which the affinity ligand has been immobilized. The method involves five steps: i) in the loading step, a solution containing the compound of interest is passed through a column that contains a highly specific ligand immobilized on the solid support; ii) as the fluid passes through the column,

the desired component binds selectively and reversibly to the ligand, while most impurities pass unhindered; iii) any residual impurities are removed by flashing the column with an appropriate buffer solution in the washing step; iv) the compound of interest now purified but still bound, is recovered by passing a solution that disrupt the ligand-ligand binding interaction (by changing ionic strength or pH for instance) through the column; v) in the last step the affinity ligand is regenerated by a regeneration buffer [4].

There are some drawbacks for large-scale industrial applications due to the fact that: i) affinity chromatography is a discontinuous process; ii) binding kinetics are slow because of restricted protein diffusion inside porous particles; iii) low flow rates and low column heights have to be employed in order to avoid compression and compaction of packed porous beds due to the poor mechanical strength of the porous particles which results in low productivity; iv) difficulties in the scaling-up of the process; v) difficulties to operate in sterile conditions. A large-scale affinity binding process is needed to overcome the drawbacks of affinity chromatography. Lots of efforts have been attempting to combine affinity binding with some processes that are easy to scale-up.

Affinity-based membrane filtration includes affinity filtration and affinity based membrane chromatography separation.

In *affinity cross-flow filtration* a ligand is coupled to a soluble macromolecule or colloidal particle, large enough to be retained during the filtration step [5]. Since desired compound binds to the ligand, the affinity complex will also be retained. The affinity filtration process combines affinity binding to colloidally suspended solid particles with cross-flow membrane filtration. In this process a five step procedure similar to the classical affinity chromatography method is required: i) a mixture of substances is loaded into the system whereby the desired component binds to the affinity ligand carrier; ii) the membrane retains the target molecule due to the formation of the affinity complex with a size larger than the pores of the membrane; iii) the isolated target molecule-carrier particle complex is treated with an appropriate eluent in order to dissociate the complex; iv) the target molecules are separated from the carrier particles and pass freely through the porous membrane; v) the carrier particles are regenerated and reused.

In *membrane chromatography* separation processes the ligand is immobilized on the membrane surface [6]. When the solution containing the compound of interest is filtrated, the desired molecule binds selectively and reversibly to the ligand immobilized on the membrane surface. In case of sterical hindrance a so called “spacer molecule” can be introduced between the polymeric support and the ligand. These spacers permit a freer rotation of the ligand and thus provide more

opportunities for the orientations necessary for ligand-ligand complex formation to occur. The conditions of the surrounding milieu determine the stability of the ligand-ligand complex. Either specific or non-specific changes in the physical or chemical environment conditions can disrupt the biological complexes. This method resembles affinity membrane separation with a very short affinity chromatography column [7]. The general principle of membrane chromatography is illustrated in Figure 2.1.

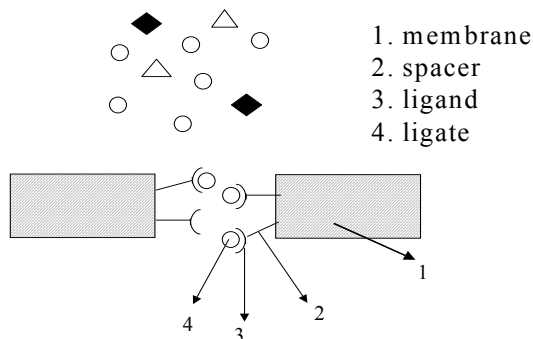


Figure 2.1 The principle of membrane chromatography

2.2 Adsorptive membranes preparation

Three main steps are usually involved in the preparation of adsorptive membranes used in affinity separation processes [8]: i) basic membrane preparation; ii) functionalization (activation) of the basic membranes; iii) spacer arms and ligand molecules coupling on the activated porous membrane surface. The preparation of basic materials is essential for the performances of the separation process.

2.2.1 Preparation of basic microporous membranes

Numerous natural and synthetic polymers such as: polysaccharides (agarose, cellulose and its derivatives, dextran, starch, chitin, chitosan), polyamide, polysulfone and its derivatives, polyethylene and polypropylene, acrylic copolymers, polycarbonates, polyvinylalcohol, have been explored as potential supports in affinity separation processes. The substrates to which the ligand molecules are to be bound, should fulfill several conditions [9]: i) high macroporosity with large internal and external surface areas to maximize interaction of matrix-bound ligands with the ligate during the binding step; ii) high mechanical stability and resistance to compaction under pressure; iii) high chemical and physical stability

under harsh conditions used for the ligand coupling and ligand–ligate complex formation reactions; iv) hydrophilic surfaces to minimize the non-specific adsorption of bioactive species, and to minimize competition for the desired ligates; vi) high density of reactive groups for subsequent coupling of spacer arms and ligand molecules. Since hardly any material can fulfill these conflicting requirements the choice of the support for a particular application is very often a compromise among the above mentioned conditions.

Very few materials could be used as membranes mainly because they are too weak to become self-supporting as thin films. Most of the available commercial microporous membranes such as poly(sulfone), poly(ether sulfone), polyamide, cellulose, poly(ethylene), poly(propylene), poly(vinylidene difluoride), are prepared by *phase inversion* processes. The concept of phase inversion in membrane formation has been introduced by Kesting [10] and can be defined as follows: A homogeneous polymer solution is transformed into a two-phase system in which a solidified polymer rich phase forms the continuous membrane matrix and a polymer lean phase fills the pores. Phase inversion is induced by evolving thermodynamic instability, either by a change in the composition or the temperature of the polymer solution. The following phase inversion processes can be distinguished: i) precipitation from the vapor phase; ii) precipitation by controlled evaporation; iii) thermal precipitation; iv) immersion precipitation.

Widely used nowadays, the *immersion precipitation* technique was introduced by Loeb and Sourirajan [11] for the preparation of skinned reverse osmosis membranes. They applied the method to develop asymmetric membranes consisting of a very dense top layer supported by a sponge-like porous structure. The formation of symmetric or asymmetric membranes by the immersion precipitation process is based on the phenomena of liquid-liquid phase separation. The basic system for the immersion precipitation process is a ternary system consisting of a polymer, a solvent and a nonsolvent for the polymer. The solvent and nonsolvent must be miscible. A concentrated solution of the polymer in the solvent is immersed in a bath containing the nonsolvent. The nonsolvent diffuses into the polymer solution while the solvent diffuses out. By the changes in composition, the polymer solution becomes unstable and liquid-liquid demixing starts. If the concentration of the polymer is high enough, nuclei of the polymer lean phase are created. These nuclei grow out into pores while the surrounding polymer solution gradually gets more concentrated in polymer, until a solidification process inhibits any further morphological changes. Solidification processes that may occur are gelation by crystallization of the polymer, vitrification

by crossing the glass transition region or a somewhat undefined process called aggregate formation.

The actual morphology of the resulting membrane depends strongly on the thermodynamic and kinetic properties of the system studied. In a quasi-ternary system, membrane structures can be obtained that have permeation properties ranging from microfiltration to gas separation. The performance of some of these membranes can be dramatically improved by using a fourth component in the system: a second solvent or polymer or a non-solvent miscible with the nonsolvent used in the coagulation bath is added to the polymer solution [12, 13]. For microfiltration processes, the resulting membrane morphologies are far more regular and show higher permeabilities while retaining the required retention properties.

Most commercial microporous membranes are either hydrophobic and/or relatively inert. Thus, the basic membranes must be modified to acquire functional groups such as hydroxyl, carboxyl, halogen, or amine groups (see Table 2.1) necessary for spacer arm and ligand coupling. The major methods employed to prepare modified membranes are based on:

- **coating techniques** usually deposit a hydrophilic layer such as hydroxyethyl cellulose, polyvinyl alcohol, chitosan, polyacrolein or polyethylenimine on a hydrophobic one [14-17]. The coating layers prepared are however not durable and stable and can be easily leached out. To overcome this drawback, the membranes (polyvinylidene fluoride, polytetrafluoroethylene, polycarbonate polyethersulfone or nylon) was coated with a mixture containing a functional monomer (hydroxyalkyl acrylate or methacrylate), a polymerization initiator (ammonium or potassium persulfate) and a cross-linking agent (difunctional acrylates, methacrylates or acrylamides) and exposed to radical polymerization by heating, UV or γ -radiation [18]
- **grafting polymerization:** hydrophobic and inert microporous membranes such as poly(ethylene) and poly(propylene) were grafted with monomers like sodium styrenesulfonates, vinyl acetate, 2-hydroxyethyl methacrylate to introduce various ion exchange groups (-CH₂OH, -NH₂, -SO₃H, -COOH) or with glycidyl methacrylate to acquire reactive epoxide groups that can be further modified with ionic exchange groups, metal chelate groups or hydrophobic groups (phenyl, butyl, phenylalanine, tryptophan) [19-21].
- **other methods** include a co-casting of a hydrophobic and a hydrophilic polymer that contains amine, imine, hydroxyl or carboxyl groups [22, 23].

The modification of commercially available membranes usually involves multiple steps and harsh modification conditions. Therefore, a different procedure based on in situ copolymerization of two different monomers was proposed to prepare mechanical stable microporous membranes with functional groups in a single step. One of the monomers such as vinylpyridine, acrylamide, vinyl alcohol, glycidyl methacrylate and its derivative, serve as the core support material for functional groups. The second monomer (divinylbenzene, ethylene dimethacrylate, methylen bisacrylamide) functions as a crosslinker and offers mechanical strength. Inorganic salts or organic solvents are usually used as porogen agents.

Porous sheets loaded with specific binding particles are another subclass of materials used as matrices in affinity processes. The incorporation into a porous polymeric membrane of functional particles such as silica and its derivatives, styrene/divinylbenzene based ion exchange resins, fibrous cellulose derivatives, results in adsorptive structures, which can be applied to isolate macromolecules such as peptides, proteins, nucleic acid or other organic compounds from complex mixtures [24-27]. Such material is either limited by its three dimensional size of the housing it is cast into (Zip Tip, Millipore) or is in the form of a sheet (Empore; Acti-Mod, FMC Bioproducts; Biorex, Bio-Rad). However, these materials differ from the classical affinity membranes since the binding process takes place at the small particles embedded into the porous matrix and not at the pore wall itself. Nevertheless these systems show hydrodynamic advantages similar to those of the adsorptive chromatographic membranes.

Most suitable particles display, in combination with the porous matrix morphology, rapid adsorption kinetics, a capacity and selectivity commensurate with the targeted application and allow for desorption of the molecule with an appropriate agent. The affinity of suitable adsorptive particles for specific molecules can be defined in terms of hydrophobic, hydrophilic or charged functionalities, in particular ion exchange functionalities, molecular (imprinted) recognition, or other specific interactions. Therefore, the use of particle-loaded membranes and particle-embedded glass fiber disks, referred generally as disk technology, have become a widely used laboratory technique to isolate and concentrate selected analytes from a gas, or liquid prior to chromatography analyses.

2.2.2 Modification of basic microporous membranes and ligand coupling

If the selected basic membrane does not possess functional groups it can be activated to acquire the reactive groups necessary for spacer arms and ligand molecule coupling using similar methods as for particulate materials (beads or gels)

in column chromatography). The spacer arms and the affinity ligand molecules may be directly or indirectly linked to residual functional groups of the membrane by any procedure that substantially retains the mechanical and hydraulic membrane properties while substantially retaining the binding activity of the affinity ligand. The functional groups of the polymer support that can be used for the covalent binding of spacer arms or ligand molecules are presented in Table 2.1.

FUNCTIONAL GROUP	POLYMER
Hydroxyl groups	Polysaccharides, PVA, inorganic glass;
Carboxyl groups	Poly(acrylic acid), poly(glutamic acid), CM-cellulose, poly(acryl amide);
Amino groups	Aminoetylated polysaccharides and silica gel, poly-(p-aminostyrene), synthetic polymers;
Aldehyde and acetal groups	Polymers reacted with glutaraldehyde, poly-saccharides reacted with periodate;
Mercapto- groups	Modified polymers;
Amide groups	Polypeptides such nylon;
Halogen groups	Polymers treated with cyanuric chloride, bromoacetyl bromide, iodoalchyl alcohol, polymers containing 3-fluoro-4,6-dinitrophenyl groups.

Table 2.1 Functional groups of polymers used for the covalent binding of spacer arms or ligand molecules

Polymers containing *hydroxyl groups* including polysaccharide, poly(vinyl alcohol) and its copolymers, poly(hydroxyethyl methacrylate), silica, porous glass can be used as material supports not only for direct ligand coupling via e.g., cyanogen bromide, triazine or cyclic carbonate reaction, but also for modification to form other reactive functional groups (carboxyl, carbonyl, mercapto) [28]. Polymers containing *carboxyl groups* activated as acylazide, acid anhydride, active ester by condensation reagents and four centered reaction can be used for ligand immobilization employing various coupling reactions via amino groups of the protein to form an amide bond [29]. Poly(acrylic acid) and poly(methacrylic acid) are typical polymers used for these activations. They can be applied as homopolymer or copolymers with acrylamide. Poly(isocyanate styrene), poly(isothiocyanate styrene) or copolymers containing these styrene units and isocyanate or isothiocyanate groups (prepared by the reaction of polymers containing amino groups with phosgene or thiophosgene) have been used as

carbamylating or thiocarbamylating reagents for protein immobilization [30, 31]. Polymers containing *amide bonds*, polyacrylamide and polyamides are useful hydrophilic and electrically neutral supports for ligand coupling. Widely used as supporting polymers for protein immobilization are polymers that contain *aldehyde or acetal functional groups* [32]. Polymers containing *mercapto functional groups* such as glutathione-agarose or thiolated-agarose [33, 34] were used for specific reversible coupling to protein mercapto groups (cystein) via thioldisulfide interchanging reactions. Polyacrylamide supports can be chemically modified by reaction with hydrazine or with ethylenediamine to obtain the corresponding acyl hydrazide or aminoethyl derivatives. Polymers containing *3-fluoro-4,6-dinitrophenyl groups* such as poly(methacrylic acid-co-methacrylic acid 3-fluoro-4,6-dinitroanilide) or poly (methacrylic acid-co-methacrylic acid 3-fluoro-4,6-dinitrostyrene) can be used for coupling via arylation with the amino groups of different biomolecules [28].

The most common activating reagents for natural and synthetic materials are glutaraldehyde, carbodiimide, cyanogen bromide, epoxy or bioxirane, cyanuric chloride, carbonyl diimidazole, N-hydroxy succinimide esters, tosyl and tresyl chloride.

- **the cyanogen bromide reaction** is the most extensively used coupling method. The hydroxyl-containing polymer is activated with cyanogen bromide at pH 10-11.5. Compounds containing free amino groups can be attached covalently to the activated polymer at mild alkaline pH values. The cyanogen bromide activation reaction is considered to proceed via the 2,3-transimidocarbonate intermediate, leading to the formation of three possible types of bonds with protein amino groups: N-substituted imidocarbonate, N-substituted carbamate, N-substituted isoureas. The cyanogen bromide method was used for immobilization of different proteins on cellulose, agarose, dextran and copolymers of hydroxyethyl methacrylate with acrylamide [35-38].
- **the glutaraldehyde reaction** has been extensively used for the immobilization of various ligands such as proteins, enzymes and cells, on amine, amide or hydroxyl containing support materials, since it is inexpensive and easy to use [39-41]. Glutaraldehyde (GA) exists in different forms depending on the experimental conditions. At low pH, GA is in dilute solution present as monomer in its free aldehyde form, as hydrate or as hemiacetal, while it polymerizes at higher concentrations into oligomeric hemiacetals. All of these species can react with proteins and lead to immobilization. Under alkaline conditions, GA undergoes aldol condensation forming α,β -unsaturated multimeric aldehydes, which can also react with proteins either via the

formation of a stabilized Schiff base or the formation of a Michael adduct [41]. A large number of possible prescription reactions are described in the literature for ligand immobilization using glutaraldehyde. Activation of the support is often conducted at low pH to catalyze acetal formation, while the coupling of the biomolecule is carried out at elevated pH to promote the nucleophilic attack onto the carbonyl group improving thus the immobilization yield.

- **the triazine method** has been used mainly for polysaccharide and vinyl alcohol supports: cellulose, agarose, cross-linked dextran [42, 43]. Dichloro-s-triazinyl derivatives that react rapidly with protein are prepared by reaction with 2,4,6-trichloro-s-triazine (cyanuric chloride). The remaining chlorines may be treated with an amine to prevent further reaction, including polymer cross-linking. As the reaction of the second chlorine with protein is very fast, it is preferable to replace the second chlorine and use the third chlorine for the coupling reaction. Monochloro-s-triazinyl derivatives can be prepared by allowing the polymer to react initially with cyanuric chloride add then with a low-molecular-weight amine under controlled conditions to leave the third chlorine unreacted. It is, however, preferable to use 4,6-dichloro-s-triazine derivative, 2-amino-4,6-dichloro-s-triazine or the so-called Procion M dyes.
- **cyclic trans-2,3-carbonate method** has been first used for immobilization of different biomolecules on cellulose materials [44]. The reaction of cellulose with ethyl chloroformate in anhydrous organic solvents gives cyclic trans-2,3-carbonate derivative of cellulose. The trans-2,3-carbonate group is analogous to the cyclic trans-2,3- imidocarbonate structure, which is assumed to be the reactive intermediate in cyanogen bromide activation. Nucleophilic attack on the cyclic carbonate by a ligand containing amino group leads to ring opening and the formation of N-substituted carbonates.
- **the Curtis azide method**: the polymer containing carboxyl group is modified to an methyl ester, which is further reacted with hydrazine hydrate to form a hydrazide derivative. The hydrazide is then reacted with an aqueous sodium nitrate solution to give the azide derivative, which can be further reacted with a ligand amino group under mild alkaline conditions [45].
- **the coupling reagents method** involve water-soluble carbodiimides and similar coupling reagents that react with carboxyl groups of the polymer at slightly acidic pH values to give O-acyl isourea derivatives. These highly reactive intermediates either condense with amines to yield the corresponding amides or rearrange to an acyl urea. The most widely used water-soluble carbodiimides for the activation of carboxyl groups are 1-cyclohexyl-3,2-(4-N-

methylmorpholinium) ethyl carbodiimide tosylate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Other coupling reagents N-alkyl-5-phenylisoxazolium salt and N-ethoxy carbonyl-2-ethoxy-1,2-dihydroquinoline can be used in a similar manner [46] .

- **the four-component condensation reaction** combines the carboxyl and amino groups to form an amide bond in an aqueous medium at neutral pH and allow for considerable versatility and high selectivity. This reaction can be used both for carboxyl and amine-containing polymers [47].
- **the diazotization reaction** can be applied to the polymers containing arylamino functional groups for different proteins coupling [48, 49]. Arylamino-containing polymers are reacted with nitrous acid to form polymers containing aryldiazonium functional groups. The electrophilic aryldiazonium ion mainly attacks activated aromatic rings, such as phenols of tyrosine or imidazole of histidine to form the corresponding azo derivatives. Tyrosine and histidine react at mild alkaline pH values (8 to 9) at comparable rates to form monoazo derivatives. Amino groups (α -amines and the ϵ -amino groups of lysine) react under similar conditions with 2 mols of diazonium salt to give the disubstituted bis-azo derivatives known as triazenes.

2.3 The affinity ligands

In general, affinity binding between a ligand and a protein is more complex than protein adsorption on a plain surface. The ligands normally are positioned at a small distance from the solid surface and are able to form a sterical contact with the biomolecule. The ligands may possess also various functional groups that bind with the protein molecules through different types of interaction. Depending on the product to be isolated the ligands can be for instance a hormone, an inhibitor, an antigen, a specific binding protein or a co-factor, specifically chosen for an affinity adsorption process [50].

The ligand molecules can be divided in two groups: i) specific ligands, which show specificity for only one complementary biomolecule; ii) group ligands that are based on biological recognition parameters but, which are not targeted to a very specific conformation or sequence of the ligate. A group specific ligand may be a large macromolecule such as lectin, protein A or protein G. They may be also small co-enzymes such adenosine monophosphate (AMP) or adenosine triphosphate (ATP), nicotinamide-adenine dinucleotide (NAD) or nicotinamide-adenine dinucleotide phosphate (NADP). For instance, a large number of metabolic toxins such as free

fatty acids, endotoxins of gram-negative bacteria, mercaptans as well as medications like nortriptyline, amitriptyline, diazepam, bromazepam, bound preferentially to the albumin fraction of blood plasma.

Affinity ligands can be classified into biospecific and pseudo-biospecific ligands [6]. The *biospecific ligands*, although possessing high specificity with proteins, have certain deficiencies: high cost, low stability for potential large-scale application and difficulties in immobilization. Therefore, ligands with *pseudo-biospecific* affinity have been recently employed. They can be distinguished by biological (amino acids, specially histidine, lysine, tryptophan) or non-biological molecules (hydrophobic side chains, triazine dyes, metal ions). Phenylalanine (Phe) or tryptophan (Trp) containing immunoabsorbers based on polyvinyl alcohol materials have been used for rheumatism and myasthenia gravis therapy. Phe has been used as a ligand for the purification of serum proteins and enzymes. Affinity chromatography using immobilized metal ions is a highly efficient and flexible method for protein fractionating. The immobilized metal ligand has a high affinity for a group of proteins whose exposed surface contain histidine, cysteine or tryptophan amino-acids residues. The behavior of histidine for affinity chromatography using immobilized metal ions of proteins was most carefully investigated [7]. The most efficient ions are Cu^{2+} , Ni^{2+} , Zn^{2+} , Co^{2+} , Fe^{2+} . These metal ions were immobilized by chelation with an iminodiacetate group which has been covalently attached to an agarose or poly(vinyl alcohol) matrix. Immobilized dyes have been found to act as pseudo-affinity sorbers for a large number of biological molecules. The dyes are chemically stable, relatively inexpensive and have binding coefficients in the range needed for ready elution of ligates. Triazine linked dyes have been used to mimic coenzymes which bind a number of dehydrogenases, hexokinases, alkaline phosphatase, ribonuclease and carboxypeptidase. The Procion Blue binding of albumin is a well known method for purifying proteins from contaminating transferrin, or a protein of similar physico/chemical properties that often contaminates albumin. Immobilized Cibacrom Blue F3G-A has been used for the purification of over 80 enzymes and proteins [9]. The degree of ligand attachment to the activated sites is dependent on ligand-ligand and ligand-activator interactions, charge or steric effects and the nature of the matrix starting material as well as the number of activated functional groups for ligand coupling. An excessive number of ligands coupled to the surface has to be avoided because this may otherwise lead to crowding and interferes with ligate recovery.

2.4 Spacers molecules

Ligand-ligate complexes able to form in solution may not be able to form when one of the components is immobilized on a porous support. The interference may

derive from the nearby support material itself or from other adsorbed proteins (especially if the support participates in nonspecific bonding). Complex formation is inhibited because the ligate and ligand must compete with other proteins for the restricted space available adjacent to the immobilizing polymer. Impaired diffusion of the ligate to the ligand site due to the channel size or tortuosity may also cause interferences with the complex formation and the requirements for spatial orientation may not be achievable [9].

The employment of a molecular spacer facilitates molecular interactions between immobilized ligands and the targeted molecules. Relatively short aliphatic chains (4 to 10 carbon atoms in length) are ineffective for interactions involving one or more macromolecules, but longer aliphatic chains alter undesirable the hydrophobicity of the membrane surface. The length of the spacer molecule is often experimentally determined. Free hydroxyl groups of a polysaccharide or vinyl alcohol copolymer support can be linked to a protein ligand via a bifunctional spacer containing at least one terminal primary amino or hydrazido functional group for reaction with the membrane hydroxyl groups. The remaining terminal functional group of the spacer is selected for reaction with free amino or carboxyl functions of the ligand, or with free hydroxyl groups of a glycoprotein ligand. Convenient spacer molecules for these applications include diamine and dihydrazides such as C₁-C₆-alkyldiamines and C₁-C₆-dihydrazides and β-alanine hydrazide that provide free amino and hydrazido groups for reaction with the ligand. They can also be C₄-C₆-alkylamino acids, which provide free carboxyl groups for reaction with ligand. The immobilization of different enzymes with a spacer such as 6-aminocaproic acid yielded kinetic and structural characteristics more similar to the free enzyme, while providing increased stability and reusability relative to the latter. Triazine dyes (Cibacron Blue F3GA or one of the Procions) were coupled using polyethyleneimine (PEI) as a spacer to nylon membranes and their adsorption capacities for lysozyme, bovine serum albumin, malate dehydrogenase or glucose-6-phosphate dehydrogenase were investigated. The use of a long spacer molecule reduces steric interference at the surface and allows multiple layering of large macromolecules at the membrane surface. Polyethylene glycol with a suitable chain length generally between 50 and 250 carbon atoms can be covalently coupled to the membrane surface through its amino groups by a proper immobilization method. The advantages of polyethylene glycol as a spacer molecule are its hydrophilic nature and biological inertness. These qualities are quite beneficial in a device that contacts blood or plasma.

2.5 Advantages and applications of membrane chromatography

Considering the general properties of microporous membranes in comparison with particle beds, the main advantages are the low resultant flow resistance (higher porosity and reduced bed heights) and fast mass transfer due to convection that is, unlike to porous particles, not limited by diffusion. In membrane-based purification systems, high flow rates can be applied due to the high porosity and high mass transfer capability. Short diffusional distances allow optimal utilization of the immobilized ligand, which is situated at the inner surface of the membrane pores. Because of the short time that the ligand and product are exposed to harsh elution conditions, the possible denaturation of ligand and/or product is decreased as compared to packed columns. The membrane chromatography offers the possibility to operate under sterile conditions with good reproducibility [7, 16].

Microporous adsorptive membranes and related systems such as single or staples flat-sheet membranes, hollow-fiber, spiral-wound and cassette devices are already commercially available. Few of these systems (Sartobind, Sartorius; MemSep, Millipore; Quik Bind, Sepracor; Biorex, Bio-Rad; Lowprodyne, Pall; AbSorbent, Genex; ActiDisc, FMC Bioproducts; MAC, Amicon) have been already developed for a small number of technical applications [8, 9, 50]. The most widely used applications for protein purification are presented in Table 2.2.

2.6 CONCLUSION

Affinity membrane chromatography uses microporous membranes that contain biospecific ligand molecules attached to their inner pore surface, as adsorbents. As a result of the convective flow of the feed solution through the porous structures the mass transfer resistance is tremendously reduced and binding kinetic is usually dominating the adsorption process. This results in rapid processing and improved adsorption, washing, elution and regeneration steps, and minimize the probability of protein denaturation. In theory, the adsorptive membrane efficiencies should exceed those of granular beds by a factor of ten or even higher. However, a number of anomalous mass transfer effects such as non-uniform flow, dead spaces and extra-column dispersion have been observed. The advantages of membrane chromatography have been confirmed by many successful applications. Nevertheless there is scope for further improvement of the adsorptive membrane design by increasing the binding capacity, extension of the supplied ligands and optimization of the hydrodynamics, and to assess the use of membrane chromatography in large-scale processes.

Table 2.2 Support materials and affinity ligands used for protein purification by affinity separation processes.

SUPPORT	LIGAND	LIGATE
- Modified cellulose membranes - Hydroxyethyl cellulose-coated polysulfone - Regenerated cellulose	- Cibacron Blue Active Red K2BP Soybean trypsin inhibitor - Protein A - Cibacron Blue F3GA Monoclonal BSA antibody Recombinant protein G	- Lysozyme Alkaline phosphatase Porcine trypsin - Human IgG - Alkaline phosphatase BSA Bovine IgG
- Chitosan - Chitosan/polyethyleneimine - Chitosan coated (sulfonated) polyethersulfone	- Protein A BSA Polylysine - Cibacron Blue - Protein A Cibacron Blue	- Human IgG Bilirubin Bilirubin - HSA - IgG Human serum albumin
- Polyamide and polyacrylamide	- Aminobenzamide Aminobenzamide Modified C-reactive protein Protein A Cibacron Blue	- Urokinase Trypsine Human IgG Human IgG Bilirubin
- Poly(methyl methacrylate) and poly(glycidyl methacrylate)	- Protein A/G	- Human and mouse Ig G
- Poly(ether-urethane-urea)	- Protein A	- IgG
- Modified poly(caprolactam)	- Recombinant protein A/G Protamine	- Human IgG Heparin
- Poly(ethylene vinyl alcohol)	- L-histidine Poly(L-lysine)	- Human IgG Heparin
- Modified polyvinylidene difluoride	- Triphosphate isomerase Protein A	- Human IgM IgG
- Epoxy membranes	- Thiophilic ligands Collagen	- Monoclonal antibodies IgG ₁ IgG _{2a} Liver membrane proteins
- Polyetherimide coated titania	- Cibacron Blue F3GA	- HSA
- Poly(ethylene grafted glycidyl methacrylate)	- L-phenylalanine Tryptophan IDA-Cu ²⁺ BSA	- Bovine γ -globuline Bovine γ -globulin BSA L,D-tryptophan
- Modified nylon and nylon-methacrylate composite membranes	- Protein A/G Triazine dyes α -Cobratoxin L-histidine	- IgG BSA, lysozyme, malate dehydrogenase, glucose-6-phosphate dehydrogenase Nicotinic acetylcholine Human IgG
- Native and modified glass membranes	- IDA-Ni ²⁺ IDA-Cu ²⁺ BSA	- Recombinant fusion protein α -chymotrypsinogen, lysozyme, β -lactoglobulin, cytochrom C, ribonuclease A L,D-tryptophan

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CHAPTER 3

PREPARATION OF ETHYLENE VINYLALCOHOL COPOLYMER (EVAL) MEMBRANES SUITABLE FOR LIGAND COUPLING IN AFFINITY SEPARATION^{*)}

ABSTRACT

Hydrophilic microfiltration membranes with functional groups that can be used as coupling sites for ligands are of central interest in affinity separation, especially in view of biomedical applications. In this study we employed ethylene vinyl alcohol copolymer (EVAL) to prepare macrovoid-free open cellular-type membranes with a high internal surface area and interconnectivity that can chemically be modified in aqueous and organic media. To tailor the required membrane morphology, we investigated the ternary water/DMSO/EVAL system and quaternary systems using a series of n-alcohols (n=2-12) as non-solvent additives in the casting solution. Addition of solvent (DMSO) to the coagulation bath (water) performed in the ternary system to delay the onset of liquid-liquid demixing, resulted in structures dominated by solid-liquid demixing before macrovoid formation was completely suppressed. The symmetric particulate membranes obtained did not display the necessary mechanical strength. Addition of medium chain alcohols (n=7-8) in the casting solution yielded macrovoid-free cellular membranes with a significantly higher pore interconnectivity and structural integrity upon drying. A model is proposed to understand the influence of the alcohol chain length and concentration on the membrane morphology.

^{*)} This chapter has been accepted in J. Membrane Sci.

3.1 INTRODUCTION

Polymeric membrane materials with hydrophilic and/or functionalizable groups have gained increasingly more attention in recent years, especially in biological and biomedical applications. Incorporation of *hydrophilic groups* reduces the hydrophobic interaction between the biological compound and the membrane surface and increases the water-wettability of the membranes. Especially in protein separation, hydrophobic interactions between proteins and hydrophobic surfaces are generally responsible for non-selective (irreversible) adsorption and membrane fouling. *Functionalisable groups* can principally serve as coupling sites for ligands in affinity separation, which has become the preferred method for separation and purification of proteins and other ligates from biological fluids [1]. Specific interactions between the ligand and the ligate lead to a high selectivity for the species targeted. Alternative methods to obtain membranes with hydrophilic and chemically modifiable surfaces via physical or chemical post-treatment of hydrophobic membranes, often result in unwanted and irreproducible inhomogeneities [2]. Thus the main limitation for supports in affinity separation presently encountered lies in the availability of functional groups suitable for ligand coupling. This imposes an increased need for the development of hydrophilic microfiltration membranes with suitable functionalizable groups.

Ethylene-vinyl alcohol (EVAL), a semi-crystalline random copolymer consisting of *hydrophobic* ethylene and *hydrophilic* vinyl alcohol segments, has become a promising biomedical material, since it is water insoluble and possesses at the same time an excellent blood compatibility. EVAL displays a good mechanical strength in the wet state, has a high thermal stability and chemical and biological resistance, and is thus easy to sterilize using, e.g., γ -radiation [3]. Therefore, EVAL hollow-fiber membranes have been utilized in various kinds of blood purification devices including plasmapheresis and hemodialyzers [4]. Furthermore, EVAL has widely been used as food packaging material due to its excellent gas barrier properties and harmlessness to health. Recently, particulate EVAL membranes have been investigated for possible application in biomedical application such as plasma protein separation [5].

In the present work, we developed open cellular-type EVAL microfiltration membranes that can be used in affinity separation. The hydroxyl groups allow further functionalization in order to couple different ligands and to decrease non-specific adsorption via hydrophobic interaction. This minimizes the competition for the desired ligate and leads to higher selectivities. Activation of the alcohol groups in order to couple, e.g., proteins to the membrane surface, can principally be performed in organic as well as aqueous environment. Application in affinity

separation requires the preparation of open-porous membranes with a large internal surface area, high interconnectivity and mechanical stability. A maximal internal surface area is required to yield high adsorption capacities. To prepare the EVAL-membranes we used as a starting point earlier work of Young and co-workers, who employed EVAL mainly for the preparation of "particulate" membranes. To obtain the required membrane structure, the ternary water/DMSO/EVAL system and quaternary systems using n-alcohols with different chain lengths as additives are investigated. Possible mechanisms for the precipitation of EVAL membranes in the presence of these weak non-solvent additives are proposed in section 3.2. However, this work does not pretend to explain the complexity of membrane formation in the quaternary system, this contribution is experimental in its nature and correlates experimental observations.

3.1.1 Morphological tailoring of membranes prepared by immersion precipitation

Diffusion induced phase separation (*immersion precipitation*) has become a standard technique to prepare polymeric porous membranes [6, 7]. A homogenous polymer solution is cast as thin film and subsequently immersed into a non-solvent bath. The diffusional exchange of solvent and non-solvent brings the film solution into an instable state resulting in phase separation. Phase separation occurs by liquid-liquid (*l-l*) and/or solid-liquid (*s-l*) demixing, depending on the type of polymer and the precipitation conditions employed [8, 9].

For amorphous polymer systems, liquid-liquid phase separation occurs by nucleation and growth of the polymer-lean phase when the film composition enters locally the meta-stable region between the binodal and the spinodal (unstable with respect to *l-l* demixing). Pores are in this case formed from droplets of the polymer-lean phase that grow further by coalescence processes until the polymer-rich phase eventually solidifies and becomes the membrane matrix [6]. The (partial) phase inversion process performed can result in a variety of different cellular morphologies including open and closed sponge or finger-like pores. The (cellular) morphology depends strongly on the diffusion kinetics and thus on the rate of transfer of solvent and non-solvent [7, 10]. For a semi-crystalline polymer system the situation is more complex, since both *l-l* and/or *s-l* demixing can occur resulting in an enriched membrane morphology. The final membrane is determined by the thermodynamic and kinetic aspects of the system involved [8, 11].

Ternary mass transfer models have been used to calculate the diffusion trajectories and the (polymer) concentration profiles within the film in the first stages of

immersion [11-14]. Generally, two types of demixing are distinguished, namely *instantaneous* and *delayed* demixing with respect to *l-l* phase separation. Instantaneous *l-l* demixing occurs when the composition of the film falls immediately after immersion locally within the binodal envelope. This is normally the case when a (strong) non-solvent with a high affinity to the solvent is used in the coagulation bath. The corresponding polymer concentration profile shows a steep increase near the film/bath interface leading to asymmetric open cellular membranes with typically a thin dense top layer (skin) and possibly macrovoids in the porous support layer [9, 11]. If the demixing is delayed, compositional changes in the film will not be disrupted by phase separation. Desolvation can thus proceed for a longer time without triggering demixing resulting mainly in a higher polymer concentration throughout the polymeric film. In this case, the polymer concentration profile within the film becomes less steep and more symmetric structures are obtained. Delayed demixing conditions generally suppress macrovoid formation and lead for amorphous polymer systems to the formation of a thick dense top layer. Additional morphological fine-tuning has been performed by adjusting the temperature of the precipitation bath or addition of a fourth component to the casting solution. For semi-crystalline polymer systems, delayed conditions suppress *l-l* demixing and thus favors *s-l* demixing by allowing crystallisation to take place [8,11].

3.1.2 Asymmetric and particulate EVAL membranes

Young and coworkers [15-22] have performed the most extensive study on the preparation of porous EVAL membranes. In order to obtain a better understanding of the different membrane formation processes taking place, they investigated the thermodynamic (phase diagrams) and kinetic (onset of demixing) properties for the *non-solvent*/DMSO/EVAL system. For all non-solvents investigated the binodals fall (at room temperature) within the crystallization lines [15, 17, 20]. In the region that is metastable with respect to *s-l* demixing a gel is observed that formed from the collapsed polymer coils and consists of interconnected submicrometer-sized spherically shaped particles [15]. Since in the immersion process, the casting film composition changes such that the isothermal crystallization line is passed first, one would expect, from the thermodynamic point of view alone, that crystallization dominates the phase inversion process. Young et al. showed that symmetric particulate membranes, typical for membranes obtained by *s-l* demixing, are only formed if *l-l* demixing is sufficiently suppressed. The membrane morphology is thus mainly kinetically controlled. When the time between immersion and the onset

of phase separation is too short, nuclei of the crystalline phase have no chance to form and grow.

In the ternary non-solvent/DMSO/EVAL system using water as strong non-solvent for EVAL, immersion of the cast film led to instantaneous demixing due to the high affinity between water and DMSO. The resulting asymmetric membranes showed features characteristic for fast l-l demixing. They consisted of a very thin continuous, relatively dense top layer (skin) and a cellular porous sublayer with large finger-like macrovoids and displayed low water fluxes in the range of 20-60 l/h/m²/bar. Calculated polymer concentration profiles showed a steep increase of the polymer concentration near the film surface [18]. Crystallization was assumed to occur only in the later stages when the polymer-rich phase became highly supersaturated. Traces of the crystallization process were only visible in the cell walls.

Particulate structures, typical for s-l demixing membrane formation, were in the ternary system only obtained when 63% DMSO was added to the coagulation bath and the demixing process thus delayed by more than about 15s [18]. The skinless symmetric membranes obtained consisted of a bicontinuous porous structure built up by interlinked submicrometer-sized polymer particles. The membranes were assumed to form by homogeneous nucleation and growth of the polymer particles over the entire cross-section. If a weak non-solvent with a lower affinity to DMSO, such as 2-propanol or 1-octanol, was used instead of water, delayed conditions and particulate structure could be obtained without DMSO addition in the coagulation bath [5, 17, 22]. Symmetric particulate membranes obtained by precipitation in a coagulation bath containing a weak non-solvent or a sufficient amount of DMSO, displayed higher pure water fluxes in the range of 90-140 l/h/m²/bar [16] but suffer, however, from brittleness. Alternatively, crystallization has been induced by either performing a solvent evaporation [23, 24] or a non-solvent vapor sorption step [25] before immersing the cast film into the coagulation bath. Phase separation then took place by a slow s-l demixing leading to particulate membrane with water permeabilities in the range of 300-350 l/h/bar/m². Thermally induced phase separation (TIPS) process represents an alternative way to produce particulate EVAL membranes [26, 27]. The water permeabilities are in the range of 720-1080 l/h/bar/m². The membrane structure, similar to the particulate membranes prepared by Young et al. make us to believe that the mechanical strength of these membranes is also rather low for industrial applications.

For all the different EVAL systems studied, Young et al. observed a traversing from asymmetric cellular membranes with large macrovoids over macrovoid-free sponge type pores with a more open skin to symmetric particulate structures by adjusting the precipitation conditions from instantaneous to delayed demixing

conditions. With respect to affinity separation, the cellular membranes prepared previously display a too low water permeability and the particulate membranes a too low mechanical stability. Furthermore, membranes that can be easily functionalized in organic media are needed. To be able to utilize a large variety of surface modification methods in aqueous as well in organic solvents, the membranes should be able to withstand a drying procedure without pore collapse.

3.2. EXPERIMENTAL

3.2.1 Materials

As membrane material, EVAL (a random copolymer of ethylene and vinyl alcohol) with an average ethylene content of 44 mol% was purchased from Aldrich and used without further modification. Dimethylsulfoxide (DMSO) was employed as solvent. *n*-alkyl alcohols with varying hydrocarbon chain length ranging from 2-12 C atoms were investigated as non-solvents and additives in the casting solution. Water was used as non-solvent in the coagulation bath. DMSO (Merck), ethanol (Merck), 1-propanol (Merck), 1-butanol (Merck), hexane (Merck), 1-hexanol (Fluka), 1-heptanol (Fluka), 1-octanol (Fluka) 1-decanol and 1-dodecanol (Merck) were used as received. Ultrapure water was prepared using a Millipore purification unit Milli-Q plus. All concentrations are stated in wt%.

3.2.2 Preparation of EVAL-membranes

All membranes were obtained by immersion precipitation. The membranes were prepared by casting homogeneous solutions of EVAL in DMSO at room temperature on a glass plate, which was then immediately immersed into a coagulation bath. The concentration of EVAL was varied between 10 and 20 %, but membranes prepared from solutions of 10% EVAL were mainly used for further characterization. In order to improve the membrane morphology, films were cast with and without additives (in concentrations of 5-25%) in the casting solution and precipitated in an aqueous coagulation bath containing different amounts of DMSO (up to 75%) at temperatures between 20-50°C. The membranes were washed to remove traces of additive and/or DMSO and dried either by replacing the water consecutively with organic solvents of different polarity (for the membranes prepared without additives) or directly in air (for the membranes prepared in the presence of additives).

The preparation conditions for the membranes described in detail in this paper are presented in Table 3.1. Samples were identified in the way that first the concentration of DMSO in the coagulation bath is given, followed by the bath temperature and if applicable the concentration of additive (octanol) in the casting solution. A polymer concentration of 10%wt was used in the casting film.

Membrane	DMSO in the coagulation bath, %	Temperature of the coagulation bath, °C	Additives in the casting solution, %
E/0/20	0	20	0
E/0/50	0	50	0
E/0/50/O10	0	50	10% octanol
E/0/50/O20	0	50	20% octanol
E/25/20	25	20	0
E/25/50	25	50	0
E/50/20	50	20	0
E/50/50	50	50	0
E/75/20	75	20	0
E/75/50	75	50	0

Table 3.1 Preparation conditions for the investigated membranes, cast from solutions with a polymer concentration of 10% EVAL in DMSO.

3.2.3 Characterization of EVAL-membranes

For characterization by *scanning electron microscopy*, pieces of the membranes were frozen in liquid nitrogen and broken in order to expose the cross-sectional areas. The dried samples were coated with platinum using a Jeol JFC-1300 Auto Fine Coater and examined using a Jeol JSM-5600 LV Scanning Electron Microscope.

Light transmission experiments were performed in order to determine the delay time of the casting solution before phase separation sets in. Details of the experimental setup are described in [28]. Membranes were cast on a small glass plate and immediately immersed into the coagulation bath. Light transmittance was measured by placing a collimated light source above and a detector below the coagulation bath and recorded as a function of time using a data acquisition system. The light intensity profile was plotted as a function of time. When phase separation sets in, the incident light becomes scattered by the inhomogeneities appearing in the polymeric film causing a decrease in light transmittance. The final value recorded was used to normalize the light transmission decay. Assuming that all membranes investigated display a similar thickness and porosity (as will be shown in Section 3.3.2), the light transmittance decay can be used as a measure for the demixing rate.

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

The *pore size distribution* was determined using a Coulter^R Porometer II. The membranes were impregnated with Porofil (Aldrich) and the pressure determined needed to expel the liquid from the pores. The resulting gas flux was measured as a function of the applied pressure and used to calculate the pore size distribution.

The *pure water flux* was determined using a dead-end ultrafiltration cell connected to a gas cylinder of compressed nitrogen to apply the feed pressure. The filtration experiments were carried out at room temperature and a transmembrane pressure of 1 bar. The pure water flux was determined after steady state conditions were reached.

3.3 RESULTS AND DISCUSSION

3.3.1 EVAL membranes prepared in the ternary water/DMSO/EVAL system

DMSO is one of the few solvents for EVAL that allows the preparation of suitable casting solutions and can be employed in immersion precipitation with water as non-solvent. The type of phase separation that dominates membrane formation for semi-crystalline polymer systems depends on the phase behaviour, and thus the positions of the binodal and the crystallization isotherm, and also on the kinetics of the phase transitions. Since we were interested in open cellular-type porous membranes with high porosity and mechanical strength, it was necessary to investigate in detail the low polymer concentration region and to avoid crystallization, macrovoid formation, and low pore interconnectivity. A minimum viscosity is, however, required to obtain membranes with an adequate mechanical strength. Solutions of EVAL in DMSO display a sufficient viscosity for film casting at polymer concentration higher than 8%. Although solutions with 10-20% EVAL have been investigated, mostly results from solutions of 10% are presented below, since any further increase of the polymer concentration results in lower water permeability.

Membrane formation and morphology

Figure 3.1 shows a cross-section of the membrane E/0/20, which has been prepared from a solution of 10% EVAL in DMSO and coagulated in water at 20°C, under different drying conditions. Since EVAL is a polymer with a low elastic modulus, the structure of the dry and the wet membrane may differ significantly due to pore collapse upon drying. When dried directly in air (Figure 3.1A), the membrane displays a dense structure on the glass side that is probably caused by a collapse of the pores formed initially. To avoid pore collapse, different drying methods were applied. Figure 3.1B shows the membrane E/0/20 dried by exchanging water progressively with ethanol and hexane. The membrane displays characteristic features for rapid demixing, such as extended macrovoid formation. Since DMSO and water have a high mutual affinity, a fast exchange of solvent and non-solvent takes place upon immersion into the coagulation bath. The casting solution transverses the crystallization line before crystallization can set in so that liquid-liquid demixing dominates the membrane structure. The macrovoids form from nuclei of the polymer-lean phase underneath the skin layer and extend over the entire thickness of the membrane [6, 7]. The resulting asymmetric membrane, composed of a thin dense skin layer and a cellular porous support with large finger-like macrovoids (Figure 3.1B) is unwanted for application in affinity separation. Both micrographs show, although to a different extend, that the membranes obtained suffer from delamination at the mechanically weak layer in the middle of the cross-section. The weak spots in the membranes can lead to mechanical failure under pressure. Furthermore, macrovoids decrease the internal surface area of the membranes, which is especially unfavorable in affinity separation.

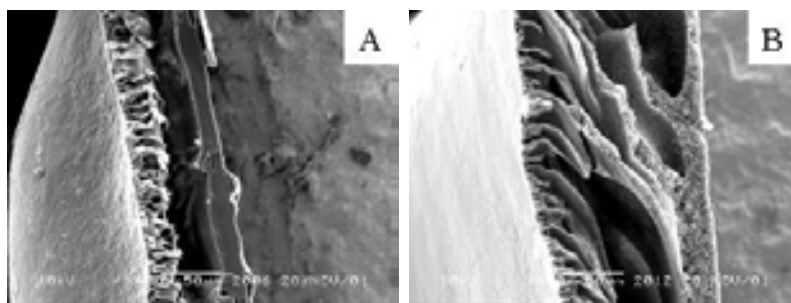


Figure 3.1. SEM micrograph of the membrane E/0/20 prepared by immersing a 10% EVAL film into water at 20°C and dried under different conditions: A) dried directly in air; B) dried by exchanging water progressively with ethanol and hexane. Both membranes are shown with top surface on the left at a magnification of 400. The size bar indicates 50 μm .

To obtain highly porous but mechanical stable EVAL membranes, the influence of temperature and the composition of the coagulation bath were further investigated. Temperature can principally have an influence on both the kinetics and the thermodynamics of the phase separation processes involved. Phase diagram studies, performed by Young et al. on the ternary water/DMSO/EVAL system, showed that the crystallization line depends strongly on temperature, while the position of the binodal is only little affected [21]. Upon increasing temperature, the crystallization line is shifted towards the water/EVAL side of the Gibbs phase triangle (lower DMSO content), whereby the distance between both demixing lines decreases until they finally intersect around 65°C. *Liquid-liquid* demixing is therefore expected to dominate membrane formation at low polymer concentrations (low supersaturation) and temperature higher than 65°C. Figure 3.2A presents a membrane precipitated in water at 50°C. The obtained membrane displays a dense top layer and large voids across the entire cross-section of the membrane, indicating a fast demixing process. In the regions surrounding the macrovoids, a porous structure is formed.

Macrovoids that are assumed to form by simultaneous inflow of solvent and non-solvent to nucleation sites underneath the skin layer. Macrovoid growth takes place as long as the surrounding polymer solution remains stable against phase separation [6, 29]. Macrovoid formation is generally suppressed or eliminated under delayed demixing conditions, such as using a solvent/non-solvent pair with a low affinity or by addition of solvent to the coagulation bath. When phase separation starts to set in, the composition in front of the first nuclei has already become unstable against *l-l* demixing and new nuclei can form. Adding increasing amounts of solvent into the coagulation bath decreases the rate of solvent/non-solvent exchange, which should result in increasingly more delayed demixing, enabling the preparation of membranes with significantly different morphologies (Figure 3.2).

The cross sections in Figure 3.2 demonstrate clearly that an increase of the DMSO concentration in the coagulation bath suppresses macrovoid formation. At 25% DMSO in the precipitation bath, an asymmetric EVAL membrane is formed, which still consists of a nearly dense skin and cellular pores with macrovoids that cover most parts of the sublayer (Figure 3.2B). The number of voids per unit of membrane volume decreases with increasing solvent concentration in the coagulation bath. At 50% of DMSO macrovoids are only observed in the upper half (Figure 3.2C). The membrane surface morphology has also changed significantly, whereby pores occur in the skin layer. The same trend can be observed in the polymeric walls surrounding the macrovoids. The pore interconnectivity remains, however, low. At 75% DMSO, symmetric membranes

were obtained constituting of submicron diameter particles packed into a bicontinuous porous structure (Figure 3.2D). Under high delayed conditions, the polymer solution may become saturated with respect to crystallization while it remains stable against *l-l* demixing. In this case the thermodynamically favored process dominates the phase separation process. Preparation of symmetric particulate membranes requires a more homogeneous polymer concentration allowing for a uniform distribution of nuclei over the entire film cross-section. The crystalline nuclei can then grow simultaneously until their fronts attach and they join adjacent particles to form a skinless and microporous structure. The shape of the particles depends on the evolution of the crystalline phase and may range from leafy to spherical morphologies [8, 30, 31]. At a polymer concentration of 10% the number of crystalline particles is rather low and the size of the pores that are formed by the interstitial space of the aggregated particle network lies in the micrometer range. Due to the high amount of solvent in the coagulation bath the membranes prepared at this low polymer concentrations are mechanically relatively weak.

Onset of phase separation and type of demixing

In order to obtain a better insight into the phase separation processes taking place during the formation of the membranes, light transmission experiments were performed. Figure 3.3 presents the measured delay times for demixing (t_d) as a function of the amount of DMSO in the coagulation bath. Since water is a strong non-solvent for EVAL, onset of demixing was observed at $t_d < 1$ s indicating that phase separation takes place instantaneously after demixing. Due to the high affinity between water and DMSO, large amounts of DMSO in the coagulation bath are necessary to obtain delayed demixing conditions. When the amount of DMSO is increased from 50 to 75% the induced delay time rises from $t_d = 13$ s to $t_d = 80$ s. This extended delay of demixing allows the formation of crystalline nuclei and membranes whose morphology results from *s-l* demixing. Membranes with particulate morphologies were obtained when the concentration of DMSO in the bath is higher than 60%.

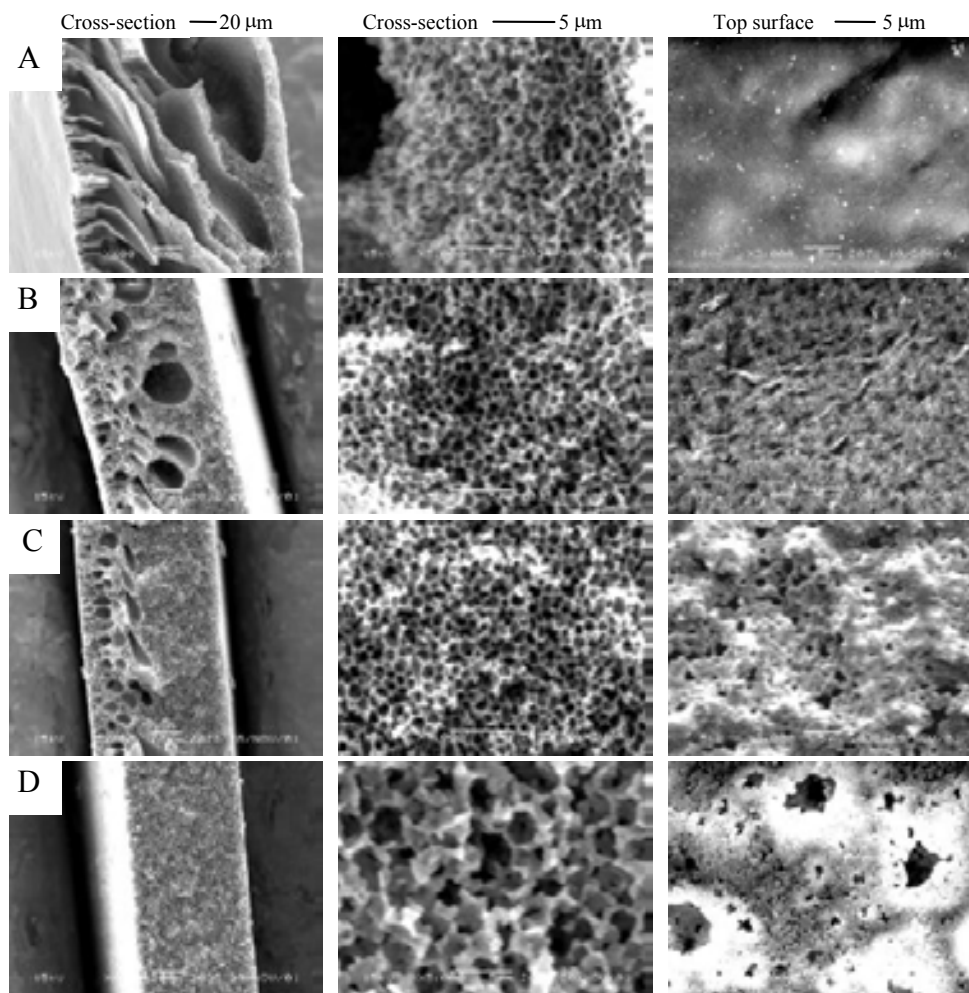


Figure 3.2 SEM-micrographs (cross-sections and top surfaces) of membranes prepared from 10% EVAL in DMSO using varying amounts of solvent in the coagulation bath at 50°C: A) E/0/50, B) E/25/50, C) E/50/50, D) E/75/50. All membranes were dried by replacing water progressively with ethanol and hexane. The different sections are displayed at the same magnification. For the cross-section micrographs (left columns), the top surface is shown on the right.

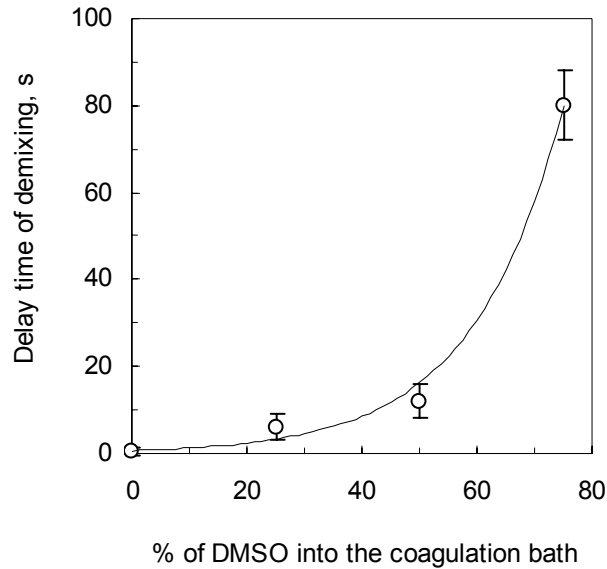


Figure 3.3 Measured delay times before phase separation occurs in polymeric films that consisted of 10% EVAL in DMSO as a function of the DMSO content in the coagulation bath at 50°C. The SEM-micrographs of the corresponding membranes are shown in Fig. 3.2.

Under instantaneous demixing conditions the polymer concentration across the casting film has hardly changed before phase separation sets in. Concentration profiles obtained from mass transfer models show generally a steep increase in the polymer concentration near the surface. The high polymer concentration at the interface is responsible for the formation of a thin dense skin that is often accompanied by membrane-spanning macrovoids. Its actual value (position at the bimodal) is given by the equilibrium condition and depends on the concentration of polymer in the film and solvent in the coagulation bath [6]. If DMSO is added to the coagulation bath, the driving force for the solvent outflow is reduced, the interfacial polymer concentration decreases and the profile becomes less steep [12]. The macrovoid formation becomes increasingly unfavorable and a braking up of the skin layer is induced.

Membrane characterization

The membranes prepared from the ternary water/DMSO/EVAL system show in general a strong tendency to collapse. This makes it necessary to perform a tedious solvent exchange treatment or to keep the membranes wet. The later has as consequence for further ligand coupling that only surface activation chemistry based on aqueous solutions can be applied. Furthermore, mechanically stable membranes could at the investigated polymer concentration only be obtained for membranes with cellular morphology.

The porosity of the membranes, measured from swelling experiments, reflects the morphological changes obtained by increasing the amount of DMSO in the coagulation bath. Membrane E/0/50 has a porosity in the range of 80-85%. Upon increasing the DMSO concentration in the coagulation bath, the membrane structure has changed; the skin layer becomes more open and fewer macrovoids are formed. The membrane porosity decreases to 75% for the membranes coagulated in a 50% DMSO in the precipitation bath. For the membranes precipitated in 75% DMSO, the presence of particulate structure goes along with an increase in the membrane porosity to 75-80%.

The pure water permeability for membranes prepared from a solution of 10% EVAL is presented as a function of the composition of the coagulation bath at 50°C (Figure 3.4). In accordance with the observations from the SEM studies, the water flux increases by increasing the DMSO content in the coagulation bath. A similar trend, but lower water permeabilities are, however, observed for the membranes prepared using a coagulation bath at 25°C. The membranes with 25-50% DMSO in the precipitation bath display an asymmetric structure. Due to the poor pore interconnectivity, the water flux is relatively low even if the skin layer becomes more open. The water permeability is higher in the case the membranes are precipitated in 75% DMSO, because of the improved interconnectivity and the larger pore sizes of the particulate structure. Unfortunately, these membranes are mechanically weak. Therefore, the membrane E/50/50 prepared at 50°C with 50% DMSO in the coagulation bath has been characterized in more detail and used for comparative studies described in *Section 3.3.3*.

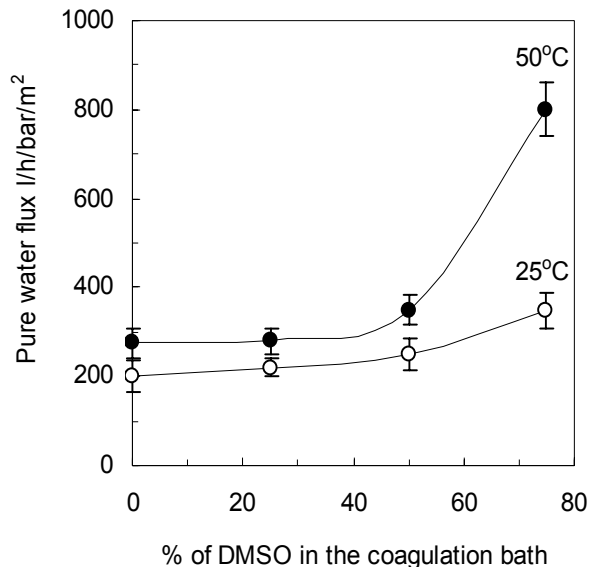


Figure 3.4 Pure water permeability measurements of membranes prepared from 10% EVAL in DMSO as a function of the DMSO content in the coagulation bath at 25 and 50°C. The lines are included to guide the eye.

3.3.2 EVAL membranes prepared in quaternary systems water/n-alcohol/DMSO/EVAL

As can be seen from the previous section, the actual parameter gap to tailor a more symmetric open-porous cellular membrane is quite small. At 50% DMSO in the coagulation bath macrovoid formation is not completely suppressed, while at 60% DMSO crystallisation already determines the membrane morphology. It is therefore difficult to adapt the preparation conditions such that both, the low interconnectivity of the cellular membranes obtained and their tendency to collapse upon drying, can be improved simultaneously. In order to overcome the limited number of parameters that allow tuning of the membrane morphology in a ternary system non-solvent/solvent/polymer, we extended our investigation to four component systems. Principally, extension to a four components system offers more possibilities to tailor the membrane structure and to suppress macrovoid formation [32-39]. Therefore, addition of salts [35], complexing agents [36], co-solvents and non-solvents with low or high molecular weight [33, 34, 37-39] into the casting solution has been investigated.

It has generally been observed that macrovoid formation can be suppressed by addition of a weak non-solvent into the casting solution, which favors destabilization of the polymer solution in front of the first nuclei formed [6, 29, 40] and that secondary phase inversion processes can increase the pore interconnectivity [6, 8]. It is assumed that the dense skin, formed under instantaneous demixing conditions, hinders the inflow of water. If the polymer solution in front of the first formed nuclei remains therefore stable against *l-l* demixing, macrovoids can grow out of these first born nuclei by influx of solvent from the surrounding polymer solution [29, 40]. Addition of a non-solvent favors destabilization of the polymer solution and phase separation sets in before these nuclei have grown in size. In the studies performed previously [6, 29, 33], the non-solvent used as additive possesses usually a high activity to water and does therefore not hinder the water from diffusing into the polymeric film.

In the present study, we investigated n-alcohols with different hydrocarbon chain length n ($n=2-12$) as additives in the casting solution, since we found that addition of the n-alcohols to the polymer solution results in cellular membranes that do not collapse upon drying. This allows surface modification based on reactions carried out in aqueous solutions as well as in organic solvents. Isopropanol and n-octanol have already been employed as weak non-solvents in the coagulation bath in the preparation of EVAL membranes [19]. In these cases *l-l* demixing was delayed to such extent that symmetric membranes with particulate structures were obtained.

Membrane morphology

Figure 3.5 displays the cross-sections of membranes prepared by immersing solutions containing 10% EVAL and 10% of n-alcohol into a water coagulation bath at 50°C. The aliphatic chain length of the n-alcohol was varied between 2 and 12 C atoms. For alcohols with a short hydrocarbon chain ($n=2-4$), the phase inversion process is still governed by fast *l-l* demixing. The resulting membranes (Figure 3.5A–C) possess large voids across the entire cross-section and a porous structure between them. The top surface as well as the macrovoids are covered by a dense layer. If the hydrophobic character of the alcohols increases, the number of macrovoids per unit of membrane volume decreases. Membranes prepared using alcohols with $n=6-8$ as additive, have fewer macrovoids (Figure 3.5D–F) and show pores on the top layer and in the walls surrounding the macrovoids. A further increase in alcohol length ($n=10-12$) leads to the opposite effect. An increased number of macrovoids across the cross-section with an open-porous structure around the voids can be observed in Figure 3.5G–H for membranes prepared in the presence of decanol and dodecanol. These results suggest that there is an optimum

chain length-dependent alcohol concentration to obtain symmetric open-porous membranes. All membranes prepared with the n-alcohols in the casting solutions do not show a particulate morphology.

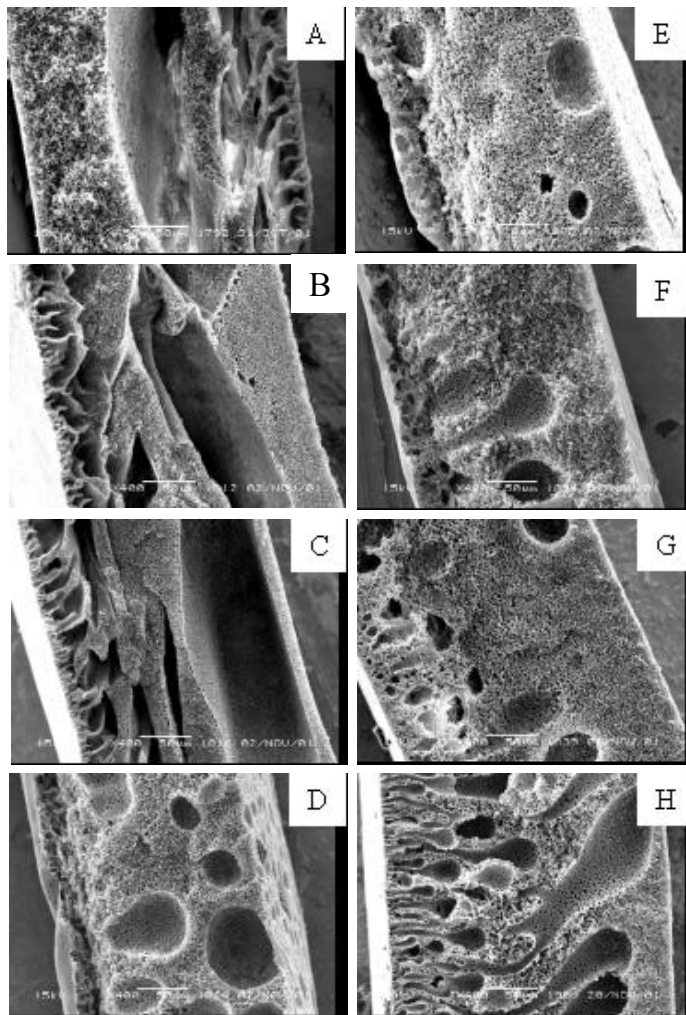


Figure 3.5 SEM-micrograph cross-sections for membranes prepared from 10% EVAL in DMSO using 10% of n-alcohols as additive in the casting solution: A) ethanol; B) 1-propanol; C) 1-butanol; D) 1-hexanol; E) 1-heptanol; F) 1-octanol; G) 1-decanol; H) 1-dodecanol. All membranes were prepared by immersing the cast film in water at 50°C and dried directly in air. The cross-sections are displayed with the top surface on the left at the same magnification of 400. The size bar corresponds to 50 μm .

Delay time adjustment

Light transmission measurements performed for the same series of membranes reflect the trend observed for the membrane morphology (Figures 3.6 and 3.7). Addition of short chain alcohols ($n < 4$), which are either completely or to a large extent miscible with water, yields only short delay times ($t_d < 3$ s) so that membrane formation takes place under instantaneously or fast demixing conditions. The decay in light transmittance resembles that recorded for the ternary system (membrane E/0/50). If the hydrophobicity of the alcohol is further increased a maximum around $n = 7-8$ is observed. For these alcohols the transmittance remains almost 100% for up to 9s. When the aliphatic chain of the alcohols is further increased, shorter delay times are recorded (Figure 3.6).

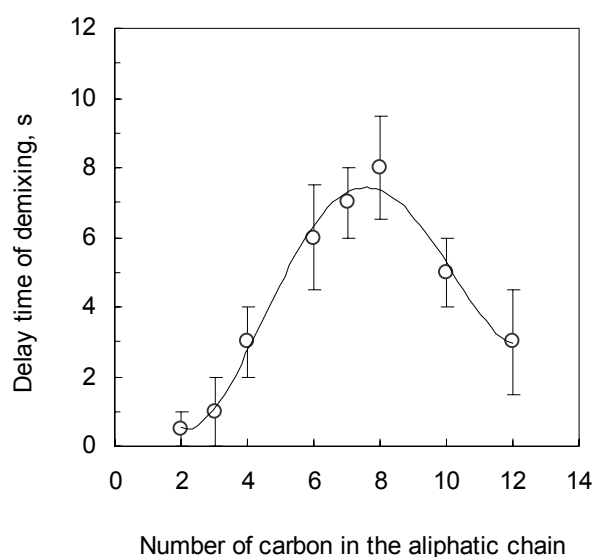


Figure 3.6 Onset of demixing as a function of the alcohol chain length. The SEM-micrographs of the corresponding membranes are shown in Fig. 3.5.

The delay time measured for dodecanol is of the same range as that for butanol, but the recorded transmittance is higher and thus indicates a different phase separation process. At the maximum, the delay time ($t_d = 9$ s) is significantly shorter than the delay time of membranes for which particulate structure formation has been observed in the ternary system. Crystallization could only set in when the phase

separation process has been delayed to $t_d > 30s$ by the addition of DMSO to the coagulation bath. For the fully developed particulate structure shown in Figure 2D a delay time of $t_d = 80s$ was measured which is 10 times as high.

It has been shown previously [41] that a lower miscibility between the solvent and the non-solvent can reduce the tendency of the casting solution to phase separate due to a lower influx of non-solvent (water) into the polymer solution. In our case, we foresee that the diffusion of water into the cast polymer film becomes increasingly hindered if the hydrophobic character of the added alcohols increases. As a result the demixing process responsible for membrane formation is slowed down for the membranes prepared in the presence of long aliphatic chain alcohols. Since the mutual miscibility between water and n-alcohols changes mainly in the range of $n=3-6$, we also expect a pronounced increase in the delay time in this region. Increasing the alcohol chain length ($n > 8$) should therefore not lead to a significant further increase in the delay time. At these induced delay times ($t_d \sim 10$) the non-solvent character of the alcohol for the EVAL polymer becomes more important and might prevail. The polymer solution becomes then unstable with respect to *l-l* demixing. The lowest rates for the decrease in light transmittance (which we assume to indicate the velocity of the membrane formation process) were obtained for the membranes prepared employing alcohols with $n=6-8$ (Figure 3.7).

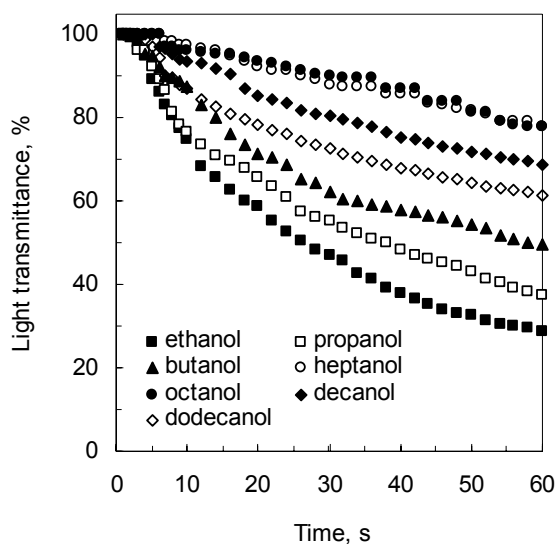


Figure 3.7 Light transmittance recorded after immersing polymeric films consisting of 10% EVAL in DMSO and 10% of different n-alcohols as additives, into water at 50°C.

Precipitation of EVAL in the presence of *n*-alcohols

In order to obtain a better insight into the phase separation processes involved, Figure 3.8 visualizes schematically the two different situations encountered during the membrane formation process in the quaternary water/alcohol/DMSO/EVAL system. Alcohols with a short hydrocarbon chain ($n=2-3$) are miscible with the non-solvent (water) used as coagulant. After immersion, solvent diffuses into the coagulation bath whereas water diffuses into the cast film. Since these alcohols are completely miscible with water, they will also diffuse into the coagulation bath (Figure 3.8A). Demixing occurs instantaneous and membranes with large voids across the entire cross-section are obtained. The non-solvent character of the alcohol itself is thereby unimportant as long as its concentration remains low enough to maintain a casting solution, which is stable against *l-l* and *s-l* demixing.

Alcohols with $n \geq 6$ are immiscible with water. When the cast film is immersed into the bath, solvent diffuses into the coagulation bath, but the with water immiscible non-solvent additive remains inside the cast film (Figure 3.8B). Water diffusion into the polymeric film is hindered by the presence of these alcohols. This lowers the coagulation rate since the inflow of water in the polymeric film is hampered. Close to t_d the concentration profile has changed so far that the polymer solution becomes locally instable with respect to *l-l* demixing and phase separation sets in. The mutual solubility of water and *n*-alcohol depends on the hydrophobicity of the alcohol. The hydrophobic character of the alcohol increases strongly in the range of $n=4-6$ but is less effected at higher chain lengths. The water inflow and thus the induced delay time rise with the increasing hydrophobicity. The more solvent flows out the film before phase separation sets in, the higher becomes the concentration of alcohol in the remaining polymeric solution. Phase separation takes place under delayed conditions and macrovoid formation is therefore less favored if t_d increases.

Whether macrovoid formation is only suppressed due to the delayed demixing conditions or also by the presence of the water insoluble alcohols in the film is not possible to say. Principally, macrovoid formation caused by instantaneous demixing can also be reduced by addition of a weak non-solvent to the casting solution [6, 20, 21]. It is assumed that the dense skin, formed under instantaneous demixing conditions, hinders the inflow of water. If the polymer solution in front of the first formed nuclei remains therefore stable against *l-l* demixing, macrovoids can grow out of these first born nuclei by influx of solvent from the surrounding polymer solution [20, 21]. Addition of a non-solvent favors destabilization of the polymer solution and phase separation sets in before these nuclei have grown in size. In the studies performed previously [6, 21, 24], the non-solvent used as

additive possesses usually a high activity to water and does therefore not hinder the water from diffusing into the polymeric film. In this context, it might be noteworthy to mention that the symmetric EVAL membranes prepared employing the longer chain alcohols as additive display a higher pore interconnectivity than those prepared in the ternary system using a higher concentration of DMSO in the coagulation to delay demixing.

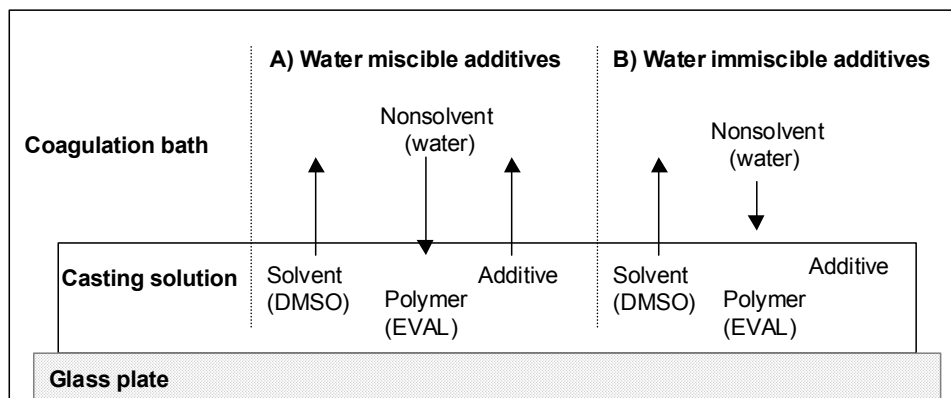


Figure 3.8 Schematic representation of the two different situations encountered in the water/n-alcohol/DMSO/EVAL system using the alcohols as additive in the casting solution: A) water miscible additives ($n \leq 4$) with exchange of both non-solvent and additive between the polymer film and the coagulation bath; B) water immiscible additives ($n \geq 6$) with no or very limited exchange of additive and non-solvent between the polymer film and the coagulation bath.

For alcohols with $n \geq 10$, the hydrophobicity only slightly increases. One would therefore expect that employing decanol or dodecanol would hardly lead to a stronger hindrance of the inflow of water into the polymeric film. Following this argument, the delay time should reach a plateau value if the alcohol chain length is increased. Since we observed a decrease in the delay time and the simultaneous reappearance of macrovoids, the non-solvent character must have become prevalent. In order to obtain an estimate of the non-solvent character of the different alcohols, we use the differences in the solubility parameters between EVAL and the alcohols employed [42]. EVAL with an ethylene content of 44% has a solubility parameter δ of $\delta = 23 \text{ MPa}^{1/2}$ (estimated by interpolation from the solubility parameters of polyethylene ($15.76 \text{ MPa}^{1/2}$) and poly(vinyl alcohol) ($25.78 \text{ MPa}^{1/2}$) [43]), while that for the alcohols varies between $\delta = 20\text{-}26 \text{ MPa}^{1/2}$, whereby δ increases with decreasing chain length of the n-alcohol. Figure 3.9 shows the modulus of the difference of the solubility parameters of the alcohols

and the polymer $|\Delta\delta_{a-p}|$ as a function of the alcohol chain length. $|\Delta\delta_{a-p}|$ displays a minimum for heptanol and octanol. A further increase in the aliphatic length results in an increased difference of the solubility parameters.

A higher value for $|\Delta\delta_{a-p}|$ indicates a stronger non-solvent character. Therefore, alcohols with a short ($n < 4$) and a long hydrocarbon chain ($n > 10$) should display a stronger non-solvent behavior in comparison with alcohols with $n = 6-8$. This would make the casting solution easier to phase separate and might lead to the formation of porous membranes with large voids across the cross-section. If the decrease in delay, observed for decanol and dodecanol, is caused by the increased non-solvent character of the additive, then one would also expect a chain length-dependent optimum in the concentration of the alcohol used. For a more detailed study we chose to use n-octanol as additive.

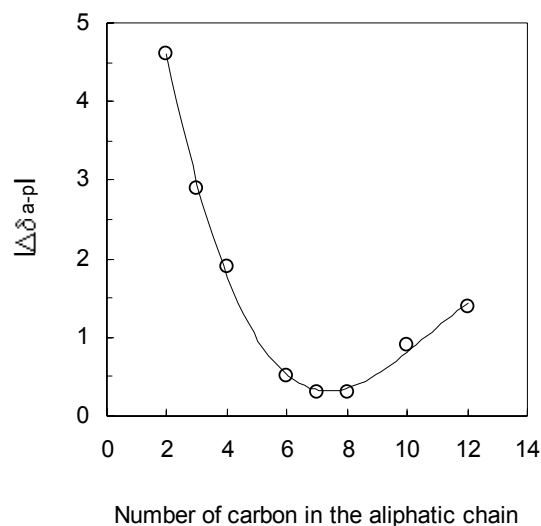


Figure 3.9 Variation of the solubility parameter difference $|\Delta\delta_{a-p}|$ obtained from [44] as a function of the hydrocarbon chain length of the alcohols used as additives in the casting solution. The line represents the best fit through the data.

It has been reported in the literature that the addition of alcohols to the casting solution can influence the membrane porosity [38, 44]. Figure 3.10 presents porosity data obtained from swelling experiments for the membranes depicted in Figure 3.5. The results indicate a slight decrease in porosity for the membranes prepared in the presence of alcohols with 7-8 C atoms in the aliphatic chain. This trend is in agreement with the data obtained by Wang et al. [44], but the minimum

they found is, however, more pronounced. In this context, it might be noteworthy to mention that the symmetric EVAL membranes prepared employing the longer chain alcohols as additive display a higher pore interconnectivity than those prepared in the ternary system using a higher concentration of DMSO in the coagulation to delay demixing.

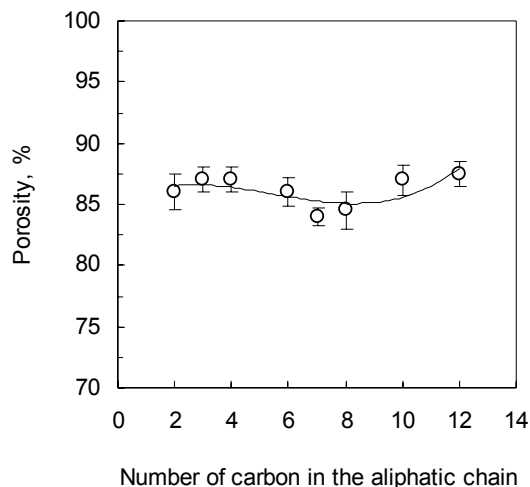


Figure 3.10 The influence of the hydrocarbon chain length of the n-alcohols used as additives in the casting solution on membrane porosity determined from swelling experiments.

3.3.3 EVAL membranes with 1-octanol as additive

In the homologous series of n-alcohols, EVAL membranes prepared with octanol as additive in the casting solution exhibited the longest delay time and a strong suppression of macrovoid formation. To prepare more symmetric, macrovoid-free, open-porous EVAL membranes, morphological fine-tuning was therefore performed by adjusting the concentration of octanol in the casting solution.

Membrane morphology and onset of phase separation

Figure 3.11 shows cross-sections of membranes prepared by precipitating polymeric films containing 10% EVAL and different amounts of octanol in water at 50°C. Increasing the octanol concentration leads to a significant suppression of macrovoids (Figure 3.11A-D). 15–20% 1-octanol in the casting solution is

sufficient to suppress macrovoid formation completely and to generate a more symmetric open-porous structure (Figure 3.11D–F). At 25% octanol macrovoids reappear (Figure 3.11F), as expected.

Smolders et al. found that during the increase of the non-solvent concentration in the immersed polymer solution, the polymer molecules change to a less expanded conformation (syneresis) before *l-l* demixing takes place [29, 40]. Additional lowering of the free energy is gained by mixing of the solvent and the non-solvent penetrating from the bath. The gradient of the non-solvent in the pores favors the diffusional solvent transport and thus the growth process. If the syneresis effect becomes less effective, growth of the voids slows down, since the diffusional transport of the solvent occurs over larger distances, and the coagulation front can proceed beyond the voids.

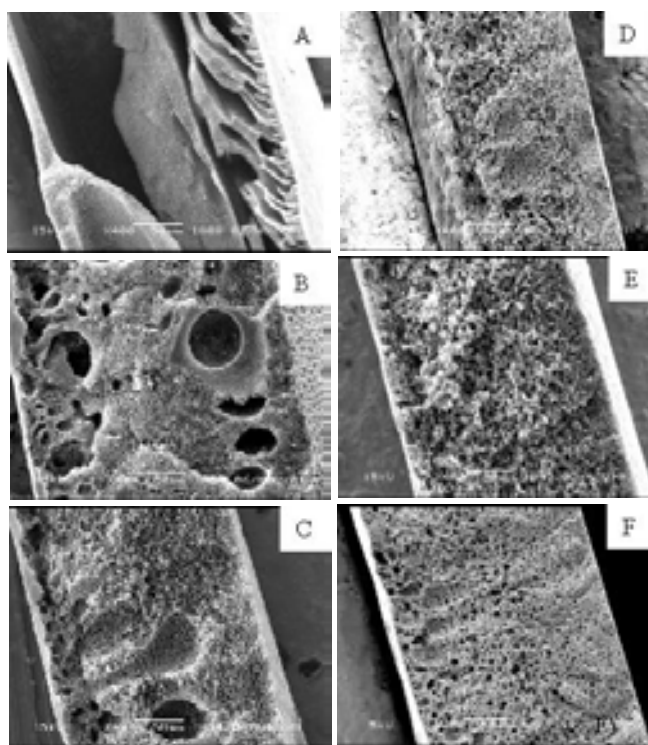


Figure 3.11 SEM-micrograph cross-sections of membranes prepared from solutions of 10% EVAL in DMSO and different amounts of 1-octanol by immersion into water at 50°C: A) no octanol; B) 5%; C) 10%; D) 15%; E) 20%; F) 25% 1-octanol. All cross-sections are presented with the top surface on the left at a magnification of 400. The size bar corresponds to 50 μm .

In Figure 3.12 the membrane prepared with 20% octanol is shown in more detail. The membrane is macrovoid-free and displays a very open pore structure and thus high pore interconnectivity. Finally, the top surface shows more pores than those prepared in the ternary system with 50% DMSO in the coagulation bath.

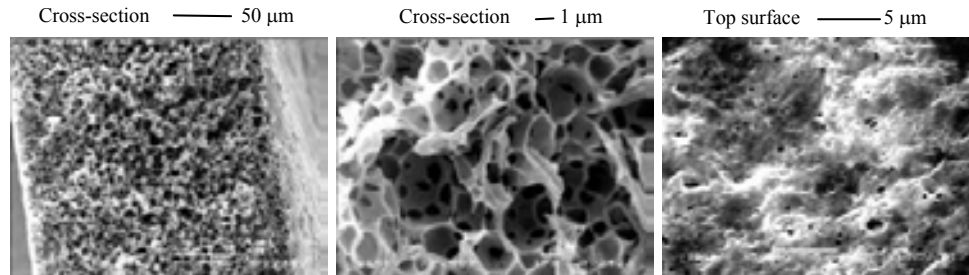


Figure 3.12 SEM-micrographs (cross-sections and top surface) for the membrane E/0/50/O20 prepared in the presence of 20% 1-octanol in the casting solution.

The influence of the octanol concentration on the phase separation kinetics is shown in Figure 3.13.

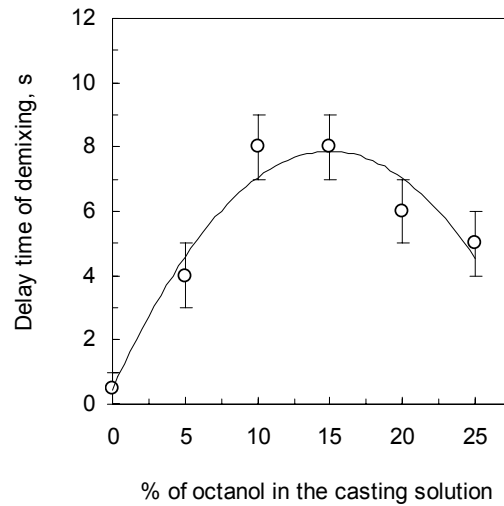


Figure 3.13 Measured delay times before phase separation occurs in polymeric films that consisted of 10% EVAL in DMSO in the presence of different amounts of 1-octanol employed as additive. A line is included to guide the eyes.

The delay time obtained by transmission experiments shows a maximum around 15% octanol. The presence of octanol in the film hinders the inflow of water and leads therefore to delayed demixing conditions. At octanol concentrations above 15% the non-solvent character becomes dominant. The compositions within the film are located closer to the binodal and the film becomes easier locally unstable with respect to *l-l* demixing. Polymer solutions with higher octanol concentrations phase separate without addition of water.

Membrane characteristics

Adjusting the octanol concentration in the casting film allowed preparing membranes with the specifications that meet the requirements for application in affinity separation. Figure 3.14 displays the influence of the octanol content in the casting solution on the pore size, determined by Coulter Porometry, and the corresponding water permeability. With increasing octanol concentration, both the mean pore diameter as well as the pure water flux increase (almost linearly). High water permeabilities of up to 1400 l/h/m²/bar are obtained due the high pore interconnectivity and increased porosity of the top layer.

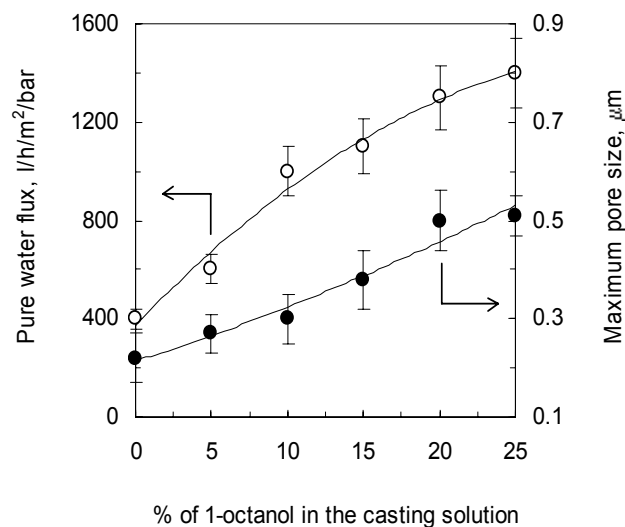


Figure 3.14 Pure water permeability and maximum pore size of membranes prepared from 10% EVAL in DMSO as a function of the concentration of 1-octanol in the casting solution.

The dependence of the pure water flux on the concentration of EVAL at a constant octanol content of 10% in the casting solution is presented in Figure 3.15. For better comparison, flux data are also shown for membranes with different EVAL content that have been prepared from the ternary system by precipitating in a coagulation bath containing 50% DMSO. For the membranes obtained from the ternary water/DMSO/ EVAL system, the measured water permeabilities are in the range of ultrafiltration membranes (20-300 l/h/bar/m²). For the quaternary system water/1-octanol/DMSO/EVAL, the water fluxes lie in the range of 100-1400 l/h/bar/m². The graph shows that for both systems the water permeability is significantly decreased if EVAL concentrations above 10% were used. Employing octanol in the casting solution leads to a major improvement of the membrane performance.

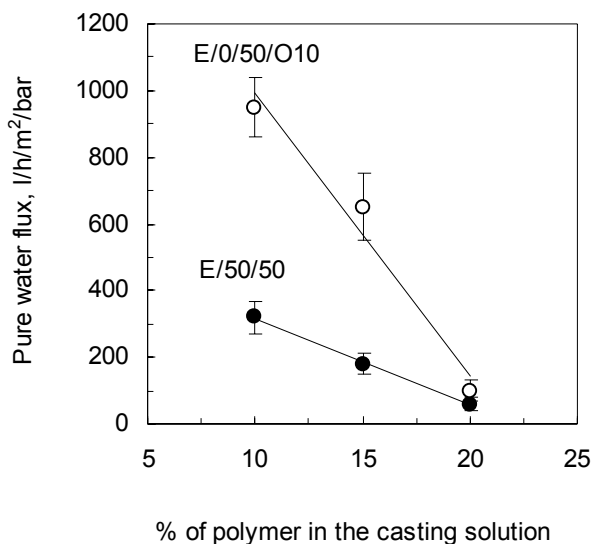


Figure 3.15 Pure water permeability for the membranes E/50/50 (ternary system) and E/0/50/O10 (quaternary system) as a function of polymer concentration in the casting solution. The quaternary system with 10% octanol as additive in the casting solution shows significantly higher water fluxes.

4. CONCLUSIONS

In this work, the ternary water/DMSO/EVAL system and quaternary systems using *n*-alcohols as additives in the casting solution have been exploited to prepare open-cellular type microfiltration membranes by immersion precipitation that can be used in affinity separation. Immersion of 10% EVAL films into water resulted in

instantaneous l-l demixing. The asymmetric membranes formed consisted of a continuous dense skin layer and a cellular porous support with finger-like macrovoids, typical for fast l-l demixing. Delaying the onset of phase separation by adding DMSO to the coagulation bath led to a suppression of macrovoid formation but also favored crystallization and thus a phase separation process that is governed by s-l demixing. Particular structures typical for s-l demixing were obtained at DMSO concentrations in the coagulation bath, which were only slightly higher than those at which residual macrovoids were still present in the porous support layer obtained by l-l demixing. This leaves only a small parameter gap to obtain cellular type macrovoid-free membranes. For the asymmetric cellular membranes prepared (E/0/50, E/25/50 and E/50/50) the water permeability was, due to the low degree of interconnectivity, too low for application in affinity separation. The particulate membranes (E/75/50) display higher water permeabilities, but possess a too low mechanical strength. Furthermore, collapse of the initially formed pores upon drying could not be completely reduced. In order to avoid the formation of closed structures, further surface modification can only be performed in the wet form. Hence activation chemistry is limited to reactions in an aqueous medium.

When alcohols were added as weak non-solvents and as additives in the casting solution, no particulate structures were observed, which left more possibilities for a directed morphological tailoring. Dry membranes could be prepared without pore collapse. Maximal suppression of macrovoids was obtained using 1-octanol as additive in the homologous n-alcohol series. Adjusting the 1-octanol concentration resulted in macrovoid-free membranes that possess a high pore interconnectivity with a water permeability of about 1000 l/h/m²/bar. A model is proposed to understand the influence of the different alcohols employed as additives in the phase inversion process. The mutual miscibility between water and n-alcohols is drastically reduced between $n=2-6$. Short chain alcohols are miscible with water and do therefore change the delay times and the membrane morphology only slightly compared to the ternary system. With increasing alcohol hydrophobicity, inflow of water into the film is hindered, resulting in a delay of l-l demixing and macrovoid suppression. Since the mutual solubility between water and the alcohol decreases only slightly for $n>8$, the increase in the delay time is expected to reach a plateau value. Otherwise, the difference in the solubility parameters between EVAL and the non-solvent additives $|\Delta\delta_{a-p}|$ shows a minimum at $n=8$. For $n>8$ the non-solvent character of the alcohols becomes dominant, leading to a decrease in the delay times observed and a (stronger) macrovoid reappearance.

Membranes obtained from the water/1-octanol/DMSO/EVAL system display a high macroporosity and a large internal and external surface area, which will be

beneficiary to the interaction of the matrix-bound ligand with the ligate during the affinity separation step. Furthermore, pore collapse has not been found therefore allowing for surface activation in organic solvents, thus enlarging the possible set of reactions significantly. But it remains a scientific challenge to give a more analytical interpretation of the effect that addition of the medium and long chain alcohols has on the membrane morphology.

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CHAPTER 4

FUNCTIONALISED ETHYLENE VINYL ALCOHOL COPOLYMER (EVAL) MEMBRANES FOR AFFINITY PROTEIN SEPARATION^{*)}

ABSTRACT

Hydrophilic microfiltration membranes with functional groups that can be used as coupling sites for ligands are of central interest in affinity separation, especially in view of biomedical applications. In this study we investigate covalent coupling of bovine serum albumin (BSA) as model ligand onto cellular-type poly(ethylene vinyl alcohol) (EVAL) microfiltration membranes. EVAL membranes prepared from the ternary water/DMSO/EVAL system are only suitable for activation and coupling reactions in aqueous media. Using glutaraldehyde and oxiran to activate the secondary alcohol groups of the vinylalcohol segments yielded a BSA-immobilisation per internal area of 0.1-0.2 $\mu\text{g}/\text{cm}^2$ (4-8 mg/g per membrane mass). Preparing microfiltration membranes from the quaternary system water/1-octanol/DMSO/EVAL, using 1-octanol as nonsolvent-additive in the casting solution, enables to perform surface functionalization reactions in organic media as well as surface activation by a low-pressure glow discharge treatment. 0.3-0.45 $\mu\text{g}/\text{cm}^2$ BSA per internal area (16-18 mg/g per membrane mass) was covalently coupled onto the porous membranes by applying trichloro-s-triazine and sulfonyl chloride activation reactions, while a BSA-immobilisation of 0.5-0.55 $\mu\text{g}/\text{cm}^2$ (20-22 mg/g per membrane mass) was reached via plasma activation. To determine the degree of BSA-immobilisation, the internal surface area of the membranes prepared was measured by BET. The formation of a BSA-monolayer is assumed on the pore surface as maximum immobilisation. Such membranes can function as adsorptive devices for endotoxin removal from blood or blood plasma.

^{*)} This chapter has been submitted to J. Membrane Sci.

4.1 INTRODUCTION

The *affinity based membrane separation* process was originally designed to bypass the fundamental limitations of packed-bed adsorbers and has recently become an attractive and competitive method for purifying proteins and other biomolecules from biological fluids [1]. In such membrane-based purification systems, high flow rates can be applied at a lower pressure due to the high membrane porosity and high mass transfer capacity. The short diffusional distances allow for an optimal utilization of the immobilized ligands that are covalently coupled to the inner surface of the membrane pores. Common limitations such as pressure drop, channeling and intra-bed diffusion are minimized by convection through the small pores of the membranes. Furthermore, ligand and product are, compared to packed columns [2], only for a short time exposed to harsh elution conditions, which decreases their possible denaturation.

Application in affinity separation asks, especially in the biomedical field, for hydrophilic microfiltration membranes with appropriate chemical groups on the pore surface that can serve as coupling sites for suitable (bio)ligands. Specific interactions between the ligand and the ligate lead to a high selectivity for the species targeted. For membranes with hydrophilic surfaces, the hydrophobic interaction with the biological compounds (ligates) is reduced, while the water-wettability increases. The latter allows for a lower operating pressure gradient. Especially for protein separation, hydrophobic interactions between proteins and the hydrophobic surfaces are generally responsible for non-selective (irreversible) adsorption and membrane fouling.

A number of different methods has been described in the literature to prepare membranes with hydrophilic and/or functionalizable surfaces, these include: i) coating of hydrophobic membranes with a hydrophilic layer such as hydroxyethyl cellulose or polyethyleneimine; ii) copolymerization of two different monomers which contain reactive groups; iii) employment of copolymers consisting of hydrophilic and hydrophobic segments; iv) chemical or physical post-treatment of hydrophobic membranes [3-5]. Surface modification of commercial microfiltration membranes can be performed by attachment of functional groups, such as $-OH$, $-NH_2$, $-SO_3H$, $-COOH$, $-CONH_2$ or epoxy, by chemical modification or graft polymerization [6, 7]. Furthermore, polymeric surfaces have been modified by oxidation with ozone or by exposure to an electron or ion-beam, ultrasonic etching, UV or laser irradiation [8-10]. A variety of functional groups has been introduced by applying gas discharge techniques (plasma treatment) operated at low or ambient pressure [11, 12]. However, the methods applied to obtain

membranes with hydrophilic and chemically modifiable surfaces via physical or chemical post-treatment of hydrophobic membranes often result in unwanted and irreproducible inhomogeneities [13]. Thus the main limitation for supports in affinity separation presently encountered lies in the availability of membranes with functional groups suitable for ligand coupling. This imposes an increased need for the development of hydrophilic microfiltration membranes with suitable functionalizable groups.

In the present paper, we investigate the immobilization (by covalent binding) of bovine serum albumin (BSA) as model protein onto cellular poly(ethylene vinyl alcohol) (EVAL) microfiltration membranes, whose preparation has been examined in a previous paper [14]. Employing EVAL as polymeric membrane material allows principally the preparation of hydrophilic microporous membranes. Immobilization of proteins can be performed via covalent binding of their amino- or acid groups by modification of the secondary alcohol groups of the vinylalcohol segments. In the following, we first give some background information on EVAL microfiltration membranes with respect to their application in affinity separation of biomedical systems and on serum albumin, before an outline of the paper is given.

4.1.1 EVAL microfiltration membranes

Ethylene-vinyl alcohol (EVAL), a semi-crystalline random copolymer consisting of *hydrophobic* ethylene and *hydrophilic* vinyl alcohol segments, has become a promising biomedical material since it is water insoluble and possesses at the same time a good blood compatibility. EVAL displays a good mechanical strength in the wet state, has high thermal stability, good chemical and biological resistance, and is thus easy to sterilize using, e.g., γ -radiation [15]. EVAL hollow-fiber membranes have been utilized in various kinds of blood purification devices including plasmapheresis and hemodialyzers [16]. Recently, particulate EVAL membranes have been investigated for possible application in plasma protein separation [17], microfiltration [18, 19], islets for artificial pancreas [20], and as support for growth of neuronal cells [21].

So far, only a few studies have been performed on the surface activation and functionalization of EVAL membranes and their application in affinity based separation processes. Nakamae and Miyata found an asymmetric distribution of the polymer segments by performing electron spectroscopy on the surface of (ultra-thin) EVAL microfiltration membranes, prepared by immersion precipitation [22, 23]. OH-groups were enriched on the membrane surface facing the coagulation bath. A higher concentration of ethylene-groups was found at the bottom surface if

the membranes were cast on hydrophobic substrates. These membranes were further investigated for enzyme immobilisation. β -cyclodextrin was coupled by glutaraldehyde crosslinking to study the catalytic activity for ester hydrolysis [24]. Urease-immobilized membranes were obtained by covalent binding of urease, which catalyses the decomposition of urea, onto cyanuric chloride-activated EVAL membranes [25]. Commercial EVAL hollow fibre membranes have been employed for protein immobilisation. L-histidine has been coupled onto EVAL-fibres activated with epichlorhydrin or butanediol diglycidyl ether to prepare an affinity support for immunoglobulin G purification [26]. Furthermore, histidine-immobilised hollow fibre membranes have been employed for endotoxin removal from snake antivenom serum solutions [27].

In Chapter 2 we performed a systematic study on the preparation of EVAL microfiltration membranes with cellular morphology that can be applied in affinity separation[14]. Based on earlier studies by Young at al. we used the *ternary* water/DMSO/EVAL system as starting point. Macrovoid-free microporous membranes were obtained by addition of substantial amounts of DMSO to the coagulation bath. The flat membranes prepared are difficult to dry and suffer from pore collapse. They are therefore only suitable for membrane modification in an aqueous medium. Unaltered, completely dryable membranes with higher permeability could be prepared using 1-octanol as nonsolvent-additive in the casting solution. This allows surface activation in organic solvents and in the dry state and enlarges thus significantly the possible set of surface modification reactions. Membranes obtained from the *quaternary* water/octanol/DMSO/EVAL system display a high porosity and a large internal and external surface area, which is beneficiary for the interaction of the matrix-bound ligand with the ligate during the affinity separation step.

4.1.2 Bovine Serum Albumin (BSA)

BSA has often been employed as model protein in biochemical and physico-chemical investigations, not for the last reason in view of its slow denaturation and low costs. A large number of investigations focused on the immobilization of albumin on natural or synthetic polymers, which were studied as sorbents for, e.g., removal of bilirubin from blood plasma [28, 29] or to separate enantiomers [30, 31]. Enantiomers of aminoacid derivatives and drugs can be separated due to the stronger adsorption of the L-form at the ligate specific BSA coupling site.

Although there exists some discrepancy in the literature about the actual structure of BSA, it is presently accepted that serum albumin forms an oblate rotational

ellipsoid with major semi-axis of 70Å and minor semi-axes of 20Å. Based largely on hydrodynamic measurements and small-angle X-ray scattering, a model has been postulated [35], which is in good agreement with measurements performed on monomeric BSA. In this model, albumin is assumed to have the shape of a trimmer that consists of two spherical subunits with a radius of 19Å and one with a radius of 26.6Å. Relevant properties of BSA are summarized in Table 4.1.

Molecular mass (g/mol)	67000-69000
Isoelectric point (pI)	4.8-5.2
Dimensions (Å)	40×40×140
Intrinsic viscosity (ml/g)	3.7-4.2
Radius based on sphere (Å)	26.6
Density (kg/m ³)	1.360

Table 4.1 Relevant properties of bovine serum albumin (BSA)

Despite the high values for BSA captured by the ion exchange groups containing polymer brushes (17-190 mg/g BSA) [31-33], used, e.g., for enantiomers separation, the preparation of these membranes is quite complicated and time consuming. It includes: i) electron beam irradiation of the trunk polymer, ii) grafting of GMA as a precursor monomer, iii) conversion of the epoxy groups to diethylamino groups, iv) hydrophilization of the remaining epoxy groups, and v) adsorption of BSA onto the adsorber by permeating a protein solution through the membrane. Lower BSA-immobilization per gel mass of 5.2-6.8 mg/g have been obtained for epoxy-activated gels prepared by cross-linking of polybutadiene-hydroxyethyl methacrylate copolymer and epichlorhydrin used in separation of bilirubin [28]. Values of 3-50 mg macromolecule/ml membrane are reported in literature for the protein binding capacities of various membrane adsorbers [34].

4.1.3 Outline

In the present study, we investigate the coupling of BSA, used as model protein, onto the surface of the microporous EVAL membranes. In order to enhance high degree of ligand coupling, different methods for membrane activation are studied and the optimal conditions for both activation and coupling reactions determined. The paper is organized as follows: In the Experimental part, details are given for

the preparation, characterization and modification of the membranes. In the *Results and discussion* section, we will first give a characterization of the two different types of EVAL membranes employed. Membrane pre-activation and protein coupling are discussed separately for the different types of modifications used. An outlook for possible applications of the BSA-immobilized EVAL membranes is finally given in the *Conclusions*.

4.2 EXPERIMENTAL

4.2.1 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. Dimethylsulfoxide (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. Surface modification of the secondary alcohol-groups of the vinylalcohol segments was performed with glutaraldehyde (GA, Sigma) and 1,4-butandiol diglycidylether (Aldrich) in aqueous solution as well as trichloro-s-triazine (sTT, Aldrich) and p-toluenesulfonyl chloride (Aldrich) in dried dioxane (Merck). Glutaraldehyde has been purified over activated carbon to remove the polymeric compounds until a single adsorption peak was observed at 280nm. Dioxane was dried overnight using a molecular sieve with 4Å pores (25 g per liter of reagent). Buffer solutions were freshly prepared in ultrapure water. As a model protein, BSA (fraction V, Sigma) was coupled to the activated OH-groups. Sodium dodecyl sulfate (SDS, Acros) was used to remove physically adsorbed BSA from the membrane surface. Bradford reagent (Sigma) was employed in determining the protein concentration. All other chemicals were used as received. Ultrapure water was prepared using a Millipore purification unit Milli-Q plus. All concentrations are stated in wt%.

4.2.2 Membrane preparation

EVAL microfiltration membranes were prepared by immersion precipitation either from the *ternary water/DMSO/EVAL system* or from the *quaternary system water/1-octanol/DMSO/EVAL*. EVAL-membranes prepared from the ternary system (0% 1-octanol) using 50% DMSO in the coagulation bath (E/50/O0) showed generally a strong tendency to collapse upon drying. The membranes are identified in the way that first the concentration of DMSO in the coagulation bath

is given, followed by the concentration of 1-octanol in the casting solution. Since a solvent-exchange treatment with ethanol and hexane could not completely suppress the occurrence of pore collapse, the membranes prepared were stored at room temperature in ultrapure water before further use. Microporous EVAL-membranes that could be completely dried without any evidence of pore collapse were prepared in the quaternary system with 20% n-octanol as nonsolvent-additive in the casting solution even without DMSO into the coagulation bath (E/O/O20). Proper preparation conditions were described in detail in a previous paper [14].

4.2.3 Membrane characterization

The *morphology* of the membranes was investigated by scanning electron microscopy. Pieces of the membranes were frozen in liquid nitrogen and broken in order to expose the cross-sectional areas. The dried samples were coated with platinum using a Jeol JFC-1300 Auto Fine Coater and examined using a Jeol JSM-5600 LV Scanning Electron Microscope.

The *pure water flux* was determined using a dead-end ultrafiltration cell connected to a gas cylinder of compressed nitrogen to apply the feed pressure. The filtration experiments were carried out at room temperature at a transmembrane pressure of 1bar. The pure water flux was determined after steady state conditions were reached.

For the *molecular weight cut-off* (MWCO) determination, the membranes were mounted in a dead-end ultrafiltration cell and a freshly prepared dextran solution (0.3% dextran + 0.03% ethylene glycol in water) was permeated at room temperature and transmembrane pressures of 1bar, under 300rpm. The feed and the collected permeate samples were degassed using a eluent degasser ERC 3315 and injected into a gel permeation chromatographic system with a refractive index detector Shodex RI 71. Using a WINGPS 6 software package (Polymer Standards Service), a sieve curve was calculated, showing the permeate concentration relative to the concentration of the feed solution as a function of the molecular weight. From the obtained molecular weight distribution in the feed and the permeate solutions, the MWCO value was calculated.

BET-measurements to determine the internal surface area of the membranes were performed with an ASAP 2400 nitrogen adsorption apparatus (MICRIMERITICS). A sample tube was filled with a known amount of EVAL membrane and degassed by heating at 300°C and evacuation for at least 4 hours. The volume of nitrogen adsorbed onto the sample at the temperature of liquid nitrogen (-190°C) was measured as a function of the relative pressure. Using an ASAP 2400 software package the nitrogen-monolayer capacity was calculated and used to determine the BET-surface area available for the nitrogen molecules.

4.2.4 Membrane activation

EVAL-membranes E/50/O0 prepared from the ternary water/DMSO/EVAL system suffered from pore collapse upon drying and could therefore only be modified in an aqueous medium. The membranes E/0/O20 prepared using 1-octanol as nonsolvent-additive in the casting solution could be completely dried without suffering from pore collapse. The latter were activated in an organic medium or by applying a CO₂-plasma. In order to confirm the presence of the different functional groups on the surface of the activated membranes, Fourier transformed infrared (FT-IR) spectra were recorded using an FTS 60 BioRad spectrophotometer.

Surface activation in aqueous solution

To immobilize BSA on the microporous EVAL-membranes E/50/O0, an aqueous glutaraldehyde solution was employed in the presence of mineral acid used as catalyst. Aqueous glutaraldehyde solutions (free of polymeric species) with different initial concentrations ranging from 0.25% to 2.5% were freshly prepared and the pH was adjusted with H₂SO₄. Circular membrane pieces were placed into the glutaraldehyde solutions at room temperature and reacted for different periods of time up to 24h. Small samples of glutaraldehyde solution were taken out of the reaction mixture at short time intervals and the concentration of unreacted glutaraldehyde was determined at $\lambda=280$ nm using a PU 8720 UV/VIS spectrophotometer. After the activation step, the membranes were washed with a 0.1 M phosphate buffer at pH 8 to remove the unreacted compounds. The modified membranes were stored in a 0.1M phosphate buffer at pH 8 and 4°C.

Besides the reaction with glutaraldehyde, surface activation via reaction of the OH-groups with oxiran has also been investigated. For this purpose, circular pieces of EVAL membranes were added to an aqueous solution containing 10 ml 1,4-butandiolglycidylether, 5 ml 1M NaOH and 30 mg NaBH₄ to prevent the oxidation of the di-epoxides. The reaction was performed at room temperature for 6–8 hours. A limited reaction time is necessary to avoid alkaline hydrolysis of the epoxide in to its glycol. The membranes were washed with a 0.1M phosphate buffer at pH 8 and deionized water in order to remove the unreacted components and stored at 4°C in 0.1M phosphate buffer at pH 8.

Surface activation in organic medium

Circular pieces of the EVAL-membrane E/0/O20, prepared in the quaternary system water/1-octanol/DMSO/EVAL, were first soaked in 3N NaOH for 30 min

at 25°C. Excess of NaOH solution was removed by draining. The alkaline membrane was placed into a 10% solution of sTT in dry dioxane at room temperature. In order to investigate the optimum parameters, the activation reaction was performed at different pH for different periods of time. After the activation step, the membranes were washed consecutively with dioxane/water mixtures of 100:0, 75:25, 50:50, 25:75, 0:100 v/v, and acetone. The modified membranes were stored in 10% acetic acid solution at 4°C for one month without losing their coupling capacity.

Additionally, surface activation in organic medium was also performed via reaction with an aromatic sulfonyl chloride. Circular membrane pieces (E/0/O20) were transferred into 10 ml p-toluenesulfonyl chloride 30% in dry dioxane. 0.1ml dry triethyl amine was added to the reaction mixture and activation performed for 1h at 20°C. The activated material was washed consecutively with dioxane:2mM HCl mixtures of 75:25, 50:50, 25:75, 0:100 v/v, acetone and demineralized water to remove the unreacted components and stored at 4°C.

Surface activation via CO₂-plasma treatment

A plasma reactor consisting of a glass tube with an internal diameter of 9.5 cm and a length of 150 cm was used for membrane activation via a low-pressure glow discharge treatment. A hot electrode was placed in the center of the reactor together with two cold electrodes at a 30 cm distance on both sides. One side of the reactor was connected to a gas inlet system equipped with a flow control. Circular membrane E/0/O20 pieces with a diameter of 9 cm, washed in acetone and dried in the vacuum oven at 30°C, were introduced into the plasma reactor. The reactor was evacuated until a pressure of 0.005 mbar was reached, before a CO₂ gas flow of 10 cm³/min was established and the plasma induced (0.15 mbar, 75W). After 15 min of plasma treatment the reactor was brought to an atmospheric pressure with air. A more detailed description of the system can be found in [36].

4.2.5 Protein coupling

The amount of protein covalently bound onto the EVAL-membranes was determined by measuring the difference between the protein concentration in the initial solution used for immobilization and the protein concentration in the solution after performing the modification reaction. The amounts of protein desorbed in SDS were subtracted to obtain the values for covalent protein coupling. The protein concentration was determined using the Bradford method [37] at 595 nm

with a PU 8720 UV/VIS spectrophotometer. Values for BSA-immobilization are given either per mass of membrane in mg/g or per geometrical surface area in $\mu\text{g}/\text{cm}^2$. To obtain a better insight into the BSA-adsorption BSA-immobilization was calculated per internal surface area ($\mu\text{g}/\text{cm}^2$), using the surface area obtained by BET measurements.

Bovine serum albumin (BSA) was used as model protein for ligand coupling. The pre-activated membranes were immersed in a protein solution (1 mg/ml BSA) at pH values varying between pH 4-8 (depending on the functional group induced in activating the membrane) and incubated at 4°C for 24h. After BSA-coupling, the membranes were washed with phosphate buffer and deionized water in order to remove the unreacted protein. The protein that was not covalently immobilized but physically adsorbed on the membrane surface was desorbed by immersing the membrane in a 3% sodium dodecyl sulfate solution for up to 24 hours.

4.3 RESULTS AND DISCUSSION

In this study, we employed two different types of EVAL microfiltration membranes, whose preparation is described in a previous article [14], for the preparation of protein (BSA)-immobilized affinity separation membranes. To optimize the BSA-immobilization a number of different pre-activations have been investigated which allow the coupling of BSA via the aminogroups of the protein onto the modified vinylalcohol segments of the EVAL membranes. Surface activation has been performed: i) in aqueous media by reaction with glutaraldehyde (GA) or oxiran, ii) in organic environment via reaction with triazine (sTT) and sulphonyl chloride, or iii) in the dry state by applying a CO₂-plasma (low-pressure glow discharge). For the latter two activations, completely dry membranes are a prerequisite. Therefore, the membranes prepared in the ternary water/DMSO/EVAL system could only be investigated for surface modification with glutaraldehyde and bioxiran. Membranes obtained in the quaternary water/1-octanol/DMSO/EVAL system were employed for surface modification in organic environment and in dry state.

4.3.1 Membrane characterization

Activation by CO₂-plasma treatment does not only introduce new functional groups on the membrane surface (changes in the chemistry of the surface), but causes also changes in the membrane morphology and thus the membrane performance. In the following, we therefore give a detailed characterization for the membrane prepared

in the ternary system (E/50/O0) and for the membrane prepared in the quaternary system (E/0/O20) before and after CO₂-plasma treatment.

Figure 4.1 shows the cross-section, the top and bottom surfaces of E/50/O0 membranes. The membranes are asymmetric and consist of an almost dense skin layer and a cellular microporous support with relative low interconnectivity. The average pore size lies around 0.2 μm. The bottom surface shows a morphology typical for liquid-liquid demixing near the glass surface [38]. Residual macrovoids are present in the upper half of the cross-section.

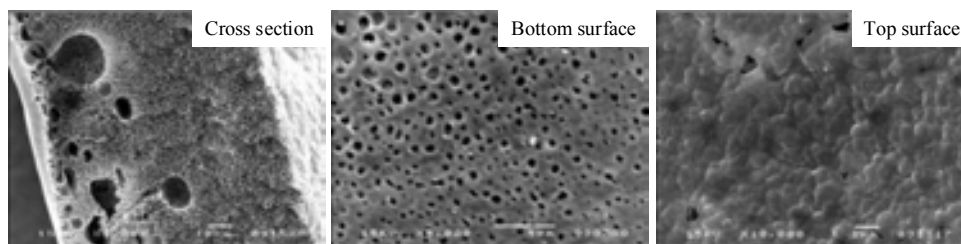


Figure 4.1 SEM micrograph of the membrane E/50/O0 prepared from 10% EVAL in DMSO, using 50% DMSO as coagulation bath: The membranes were dried by replacing water progressively with ethanol and hexane.

For application in affinity separation the presence of macrovoids in the sublayer is unwanted, because it causes mechanical instability and decreases the internal surface area. Since EVAL is a semi-crystalline polymer, conditions that lead to a suppression of macrovoids favor often also crystallization and the formation of particulate structures. This makes the parameter space for adjusting the preparation conditions quite small for the ternary systems. Particulate structures are unfavorable in affinity separation, since they possess a low mechanical stability and can lead to entrapment of small molecules within the pores of the crystalline particles that form the bicontinuous network of particulate microfiltration membranes [39]. The E/50/O0 membranes are difficult to dry and suffer from pore collapse. Pure water fluxes measured lay the range of 300-400 l/h/m²/bar. These, for microfiltration membranes, quite low values are caused by the low degree of interconnectivity and the dense top surface.

In order to obtain a better membrane tailoring, we have investigated the influence of n-alcohols, used as nonsolvent-additive in the casting solution, on the membrane morphology [14]. Figure 4.2A shows the morphology of the E/0/O20 membrane, prepared with 20% 1-octanol in the casting solution and water as coagulation bath. The membrane is completely free of macrovoids and shows an open porous structure

with high pore interconnectivity. The average pore size lies around $0.3\ \mu\text{m}$. Both, the top and the bottom surfaces show a more open structure than the E/50/O0 membranes. The water permeability is also significantly higher reaching values of $1400\ \text{l/h/m}^2/\text{bar}$. The E/0/O20 membrane can be dried completely without pore collapsing, which enlarges the number of possible surface modifications for protein coupling. Figure 4.2B shows the E/0/O20 membrane after CO_2 -plasma treatment for 15 min on both sides. Due to the plasma etching, the surface roughness especially of the toplayer increases dramatically. Comparing micrographs 4.2A and 4.2B indicates that the interior of the membrane support layer is not affected by the plasma treatment in a significant way.

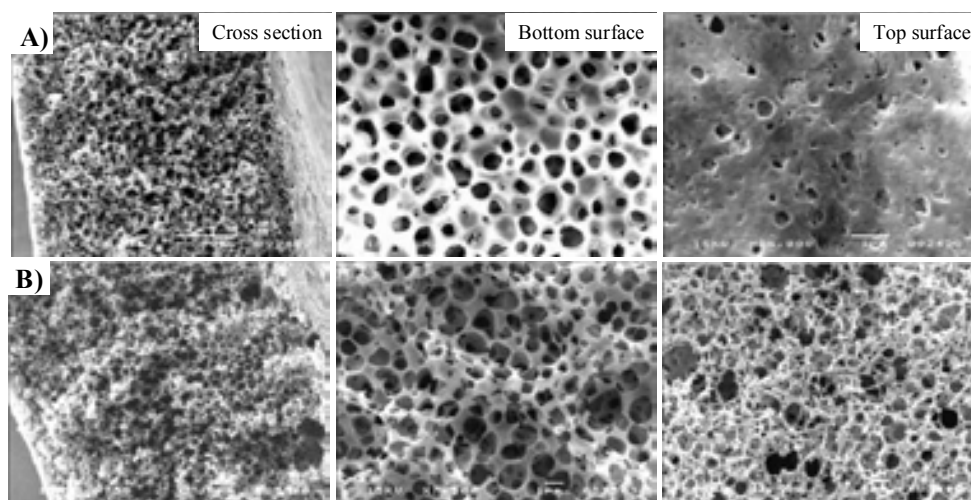


Figure 4.2 SEM micrograph of the membrane E/0/O20 prepared from 10% EVAL in DMSO, using 20% 1-octanol as additive in the casting solution and water as coagulation bath at 50°C : before (A) and after (B) plasma treatment.

The comparison between Figures 4.2A and 4.2B shows that the toplayer is completely removed during plasma treatment. The underlying interconnected porous structure of the support layer becomes visible. Both, glass and the air surfaces are very open and display pores of around $0.2\text{--}0.5\ \mu\text{m}$. Due to the removal of the toplayer, the water permeability of the CO_2 -plasma treated membrane increases by a factor of 5, to values of $5500\pm 1000\ \text{l/h/m}^2/\text{bar}$. Unfortunately, the variation in the value is quite large due to the critical control of the plasma conditions (Figure 4.3). Using a dextran solution of 282 kDa average molecular weight with a polydispersity of 2.20, both the untreated and treated membranes present no retention. No entrapment of small molecules within the pores of the

microfiltration membranes was observed, therefore it was concluded that the membrane cutoff is higher than 500 kDa.

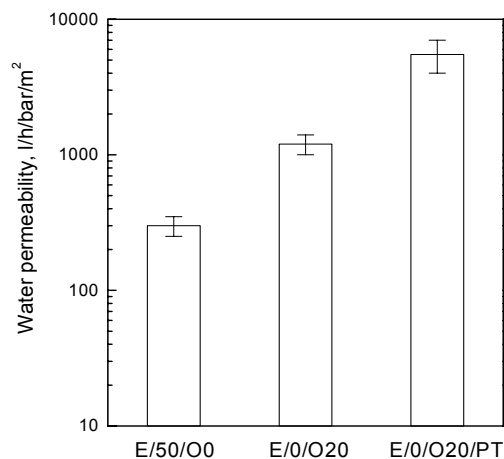


Figure 4.3 Pure water permeability measurements for the membranes E/50/O0 and E/0/O20 before and after plasma etching.

4.3.2 Membrane activation

Membrane activation has been performed in aqueous media (by reaction with GA and oxiran), organic media (by reaction with sTT and tosyl chloride) and in the dry state (by CO₂-plasma treatment). In this section, only the more effective reactions for each type of activation are described in detail. Also the optimal results of the reactions with oxiran in aqueous solution and tosyl chloride in dioxane are presented in Table 4.2.

Membrane activation with glutaraldehyde

Glutaraldehyde (GA) has extensively been used for the immobilization of proteins, enzymes and cells, since it is inexpensive and easy to use. GA exists in different forms depending on the experimental conditions. At low pH, GA is in dilute solution present as monomer in the free aldehyde form, as hydrate or as hemiacetal, while it polymerizes at higher concentrations into oligomeric hemiacetals. All of these species can react with proteins and lead to immobilization. Under alkaline

conditions, GA undergoes aldol condensation forming α,β -unsaturated multimetric aldehydes, which can also react with proteins either via the formation of a stabilized Schiff base or the formation of a Michael adduct [40]. A large number of possible prescriptions are described in the literature for protein immobilization using glutaraldehyde. Activation of the support is often conducted at low pH to catalyze acetal formation, while the coupling of the protein is carried out at elevated pH to promote the nucleophilic attack onto the carbonyl group improving thus the immobilization yield. For the latter step the pH was usually adjusted about 2-3 pH-units higher than the isoelectric point ($pI_{BSA}=4.8-5.2$), to avoid protein denaturation.

The reaction scheme for the acetal formation, which we applied for the activation of the EVAL membranes is presented in Figure 4.4. GA reacts with two OH-groups of the EVAL membrane. In this way, the less active secondary alcohol group of the vinylalcohol segments is transformed into a more functional aldehyde group. The latter can react with the primary and secondary amino groups of the protein to form a Schiff base.

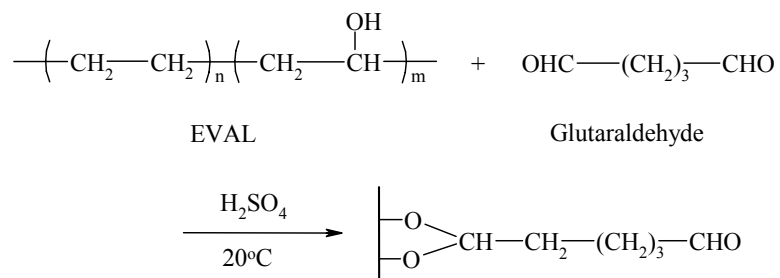


Figure 4.4 Reaction scheme for the activation of EVAL with glutaraldehyde.

Figure 4.5 shows the FT-IR spectra of the glutaraldehyde modified membrane E/50/O0 after drying in a vacuum oven together with that of the unmodified membrane. The new appearing absorption band at 1720 cm^{-1} is assigned to the C=O vibration of the aldehyde group and indicates the presence of aldehyde groups on the membrane surface.

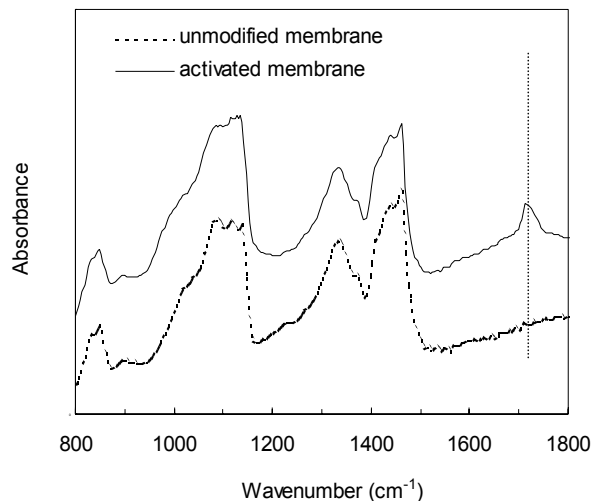


Figure 4.5 FT-IR spectrum of an EVAL membrane E/50/50 before and after modification with glutaraldehyde reaction.

A freshly prepared aqueous glutaraldehyde solution displays a single absorbance band at 280 nm [41]. To investigate the kinetics of the GA reaction, we measured the variation in time of the absorbance of the reaction mixture at 280 nm. The absorbance decreased as a function of time and reached a minimum value after approximately 12h. The yield of the OH-group activation of the EVAL membrane was determined from the difference between the initial GA concentration and the concentration of GA in the reaction mixture at different times. It can be seen from Figure 4.6A, that the membrane activation reaction is completed after 12h, when approximately 30% of the hydroxyl groups were transformed into aldehyde groups. Membranes used for the BSA coupling were activated with GA for 24h.

To investigate the effect of the initial glutaraldehyde concentration on the activation of the EVAL membrane, the GA concentration was varied between 0.25 and 2.5%. The pH of the solution was adjusted with H₂SO₄ at pH 3. The absorbance of the different reaction mixtures at 280nm was measured after 24h. Figure 4.6B depicts the modification yields as a function of the initial GA concentration. The maximum amount of activation was obtained for an initial glutaraldehyde concentration of 1%. This concentration was used for further coupling experiments. Similar results were obtained for the modification of the membrane E/0/O20 via glutaraldehyde reaction (data not shown).

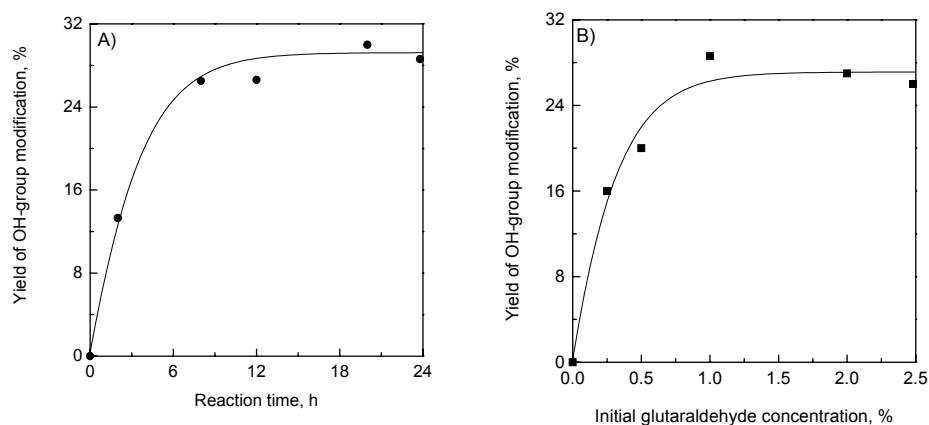


Figure 4.6 Time (A) and GA-concentration (B) dependence of the modification yield for the activation of EVAL membranes by reaction with glutaraldehyde (GA).

Membrane activation with trichloro triazine

Triazine has mainly been used to activate polysaccharide supports and their amino-derivates but was also employed to modify other polymers such as collagen, keratin, aminated polystyrene, anion-exchange resins, polyvinyl alcohol, and polyethylene glycol [42]. Polymeric derivatives of dichloro-s-triazinyl have been obtained by reaction with 2,4,6-trichloro-s-triazine (sTT) and react rapidly with the amino groups of the protein in the consecutively performed immobilization step. Figure 4.7 shows the reaction scheme for the activation of the vinylalcohol groups of the EVAL membrane with sTT.

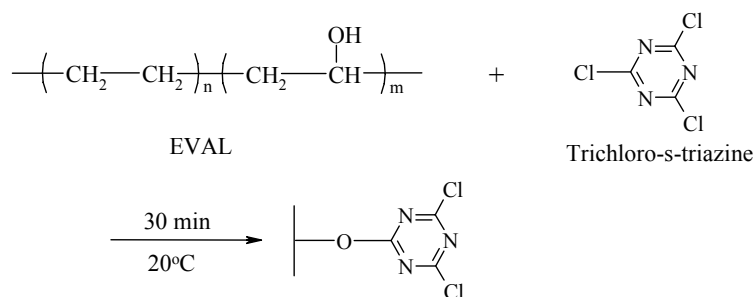


Figure 4.7 Reaction scheme for the activation of EVAL with trichloro-s-triazine.

The FT-IR spectrum of the triazine-activated membrane E/0/O20 is shown in Figure 4.8. Coupling of sTT to the membrane surface is confirmed by the absorption bands at 1720 cm^{-1} , attributed to the substituted benzene rings, 1640 cm^{-1} , assigned to the C=N groups, 1505 cm^{-1} and 1545 cm^{-1} characteristic for triazine, and 1050 cm^{-1} and 1310 cm^{-1} for the carbon ring of the cyclic compound. The optimal conditions for the membrane activation, such as sTT concentration and pH, were investigated by determining the optimum in the BSA adsorption and are therefore described in section 4.3.3.

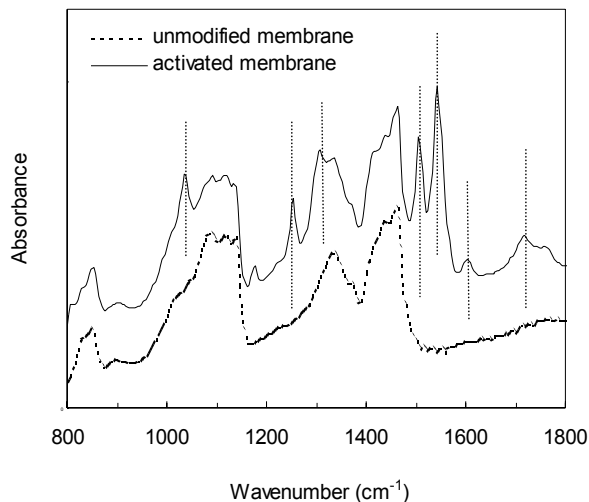


Figure 4.8 FT-IR spectrum of an EVAL membrane E/0/O20 before and after modification by reaction with trichloro-s-triazine.

Membrane activation by CO₂ plasma treatment

CO₂-plasma treatment of the EVAL membranes has been performed to incorporate oxygen-containing functional groups, such as hydroperoxide, hydroxyl, epoxide, carbonyl, and carboxylic acid groups, in the membrane surface. Figure 4.9 shows the FT-IR spectrum of an EVAL membrane E/0/O20 activated by plasma treatment for 15 min. The appearance of new functional groups was confirmed by the broad absorption band at $1650\text{--}1750\text{ cm}^{-1}$, which is attributed to the C=O groups in carboxylic compounds, such as esters, aldehydes, ketones or carboxylic acids.

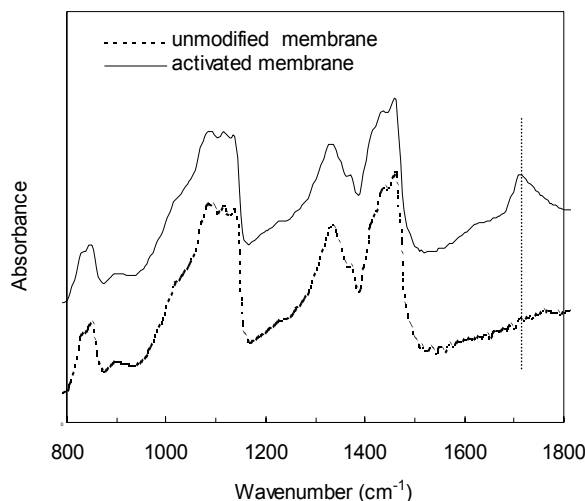


Figure 4.9 FT-IR spectrum of an EVAL membrane E/0/O20 before and after CO₂ plasma treatment.

4.3.3 Protein coupling

Immobilization of proteins can principally occur through i) electrostatic and/or hydrophobic attraction, ii) entrapment or encapsulation, or iii) covalent binding to polymeric supports by reaction of the functional groups with the protein. Application in affinity separation requires immobilized ligands that cannot leak out. A number of studies have been performed on the adsorption and immobilization of BSA on a variety of different substrates. BSA adsorbs generally into a monolayer with the longitudinal axis perpendicular to the sorbent surface. A BSA-immobilization per internal surface area of 0.6-0.84 $\mu\text{g}/\text{cm}^2$ has been found for the formation of an adsorbed monolayer of BSA molecules [43, 44]. Only when very hydrophobic sorbent surfaces are employed, the proteins can adsorb (or be coupled) in multilayer. The adsorbing macromolecules rearrange at the interface, since adsorption has become energetically more favorable [45].

We performed coupling of BSA onto E/50/O0 membranes that have been activated in aqueous solutions by reaction with glutaraldehyde or oxiran and onto E/0/O20 membranes in dioxane with trichloro-s-triazine (sTT) or p-tolenesulfonyl chloride, or in the dry state via CO₂-plasma treatment. Figure 4.10 shows the reaction scheme for BSA coupling on EVAL membranes activated by either glutaraldehyde or trichloro-s-triazine, respectively.

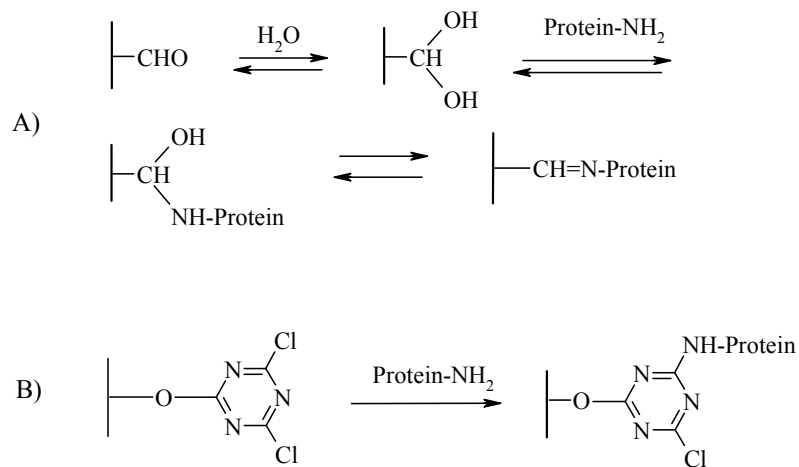


Figure 4.10 Reaction scheme for protein coupling onto EVAL membranes activated with glutaraldehyde (A) and trichloro-s-triazine (B).

All optimal values obtained for BSA-immobilization performed by the different activation reactions used, are summarized in Table 4.2, but only the reactions leading to the higher immobilizations are described in more detail. Values for the BSA-immobilization are given per membrane mass (in $\mu\text{g/g}$) and per internal surface area (in $\mu\text{g}/\text{cm}^2$). For the latter the internal surface area of the E/50/O0 and E/0/O20 membranes employed has been determined by BET-measurements. Furthermore, it has been taken into account that the accessible internal surface area is not the same for BSA and for N_2 -molecules, used for determining the BET-surface area. Since for the glutaraldehyde-activated membranes the optimal conditions for the activation step are already described in section 4.3.2, emphasis will only be laid on the GA-coupling results and the calculation of the BSA-immobilization per internal surface area. For activation with sTT, optimal conditions for both (pre)activation and coupling are described. Time and pH dependence are investigated for membranes activated by CO_2 -plasma treatment.

BSA-coupling on glutaraldehyde activated EVAL membranes

The membranes activated with glutaraldehyde were immersed in a BSA solution (1 mg/ml in 0.1 M phosphate buffer) at pH 7, incubated at 4°C for 24h and treated as described in section 4.2.5. Physically adsorbed BSA was removed by desorption in SDS solution and amounted up to 2mg per mass membrane. The values

measured for covalently coupled BSA per mass of membrane lay between 4-8 mg/g. This value corresponds to a BSA-immobilization per geometrical membrane surface of 40-80 $\mu\text{g}/\text{cm}^2$. To obtain a better sight into the BSA-adsorption, the internal surface area of the membrane E/50/O0 has been determined by BET-measurements. The BET analysis revealed a surface area of 8 m^2/g for pores lying in the range of 17Å up to 3 μm . The surface area available for the small N_2 molecules is probably larger than the surface area available for the much larger BSA macromolecules. For determining the protein-coupling yield per internal surface area, we took therefore the internal surface area into consideration that was obtained from the BET-measurements for pores with a diameter larger than that of BSA. The latter corresponds to approximately half of the total internal area, leading to a BSA-immobilization per internal surface area of 0.1-0.2 $\mu\text{g}/\text{cm}^2$, see Table 4.2. This value is by a factor of 5 smaller than that expected for the formation of a BSA monolayer. How far this lower value is caused by the fact that one glutaraldehyde molecule binds to two OH-groups of the vinylalcohol segments is difficult to estimate. A lower immobilization value might also be caused by glutaraldehyde oligomerisation that might occur simultaneously during the activation step.

BSA-coupling on triazine chloride activated EVAL membranes

The membranes activated with sTT were immersed in BSA solution (1mg/ml in buffer solution) and incubated at 4°C for 24 hours. BSA-immobilization was calculated in the same way as for the GA-activated membranes. For the E/0/O20 membranes a total surface area of 10 m^2/g was found by BET-measurements. For the calculations of the immobilization a surface area of 4 m^2/g was used, which corresponds to the surface formed by pores $>53\text{Å}$. Figure 4.11A presents the amount of BSA coupled onto EVAL membranes as a function of the pre-activation time in 10% cyanuric chloride. BSA coupling has been performed in a 0.1M phosphate buffer at pH 7. Although the membrane activation seems to be complete within 20 min, activation steps of 30 min were performed in the further experiments.

The effect of pH on the amount of BSA bound on the polymeric surface was investigated by incubating the membranes, activated for 30 min in triazine chloride, at 4°C for 24h in solutions of 1 mg/ml BSA in different 0.1 M buffer solutions (acetate buffer pH 4-5, phosphate buffer pH 6-7, Tris buffer pH 8-9). Figure 4.11B shows a maximum BSA-immobilization per internal surface area of 0.4-0.45 $\mu\text{g}/\text{cm}^2$ (16-18 mg/g per mass of membrane) at pH 5-6 (Table 4.2). At higher values less protein is immobilized on the porous membrane surface. The

latter is probably caused by hydrolysis of the reactive cyanuric chloride groups. A similar behavior has been observed by Smith et al. The most favorable pH values for BSA binding obtained by them lay below pH 7, since the reaction rate of free sTT with the protein increases more slowly at increased pH than the rate of triazine hydrolysis [46].

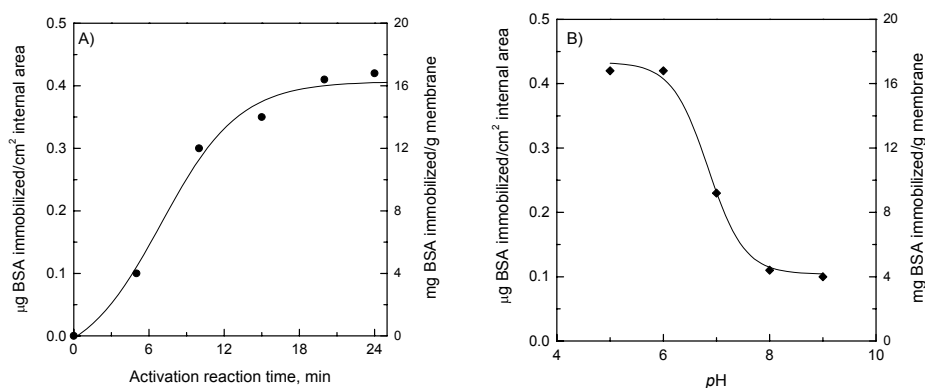


Figure 4.11 Yield of BSA-immobilization for trichloro-s-triazine activated EVAL membranes: effect of the pre-activation time with trichloro-s-triazine (A); effect of pH on the BSA coupling reaction (B).

BSA-coupling on EVAL membranes activated by CO_2 -plasma treatment

The membranes pre-activated by plasma treatment were immersed into a protein solution (1mg/ml BSA) at pH 4.5 and incubated at 4°C for up to 24h. The amount of protein bound onto the EVAL membranes is presented in Figure 4.12A as a function of the reaction time. Since the internal membrane morphology is not significantly changed by the CO_2 -plasma treatment (see Figures 4.2), we used the same internal surface area as for the untreated E/O/O20 membrane. BSA coupling is completed after 8h and reaches a maximal value per internal surface of 0.5-0.55 $\mu\text{g}/\text{cm}^2$ (20-22 mg/g per membrane mass), see Table 4.2. For the other experiments performed, the pre-activated membranes were reacted with BSA solution for 24h.

The effect of pH on the BSA-immobilization was investigated by immersing the plasma treated membranes in solutions of 1 mg/ml BSA in different 0.1 M buffer solutions (acetate buffer pH 4.5, phosphate buffer pH 6-7, Tris buffer pH 9) at 4°C

for 24h. Figure 4.12B shows that BSA-coupling onto CO₂-plasma treated membranes, does not significantly depend on the pH in the range investigated of pH 4.5-9.

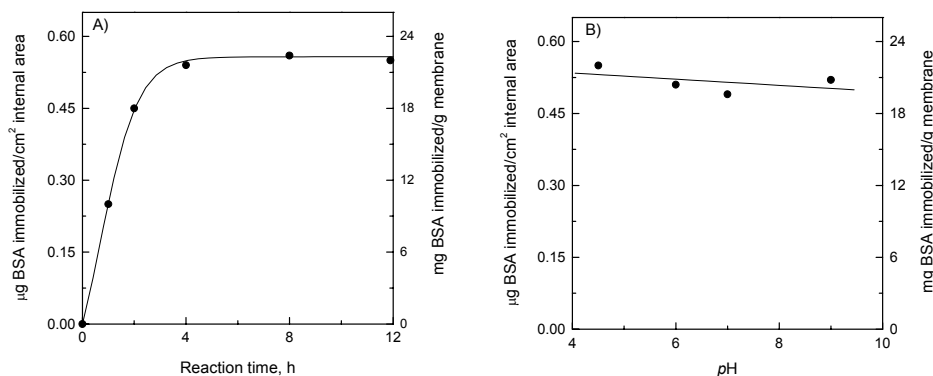


Figure 4.12 Yield of BSA-immobilization after pre-activation by CO₂-plasma treatment: effect of coupling reaction time (A) and pH (B).

Table 4.2 summarizes the optimal BSA-immobilizations by covalent binding obtained for the different activation reactions employed. The values presented do not include physically bound BSA. The immobilization per internal surface area has been calculated using the BET-surface area as described above.

	Membranes prepared in the ternary system EVAL/DMSO/water		Membranes prepared in the quaternary system EVAL/DMSO/octanol/water		
Coagulant	50% DMSO, 50°C		water, 50°C		
Modification	Aqueous media		Organic solvents		CO ₂ plasma treatment
	Aldehyde method	Oxirane method	Triazine method	Sulphonyl method	
BSA coupling, mg BSA/g	4-8	4-6	16-18	8-12	20-22
BSA coupling, $\mu\text{g BSA/cm}^2$	0.1-0.2	0.1-0.15	0.4-0.45	0.2-0.3	0.5-0.55

Table 4.2 Maximum BSA-immobilization by covalent binding onto differently pre-activated porous EVAL membranes.

For the E/50/O0 membranes activated in water with either glutaraldehyde or oxiran, lower values are principally obtained. The yield of BSA-immobilizations is by a factor of 2-5 higher for the E/O/O20 membranes. The highest values are obtained for the CO₂-plasma pre-activated membranes. The BSA-immobilization per internal surface area of 0.5-0.55 μg/cm² comes close to those expected for monolayer BSA adsorption. The physical adsorption of BSA amounts generally to about 3±1 mg per mass membrane.

4.4 CONCLUSIONS

In this work protein-immobilized EVAL microfiltration membranes have been prepared that are of specific interest for a number of different applications in affinity separation, especially in the biomedical field. It is already known that a number of metabolic toxins such as mercaptans, free fatty acids, endotoxins of gram negative bacteria, as well as many medications such as nortriptyline, amitriptyline, diazepam, bromazepam, are bounded to proteins in the bloodstream, especially to the albumin fraction of blood plasma. Bioaffinity separation principally asks for membranes that show a good compatibility to the targeted biological fluids, provide coupling sites for (bio)ligands and possess a hydrophilic surface to reduce non-specific adsorption caused by hydrophobic attraction. Due to its good blood compatibility and very low water solubility, EVAL has become a promising biomedical membrane material, but has so far not been investigated for (bio)affinity separation.

We studied two different flat, cellular type EVAL microfiltration membranes, whose preparation has been investigated in a previous paper [14], for covalent coupling of bovine serum albumin used as model bio-ligand. Covalent binding of BSA via its amino-groups onto the surface of EVAL requires a pre-activation of the secondary OH-groups of the vinylalcohol segments. Surface activation was performed for E/50/O0 membranes, prepared from the ternary water/DMSO/EVAL system, in aqueous solution by reaction with glutaraldehyde and oxiran. EVAL membranes prepared in the quaternary system with 20% 1-octanol as nonsolvent-additive in the casting solution (E/O/O20) can be completely dried without altering, which enlarges the possible set of activation reactions. The E/O/O20 membranes were activated in dioxane by reaction with triazine or sulfonyl chloride or in the dry state by applying a CO₂-plasma.

To enhance maximum protein coupling, the optimum conditions for membrane activation and BSA-coupling were determined for each activation method applied. The effect of the reaction time for pre-activation and coupling, the initial

concentration of the activation reagents and the pH for BSA-coupling on the amount of immobilized protein were investigated in detail. Immobilization of BSA on the EVAL membranes is strongly influenced by the activation method employed and the time for the pre-activation reaction. The highest BSA-immobilizations are obtained for the E/O/O20 membranes prepared from the quaternary system water/1-octanol/DMSO/EVAL after pre-activation with triazine (sTT) in dioxane or after CO₂-plasma treatment in the dry state (see Table 2). The maximal value for BSA-immobilization per internal area of 0.5-0.55 μg/cm² for the plasma-treated membranes reaches values reported in the literature for the formation of a complete BSA monolayer. These membranes possess a high interconnectivity, a water permeability in the range of 5500±1000 l/h/m²/bar making them suitable for applications in membrane based affinity separation processes.

Possible applications for the BSA-immobilized membranes lie in the separation of unconjugate bilirubin and/or D,L-tryptophan. Such membranes can also function as adsorptive devices for a large number of metabolic toxins such as free fatty acids, endotoxins of gram-negative bacteria, mercaptans as well as medications like nortriptyline, amitriptyline, diazepam, bromazepam, which bound preferentially to the albumin fraction of the blood plasma. Further applications open up by coupling of proteins via their amino-groups that have already been successfully employed as bio-ligands in affinity chromatography.

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CHAPTER 5

PREPARATION OF MIXED MATRIX ADSORBER MEMBRANES FOR PROTEIN RECOVERY^{*)}

ABSTRACT

This paper presents a generic technology allowing the incorporation of functional entities into a porous substrate. Various ion exchange particles were incorporated into an EVAL porous matrix by an immersion phase separation process and a heterogeneous matrix, composed of solid particles surrounded by a polymeric film, was formed. The developed concept is flexible and offers the possibility to easily adjust the geometry, the adsorption capacity, as well as the functionality of the structure. A series of fibers as well as flat membranes bearing an adsorptive function and high protein binding capacities were prepared. The membranes were characterised with respect to their morphology, porosity, permeability and adsorption capacity. High values of the protein adsorption capacity (135 mg BSA/g membrane) were obtained by static adsorption experiments. In a sequential desorption step by changing the pH and/or the ionic strength of the eluent, up to 90% protein recovery can be obtained. Dynamic capacity experiments were performed by flowing the protein solution through a stack of 10 membranes. The protein mass adsorbed per unit of membrane bed was calculated at a breakthrough concentration 10% of the feed concentration. The adsorber membranes can be reused in multiple adsorption/desorption cycles without significant loss of performance.

*) This chapter has been submitted to J. Membrane Sci.

5.1 INTRODUCTION

A new method to prepare structures of any geometry and large variety of functionality will be described in this paper. The motivation to develop a new platform technology for protein recovery and separation lies in the shortcomings of classical technologies. We propose to incorporate the functionality by dispersion of particles in a polymeric porous structure, which is formed by phase inversion. A slurry of dissolved polymer and particulate material is cast as a flat film or spun into a fiber and then solidified by a phase inversion process. The adsorber membranes prepared via this route contain particles tightly held together within a polymeric matrix (25-75% sorbent by weight). The polymer used should not interfere with the activity of the particles. Such adsorber membranes can be prepared in different shapes and can be operated either in stack of macroporous flat membranes or as a bundle of solid or hollow fiber membranes. They may also serve as a platform to which an end-user can couple the specific ligate needed. This has been recently pointed out by E. Klein [1] as one of the significant opportunities in membrane chromatography. A schematic representation of the technology platform is presented in Figure 5.1. We named the platform "mixed matrix" in analogy to its pendant in gas separation because the functional particles are embedded into a porous polymeric support. The reader may note that the function of polymer binder is to be highly porous, having a high degree of pore interconnectivity as well as having a low adsorption tendency towards the desired products. By choosing the particles and the binder one can establish various function such as ion exchange, adsorption, catalysis and hybrid such as reactive chromatography.

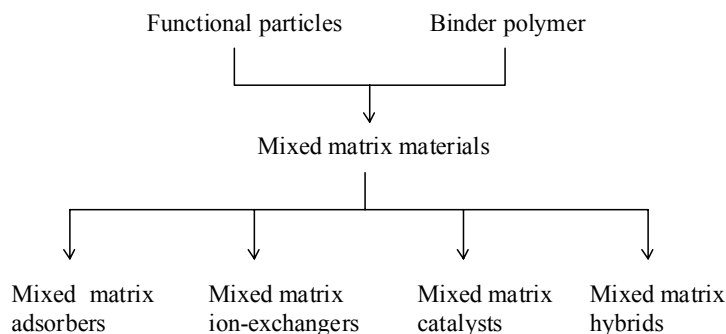


Figure 5.1 Schematic representation of the mixed matrix membrane preparation method.

We demonstrate that the incorporation of ion-exchange particles into a porous polymeric membrane results in an adsorptive structure, which can be applied to

isolate biomolecules. In particular such an application concerns the isolation of macromolecules such as peptides, proteins, nucleic acid or other organic compounds. Most suitable particles have, in combination with the porous matrix morphology, rapid adsorption kinetics, a capacity and selectivity commensurate with the application and allows for desorption of the molecule with an appropriate agent. The affinity of suitable adsorptive particles for specific molecules can be defined in terms of hydrophobic, hydrophilic or charged functionalities, in particular ion exchange functionalities, molecular (imprinted) recognition, or other specific interactions.

Ethylene vinyl alcohol copolymer (EVAL) is a good candidate as polymeric material for the preparation of membrane chromatographic supports since it displays a good mechanical strength, has high thermal stability, good chemical and biological resistance, and is easy to sterilize using, e.g., γ -radiation. Lewatit ion exchange resins CNP 80 were incorporated as particulate material into an EVAL porous structure to prepare heterogeneous membranes with high protein adsorption capacity, in various geometries (flat sheet membranes and solid or hollow fiber). An 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) these resins are economic feasible in protein separation processes [2]. The performance of the prepared ion exchange mixed matrix membranes as protein adsorber was investigated using bovine serum albumin (BSA) as a model protein.

5.2 THEORY

5.2.1 Membrane chromatography

Despite their large static adsorption capacity, conventional stationary phases involved in protein chromatographic separations are generally not suitable for operating with high linear velocities of the mobile phase. Their main drawback is the compression and compaction at high velocities [3]. The pressure drop over the column is high even for low flow rates, and increases during the process time due to bed consolidation and plugging. For efficient use of adsorptive sites, a certain residence time dependent on the particle size is required: the shorter the diffusion distance, the more rapid can be the flow of the feed solution. This leads to the development of smaller-diameter chromatographic supports, with the drawback that smaller particles pack more densely and create higher-pressure drops. The pressure drop in conventional chromatographic columns with particles with a

diameter of 2 μm is usually high, up to 25 MPa [4]. The use of monodisperse, non-porous, rigid particles as chromatographic media decreases the pressure drop, but usually leads to lower protein binding capacities, low reproducibility and high process costs [5]. Because of the higher-pressure drop, shallower adsorption columns with larger cross-section were developed. In fact, the ideal column geometry has *an infinitely short bed height* in order to minimize operating pressure and maximize throughput and *an infinite width* so that the ligand loading and binding capacity are maximized. In reality microporous adsorber membranes approach this ideal.

In the recent years there has been a considerable interest in developing membrane chromatography systems that function as a short, wide chromatographic column in which the adsorptive packing consist of one or more microporous membranes [6]. During the separation of macromolecules from a liquid phase by adsorption into the porous media, the diffusion of the molecule into the adsorbent determines the adsorption limits. In contrast to packed chromatographic beds, the main advantage of adsorber membranes is that the transport through the porous structure is controlled by convective instead of diffusional transport. This results in shorter process times and minimizes the denaturation of the products. The only drawback of adsorber membranes compared with packed beds is a lower dynamic binding capacity. The solute molecule, which flows preferentially through large pores quickly, saturates the adsorptive-sites on such large diameter and low surface area, pores. Subsequent flow through the adsorptive saturated large pores does not result in any further capture of solute. Meanwhile, residual capacity for the adsorber located in small pores, which exhibit high resistance to flow, is not complete utilized.

An ideal membrane support for application in chromatographic separation processes should be microporous to provide free interactions of biomolecules with the support and should be hydrophilic since the hydrophobic interactions between proteins and hydrophobic membrane surfaces are generally responsible for non-selective (irreversible) adsorption and membrane fouling. The membrane should be chemically and physically stable to resist to the conditions of adsorption, elution and regeneration and should possess functional groups which provide interactions (e.g. ion-exchange, affinity, hydrophobic) between the support and the solute molecules.

5.2.2 Mixed matrix adsorber membranes

Solid-phase extraction using particle-loaded membranes and particle-embedded glass fiber disks, referred generically as disk technology, became a widely used laboratory technique to isolate and concentrate selected analytes from a gas, or liquid prior to chromatography analyses [7, 8]. Nowadays, methods are known to prepare porous polymeric supports comprising particulate material from an appropriate mixture of starting components. Such a material is prepared by a casting process and either is limited in its three dimensional size by the housing it is cast into (Zip Tip, Millipore) or is in the form of a sheet (Empore) [9, 10]. Other preparation routes for porous polymeric fibers with particulate material incorporated require an additional process step to introduce the desired porosity [11]. After the step of preparing the matrix comprising particulate material either particulate material is removed from the non-porous fiber or the non-porous fiber is stretched resulting in porous fibers. Only in the latter case a microporous fiber comprising particles having a certain (sorptive) function is obtained. Disadvantages of the known porous polymeric membrane preparation processes are that they involve additional process steps after the formation of the fiber to come to a final product. It is therefore desirable to have a more efficient preparation process.

Depending on the actual process steps needed to come to the final product, suitable starting materials with properties that can sustain the conditions of the additional process steps have to be selected. Obviously, such a requirement limits the type of polymeric material that can be used. Furthermore it puts limitations on the type of particulate material that can be comprised in the polymeric matrix. A very high degree of particle loading will reduce the mechanical strength of the mixed matrix and therefore restrict the stretching procedure. The force required to reach sufficient stretching of the matrix material limits the degree of loading. By stretching of the particle comprising material the particulate material can drop out of the porous structure to be formed. In processes involving melt extrusion, only particulate material that can sustain temperatures required to melt the matrix polymer, usually are above 200°C [12], can be applied.

5.2.3 Protein adsorption

The use of solids for the removal of specific components from liquid mixtures has been widely used since ancient times. Nowadays it has become a useful tool for purification and separation processes with thousands of applications in the biological and biomedical fields, in biotechnology, or food industry. The separation

process known as adsorption, involves the preferential partitioning of components (adsorbate molecule) from the liquid phase onto the surface of a solid substrate (adsorbent) mainly by van der Waals and electrostatic forces [13].

It is generally acknowledged that protein adsorption is a process driven by different factors. Surface functionality, hydrophobic interactions as well as electrostatic interactions play an important role in protein adsorption and retention. When charged surfaces are involved in the adsorption process, beside the hydrophobic interactions, the pH and the ionic strength of the protein solution are important parameter to consider. In addition, there are many factors such as protein concentration, structural stability of protein, isoelectric point, surface morphology, domain composition, that can influence the protein adsorption process. Although the basics of adsorption are known, it is still not possible to predict the process of protein adsorption onto a polymeric surface even if the protein and the surface characteristics are well known.

With proteins, two important steps in the adsorption process are distinguished: i) the transport of the protein to the adsorbent surface, which is a function of protein concentration, diffusion coefficient, and flow conditions; ii) the interaction of the protein with the adsorbent surface, important for the protein desorption. Denaturation of protein molecules on the adsorbent surface might increase the contact area between the protein and the surface and therefore reduce the protein ability to desorb [14].

The adsorption of proteins is a semi-reversible process and a model, which describes both adsorption/desorption and exchange of proteins, has been not completely developed up to now. Several models based on solving the kinetic and the transport equations coupled by the boundary conditions were proposed to describe the protein adsorption onto solid surfaces.

In order to model the *adsorption kinetic*, an expression for the variation in time of surface concentration has to be designed. Different kinetic models e.g., the linear, the Langmuir, the bi-Langmuir, the Freundlich, the Freundlich-Langmuir isotherms have been proposed to describe the adsorption of solutes from a liquid solution onto a solid surface. The most common used model for the protein adsorption kinetic onto ion exchangers is the Langmuir adsorption isotherm, which assumes that adsorption is a reaction between the adsorbate molecule and the adsorption site. This kind of model implies that the adsorption is completely reversible and no permanent interactions take place between adsorbed molecules. The effect of *mass transfer* on the total rate of protein adsorption can be investigated from the change in protein bulk concentration as a function of place and adsorption time. Transport of protein molecules from an aqueous solution to a polymeric surface is caused by fluid flow and by diffusion. Analytical solution for the general case of protein

adsorption onto a polymeric surface does not exist, thus numerical solutions have to be obtained.

In protein adsorption studies reported in the literature, the initial stage in the adsorption process has often been considered mass transport limited. Quantitative measurements of the rate at which a diffusion process occurs are usually expressed in terms of diffusion coefficients. The flux of proteins through the aqueous protein solution-adsorber membrane interface can be well described considering just the protein diffusion from a semi-infinite medium towards a flat plate. In this simple case, the maximum amount of adsorbed protein after a certain time t can be calculated from equation (1) obtained by integrating Fick's second law equation. From this equation one can see that in the diffusion limited case there is a linear relationship between the adsorbed amount of protein and the square root of time. Using the initial gradient of the protein adsorption curve plotted against the square root of time, an effective diffusion coefficient is possible to be calculated according to:

$$\frac{M_t}{M_\infty} = \frac{4}{\sqrt{\pi}} \cdot \sqrt{\frac{D \cdot t}{l^2}} \quad (1)$$

where M_t and M_∞ are the mass uptake at time t and infinite time respectively, D is the effective diffusion coefficient and l is the membrane thickness [15].

5.3. EXPERIMENTAL

5.3.1 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. Dimethylsulfoxide (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. Lewatit ion exchange resins CNP 80 kindly supplied by Caldic, Belgium were used as adsorbent particles. Buffer solutions were freshly prepared in ultrapure water. BSA (fraction V, Sigma) was used as a model protein in the adsorption/desorption experiments. Bradford reagent (Sigma) was employed in protein concentration determination. All other chemicals were used as received. Ultrapure water was prepared using a Millipore purification unit Milli-Q plus.

5.3.2 Adsorbent preparation

The Lewatit CNP 80 resin beads, with a size of 0.4-1.6 mm, were washed with demiwater in a stirred vessel until neutral pH and dried at 60°C in a conventional oven. Before incorporation into the polymeric matrix, the IER were grinded and sieved to obtain fractions with different particle sizes: i) 20-40 µm; ii) smaller than 20 µm. The particle size distribution of different particle fractions was measured using a Microtrax X-100 from Leeds & Northrup, after 3 minutes of ultrasonically treatment with a Branson Sonifier 450 in order to break down the formed agglomerates.

5.3.3 Membrane preparation

Different amounts and sizes of Lewatit ion exchange particles were added to a solution containing 10%wt EVAL in DMSO in order to obtain membranes with different adsorptive properties. 10% 1-octanol was added to the casting solution in order to improve the membrane morphology [16]. The mixtures were stirred over night to break the clusters of particles. Our goal was the preparation of mechanically stable adsorber membranes consisting of submicrometer sized pores, with high interconnectivity, containing accessible adsorbent particles needed to obtain high protein adsorption capacities.

All membranes were made by immersion precipitation. For the preparation of flat sheet adsorber membranes the polymeric solution was cast on a glass plate and immediately immersed into a water coagulation bath at 40°C. For solid and hollow fiber preparation, the dope solution was pumped through a triple spinneret and, after a short residence time in an air gap, immersed into the water bath at room temperature. For the preparation of solid fibers no bore liquid was pumped through the inner needle. The outer orifice can be used to pass different solvent mixtures to modify the fiber morphology e.g., to eliminate the formation of a skin layer at the outer surface of the fiber [17]. All prepared membranes were washed to remove the additives and solvents and afterwards dried in air. Table 5.1 summarizes the preparation conditions for the membrane adsorbers characterized in this study. Samples were identified in the way that first the sieve fraction is given, in terms of maximum particle size, followed by the amount of ion exchange resins loaded into the casting solution.

Membrane adsorber	Sieve fraction, μm	Resin loading ^{*)} (%)
M _{20/25}	<20	25
M _{20/50}	<20	50
M _{20/65}	<20	65
M _{40/65}	20-40	
M _{20/75}	<20	75

Table 5.1 The conditions for the mixed matrix adsorber membranes preparation.

^{*)} An important parameter for the adsorbers' preparation is the *resin loading* defined as (2) where W_r is the amount of ion exchange resins and W_p is the amount of EVAL polymer in the casting solution (g).

$$R_{loading} = \left(\frac{W_r}{W_p + W_r} \right) \times 100 \quad (2)$$

5.3.4 Membrane characterization

For *scanning electron microscopy*, pieces of membranes were frozen in liquid nitrogen and fractured in order to visualize the 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 water uptake of a calibrated volume of a piece of dry polymer. Average values were obtained from three different samples.

The *permeation rates* through the flat sheet adsorber membranes were determined using a nitrogen pressurized stirred dead-end filtration cell. Solutions of 50 mM acetate buffer pH 4.5 or 50 mM phosphate buffer pH 7 were permeated through a stack of 10 EVAL ion-exchange membranes.

For the *retention coefficient* measurements, a freshly prepared BSA solution (1 mg/ml in 50mM acetate buffer) was permeated through the membrane. The BSA concentration in the feed and the permeate samples was determined by measuring the absorbance at 280 nm with a spectrophotometer PU 8720 UV/VIS. All the filtration experiments were carried out at room temperature at a transmembrane pressure of 0.1 bar, under 200 rpm.

The *static protein adsorption capacity* of porous adsorber membranes was determined with bovine serum albumin. A known amount of membrane was equilibrated with a BSA containing acetate buffer, pH 4.5 and the protein uptake per membrane volume was measured. Protein concentrations of 1-2 mg/ml were employed in the experiments as a realistic concentration for many commercial separation processes. At a pH lower than its isoelectric point, the protein is positively charged, meanwhile the adsorber membrane having a negative net charge. The protein adsorbs into the mixed matrix adsorber membrane reducing the BSA concentration in the bulk solution until equilibrium is reached. For minimal disturbances in the equilibrium values, only small discrete samples of 0.1 ml protein solution were taken at short time intervals from the bulk solution and measured at 595 nm using the Bradford method [18]. The adsorbed protein (mg BSA/g membrane) was calculated using a depletion method. Equivalent mass, volume and surface measurements allow us to express the binding capacity in alternative units, e.g., mg BSA/ml membrane, mg BSA/cm² membrane.

The isolated BSA- adsorber membrane complex was consecutively treated with different eluents in order to dissociate the complex. To determine the optimal conditions for the BSA desorption eluents with different pH and ionic strength (50 mM acetate buffer pH 4.5, 50 mM acetate buffer pH 4.5 + 1M NaCl, 50mM Tris buffer pH=9, and 50mM Tris buffer pH=9 + 1M NaCl) were used. At a pH higher than the BSA isoelectric point, the protein becomes negatively charged and is therefore released from the matrix passing freely through the porous membrane in the bulk solution. It is a feature of ion-exchange resins that due to Debye screening effect the ionic interaction decreases with increasing the ionic strength. Therefore, by increasing the ionic strength bulk phase, the protein adsorbed onto the adsorber membrane can be eluted. The BSA concentration in the elution step was monitored at 280 nm as previously described. The particle-loaded adsorber membranes were regenerated with 10% HCl, washed with demiwater till neutral pH and reused in a new adsorption/desorption cycle.

The *adsorption isotherm* for the adsorbers prepared by incorporation of ion exchange resins was obtained by incubating the same amount of membrane with different initial amounts of BSA to reach different equilibrium concentrations. It has been assumed that the adsorption isotherm follows the Langmuir type isotherm, which can be written as:

$$\frac{1}{q^*} = \frac{1}{q_m} + \frac{K_d}{q_m} \times \frac{1}{C^*} \quad (3)$$

Since the parameters q^* (the protein concentration into the adsorber membrane) and C^* (the protein concentration in the bulk solution) at equilibrium are experimental data, a plot of $1/q^*$ versus $1/C^*$ allows to determine the dissociation constant K_d and the maximum adsorption capacity q_m using a linear curve fitting.

The *dynamic adsorption performances* of the flat sheet EVAL adsorber membranes were measured at constant permeation rate using a stirred dead-end filtration cell. To overcome the limited adsorption capacity of single adsorptive membrane, 10 sheets of membrane were stacked. The obtained configuration permits rapid, low-pressure adsorption of protein in either batch or continuous recycle mode. The desired product is concentrated substantially through its adsorption into the membrane. 1 mg/ml BSA solution in 50 mM acetate buffer pH 4.5 was permeated through the stack of 10 membranes with a flow rate of 10 l/h/m². The permeate was collected and fractionated using a fraction collector LKB Frac-100 from Pharmacia and the BSA concentration in the permeate samples was determined spectrophotometrical at 280 nm. The protein mass adsorbed per unit of membrane bed was calculated by numerical integration over the filtration run till a breakthrough concentration 10% of the feed concentration was reached.

5.4 RESULTS AND DISCUSSIONS

The *results and discussion section* first describes the morphological characterization of the prepared adsorber membranes. Then the results of the influence of the amount and the size of the particulate material entrapped into the porous matrix on the morphology of the EVAL adsorber membranes are presented. A detailed membrane characterization including porosity and transport properties, protein retention experiments as well as protein adsorption capacity in both static and dynamic mode is finally presented.

5.4.1 Adsorber membrane morphology

An important parameter for the preparation of adsorber membranes is the amount of resins incorporated into the polymeric matrix. By increasing of the resin loading, the amount of adsorptive sites increases and a higher protein adsorption capacity is expected. The morphologies of the adsorber membranes prepared in the solid fiber geometry with different loading capacities of Lewatit CNP 80 ion exchange resins are shown in Figure 5.2.

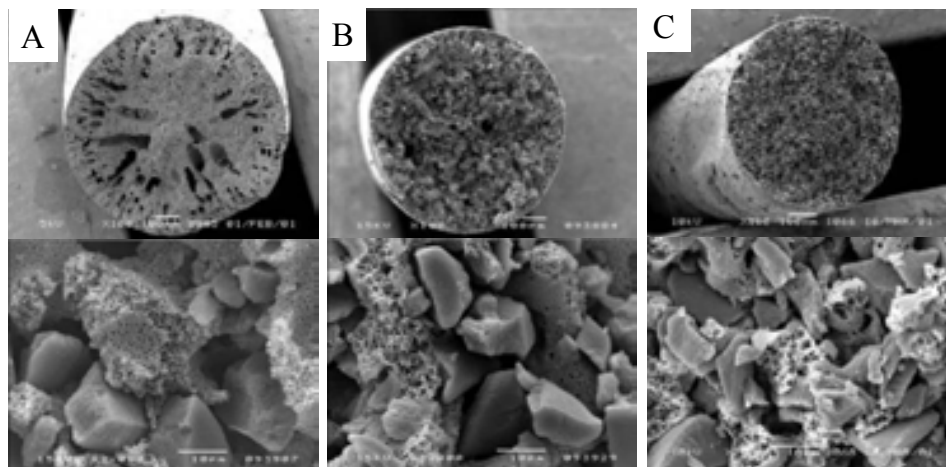


Figure 5.2 SEM micrograph of the adsorber membranes prepared in the solid fiber geometry using: A) 33%; B) 50%; C) 75% Lewatit CNP 80 ion exchange resins incorporated into the EVAL polymeric support. Magnification $\times 100$ and $\times 2000$, the white horizontal bar indicates $100\mu\text{m}$ and $10\mu\text{m}$ respectively.

For low loading capacities (Figure 5.2A), macrovoids appear across the adsorber membrane structure and therefore the membrane $M_{20/25}$ is not expected to have optimum morphology for protein adsorption. The presence of macrovoids in the membrane structure is undesirable, since the macrovoids can cause weak spots in the membrane leading to mechanical failure under pressure. Furthermore, the presence of macrovoids creates channels and leads to a lower dynamic adsorption capacity due to a lower effective adsorptive area. The number and size of voids per unit of membrane volume decreases with increasing the resin loading. We interpret the solid particles to act as a nucleus in the casting solution limiting the grow size of the macrovoids. The solid particles also increase the viscosity of the polymer solution and therefore decrease the macrovoid formation. At loading higher than 50%, macrovoids are not observed across the adsorber membrane structure (Figure 5.2B). A further increase in the resin content leads to an increase of viscosity, which restrict the resin loading to 80%. For the membrane $M_{20/75}$, the ion exchange resins occupy a very high percentage of the membrane volume, leading to high area available for the adsorption process (Figure 5.2C). Despite the proper structure, the membrane $M_{20/75}$ is difficult to use in the adsorption experiments due to its low mechanical strength. For the mixed matrix adsorber membrane preparation, different *particle sizes* were tested. The structure for the adsorbers prepared by incorporation of 65% resins with different sieve fractions differs for both fiber and flat membranes (Figure 5.3).

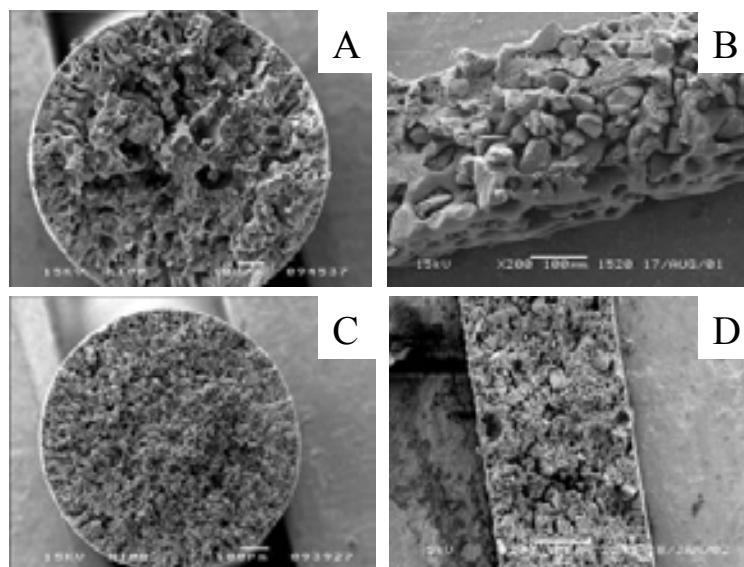


Figure 4.3 The effect of particle sieve fraction of the particulate material entrapped into the porous matrix on the morphology of the EVAL membrane adsorbers prepared in both flat membrane and solid fiber geometry: A-B) 20-40 μm ; C-D) 0-20 μm . Magnification $\times 100$ for the solid fibers and $\times 200$ for the flat membranes, the white horizontal bar indicates 100 μm .

For particles with diameter larger than 20 μm , finger-like macrovoids can be observed across the fiber thickness (Figure 5.3A). The low adhesion between the resins and the polymeric matrix can cause the resins falling out during the protein adsorption experiments (Figure 5.3B). Using ion exchange resins with diameter smaller than 20 μm a adsorber membrane with more uniform structure and large ion exchange surface available for protein adsorption was obtained (Figures 5.3C and 5.3D).

Figure 5.4 presents a more detailed structure for the flat adsorber membranes prepared by incorporation of 65% ion exchange resins with a diameter smaller than 20 μm . The membrane shows pores larger than 0.1 μm on both, the glass (Figure 5.4C) and the air surface (Figure 5.4D). The porous substructure shows interconnected pores without evident finger-like macrovoids across the entire cross-section (Figures 5.4A and 5.4B). 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.

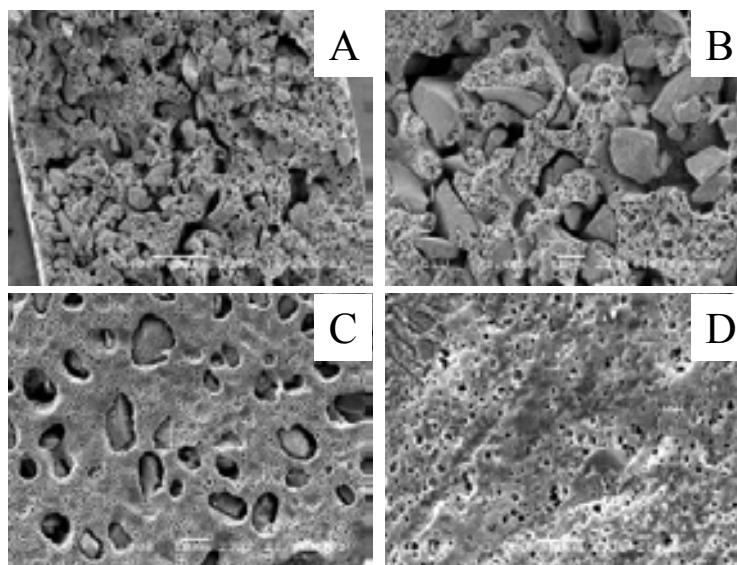


Figure 5.4 SEM photomicrograph for the membranes prepared from 10% EVAL in DMSO in the presence of 10% 1-octanol as additive by incorporation of 65% ion exchange particles into the polymeric support: A) cross-section, magnification $\times 400$, the white horizontal bar indicates $50\mu\text{m}$; B) cross-section, magnification $\times 1000$, the white horizontal bar indicates $10\mu\text{m}$; C) bottom-surface magnification $\times 1000$, the white horizontal bar indicates $10\mu\text{m}$; D) top-surface, magnification $\times 3000$, the white horizontal bar indicates $5\mu\text{m}$.

5.4.2 Adsorber membrane performance

Porosities in the range of 75% were obtained from swelling experiments for both flat and fiber geometry, with no significant differences for various amounts and sizes of ion exchanger incorporated into the polymeric structure. Due to the high pore interconnectivity and porosity of the top layer, high permeation rates through a stack of 10 adsorber membranes ($\approx 300 \text{ l/h/m}^2/\text{bar}$) were obtained. The maximum operation pressure used was 0.1 bar. The continuous porous structure of the adsorber enables the use of stacking of membranes avoiding the well-known packing problems that arise with conventional particles containing chromatographic columns and expanded bed reactors. BSA retention lower than 3% of the initial protein feed concentration by the protein-saturated adsorber membrane indicate that the adsorber membrane retains its microfiltration properties.

Protein adsorption/desorption test

Milling is a simple way to reduce the size of sorbent particles and potentially a method to increase the surface area. The size of the ion exchange resins was reduced from an average particle diameter of 480 μm to less than 20 μm . Data on particle size distribution and total surface area are presented in Table 5.2. Indeed, particles with diameters smaller than 20 μm present up to 20-fold higher BSA adsorption capacity (110 ± 20 mg BSA/g ion exchange resins) in comparison with raw ion exchange particles (see Table 5.3).

Particle diameter, μm			Specific surface area
$x_{10} = 3.9$	$x_{50} = 9.03$	$x_{90} = 16.89$	1.1 m^2/g particles (0.89 m^2/cm^3 particles)

Table 5.2 Particle size distribution and total geometric surface data for the milled ion exchange resins.

The effect of the adsorption time on the static adsorption capacity for the adsorber membranes with different amounts and sizes of Lewatit CNP 80 ion exchange resins incorporated into the polymeric matrix was investigated (Figure 5.5). Although the protein adsorption capacity still slightly increases after 24 hours all the protein adsorption experiments were performed within 24h in order to minimize the effect of protein denaturation. Figures 5.5A demonstrate that the protein adsorption capacities increases to even more than 100 mg BSA/g membrane by using particles smaller than 20 μm due to an increased adsorptive particle surface at high resin loading. Pure EVAL membranes have an adsorption capacity of around 3 mg BSA/g membrane, which is less than 3% of the total protein adsorption capacity. This leads to the conclusion that the ion exchange particles, which are incorporated into the polymeric matrix, are almost totally responsible for the adsorption capacity of the prepared mixed matrix adsorber membrane. The effect of particle sieve fraction incorporated into the polymeric matrix on the static BSA adsorption capacity is presented in Figure 5.5B. By using smaller particles the adsorption capacity of protein per gram of ion exchange resins increases due to the increased surface area available for adsorption per gram of resins.

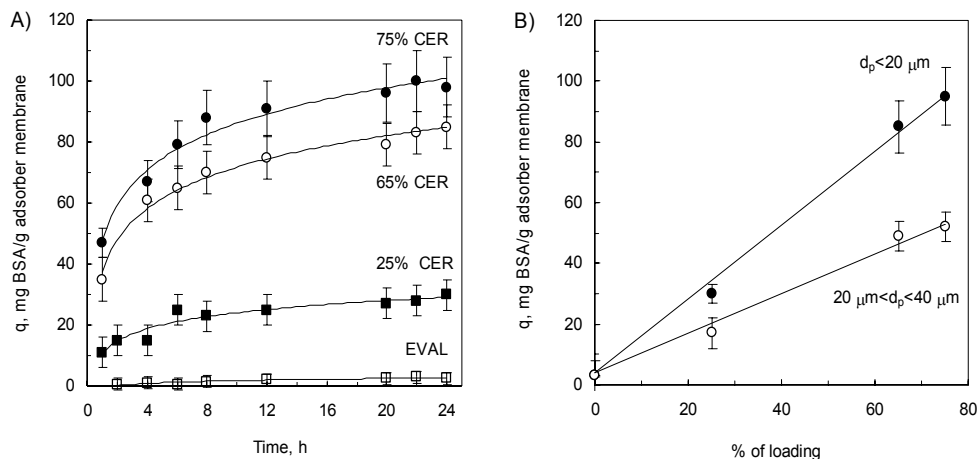


Figure 5.5 Effect of ion exchange resin loading (A) and particle sieve fraction (B) incorporated into the polymeric matrix on the BSA adsorption capacity of the prepared adsorber membranes.

The accessibility of ion exchange particles inside the porous structure was investigated by performing adsorption tests in the same experimental conditions for both suspensions of ion exchange resins and prepared adsorber membranes. Protein adsorption measurements on suspensions of ion exchange particles with diameters smaller than $20 \mu\text{m}$ leads to BSA adsorption capacity values of 110 ± 20 mg BSA per mass of ion exchange resins. Table 5.3 concludes that the adsorber membranes possess a good accessibility for the protein to the adsorptive sites. In fact, a slight increase for the equivalent value of protein adsorption capacity, to 130 ± 10 mg BSA/g ion exchange resins incorporated into the polymeric structure, was observed. This positive deviation can be explained by the formation of agglomerates in the ion exchange particle suspensions reducing the accessible adsorptive surface available for protein binding. In the case of adsorber membranes, the particles are entrapped within the polymeric matrix eliminating the possibility of aggregates formation.

Adsorber		Protein adsorption capacity	
		mg BSA/g resins	mg BSA/g membrane
<i>EVAL membrane</i>		-	3±2
<i>Ion exchange resins</i>	Raw particles, $d_p^{\text{average}} = 480\mu\text{m}$	6±2	-
	Milled particles, $d_p < 20\mu\text{m}$	110±20	-
<i>Membrane adsorber</i>	M _{20/25}	130±10	130±10
	M _{20/65}	131±11	131±11
	M _{20/75}	126±13	126±13

Table 5.3 Static BSA adsorption capacities for both suspensions of ion exchange resins and membrane adsorbers prepared by incorporation of the above mentioned particles.

About 65% resins loading for ion exchange particles smaller than 20 μm is the optimum degree of particle loading without hampering the mechanical integrity of the adsorber membrane. Therefore, in the following experiments we will focus on the adsorption properties of the adsorber membranes M_{20/65} with a thickness of approximately 300-350 μm. The measured adsorption equilibrium isotherm of BSA on the adsorber membrane appears to be of the Langmuir type, with maximum adsorption capacity of 135 mg BSA/g membrane, equivalent with 45 mg protein/ml membrane (Figure 5.6).

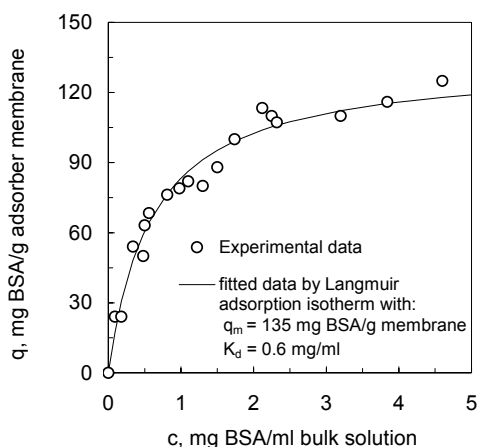


Figure 5.6 Correlation between the Langmuir model and experimental data of the BSA adsorption isotherm.

Reported capacities for several adsorptive membranes are summarized in [19] with adsorption capacities ranging from 3 up to 50 mg macromolecule/ml of membrane. This indicates that the technical concept of mixed matrix adsorber membranes shows performances equivalent to the highest values reported in the literature.

Experiments with albumin reported in literature have shown that the protein adsorbs mainly in monolayer with the longitudinal axis perpendicular to the sorbent surface. Values of about 0.6-0.84 $\mu\text{g BSA}/\text{cm}^2$ are accepted for a monolayer of BSA molecules adsorbed on polymeric surfaces [20]. When the sorbent surface is very hydrophobic the protein can rearrange at the interface, thus the adsorption becomes energetically more favorable and the formation of multilayer can occur [21].

The total geometric surface for the particles incorporated into the EVAL porous structure, available for protein adsorption, was obtained from the particle size distribution measurements. Assuming that all the particles have spherical geometry a total adsorptive surface of 1.1 m^2/g particles was obtained (see Table 5.2). From the membrane SEM micrographs one can observe that, after grinding, the resins morphology differs significantly from the spherical geometry. Including a non-uniformity factor of 1.5, responsible for the surface roughness and the deviation from the spherical shape, the total surface available for protein adsorption will be around 1.6 m^2/g particles. As already concluded from Table 5.3, the ion exchange particles incorporated into the polymeric matrix are almost totally responsible for the protein adsorption capacity of the prepared mixed matrix adsorber membranes. Therefore for quantifying the protein adsorption into the adsorber membrane no adsorption into porous EVAL polymeric matrix was taken into account. The method used to calculate the maximum amount of protein adsorbed on the porous ion exchanger surface based on the geometric area of 1.6 m^2/g particles, leads to values of 13 $\mu\text{g BSA}/\text{cm}^2$, which imply multilayer adsorption. Up to 20 BSA layers are formed at the optimum conditions. For initial protein concentrations of 2 mg/ml involved in many commercial separation processes, values of 8.1 $\mu\text{g BSA}$ adsorbed on the unit of porous ion exchanger surface (equivalent of 9 layers of BSA) were obtained.

The total area available for adsorption can be larger than the geometric area due to the porous nature of the ion exchangers. The BET data provided by the producer for the Lewatit ion exchange resins are in the range of 40 m^2/g resins. Assuming that all the porous area is available for protein adsorption, values in the range of 0.5 $\mu\text{g BSA}$ adsorbed on 1cm^2 internal area, close to the values reported for a complete BSA monolayer, are obtained.

For diffusion coefficient measurements, adsorption experiments were performed with large volumes of protein solution in order to maintain a constant solute concentration in the bulk solution during adsorption. The values of the amount of protein adsorbed into the adsorber membrane at a certain time were calculated from the desorption measurements for individual experiments. In this case a linear variation of protein mass uptake with the square root of time was obtained (Figure 5.7). Assuming that the bulk protein concentration and the diffusion coefficient determine the rate of protein adsorption, a BSA diffusion coefficient into the adsorber membrane of $3.4 \times 10^{-13} \text{ m}^2/\text{s}$ was calculated based on equation (1). Such a value is about 150 times lower than the free BSA diffusion coefficient in aqueous solution, equal with 5.3×10^{-11} [22].

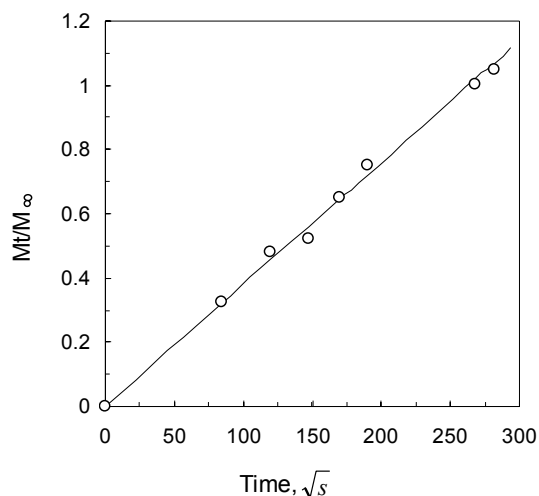


Figure 5.7 The gradient of protein adsorption curve plotted against square root of time for diffusion coefficient measurements.

Different experimental values of the BSA diffusivity are reported in the literature for various porous materials. Graham et al. [23] studied the equilibrium and the kinetic of BSA adsorption on a DEAE-resin by a batch method and reported a BSA diffusion coefficient in the resins of about 100 times smaller than in the free solution. Using composite silica-polyacrylamide gel anion exchanger Fernandez et al. [24] obtained values of $9.2 \times 10^{-13} \text{ m}^2/\text{s}$ as the BSA diffusivity. Yoshida et al. [25] reported values of 1×10^{-13} and $4.6 \times 10^{-13} \text{ m}^2/\text{s}$ for the BSA diffusion into chitosan particles, for up to 500 times smaller than the one in the free solution. The values of BSA diffusivity calculated by Miyabe and Guichon [26] in a porous ion exchanger range from 10^{-14} to $10^{-13} \text{ m}^2/\text{s}$, dependent on protein concentration. Hunter et al.

[27] reported a calculated BSA diffusivity in BRX-QP particles of $2.4 \times 10^{-13} \text{ m}^2/\text{s}$ based on the homogeneous diffusion model. The BSA diffusivity calculated by Arevalo et al. with a pore diffusion model for BSA adsorption onto Lewatit TP 207 ion exchange particles [2] was $1.5 \times 10^{-9} \text{ m}^2/\text{s}$, much higher than the values obtained by other authors, even that the free BSA diffusion coefficient in aqueous solution. The authors assumed that this may be due to the fact BSA molecule cannot penetrate the resin pores because of its high molecular size. Thus mainly a surface adsorption of BSA onto the adsorber particles is predicted. Nevertheless, the value of diffusion coefficient determined from Figure 5.7 is comparable with those obtained in the literature. It remains an experimental challenge to obtain conclusive data and to give a complete interpretation for the mechanism of BSA adsorption into the adsorber membranes. Nevertheless, we assume that a combination of the two mechanisms (adsorption onto the geometric area and adsorption onto the total internal porous surface) occurs for BSA adsorption into the EVAL ion exchange adsorber membranes.

To investigate the effect of salt concentration on the BSA adsorption, samples of membrane were exposed to protein solutions of different ionic strength. As shown in Figure 5.8, by increasing the bulk ionic strength from 20 to 200 mM, a loss of about 20% of the BSA adsorption capacity for the EVAL adsorber membranes was obtained.

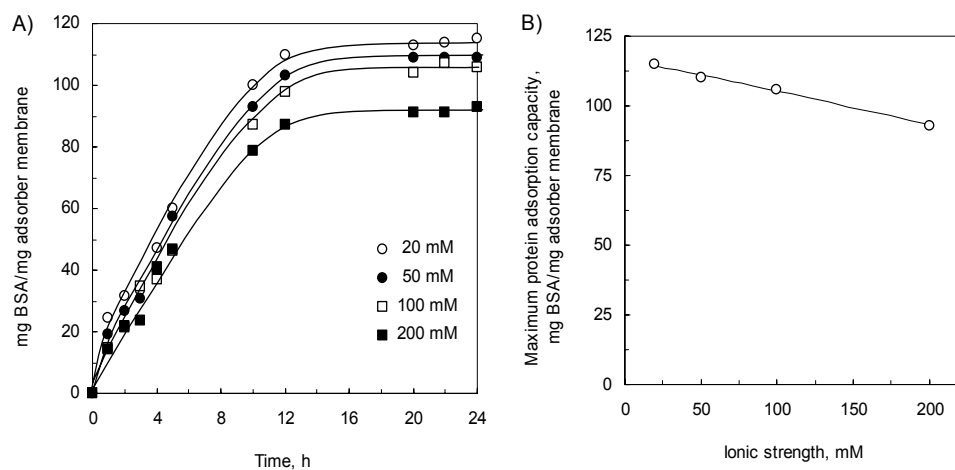


Figure 5.8 Effect of ionic strength on the BSA adsorption capacity of the adsorber membranes.

Similar phenomena have been reported in the literature [28-30] for various protein/adsorbers pairs due to: i) competition of salt counter ion against the protein ions for binding sites; ii) the salt co-ion binding shield the protein ion and the charged binding sites from each other; iii) the change of the conformation of the protein molecules; iv) the increased shrinking of ion exchange resins, which reduces the porosity and the surface area and thus the availability of binding sites. However, Conder et al. [31] observed that using a cross-linked cellulose chromatographic ion exchanger, the BSA binding capacity decreases with increasing salt concentration only for salt concentration above approximately 100 mM. At salt concentrations below 100 mM (≈ 5 g/l) the capacity changes only slightly. In contrast, significant decreases of the BSA binding capacities on Diaion HPA25 adsorbent, with more than 60%, were reported by Lan et al. for ionic strength increasing from 2 to 52 mM [28]. For the ion exchanger used in this study we see a linear decrease of the protein adsorption capacity with the ionic strength of the feed solution (Figure 5.8B). Different elution conditions were tested for BSA desorption from the adsorber membrane (Figure 5.9). The eluted amount of protein is very small for 50 mM acetate buffer pH 4.5 indicating a strong adsorption which cannot be disrupted by a gradient of salt concentration. It can be also observed that an increase of the ionic strength does not lead to a substantial protein recovery. However, by increasing the pH of the eluent to 9, around 90% BSA desorption was obtained.

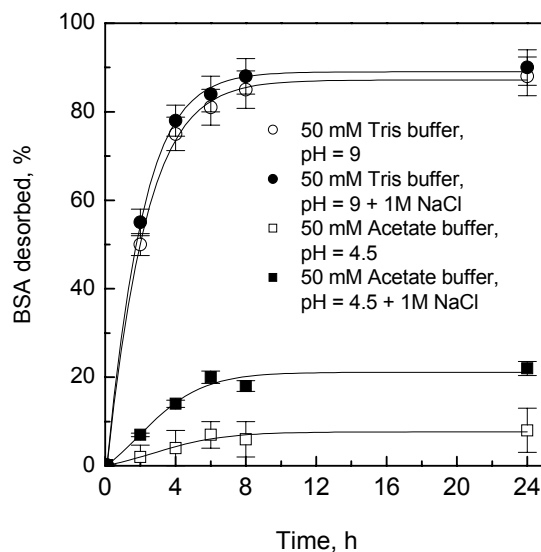


Figure 5.9 Effect of elution conditions employed in protein recovery.

A critical issue in adsorption technology is the adsorption capacity after repetitive cycles of adsorption/desorption/regeneration. After the described adsorption and desorption steps, the mixed matrix adsorber membranes were regenerated with 10% HCl, washed with ultrapure water until neutral pH and reused in a next adsorption/desorption cycle. After four sequential cycles no significant loss of performance can be observed (Table 5.4).

Adsorption/desorption/regeneration cycle number	Protein adsorption capacity, mg BSA/g membrane
I	85
II	89
III	86
IV	80

Table 5.4 Protein adsorption capacity for sequential adsorption/desorption/regeneration cycles.

In dynamic operation, the protein molecules are transported through the adsorptive membrane by a convective flow of the feed solution. The transport of the protein to the adsorptive surface, is a function of flow conditions, protein pore diffusion as well as protein intraparticle diffusion. Typical breakthrough and elution curves for a stack of 10 mixed matrix adsorber membranes are presented in Figure 5.10. The dynamic BSA adsorption capacity until the breakthrough point 10% of the protein feed concentration was 35 mg BSA/g membrane. Desorption with 50 mM Tris buffer pH 9, at a flow rate of 10 l/h/m², leads to around 85% BSA recovery.

The measured dynamic protein adsorption capacity is 2.5 times smaller than the maximum static binding capacity. This can be contributed to a non-uniform flow distribution and resistance against mass transport rate processes. This effect is also known in packed bed chromatography where, because of slow processes of adsorption/rearrangement/unfolding and axial dispersion, the dynamic adsorption is 5-10 times lower than the static binding capacity. The transport rate resistance within the adsorptive membrane is principally due to dispersive and diffusional resistances to mass transport. Also the unfolding and rearrangements of the protein at the adsorptive interface contributes to a somewhat lower adsorption capacities.

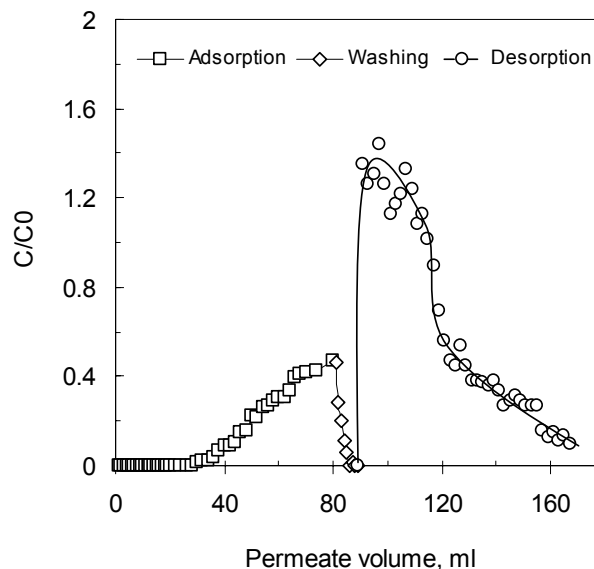


Figure 5.10 Typical breakthrough and elution curves for a module of 10 mixed matrix adsorber membranes.

The convective feed flow containing the protein molecules flows preferentially through the large pores and saturates the adsorptive-sites surrounded by the large pores. Meanwhile, adsorption in smaller pores and into the particles is more dominated by diffusional processes, which 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 so that each adsorbate molecules can diffuse to the adsorptive site and can rearrange/unfold their structure to its most favorable one before the interstitial volume continues through the membrane. Furthermore, it has been shown 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.

Detailed analysis on dynamic adsorption behaviour as well as the separation of mixtures containing proteins with similar size (BSA and Hb) using the new adsorber membrane system will be described in a forthcoming article.

5.5 CONCLUSIONS

The unique feature of the described adsorber membranes concept is the ability to create particle-loaded membranes using any type and/or size of entrapped particle and almost any type of polymeric material. The proposed adsorber membranes can be prepared under mild process conditions. The preparation method does not influence the integrity of the particulate material during the processing. Heat sensitive particles, which cannot be melt-extruded because of the elevated temperatures required to melt the matrix polymer, can be incorporated into a porous matrix by the phase inversion process without danger of deactivating the particle. Furthermore, since the particles are functionalized before the incorporation into the polymeric support, extreme conditions can be also applied for the functionalization of the resin particles.

The adsorber membranes prepared in this study combine the advantages of membrane technology (easy scale-up, low-pressure drop and high throughputs) with column chromatography (selectivity and high binding capacity). Beside this, the adsorber membranes are also less sensitive to fouling and plugging. The advantages of the proposed adsorber membranes compared with high performance liquid chromatography lies therefore in high protein binding capacities at low backpressure leading to lower denaturation. High protein recovery can be obtained by increasing the pH and/or the ionic strength of the eluent. The adsorber membranes can be reused in multiple adsorption/desorption cycles without significant loss of performance.

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CHAPTER 6

DYNAMIC BEHAVIOUR OF ADSORBER MEMBRANES FOR PROTEIN RECOVERY⁸⁾

ABSTRACT

Despite their large static adsorption capacity, conventionally gel-type stationary phases involved in protein chromatographic separations are generally not suitable for operating with high linear velocities of the mobile phase in the packed beds. Their main drawback is the compression at high velocities. In the recent years there has been a considerable interest in developing membrane chromatography systems that function as a short, wide chromatographic column in which the adsorptive packing consist of one or more microporous membranes. This study reports the use of such adsorber membranes prepared by incorporation of various types of ion exchange resins into an EVAL porous membrane for protein recovery. The obtained heterogeneous matrixes composed of solid particles surrounded by the polymeric film posses a good accessibility for the protein to the adsorptive sides. Furthermore, small particles can be embedded into porous polymeric structures without the disadvantages of classical chromatografic columns (high pressure drop, fouling and plugging sensitivity, low flow rate), but with the advantages of membrane technology (easy scale-up, low-pressure drop, high binding capacity). The adsorptive membranes feature high static as well as dynamic protein adsorption capacities for operating flow rates ranging from 200 to 400 l/h/bar/m² and ionic strength of 20–200 mM. In a sequential desorption step by changing the pH and/or the ionic strength of the eluent, up to 90% protein recovery was obtained. Next to the separation, the mixed matrix adsorber membranes function as a concentration medium since the protein can be concentrated up to 10–fold in the eluent. The adsorber membranes can be reused in multiple adsorption/desorption cycles with good adsorption performances.

8) This chapter has been submitted to *Biotechnol. Bioeng.*

6.1 INTRODUCTION

Affinity membranes have been designed to bypass the fundamental limitations of packed-bed absorbers, mainly the bead compression at high velocities. The main advantages of microporous membranes in comparison with particle beds are the low flow resistance, due to higher porosity and reduced bed heights, low sensitivity for clogging and faster mass transfer. Pressure drop and intra-bed diffusion limitations are minimised by using convective flow through the fine pores of the membrane [1]. The ideal membrane support for applications in affinity separation processes should be microporous, to provide free interactions of biomolecules with the support and hydrophilic, since the hydrophobic interactions between proteins and hydrophobic membrane surfaces are generally responsible for non-selective (irreversible) adsorption and membrane fouling. The membrane should be chemically and physically stable to resist to the conditions of adsorption, elution and regeneration and should possess a high density of functional groups which provide desirable interactions (e.g. ion-exchange, affinity, hydrophobic) between the support and the solute molecules [2].

Recently, methods were developed to prepare stable polymeric supports comprising functionalized particulate materials. These materials are either limited in its three-dimensional size by a surrounding housing (Zip Tip, Millipore) or are cast in the form of a sheet, often containing glass fibers to enhance the required mechanical properties (Empore) [3, 4]. Other routes such as melt extrusion to prepare porous polymeric fibers containing particulate material require additional process steps to introduce the desired porosity [5]. Therefore, it is desirable to have a more efficient preparation process. Solid-phase extraction process using this type of particle-loaded membranes and particle-embedded glass fiber disks, referred generically as disk technology, became a widely used laboratory technique to isolate and concentrate selected compounds out of a gas or a liquid, prior to chromatography analyses [6-7].

In a previous paper [8] we described a new concept for a single-step process preparation of ion exchange adsorber membranes having particulate material entrapped in the porous matrix as potential chromatographic systems. A mixture of dissolved polymeric and particulate material was cast/spun into a flat/fiber membrane and then solidified by a phase inversion process. The prepared adsorber membranes contain ion exchange particles tightly held together within a polymeric matrix (25-75% sorbent by weight), the latter not interfering with the activity of the particles. Such membranes can be prepared in different shapes and can be operated either as stacked microporous flat sheet membranes or as modules containing solid or hollow 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 membrane. Such adsorber membranes may also serve as a platform to which an end-user can couple the specific ligate needed. This has been recently pointed out by E. Klein [9] as one of the significant opportunities in membrane chromatography. A schematic representation of the technology platform is presented in Figure 6.1.

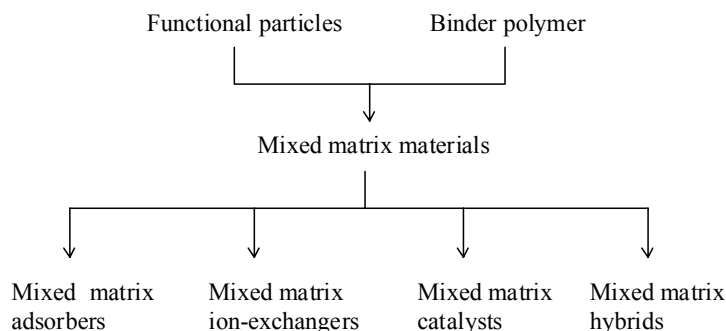


Figure 6.1 Schematic representation of the mixed matrix membrane preparation method

We named the platform "mixed matrix" in analogy to its pendant in gas separation because the functional particles are embedded into a porous polymeric support. The reader may note that the function of polymer binder is to be highly porous, having a high degree of pore interconnectivity as well as having a low adsorption tendency towards the desired product. By choosing the particles and the binder one can establish various function such as ion exchange, adsorption, catalysis and hybrid such as reactive chromatography.

The porous mixed matrix adsorber membranes have a wide variety of applications, depending upon the particle selection. For example, applications include peptide and protein isolation from fermentation broths, protein fractionation, ligands immobilisation for affinity-based separations, chromatography, immobilised catalysts and enzymes for reactions, detoxification, product protection and release systems. In this paper, we provide a broad characterisation of the new type of adsorber membranes. We focus on their application as protein adsorbers for a single protein using bovine serum albumin (BSA) as a model, by mean of static and dynamic protein binding capacities.

6.2 THEORETICAL ASPECTS

6.2.1 Background of protein adsorption

The use of solids for the removal of specific components from liquid mixtures has been widely used over the past decades. Nowadays it has become an essential tool for purification and separation processes with thousands of applications in the biological and biomedical fields, in biotechnology, or food industry. The separation process known as adsorption, involves the preferential partitioning of components (adsorbate molecule) from the liquid phase onto the surface of a solid substrate (adsorbent) mainly by van der Waals and electrostatic forces [10]. Different isotherm models e.g., the Henry, the Langmuir, the bi-Langmuir, the Freundlich, the Freundlich-Langmuir isotherms have been proposed to describe the adsorption of solutes from a liquid solution onto a solid surface. The most common used model for the protein adsorption onto ion exchangers is the *Langmuir adsorption isotherm* [11], which assumes that adsorption is a reversible reaction between the adsorbate molecule and the adsorption site. This kind of model implies that: i) all binding sites are equivalent, distinguishable and independent; ii) each binding site combine with only one solute molecule; iii) a molecule adsorbed onto one binding site does not influence the adsorption of another molecule on a neighboring binding site; iv) the adsorption is completely reversible and no permanent interactions take place between adsorbed molecules.

With proteins, two important steps in the adsorption process are distinguished: i) the transport of the protein to the adsorbent surface, which is a function of protein concentration, diffusion coefficient, and flow conditions; ii) the interaction of the protein with the solid (important for the protein desorption). Denaturation of protein molecules on the adsorbent surface might increase the contact area between the protein and the surface and therefore reduce the protein ability to desorb [12].

It is generally acknowledged that protein adsorption is a process driven by different factors; surface functionality, hydrophobic interactions, as well as electrostatic interactions play an important role in protein adsorption and retention. When charged surfaces are involved into the adsorption process, the pH and the ionic strength of the protein solution are important parameters to consider. It has been reported in the literature for various protein/adsorber pairs that an increase of the ionic strength bulk solution leads to a loss in protein adsorption capacity [13-15]. This is due to: i) the competition of salt counter ions and proteins for occupying the binding sites; ii) the salt co-ion shields the protein ion and the charged binding sites from each other; iii) the change of the ionic strength changes the conformation of the protein molecule thereby influencing the adsorption process; iv) the increase of ionic strength cause

shrinkage of ion exchange resins reducing the porosity and therefore the availability of the binding sites. In addition, there are many factors such as protein concentration, structural stability of protein, isoelectric point, surface morphology, domain composition that can influence the protein adsorption process.

Although the basics of adsorption are known, it is still not possible to predict the process of protein adsorption onto a polymeric surface even if the protein and the surface characteristics are known. Nevertheless, adsorption techniques often result in purification steps that give the greatest increase in protein purity thus these techniques, especially when adopted in chromatographic processes, have become widely used.

6.2.2 Affinity column model

Most affinity separations in chromatography are operated in frontal analysis mode. Axial and radial diffusion, sorption kinetics and non-uniformities in membrane porosity and thickness have been shown to affect affinity membrane performances such as breakthrough curve sharpness and residence time. Furthermore, the dynamic adsorption capacity was frequently reported to be smaller than the corresponding static capacity, because some adsorptive capacity remains unused mainly due to non-uniform flow distribution and resistance from mass transport rate processes. Degradation of membrane performance can be minimised by working with axial Peclet numbers greater than 40 and radial Peclet numbers smaller than 0.04 [16]. This effect can also be observed 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 [17].

Numerous mathematical models are available for the prediction of breakthrough curves in chromatography and in other adsorption-based separation processes [10]. In principle, if the parameters that characterise the intra-particle kinetic mechanism are known experimentally, it should be possible to predict the dynamic behaviour of a chromatographic system. A complete and detailed description of the mathematical model, which predict the mixed matrix adsorber membranes breakthrough curves is beyond the scope of this study, however some general models are presented below.

The transport rate resistance within the adsorptive membrane is principally based on kinetic, dispersive and diffusional resistances to mass transport. When the mass transfer resistances are small and have a minor influence on the breakthrough curve profile, the equilibrium-dispersive model (ED) is recommended. Otherwise, depending

on the nature and complexity of the system, the general rate (GR), the lumped pore diffusion (POR), or the transport-dispersive (TD) model can be used [18].

In the general rate model the axial dispersion and all the mass transfer resistances are taken into consideration. The external mass transfer of the solute molecules from the bulk phase to the external surface of the adsorbent; the diffusive transport through the porous matrix and the adsorption-desorption processes at the adsorptive sites are all contributing to the dynamic behaviour [19]. However, the general rate model is not widely used because of the large number of parameters involved in the characterization of the axial dispersion, the external mass transfer and the effective diffusion through the pores including the adsorber porosity. The use of the simpler lumped pore diffusion model also requires knowledge of the values of several of these parameters. Therefore, the simple transport-dispersive model is frequently used when the mass transfer resistances have a moderate influence on the profiles of breakthrough curves. To solve this model, only the value of the dispersion coefficient, the overall mass transfer coefficient, and the adsorber porosity, which can be derived from simple experimental measurements are needed. However it was shown that, in order to correctly describe experimental breakthrough curves with the TD model, it had to be assumed that the overall mass transfer coefficient depends on the concentration [20].

Kaczmarzsky et al. presented a comparison between the chromatographic band profiles calculated with the GR, the POR and the ED models [21]. It was showed that in high concentration chromatography, the ED or the POR models could replace the GR model in certain specific operational conditions. If these conditions are not fulfilled, important or even major differences are observed between the numerical solutions of these different models, the results closest to experimental data being those obtained with the more complex GR model.

6.3 EXPERIMENTAL

6.3.1 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. Dimethylsulfoxide (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) was 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 purification unit Milli-Q plus.

6.3.2 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. 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 [22].

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 grinded and sieved to obtain fractions with particle sizes less than 20 µm. A complete and detailed description of the ion exchange resins is beyond the scope of this study, however some general characteristics are listed in Table 6.1.

Resin type	Functional group	Ionic form	Ion exchange capacity, eq/l
CNP 80	Carboxylic acid	H ⁺	4.3
K 2629	Sulfonic acid	H ⁺	1.9
112 WS	Sulfonic acid	Na ⁺	1.75
MP62	Tertiary amine	OH ⁻	1.7
S 6328	Quaternary amine	Cl ⁻	1.2
MP 500	Quaternary amine	Cl ⁻	1.1

Table 6.1 Principal characteristics of the filling ion exchange resins

6.3.3 Membrane preparation

Ethylene vinyl alcohol copolymer (EVAL) is a potential candidate as polymeric material for the preparation of membrane chromatographic supports since it displays a good mechanical strength, has a high thermal stability, demonstrates a good chemical and biological resistance, and is easy to sterilize with, e.g., γ -radiation. To obtain membranes with protein adsorptive properties, different types of Lewatit ion exchange particles with diameters smaller than 20 µm were added to a solution containing 10%wt EVAL and 10% 1-octanol in DMSO. The 1-octanol

was added to the casting solution in order to improve the membrane morphology [23]. The mixtures were stirred over night to break down the clusters of particles. The mixed matrix adsorber membranes were prepared by immersion precipitation. For this the polymeric mixture was cast on a glass plate and immediately immersed into a water coagulation bath at 40°C.

6.3.4 Membrane characterisation

To investigate the morphology of the prepared mixed matrix adsorber membranes, the membranes were investigated by scanning electron microscopy. The membranes were also characterized by measuring the porosity, the permeation rate, as well as the static and the dynamic protein adsorption capacity.

For visualising the cross-sectional areas of the adsorber membranes pieces of membranes were frozen in liquid nitrogen and fractured. 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 volume of polymer 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 sheet membranes was determined using a pressurised nitrogen gas stirred dead-end filtration cell. Solutions of 50 mM acetate buffer pH 4.5 and 50 mM phosphate buffer pH 7 were permeated through a stack of 10 sheets adsorber membrane.

For the *retention coefficient* measurements, a freshly prepared BSA solution was permeated through the adsorber membrane. The BSA concentration in the feed and the permeate samples after adsorption saturation conditions were reached, was determined by measuring the absorbance at 280 nm with a spectrophotometer PU 8720 UV/VIS. All the filtration experiments were carried out at room temperature with a transmembrane pressure of 0.1 bar, and a stirring rate of 200 rpm.

The characterisation of porous adsorptive membranes with respect to their *static protein adsorption capacity* was determined by batch experiments with BSA. A known amount of membrane containing 65% of ion exchange resins was equilibrated with a known volume of dissolved protein and the protein uptake in 24h per membrane volume was measured. Initial BSA concentrations of 1-5 mg/ml were employed in these experiments to mimic realistic protein concentrations for many commercial separation processes. The ion exchange

particles incorporated into the porous matrix exhibit different fixed ionic charges therefore, different adsorption/desorption conditions have to be involved for each class of ion exchange membranes.

For the *cation exchange adsorber* membranes, 50 mM acetate buffer with pH 4.5 was employed in the adsorption measurements. At a pH lower than the isoelectric point (pI) the protein is positively charged, meanwhile the adsorber membrane is negatively charged. The protein is adsorbed by the ion exchange particles incorporated into the mixed matrix adsorber membranes and hence the BSA concentration in the bulk solution decreases until equilibrium is reached. The BSA concentration in the bulk was determined measuring the absorbance at 280 nm as mentioned. From the BSA mass balance the amount of adsorbed protein (mg BSA/g membrane) could be calculated. Equivalent mass, volume and surface measurements allow us to express the binding capacity in alternative units, e.g., mg BSA/g membrane, mg BSA/ml membrane, mg BSA/cm² membrane.

The isolated BSA-adsorber membrane complex was consecutively treated with 50mM Tris buffer pH 9 in order to dissociate the complex. At a pH > pI, the protein becomes negatively charged and is therefore released from the matrix passing freely through the porous membrane into the eluent. The BSA concentration in the elution buffers was monitored by the method previously described.

For the adsorber membranes with *anion exchange* resins incorporated, 50 mM phosphate buffer at pH 7 was used in the adsorption experiments. At this pH, the membrane ionic groups are positively charged, the BSA is negatively charged and thus adsorbs on the membrane. The desorption measurements were performed in 50 mM acetate buffer pH 3.5. In this conditions the BSA has the same positive charge as the ionic groups in the membrane therefore, the complex could be dissociated.

The *adsorption isotherm* for the adsorbers prepared by incorporation of Lewatit ion exchange resins was obtained by incubating the same amount of membrane with different initial amounts of BSA to reach different equilibrium concentrations. It has been assumed that the adsorption isotherm follows the Langmuir type equation, where q^* is the solute concentration into the solid system at a certain concentration C^* into the bulk solution at equilibrium.

$$\frac{1}{q^*} = \frac{1}{q_m} + \frac{K_d}{q_m} \times \frac{1}{C^*}$$

Since the parameters q^* and C^* are experimental data, a plot of $1/q^*$ versus $1/C^*$ using a linear curve fitting allows to determine the dissociation constant (K_d) and the maximum protein adsorption capacity (q_m).

The *dynamic adsorption performance* of EVAL-based mixed matrix adsorber membranes with high static adsorption capacity was evaluated at constant flow rates using a dead-end filtration cell as described before. The capacity of a single adsorptive membrane for preparative recovery may be too limited. To overcome this limitation, a number of sheets were mounted in series to achieve the adsorptive capacities necessary for characterising the sheets of adsorber membranes. This kind of configuration permits rapid, low-pressure adsorption of protein in either batch or continuous recycle mode, the intended product being concentrated substantially. A BSA solution was permeated through a stack of 5–15 adsorber membranes at a flow rate of 10–20 l/h/m². The permeate was collected and fractionated using a fraction collector LKB Frac-100 from Pharmacia. The BSA concentration in the feed and the collected permeate samples was determined spectrophotometrically as described. The protein mass adsorbed per unit of membrane bed was calculated by numerical integration over the filtration run at a breakthrough concentration 10% of the feed concentration.

The BSA adsorbed into the mixed matrix adsorber membranes was consecutively eluted with 50mM Tris buffer pH 9 in order to dissociate the complex. The BSA concentration in the elution buffer was monitored at 280 nm by the method described above. The particle-loaded adsorber membranes were regenerated with 10% HCl, washed with demineralised water until neutral pH and reused in a new adsorption/desorption cycle.

6.4 RESULTS AND DISCUSSIONS

The *results and discussion section* first describes the characterisation of the prepared EVAL-based mixed matrix adsorber membranes in terms of morphology, porosity, transport properties and protein retention experiments. For the adsorber membranes having different particulate materials entrapped in the porous matrix, emphasis is first laid on the influence of ion exchange particles type on the BSA static adsorption capacity. Measurements of the dynamic protein adsorption capacity for the optimum mixed matrix adsorber membranes in different operation conditions are finally presented.

6.4.1 Mixed matrix adsorber membrane characterisation

Different types of ion exchange resins were employed in the preparation of mixed matrix adsorber membranes. Optimum adsorption performances were obtained for the adsorber membranes prepared by incorporation of Lewatit 112WS ion

exchange resins into the polymeric matrix (see Section 6.4.3). Therefore, in the following section we only give a detailed morphological characterisation for this type of membranes. The structure of the EVAL-based adsorber membranes prepared by incorporation of 65% Lewatit 112WS ion exchange resins, with a particle size diameter smaller than 20 μm , is presented in Figure 6.2.

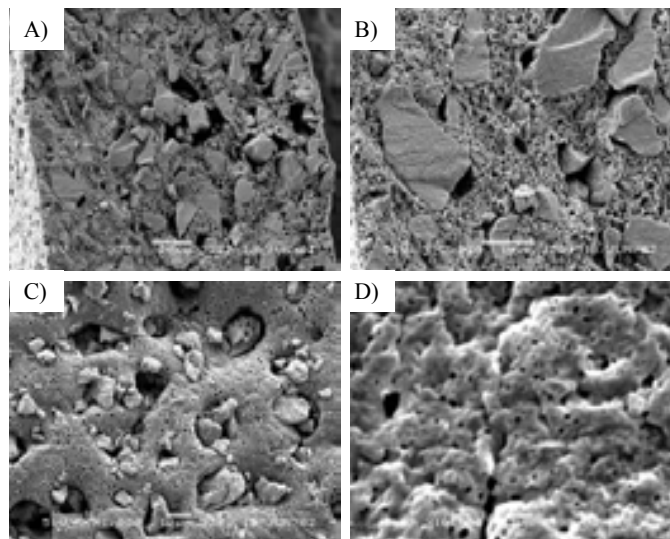


Figure 6.2 SEM photomicrograph for the membranes M_{112WS} prepared from 10% EVAL in DMSO in the presence of 10% 1-octanol as additive, by incorporation of 65% Lewatit 112WS ion exchange particles: A) cross-section, magnification $\times 750$, the white horizontal bar indicates 20 μm ; B) cross-section, magnification $\times 1000$, the white horizontal bar indicates 10 μm ; C) bottom-surface magnification $\times 3000$, the white horizontal bar indicates 10 μm ; D) top-surface, magnification $\times 10000$, the white horizontal bar indicates 1 μm .

The membrane possesses an open, interconnected porous structure, without evident finger-like macrovoids across the entire cross-section (Figures 6.2A and 6.2B). Pores larger than 0.1 μm can be observed on both the glass (Figure 6.2C) and the air surfaces (Figure 6.2D). 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.

Porosities in the range of $75\pm 5\%$ were obtained from liquid wicking experiments, with no 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 (300–400 $\text{l/h/m}^2/\text{bar}$) were obtained for operating pressure below 0.1 bar. The open porous structure of the adsorber enables the use

of stacking of membranes avoiding the well-known packing problems with conventional particles. Low BSA retention values (less than 3% of the initial protein feed concentration) were obtained after adsorption saturation conditions were reached for this type of mixed matrix adsorber membranes. This means that protein entrapping into the porous matrix during the adsorption processes can be assumed to be negligible.

6.4.2 Static adsorption capacity

Static protein adsorption capacities of the prepared adsorber membranes were studied batchwise, with BSA as a model protein. Screening adsorption experiments (see Figure 6.3) revealed high BSA adsorption capacities for the adsorber membranes prepared by incorporation of 65% Lewatit K2629 (referred as M_{K2629}) and 112WS ion exchange resins (referred as M_{112WS}) into the EVAL polymeric matrix. Therefore, the adsorption properties of mixed matrix adsorber membranes based on these resins are further investigated.

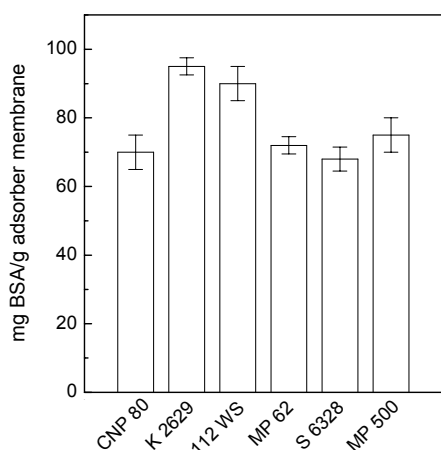


Figure 6.3 Effect of the Lewatit ion exchange resin type incorporated into the polymeric matrix on the membrane's static BSA adsorption capacity.

The fitting of the experimental results to the equation (1) is shown in Figure 6.4 for BSA adsorption into the EVAL-based mixed matrix membrane M_{112WS} and M_{K2629} . The measured protein adsorption equilibrium isotherm appears to be of the Langmuir type for both adsorber membranes, with higher protein affinity in the case of adsorber

membrane M_{K2629} . The maximum adsorption capacity was 160 mg BSA/g membrane for adsorber membranes M_{112WS} and 170 mg BSA per mass of adsorber membrane M_{K2629} , equivalent with around 52 and 57 mg BSA/ml membrane respectively. The obtained adsorption capacity values are lower in comparison with more expensive sorbents such as HyperD, (240 mg protein/ml adsorbent [24]), but similar with the values obtained by other authors. Reported capacities for several adsorptive membranes are summarised in [25] with protein capacities from 3 up to 50 mg macromolecule/ml of membrane. This indicates that the technical concept of mixed matrix adsorber membranes shows performances equivalent to the highest reported in literature.

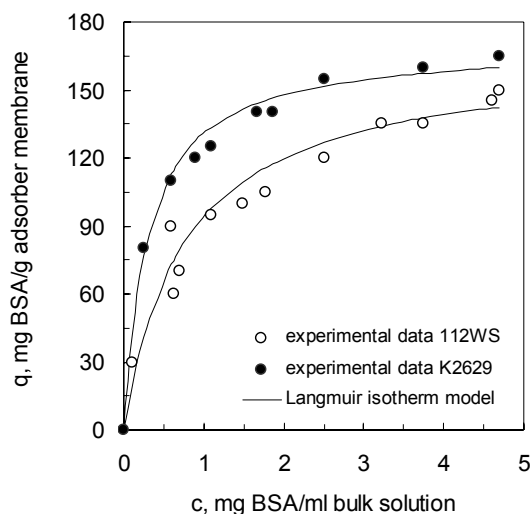


Figure 6.4 Correlation between the Langmuir model and experimental data of the BSA adsorption isotherm for the membrane adsorbers M_{112WS} and M_{K2629} respectively.

6.4.3 Dynamic binding capacity

Typical breakthrough and elution curves for a stack of 10 sheets of mixed matrix adsorber membranes M_{112WS} are presented in Figure 6.5. A solution of 50 mM acetate buffer pH 4.5 containing 1 mg BSA/ml, was permeated through the stack of 10 adsorptive membranes with a flow rate of 10 l/h/m². The obtained dynamic BSA adsorption capacity at breakthrough point 10% of the protein feed concentration was 95 mg BSA/g adsorber membrane smaller than the corresponding static capacity, because some adsorptive capacity remains unused mainly due to non-uniform flow distribution and resistance from mass transport rate processes.

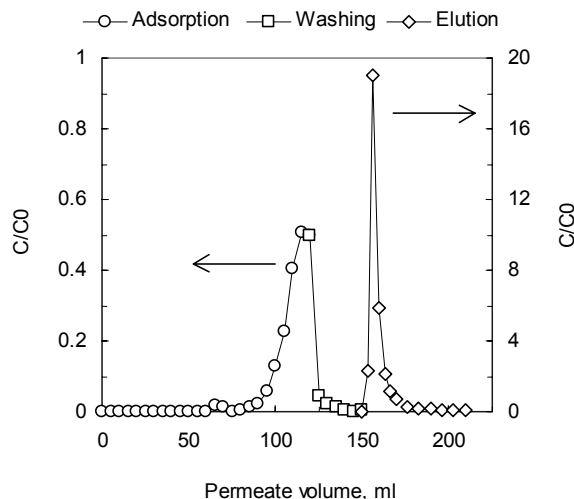


Figure 6.5 Typical BSA breakthrough and elution curves for a module of 10 mixed matrix membrane adsorbers M_{112WS} operated at an ionic strength of 50mM and a constant filtration flow rate of 10 l/h/m².

The transport rate resistance within the adsorptive membrane is principally due to disperse and diffusional resistances to mass transport and the unfolding and rearrangements of the protein at the adsorptive interface. The feed flow containing the protein molecules flows preferentially through the large pores and saturates the adsorptive-sites surrounded by the large pores. Meanwhile, adsorption in smaller pores and into the particles is more dominated by diffusional processes usually with 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 molecules to diffuse to the adsorptive site and to rearrange/unfold their structure to its most favorable one before the interstitial volume continues through the membrane. Furthermore, it has been shown 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.

Elution of the adsorbed protein-adsorber membrane complex with Tris buffer pH 9 at a flow rate of 10 l/h/m², leads to BSA recovery values up to 95%. Furthermore, the protein was concentrated on average up to 10-fold into the desorptive buffer offering perspectives for the adsorber membranes to function both as purification as well as concentration medium (Figure 6.5). In the initial stages of 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 membranes with high protein recoveries in the final concentrated volume. The breakthrough curves for the adsorber membranes M_{K2629} and M_{112WS} used in four consecutively protein adsorption/desorption/regeneration cycles are shown in Figure 6.6. The dynamic capacity at the fourth cycle was around 80% of that at the first cycle for the membrane prepared by incorporation of Lewatit 112WS resins (Figure 6.6A) and 50% by using K2629 ion exchange resins (Figure 6.6B).

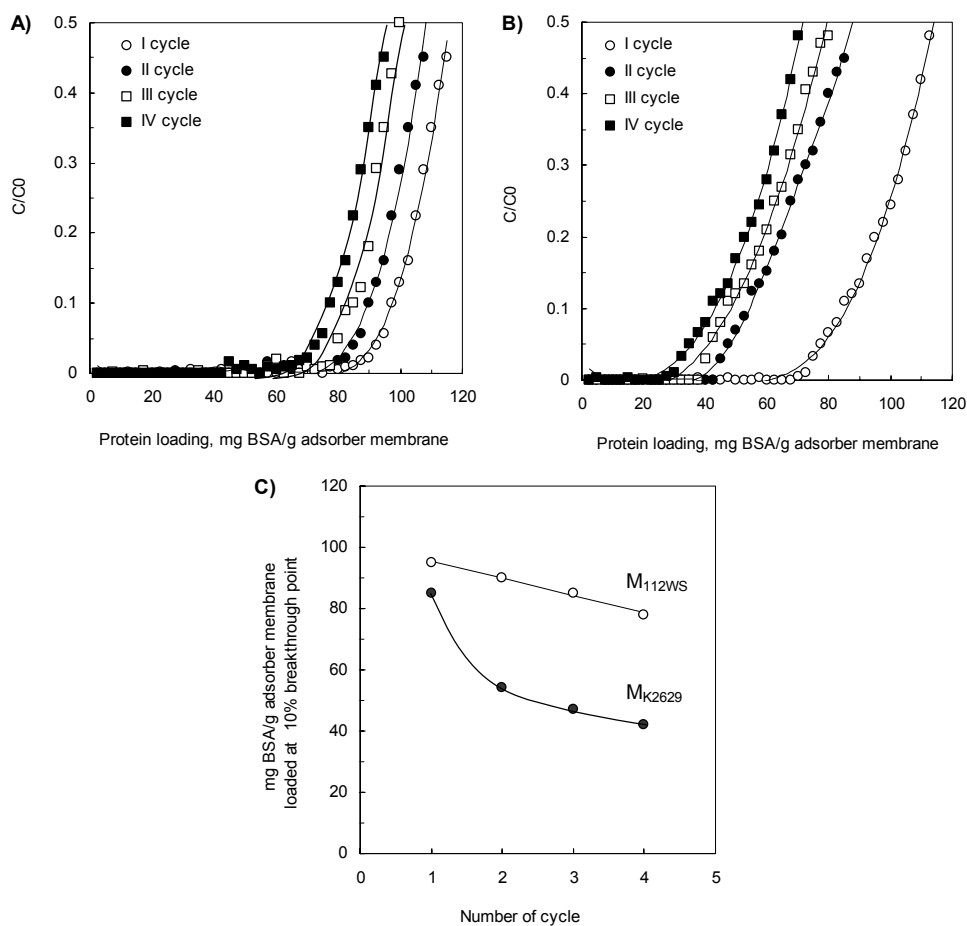


Figure 6.6 Performances of mixed matrix membrane adsorbers M_{112WS} (A) and M_{K2629} (B) in four sequential adsorption/desorption/regeneration cycles.

It is generally accepted that the dynamic adsorption capacity of the adsorber membranes decreases with increasing number of cycles due to accumulation of undesorbed BSA on the porous surface. Nevertheless we do not have an explanation at hand regarding the high loss in protein adsorption capacity for the membrane M_{K2629} in comparison with the M_{112WS} membrane (Figure 6.6C). Since the mixed matrix adsorber membranes M_{112WS} seems to offer the optimum performances, in the following section we will focus on the adsorption properties for this type of membranes.

Figure 6.7 shows the influence of the flow rate of the feed protein solution on the BSA breakthrough curves for the mixed matrix adsorber membranes M_{112WS} . Increasing the flow rate from 10 to 20 $l/h/m^2$, the dynamic capacity of the stack containing 10 membranes decreases from 95 to nearly 75 mg BSA/g adsorber membrane. The decrease of 20% in the dynamic binding amount value indicates the presence of mass transfer limitation in the adsorption process. We predict that this effect can be minimised by incorporation of ion exchange resins with smaller particle diameter into the polymeric matrix, due to lower diffusion limitations into the porous particle structure. Commercially available adsorber membranes do not show adsorption capacity-flow dependence, however literature revealed breakthrough point values depending on the filtration protein flow rates of ovalbumine [26] and lysozyme [27].

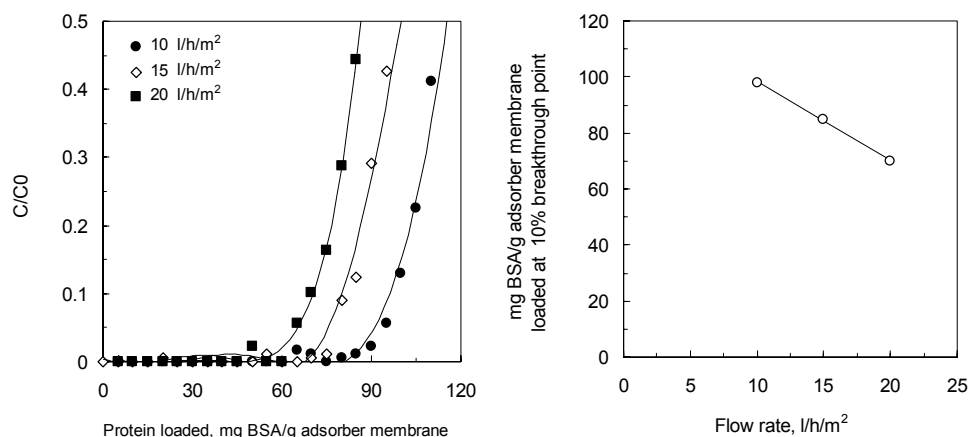


Figure 6.7 BSA breakthrough curves for a module of 10 mixed matrix membrane adsorbers M_{112WS} as a function of flow rate, at an ionic strength of 50mM.

Figure 6.8 shows the effect of ionic strength on the BSA adsorption. The data indicate a linear decrease of protein adsorption capacity with an increased ionic

strength. One can observe that increasing the bulk ionic strength from 20 to 150 mM with equivalent amounts of NaCl, a loss of about 40% of the BSA adsorption capacity for the EVAL adsorber membranes was obtained. This can be mainly due to competition of proteins and Na^+ for the adsorptive sites and conformational changes in protein molecules as already discussed in Section 6.2.1. However, Conder et al. [28] observed that using a cross-linked cellulose chromatographic ion exchanger, BSA binding capacity decreases with increasing salt concentration only for salt concentration above 5 g/l (≈ 100 mM). At salt concentrations below 100 mM the capacity changes only slightly. In contrast, significant decreases of the BSA binding capacities on Diaion HPA25 adsorbent, with more than 60%, were reported by Lan et al. for an ionic strength increasing from 2 to 52 mM [13]. However, for the ion exchangers used in this study we observed a linear decrease of the protein adsorption capacity with the ionic strength of the feed solution.

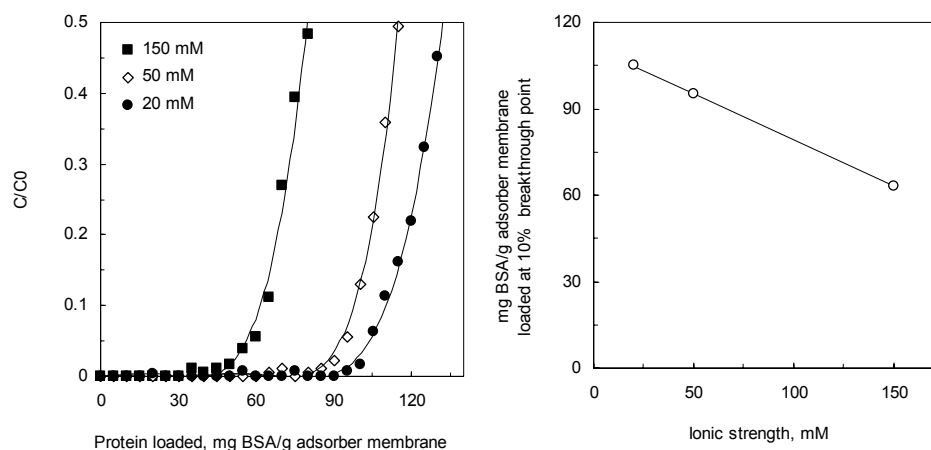


Figure 6.8 BSA breakthrough curves for a module of 10 mixed matrix membrane adsorbers M_{112WS} as a function of the feed ionic strength. The filtration flow rate was maintained at a constant value of 10 l/h/m².

Liu et al. [29] suggested that stacking more than 30 membranes averages out membrane porosity and thickness nonuniformity. To investigate the effectiveness of the thickness of the adsorber membrane height on the dynamic BSA adsorption capacities, the breakthrough curves were measured by permeating the BSA solution through a stack of 5, 10 and 15 adsorber membranes, at a flow rate of 10 l/h/m². Dynamic binding capacity values of 95 ± 10 mg BSA/g adsorber membrane were obtained for modules of 5 up to 15 sheets of mixed matrix

membranes. A slight difference from a proportional increase in the protein capacity was obtained for an increased adsorptive area (Figure 6.9). This can be a result of non-uniform flow distribution mainly due to axially directed protein solution velocity, bypassing and uneven fluid distribution. Nevertheless, the results indicate an easy scale-up for adsorption processes of the mixed matrix adsorber membranes M_{112WS} .

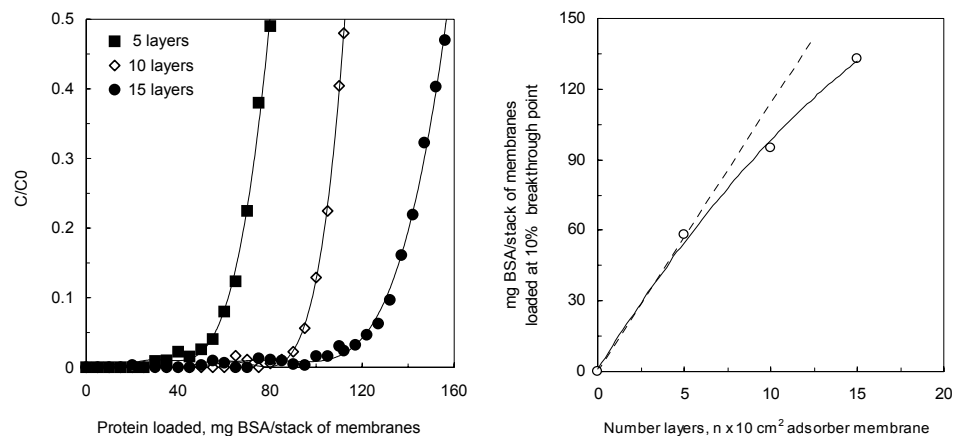


Figure 6.9 BSA breakthrough curves for modules of mixed matrix adsorber membranes M_{112WS} with different adsorptive area, as a function of number of membrane layers. The ionic strength was set at 50mM and filtration flow rate was maintained at a constant value of 10 l/h/m².

6.5 CONCLUSION

The new mixed matrix adsorber membranes feature high protein adsorption capacity (30-50 mg BSA/ml membrane) and an easy scale-up for adsorption processes. The membranes have a wide variety of applications, mainly for the removal and/or purification of compounds from complex mixture. By choosing the right eluent conditions, the desired components can be concentrated up to 10-fold. This means that beside isolation the mixed matrix adsorber membranes can also act as a concentration medium. The developed adsorber combines both the advantages of membrane technology (easy scale-up, low-pressure drop) and column chromatography (high binding capacity, high recovery).

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CHAPTER 7

MIXED MATRIX MEMBRANE ADSORBERS FOR PROTEIN SEPARATION^{*)}

ABSTRACT

The separation of two proteins of similar size, bovine serum albumin (BSA) and bovine haemoglobin (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 EVAL porous structure. The obtained heterogeneous matrices composed of solid particles surrounded by the polymeric film, featuring 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 up to 20 l/h/m² filtration flow rate, until the protein breakthrough point 10% of the feed concentration.

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7.1 INTRODUCTION

The use 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 thousands of applications in the biological and biomedical fields, in biotechnology and food industry [1]. The separation process known as adsorption involves the preferential partitioning of components (adsorbate molecule) from the liquid phase onto the surface of a solid substrate (adsorbent) mainly by van der Waals and electrostatic interactions [2].

In the recent years there has been a 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]. The affinity membranes have been designed to bypass the fundamental limitations of packed-bed absorbers, mainly the bed compression at high velocities. The main advantages of microporous membranes in comparison with packed-bed are the low flow resistance due to higher porosity, reduced bed heights, and fast mass transfer. Pressure drop and intra-bed diffusion limitations are minimised by convection through the fine pores of the membrane [4].

In a previous paper [5], we presented a new concept for the preparation of mixed matrix adsorber membranes having particulate material entrapped in a porous matrix. A slurry of dissolved polymer and 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% sorbent by weight), the latter not interfering with the activity of the particles. EVAL is a suitable candidate as polymeric material for the preparation of such adsorptive membranes 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. 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 [6].

The 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 full or hollow 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 have a wide variety of applications, depending upon 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 release systems. 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 bovine serum albumin (BSA) and bovine hemoglobin (Hb) was carried out using the new adsorber membrane system at different operational parameters. Some physical properties of BSA and Hb are given in Table 7.1 [7]. Although similar in size, the protein electrostatic properties as a function of pH differ significantly (Figure 7.1, plotted with data from [8]) thus it is possible to use their electrostatic interaction with ion exchange materials as a mean of recognition and adsorptive separation.

	BSA	Hb
Molecular mass (Da)	67000	68000
Isoelectric Point (pI)	4.7-4.9	7.1
Protein charge at:		
*) pH 5	-1.5	+20
*) pH 7.1	-14	0
Ellipsoidal diameters (Å)	40×40×140	55×55×70
Equivalent radius (Å)	36.1	31
Area occupied by each ellipsoidal molecule (nm ²)	16-56	30-39
Area occupied by each spherical molecule (nm ²)	52	38
Hydrophobic amino acids content (g amino acid/100 g protein)	44.07	54.16

Table 7.1 Physical properties of bovine serum albumin (BSA) and bovine hemoglobin (Hb).

Previous studies [9] concluded that protein separation by membrane filtration processes could only be performed with proteins differing by at least a factor of ten in molecular weight. Effective protein fractionation using membrane filtration for mixtures of albumin-myoglobin and albumin-immunoglobulins were reported in the literature [10, 11].

Recently it has been demonstrated that using electrostatic interactions, the separation factor even for two proteins of similar size BSA and 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) UF membranes. A maximum separation factor of

3.2 was obtained at low pH and the separation performance decreases by increasing the pH. Eijndhoven et al. [13] used electrostatic interactions to effectively separate BSA-Hb mixtures by membrane filtration. They obtained the highest protein separation factors (up to 70) at pH 7, ionic strength 2.3 mM and a filtration velocity of 3×10^{-6} m/s (≈ 11 l/h/m²). Nevertheless, the separation factor decreases drastically to values of 2–3 if the ionic strength increases to 16 mM and/or the filtration velocity increases over 6×10^{-6} m/s (≈ 22 l/h/m²). The BSA-Hb fractionation using a dead-end, cross-flow and vortex-flow ultrafiltration module was investigated by Shukla et al. [14] for different membrane material e.g., regenerated cellulose, poly(sulfone) and surface modified poly(acrylonitrile). The highest separation factor of 40 was obtained in a vortex-flow module housing a polyacrylonitrile membrane and operated at the isoelectric point of haemoglobin, low applied pressure (40kPa) and high feed flow (10×10^{-6} m/s, ≈ 36 l/h/m²). However, the flux decline was around 25% in 1h of filtration and the Hb recovery does not exceed 50%. The process developed by C. Causserand [7] proposed a selective protein adsorption on montmorillonite clay particles. Once the adsorption equilibrium had been reached, the mixture of BSA/Hb/clay-particle was filtrated through a polyvinylidene difluoride membrane with a nominal pore size of 0.1 μ m. Free BSA permeates through the membrane, while the Hb adsorbed on the clay particle was retained by the membrane. The maximum obtained separation factor was 6.4 for an operational flux of 1.4×10^{-5} m/s (≈ 50 l/h/m²), 1mM ionic strength and low protein concentrations (0.1 mg/ml). Increasing the ionic strength to 10 and 100 mM, the separation factor decreases to 3.6 and 1.53.

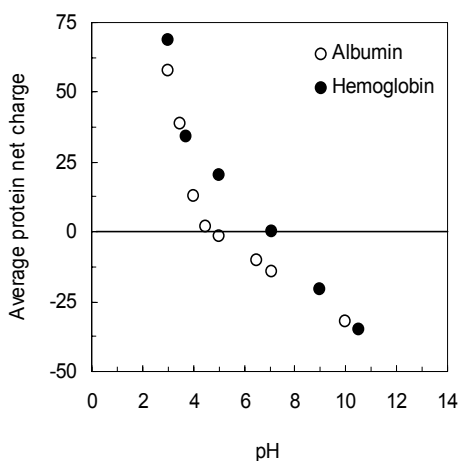


Figure 7.1 Net charge of serum albumin and hemoglobin as a function of pH.

The flexible concept of mixed matrix adsorber membranes allows the incorporation of cation as well as anion exchange particle. 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.

7.2 EXPERIMENTAL

7.2.1 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. Dimethylsulfoxide (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 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 purification unit Milli-Q plus.

7.2.2 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. 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 7.2.

Resin type	Functional group	Ionic form	pK _a	Ion exchange capacity, eq/l
112 WS	Sulfonic acid	Na ⁺	2	1.75
MP 500	Quaternary amine	Cl ⁻	9-9.5	1.1

Table 7.2 Principal characteristics of the filling ion exchange resins.

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 grinded and sieved to obtain fractions with particle sizes smaller than 20 µm.

7.2.3 Membrane preparation

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 [15]. The mixtures were stirred over night to break the clusters of particles. EVAL-based mixed matrix adsorber membranes were prepared by immersion precipitation; the polymeric solution was cast on a glass plate and immediately immersed into a water coagulation bath at 40°C. The preparation of the mixed matrix adsorber membranes is described in detail in [5].

7.2.4 Membrane characterisation

To characterise the prepared mixed matrix adsorber membranes, the membrane morphology was investigated by scanning electron microscopy. Membrane porosity, permeation rate, 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 membranes were frozen in liquid nitrogen and fractured to visualise the 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 volume of polymer 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 mM acetate buffer pH 4.5 and 50 mM phosphate buffer pH 7 were permeated through a stack of 10 sheets ion-exchange adsorber membranes.

The *dynamic adsorption performance* of EVAL-based mixed matrix adsorber membrane was measured at constant permeation rate 10 l/h/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 batch or continuous recycle mode. The intended product is concentrated substantially through its adsorption into the membrane. BSA and Hb solutions were permeated through the adsorber membranes 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 fraction collector LKB Frac-100 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 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 nm and 406 nm respectively with a spectrophotometer PU 8720 UV/VIS. Hb solutions exhibit maxima at two different wavelengths (280 nm 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 proteins was formed.

7.2.5 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}) operated at a pH lower than the protein isoelectric point, the protein is positively charged, meanwhile the ion exchange particles having 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, appropriate protein solution environment and filtration conditions have to be chosen in the way that high Hb adsorption into the membrane take place, while the BSA adsorption is minimised. The optimum pH in the permeation step was therefore considered to be slightly on the acid side of the hemoglobin ($6.5 < \text{pH} < 7.1$), since Hb was the target protein in the adsorption stage. In this pH condition, the net charge of Hb is slightly positive and compensates the negative surface charge of the sorbent. Meanwhile, BSA is negatively charged and thus low BSA adsorption is expected. Moving the pH to either side of the optimum point results in a less favourable adsorption owing to electrostatic repulsion [16, 17].

The two proteins were mixed in equal concentration and the competitive adsorption on the adsorber membrane was studied for different operational conditions. A solution of 50 mM phosphate buffer containing 1mg/ml of each protein, was permeated through a stack of 10 membranes at a constant filtration flow rate of 10 l/h/m² and the permeate was collected using the fraction collector. The BSA and Hb concentrations in the feed and the permeate fractions were determined spectrophotometrical 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 $\bar{C}_{p,BSA}$ and $\bar{C}_{p,Hb}$ are the average protein concentrations in the collected permeate fractions during filtration, calculated by numerical integration of permeation curve over the filtration run.

$$S^{BSA / Hb} = \frac{\bar{C}_{p,BSA} / \bar{C}_{p,Hb}}{C_{f,BSA} / C_{f,Hb}}$$

The proteins adsorbed into the mixed matrix adsorber membrane were consecutively eluted with 50mM borate buffer at pH 10 at a flow rate of 10 l/h/m² 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 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 mM acetate buffer at pH 5.5 was used in the permeation experiments. At this pH the BSA is negatively charged compensate the positive surface charge of the sorbent and thus adsorbs on the membrane. The net charge of Hb is positive and therefore, repelled by the anion exchange particles, 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 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 performed in 50 mM acetate buffer pH 3.5 at a flow rate of 10 l/h/m². In this 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 spectrophotometrical monitored and the protein recoveries were calculated by numerical integration over the elution curves.

7.3 RESULTS AND DISCUSSIONS

The *results and discussion section* starts with 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 then laid on the efficiency of separation of two proteins with similar sizes (BSA and Hb) for different operational conditions.

7.3.1 Mixed matrix adsorber membrane characterisation

Different types of ion exchange resins were employed in the preparation of mixed matrix adsorber membranes. The structure for the flat EVAL-based mixed matrix adsorber membranes prepared by incorporation 65% Lewatit 112WS ion exchange

resins with particle size diameter smaller than $20\ \mu\text{m}$ (C_{MMA}) is presented in Figure 7.2. The membranes possess an open, interconnected porous structure, without evident finger-like macrovoids across the entire cross-section (Figures 7.2A and 7.2B) and pores larger than $0.1\ \mu\text{m}$ on both, the glass (Figure 7.2C) and the air surface (Figure 7.2D). 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 diameter smaller than $20\ \mu\text{m}$ into an EVAL porous structure (A_{MMA}). Porosities in the range of $75\pm 5\%$ 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 ($\approx 300\ \text{l/h/m}^2/\text{bar}$) were obtained. The continuous porous structure of the adsorber enables the use of stacking of membranes avoiding the well-known packing problems that arise with conventional particles in packed bed systems.

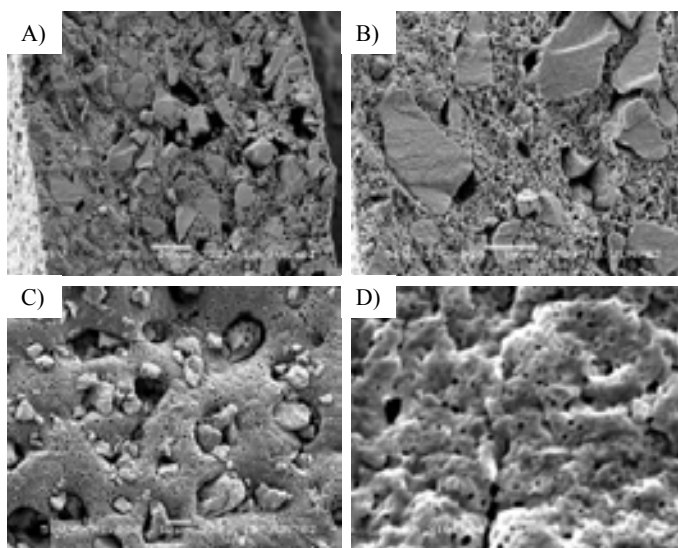


Figure 7.2 SEM photomicrograph for the mixed matrix adsorber membranes C_{MMA} prepared from 10% EVAL in DMSO in the presence of 10% 1-octanol as additive, by incorporation of 65% Lewatit 112WS ion exchange particles into the polymeric support: A) cross-section $\times 750$, the white horizontal bar indicates $20\ \mu\text{m}$; B) cross-section $\times 1000$, the white horizontal bar indicates $10\ \mu\text{m}$; C) bottom-surface $\times 3000$, the white horizontal bar indicates $10\ \mu\text{m}$; D) top-surface, $\times 10000$, the white horizontal bar indicates $1\ \mu\text{m}$.

Typical breakthrough and elution curves for permeation of single protein component solution through the stack of mixed matrix adsorber membranes C_{MMA} are presented in Figure 7.3. The obtained dynamic capacity (95 mg BSA/g adsorber membrane, equal to 32 mg BSA/ml adsorber) was smaller than the corresponding static BSA adsorption capacity (53 mg BSA/ml adsorber), 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 [8].

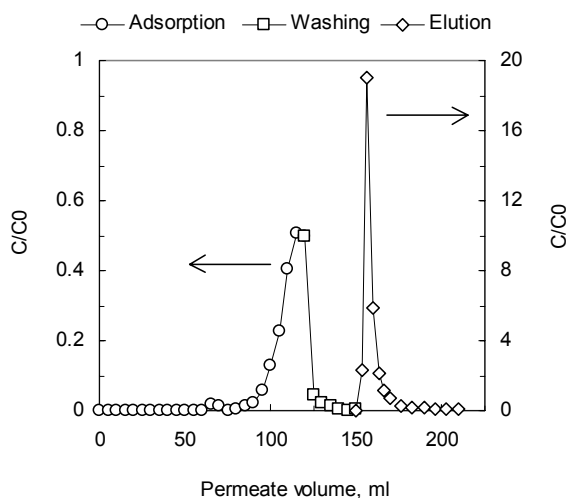


Figure 7.3 Typical BSA breakthrough and elution curves through a stack of 10 membranes C_{MMA} , at an ionic strength of 50mM and a constant filtration flow rate of 10 l/h/m².

The transport rate resistance to ideal within the adsorptive membrane is principally due to disperse and diffusional resistances to mass transport and the unfolding and rearrangements of the protein at the adsorptive interface. The feed flow containing the protein molecules flows preferentially through the large pores and saturates the adsorptive-sites surrounded by the large pores. Meanwhile, adsorption in smaller pores and into the particles is more dominated by diffusional processes usually with one order of magnitude slower. Full adsorption capacity can be achieved only if the flow rate through the adsorber membrane is slow enough so that to allow each adsorbate molecules to diffuse to the adsorptive site and to rearrange/unfold their structure to its most favorable one before the interstitial volume continues

through the membrane. Furthermore, it has been shown 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 [18] 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 elution of adsorbed protein-adsorber membrane complex (Figure 7.3), the biomolecule can be rapidly concentrated in the desorptive buffer up to 10 fold with 90% protein recovery. Thus offers perspectives for the adsorber membranes to function both as purifier and concentrator. In the initial stages of 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 membranes with high protein recoveries in the final concentrated volume.

7.3.2 Fractionation of BSA-Hb mixtures

In single component adsorption experiments at pH 4.5, close to the isoelectric point of BSA, both proteins are positively charged and therefore they adsorb almost equally strong onto the adsorber membrane C_{MMA} (90 ± 10 mg protein/g membrane, Figure 7.4A). Figure 7.4B shows the single component breakthrough curves for BSA and Hb at pH 7, close to the isoelectric point of Hb. At these conditions, the membranes adsorb a major proportion of Hb due to opposite protein/adsorber membrane charge (around 40 ± 5 mg Hb/g adsorber membrane C_{MMA}). The 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 close to the isoelectric point of Hb.

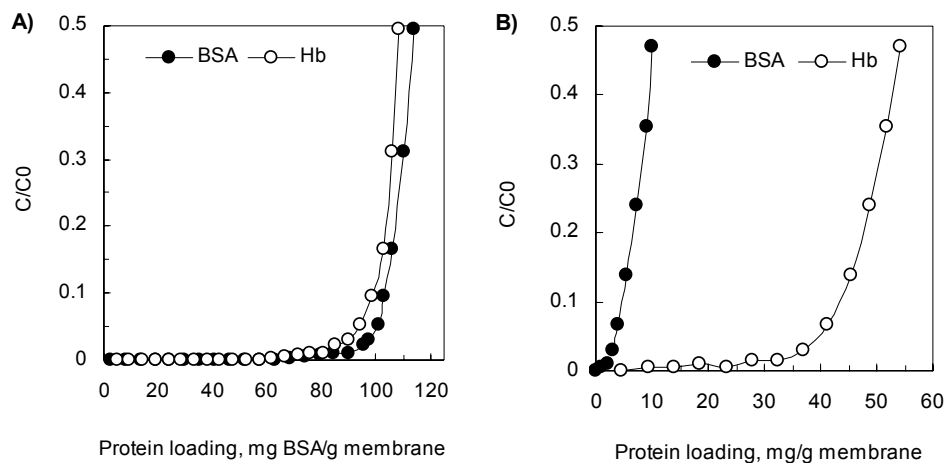


Figure 7.4 BSA and Hb breakthrough curves for single components at pH 4.5 (A) and pH 7 (B). The ionic strength was set at 50mM and the filtration flow rate through a stack of 10 membranes C_{MMA} was kept constant at 10 l/h/m².

For the BSA-Hb mixture, the permeation curve for Hb is, within the experimental errors, similar with the one for single component: only BSA shows a slight decrease in adsorption (Figure 7.5A).

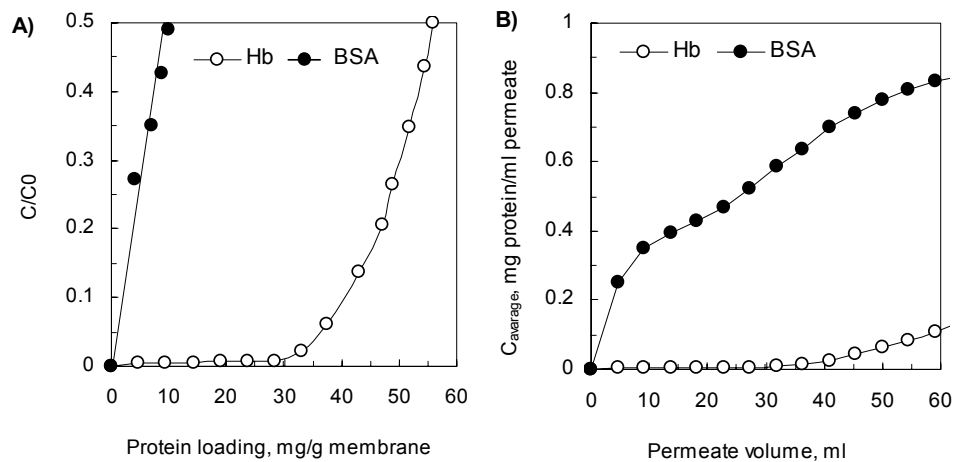


Figure 7.5 BSA and Hb breakthrough curves for a BSA-Hb mixture with ratio 1:1 at pH 7. The ionic strength was set at 50mM and the filtration flow rate through a stack of 10 membranes C_{MMA} was kept constant at 10 l/h/m².

It was concluded by other authors that Hb adsorption is not affected by the other component since BSA lacks competitive power with respect to Hb due to electrostatic repulsion [19]. The BSA passes very fast through the adsorber membrane and its average concentration into the permeate reaches 60% of the feed concentration while the Hb is still totally adsorbed into the matrix (Figure 7.5B).

The average separation factor was calculated from the adsorption experiments and is presented in Figure 7.6 at two different pH's. At the operating pH ($pI_{BSA} < pH < pI_{Hb}$), the membranes C_{MMA} adsorb a major proportion of Hb due to opposite protein/membrane charge and allow 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 of our experimental protocol. Practically no Hb was detected in the first 20 ml collected. Hypothetical Hb concentration values being 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 values of 100 for the separation factor in the beginning of the experiments).

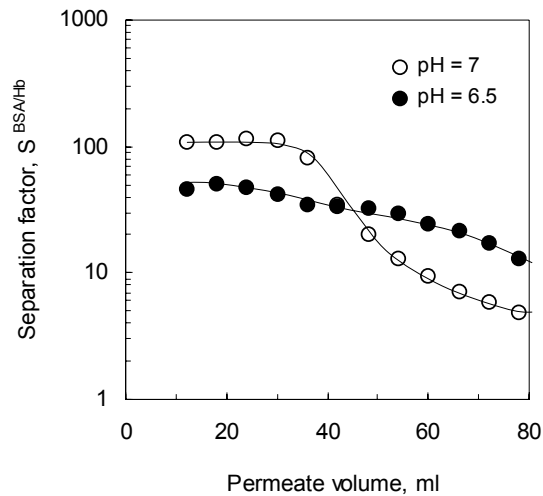


Figure 7.6. Separation factor for the BSA-Hb mixture as a function of the operating pH. The filtration flow rate through a stack of 10 membranes C_{MMA} was kept constant at 10 l/h/m^2 at an ionic strength of 50mM. The lines are included to guide the eye.

At longer filtration times the Hb starts to appear in the permeate and therefore the separation factor values decreases with increased 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 on 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 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, in 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.

Figure 7.7 presents the elution curve of a single protein for the C_{MMA} membrane-adsorbed protein complex with solution of borate buffer at pH 10. The measured protein concentrations in the collected eluent samples are plotted as a function of eluent volume for BSA (Figure 7.7A) and Hb (Figure 7.7B). The protein recoveries calculated by numerical integration over the elution curves leads to high BSA desorption values (around 80%) while lower Hb recovery up to 60% was obtained.

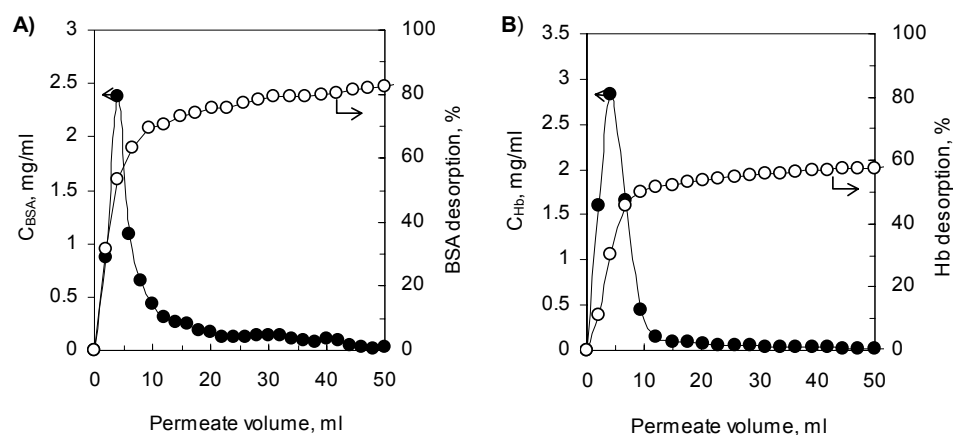


Figure 7.7 Elution curves for BSA (A) and HB (B) with 50 mM Borate buffer pH 10, at a constant filtration flow rate through a stack of 10 membranes M_{112WS} of 10 l/h/m². The lines are included to guide the eye.

Overall hemoglobin recoveries of 60% are usually reported in the literature [12, 20]. The results obtained by Kondo and Mihara [21] using a desorption protocol, which consists of an increasing pH from 7 to 10 with NaOH for 30 min with in the most favourable case 63% of Hb recovery. The denaturation of hemoglobin molecules

on the adsorbent surface might increase the contact area between the protein and the surface and thus reduces the amount of protein desorbed [22]. 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.

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 performed using the anion exchange membranes A_{MMA} .

At pH 5.5, slightly on the basic side of BSA (see Figure 7.1), the membrane A_{MMA} adsorbs preferentially the BSA due to opposite protein/membrane charge meanwhile the Hb is adsorbed to a lesser extent and passes rapidly into the permeate (Figure 7.8). The Hb average concentration in the permeate reaches almost 80% of the feed concentration while the BSA is still totally adsorbed (Figure 7.8B).

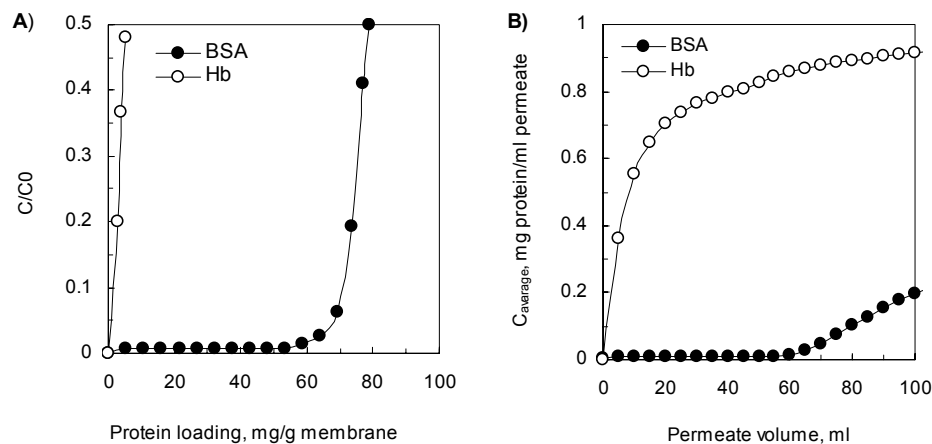


Figure 7.8 BSA and Hb breakthrough curves for a BSA-Hb mixture with ratio 1:1 at pH 7. The ionic strength was set at 50mM and the filtration flow rate through a stack of 10 membranes A_{MMA} was kept constant at 10 l/h/m².

The measurements of proteins desorption from the complex adsorbed protein-membrane A_{MMA} performed in acetate buffer pH 3.5, are presented in Figure 7.9. The measured BSA (Figure 7.9A) and Hb (Figure 7.9B) concentrations in the eluent samples were plotted as a function of eluent volume. The protein recoveries calculated by numerical integration over the elution curves leads to values of around 60% desorption for both BSA and Hb. Furthermore, the BSA was

concentrated around 5-fold into the desorptive buffer thus offering perspectives for the adsorber membranes to function as well as a concentration medium.

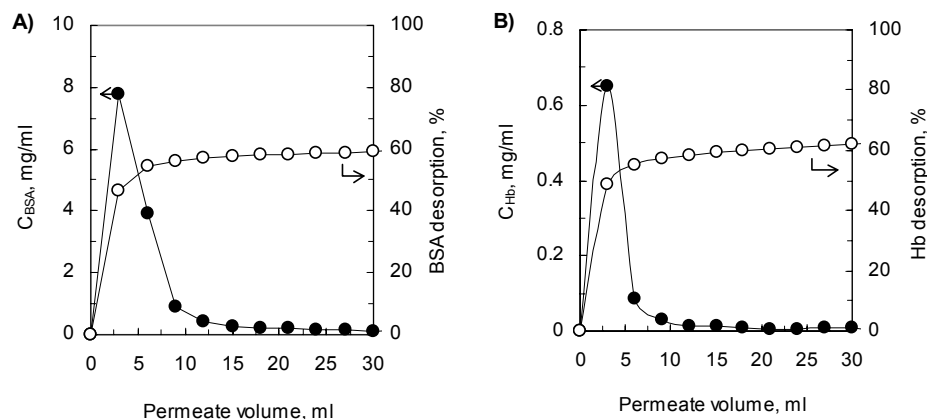


Figure 7.9 Elution curves for BSA (A) and HB (B) with 50 mM Acetate buffer pH 3.5, at a constant filtration flow rate through a stack of 10 membranes A_{MMA} of 10 l/h/m².

The effect of filtration flow rate on the separation factor for the BSA-Hb mixture is presented in Figure 10. For the C_{MMA} membrane operated with a filtration flow rate of 10 l/h/m², the average protein concentrations into the permeate were 0.64 mg BSA/ml and 0.014 mg Hb/ml, at hemoglobin breakthrough point 10% of the feed concentration (data from Figure 7.4). This result in average separation factors higher than 40. A lower average separation factor of 32 (Figure 7.10A) calculated over the filtration until the Hb breakthrough point was obtained when the filtration flow rate increased from 10 to 20 l/h/m², 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 so that to allow each adsorptive site to bind the protein before the interstitial volume continues through the membrane. The separation of BSA-Hb mixture performed with the membranes A_{MMA} , at a filtration flow rate of 10 l/h/m² until the BSA breakthrough, offers a separation factor of almost 50. If the filtration flow rate was increased up to 20 l/h/m², the separation factor decreases to a value of 35 (Figure 7.10B).

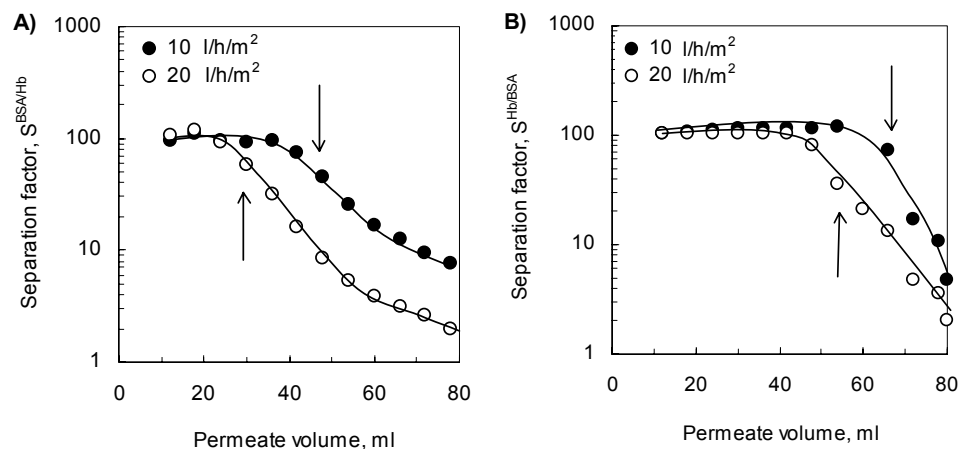


Figure 7.10 Separation factor for the BSA-Hb system as a function of the filtration flow rate, at an ionic strength of 50mM, for the mixed matrix adsorber membrane C_{MMA} (A) and A_{MMA} (B).

One needs to be aware that the arbitrary choice of the breakthrough point 10% of the feed concentration determines the protein separation performances. In industrial applications the filtration process can be stop at a lower permeate concentration, e.g. 5% breakthrough and the absolute value of the average separation factor will increase up to a double value. Nevertheless, the obtained values are at least 10 times higher than the separation factor reported by Eijndhoven et al. [13] for filtration velocity of approximately 22 l/h/m².

The results presented in Figure 7.10 were obtained for the separation of BSA-Hb mixture in phosphate buffer pH 7 at 50 mM ionic strength. 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 [5], the protein adsorption capacity slowly decreases with increasing ionic strength and we anticipate the separation factor to follow the same trend. Furthermore, drastically decrease of the separation factor due to an increase of the ionic strength from 2.3 to only 16 mM (from 70 to almost 3), were observed by Eijndhoven et al. [13]. The same trend was also found by C. Causserand [7] who observed a decrease of the BSA separation factor from 6.4 to 1.53 operated at 1mM and respectively 100 mM ionic strength.

Using the adsorber membranes C_{MMA} with physiological ionic strength conditions (150 mM) the average protein concentrations in the permeate were

0.52 mg BSA/ml and 0.033 mg Hb/ml at hemoglobin breakthrough point 10% of the Hb feed concentration. In these conditions, the calculated average separation factor over the filtration run until the hemoglobin breakthrough decreases to 15 (Figure 7.11A). Lower average separation factor (12) was also obtained for BSA-Hb fractionation using the adsorber A_{MMA} operated at physiological ionic strength (Figure 7.11B). Nevertheless, the values of the BSA-Hb separation factor obtained with the mixed matrix adsorber membranes are still higher than the ones reported in the literature for similar operating conditions.

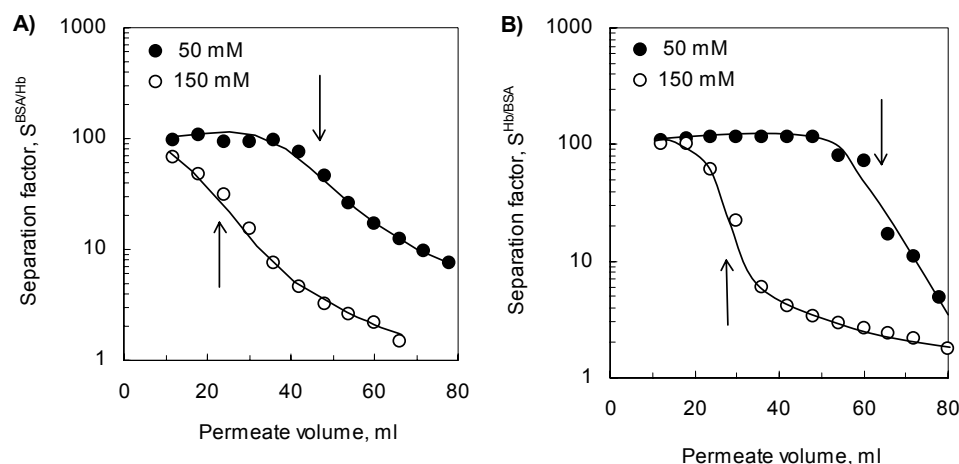


Figure 7.11. Separation factor for the BSA-Hb mixture as a function of the ionic strength. The filtration flow rate through a stack of 10 membranes C_{MMA} (A) A_{MMA} (B) was kept constant at 10 l/h/m^2 .

7.4 CONCLUSION

The mixed matrix adsorber membranes feature high protein adsorption capacities offering 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 function also 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 in less sensitive to fouling and plugging resulting in less extensive pre-treatment of the feed solution and a comparable high protein binding capacity but at low pressure drop. This leads to lower protein denaturation during separation process.

A particular use of the mixed matrix adsorber membrane is the fractionation of proteins having similar molecular weight. Selective adsorption of hemoglobin into the cation adsorber membrane C_{MMA} allows separation of BSA-Hb mixtures with an average separation factor of 40, calculated over the filtration run until a hemoglobin breakthrough of 10% of the Hb feed concentration, at 50 mM ionic strength and 10 l/h/m² 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 the ones reported in literature.

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CHAPTER 8

EVAL-BASED ADSORPTIVE MEMBRANES FOR BILIRUBIN REMOVAL^{*)}

ABSTRACT

This study focuses on the preparation of microporous adsorptive membranes used as affinity supports for bilirubin (BR) retention. BSA was used as affinity ligand for specific binding of BR molecules. The membranes were prepared directly from hydrophilic ethylene vinyl alcohol copolymers (EVAL) in both ternary water/DMSO/EVAL and quaternary water/1-octanol/DMSO/EVAL systems followed by membrane surface modification and ligand coupling. The membranes prepared from the ternary system are only suitable for activation and BSA coupling reactions in aqueous media. Using glutaraldehyde (GA) to activate the secondary alcohol groups of the vinylalcohol segments yielded to-immobilisation of 8 mg BSA per unit of membrane mass. The microfiltration membranes prepared from the quaternary system enable us to perform surface functionalization reactions in organic media. Up to 18 mg BSA per membrane mass covalently were coupled onto porous EVAL membranes activated by trichloro-s-triazine reaction. The preparation of functionalized particle loaded membranes suitable for bilirubin separation was as well investigated. Lewatit type ion exchange particles were incorporated into an EVAL porous matrix by immersion phase separation process. A heterogeneous matrix, composed of solid particles surrounded by a porous polymeric structure, with high BSA binding capacity (150 mg/g membrane) was formed. In a subsequent step the adsorber membrane was loaded with BSA and further treated with GA to chemically attach the protein into the porous matrix. The membrane stability was investigated by eluents of different pH and ionic strength. Less than 10% from the total adsorbed protein was released in the desorption medium indicating a successful protein crosslinking into the membrane. BR removal by EVAL-based adsorptive membranes was performed in static as well as in dynamic mode. The obtained BR retentions of 25 mg/g adsorber membrane are comparable with the data reported in the literature. These results allow us to conclude that this system could be an alternative for the bilirubin retention from human plasma.

^{*)} To be submitted for publication.

8.1 INTRODUCTION

In medical and biochemical industry there exists a 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. The affinity membranes were designed to bypass the fundamental limitations of packed-bed absorbers, mainly the bead compression at high velocities. Considering the general properties of microporous membranes in comparison with particle beds, the main advantages are the lower flow resistance (higher porosity and reduced bed heights) and faster mass transfer. Pressure drop and intra-bed diffusion limitations are minimized by convection through the fine pores of the membranes [1].

Various affinity ligands including enzymes, coenzymes, cofactors, antibodies, amino acids, oligopeptides, proteins, nucleic acids, oligonucleotides and dye-ligands were coupled onto modified commercially available microporous membranes and they were used as alternative chromatographic adsorbents for biomedical applications [2, 3]. An ideal membrane for biomedical application must be highly hydrophilic and have low nonspecific protein adsorption, fairly large pore size and a narrow pore size distribution. In addition, the membrane should have high biological, chemical and mechanical resistance as well as a large number of reactive functional groups [4, 5]. The efficiency of affinity chromatography supports depends on numerous factors, such as chemical structure of the matrix, type and number of reactive functional groups, swelling index, which influences the ligand immobilization reactions.

A large number of metabolic toxins such as free fatty acids, endotoxins of gram-negative bacteria, mercaptans as well as medications like nortriptyline, amitriptyline, diazepam, bromazepam, bound preferentially to the albumin fraction of the blood plasma. These interactions have been thoroughly studied [6-8] not only for their theoretical interest but also for their clinical relevance.

Bilirubin (BR), a bile pigment, is formed as a result of the catabolism of hemoglobin from aged red blood cells in all mammals [9]. Although its physiological functions in the human body are not fully understood, it has been suggested that bilirubin can serve as a chain-breaking antioxidant. Disorders in the metabolism of BR, especially common among newborn infants, may cause jaundice, a yellow discoloration of the skin and other tissues. Free BR is toxic and at a high concentration causes hepatic or biliary tract dysfunction and permanent brain damage [10]. Neurological dysfunctions called kernicterus or bilirubin encephalopathy may develop if BR concentration in plasma rises above 0.03 mg/ml. It is known that each albumin molecule can bind strongly two bilirubin molecules. There are also numerous other binding sites which bind BR loosely [11]. Furthermore, BSA immobilized onto

polymeric supports improves the material biocompatibility [12]. As a consequence, albumin has been widely used as the ligand in studies of BR removal [13-15].

Although methods are known to help the body eliminating the excess of bilirubin, many techniques have been used for BR removal directly from plasma of patients suffering from hyperbilirubinemia such as hemodialysis, phototherapy and hemoperfusion. Hemoperfusion treatment, i.e., circulation of blood through an extracorporeal unit containing an adsorbent system for BR is the most promising technique [16-28]. In most cases basic ion exchange materials have been used but uncharged polymers can also adsorb BR from aqueous media.

The affinity chromatography principle is extensively applied for removal of BR and other albumin-bound substances from plasma and whole blood. During the 1970s and 1980s, numerous papers on albumin immobilized on natural polymers like agarose, agar and charcoal have been published. Synthetic polymers, such as poly(styrene-divinyl benzene), ethylene glycol dimethacrylate-hydroxyethyl methacrylate (EGDMA-HEMA) microbeads and acrylamide have been also used as sorbents for the removal of BR directly from the plasma of patients suffering from hyperbilirubinemia. Sideman et al. [12, 16] suggested the application of hemoperfusion to the removal of the BR from jaundiced newborn babies by using albumin-deposited macroreticular resin with BR adsorption capacities ranging from 2 to 24 mg/g resin. Anion-exchange synthetic resins (IONEX) developed by Kanai et al. were clinically used in selective BR separation with 7.7 mg BR/g resin as maximum bilirubin adsorption [17]. Yamazaki et al. developed poly(styrene-divinyl benzene)-based sorbents, and successfully applied them in the treatment in more than 200 patients with hyperbilirubinemia [18]. Styrene-divinyl benzene resins were also used by Morimoto et al. [19] for plasma exchange, plasma adsorption and removal of BR from hepatectomized patients. This plasma adsorption system improves the supportive therapy for hepatic failure, especially for patients with hepatic coma and hyperbilirubinemia. Chandy and Sharma used polylysine immobilized chitosan beads for selective BR removal with up to 1.13 mg BR per mass of beads [20]. Polypeptide functionalized polyacrylamide beads prepared by Zhu et al. were used as an affinity sorbent system for BR removal with adsorption of 0.2-75 mg bilirubin/g resin [21]. Plotz et al. immobilized human serum albumin on agarose supports by using the cyanogen bromide reaction and reported high BR binding capacity [22]. Denizli et al. [24-26] have produced bioaffinity sorbents with poly(HEMA) or poly(EGDMA-HEMA) microbeads as basic carrier. Several bio-ligands (e.g. protein A, DNA, heparin, collagen) and dye-ligands (e.g., Cibacron Blue F3GA, Congo Red, Alkali Blue 6B) were incorporated into these microbeads. Bilirubin was removed from aqueous media, including

plasma with 6.8-32.5 mg/g sorbent. The use of epoxy-activated gels such as modified cross-linked polybutadiene hydroxyethylmethacrylate (PB-HEMA) for albumin immobilization and their performances as supports in affinity chromatography to retain BR was investigated by Alvarez et al. [27]. Bilirubin retention of 3.1 mg/g beads were observed at 5°C. Senel et al. [28] reported up to 48.9 mg BR retain by gram of modified polyamide hollow fibers with Cibacron Blue F3GA attached as affinity ligand on the membrane surface.

This work investigates the use of microporous BSA-immobilized adsorptive membranes for BR removal from aqueous media. In this study, we employed two different types of basic membranes whose preparation was described in details in [29, 30]. The first type of adsorptive membranes was prepared directly from hydrophilic ethylene vinyl alcohol copolymers in either ternary or quaternary systems, followed by membrane surface modification and ligand coupling. To optimize the BSA-binding into the porous structure, a number of different pre-activation reactions in aqueous media (with glutaraldehyde) as well as in organic environment (with triazine, sTT) have been investigated. The pre-activation allows the BSA coupling onto the modified vinylalcohol segments of the EVAL membranes via the aminogroups of the protein. The second class of membranes investigated in this study was prepared by incorporation of ion exchange resins as a particulate material into an EVAL porous matrix. Heterogeneous mixed matrix adsorber membranes with high BSA adsorption capacity were prepared. In a sequential step the physically adsorbed BSA was chemically attached into the porous matrix by a crosslinking reaction with GA.

8.2 EXPERIMENTAL

8.2.1 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 basic membrane material without further modification. Dimethylsulfoxide (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. Surface modification of the secondary alcohol-groups of the vinylalcohol segments was performed with glutaraldehyde (GA, Sigma) in aqueous solution as well as trichloro-s-triazine (sTT, Aldrich) in dried dioxane (Merck). GA has been purified over activated carbon until a single adsorption peak was observed at 280 nm. Dioxane was dried overnight using a molecular sieve with 4Å pores (25 g per liter

of reagent). Lewatit ion exchange resins 112WS kindly supplied by Caldic, Belgium were incorporated into the basic EVAL polymeric membranes. BSA (fraction V, Sigma) was bound as a ligand onto the pre-activated EVAL membranes. Sodium dodecyl sulfate (SDS, Acros) was used to remove physically adsorbed BSA from the membrane surface. Solid bilirubin (Fluka) was dissolved in NaOH followed by dilution with ultrapure water and used in affinity separation experiments. All other chemicals were used as received. Ultrapure water was prepared using a Millipore purification unit Milli-Q plus.

8.2.2 Membrane preparation and characterization

EVAL microfiltration membranes were prepared by immersion precipitation either from the *ternary water/DMSO/EVAL system* or from the *quaternary system water/1-octanol/DMSO/EVAL*. EVAL-membranes prepared from the ternary system using 50% DMSO in the coagulation bath (E/50/O0) showed generally a strong tendency to collapse upon drying. Since a solvent-exchange treatment with ethanol and hexane could not completely suppress the pore collapsing, the membranes prepared were stored at room temperature in ultrapure water before further use. In contrary, the microporous EVAL-membranes prepared in the quaternary system water/1-octanol/DMSO/EVAL with 20% octanol as nonsolvent-additive in the casting solution even without DMSO into the coagulation bath (E/0/O20), could be completely dried without evidence of pore collapse were. Proper membrane preparation conditions were described in detail in [29]. Samples were identified in the way that first the concentration of DMSO in the coagulation bath is given, followed by the concentration of 1-octanol in the casting solution.

Porous EVAL-based adsorptive membranes loaded with specific binding materials (E/MMA) were prepared by incorporation of functional particles into a porous polymeric structure. 65% Lewatit cation exchange particles 112WS were added to a solution of 10%wt EVAL in DMSO. 10% 1-octanol was added to the casting solution in order to improve the membrane morphology [30]. The mixtures were stirred over night to break the clusters of particles. EVAL microfiltration membrane adsorbers were prepared by immersion precipitation; the polymeric solution was cast on a glass plate and immediately immersed into a water coagulation bath at 40°C.

For membrane morphological characterization, pieces of membranes were frozen in liquid nitrogen and fractured. They were then 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.

8.2.3 Membrane activation and BSA binding

EVAl-membranes E/50/O0 prepared from the ternary system suffer from pore collapsing upon drying. They needed to be kept wet in an aqueous solution and could therefore only be modified based on aqueous chemistry. The membranes prepared using 1-octanol as nonsolvent-additive in the casting solution (E/0/O20) could be completely dried without suffering from pore collapsing and could be also activated in organic medium.

Aqueous membrane functionalization

To immobilize BSA on the microporous EVAl-membranes E/50/O0, an aqueous GA solution was employed in the presence of mineral acid as catalyst. Aqueous GA solutions of 1% initial concentrations were freshly prepared and the pH was adjusted with H₂SO₄ 0.1M. Circular membrane pieces were placed into the GA solutions at room temperature and reacted for 24h. Subsequently, the membranes were washed with a 0.1 M phosphate buffer at pH 8 to remove the unreacted compounds. The modified membranes were stored in a 0.1M phosphate buffer at pH 8 and 4°C.

For BSA coupling on the pre-activated membranes, the membranes were immersed in 1 mg/ml protein solution prepared in phosphate buffer pH 7 and incubated at 4°C for 24h. Then, the membranes were washed with phosphate buffer and deionized water in order to remove the unreacted protein. The physically adsorbed protein on the membrane surface was desorbed by immersing the membrane in a 3% sodium dodecyl sulfate solution for up to 24 hours [31]. The amount of protein covalently bound onto the EVAl-membranes was determined by measuring the difference between the protein concentration in the solution before and after coupling reaction. The amounts of protein desorbed in SDS were subtracted to obtain the values for covalent protein coupling. The protein concentration was determined spectrophotometrically at 280 nm with a PU 8720 UV/VIS apparatus.

Non-aqueous membrane functionalization

Circular pieces of the EVAl-membrane E/0/O20 were first soaked in 3N NaOH for 30 min at 25°C. Excess of NaOH solution was removed by draining. The alkaline membrane was placed into a 10% solution of sTT in dry dioxane at room temperature for 30 minutes. After the activation step, the membranes were washed consecutively with dioxane/water mixtures of 100:0, 75:25, 50:50, 25:75, 0:100 v/v,

and acetone. The modified membranes were stored in 10% acetic acid solution at 4°C before protein coupling.

The membranes activated with sTT were immersed in BSA solution (1mg/ml in acetate buffer pH 5), incubated for 24 h at 4°C and treated further as previously described. BSA-immobilization was calculated in the same way as for the E/50/O0 membranes.

Mixed matrix adsorber membranes

A known amount of cation exchange adsorber membranes E/MMA was equilibrated with a concentrated volume of dissolved BSA in 50 mM acetate buffer with pH 4.5. At this pH which is lower than the BSA isoelectric point ($pI_{BSA}=4.8$), the protein is positively charged, meanwhile the membrane is negatively charged. The protein is adsorbed into the mixed matrix adsorber membranes by the ion exchange particles and hence the BSA concentration in the bulk solution decreases until equilibrium is reached. The protein uptake per membrane mass was determined using a depletion method by measuring the absorbance at 280 nm.

In a sequential step the BSA-adsorber membrane complex was treated with 1 % GA solution at pH 4, to crosslink the BSA molecules adsorbed on the ion exchange particles and thus to minimize the protein leaching out due to changes of environmental conditions such as pH or ionic strength. The reaction time was varied from 1 to 12 hours. The membranes were subsequently exposed to desorptive solutions such as 1M NaCl and 50 mM borate buffer pH 10 for 4 and respectively 12 hours. The amount of BSA immobilized by crosslinking was calculated by subtracting the amount of protein desorbed into the desorptive buffers from the total amount of BSA adsorbed into the membrane.

8.2.4 Bilirubin removal

Bilirubin removal by E/50/O0, E/0/O20 and E/MMA adsorptive membranes was performed both in static and dynamic mode. The BR solution was prepared by dissolving the solid BR in 10 ml of 10 mM NaOH followed by dilution with ultrapure water and immediately used after preparation. Bilirubin concentrations of 0.2 mg/ml, which equal 10 times the normal bilirubin value in plasma, were employed in the adsorption experiments.

For *static adsorption experiments*, a known amount of membrane was equilibrated with BR solution. The amount of BR removed per unit of membrane mass was calculated using a depletion method by measuring the absorbance at 438 nm.

The *dynamic adsorption performance* of EVAL membrane adsorbers was evaluated at constant flow rates using a compressed nitrogen stirred dead-end filtration cell. Because the capacity of a single adsorptive membrane is limited 10 sheets were mounted in series in an ultrafiltration device to achieve the necessary accuracy in the measurements. This configuration permits rapid, low-pressure adsorption of protein in either batch or continuous recycle mode, the intended product being concentrated substantially. A BR solution was permeated through the membrane stack measuring the BR adsorbed per unit of membrane bed as mentioned above.

8.3 RESULTS AND DISCUSSIONS

In the *results and discussion section* the membranes characterization in respect to their morphology is first presented. Emphasis is then laid on the modification of the basic membranes and ligand coupling. The efficiency of BR removal from aqueous solution using the prepared adsorptive membranes is finally discussed.

8.3.1 Membrane preparation

In Figure 8.1 the cross-section, the top and bottom surfaces of E/50/O0 membrane is shown. The membranes are asymmetric and consist of an almost dense skin top layer and a cellular microporous support with average pore size of 0.2 μm and relative low interconnectivity. Residual macrovoids are present in the upper half of the cross-section.

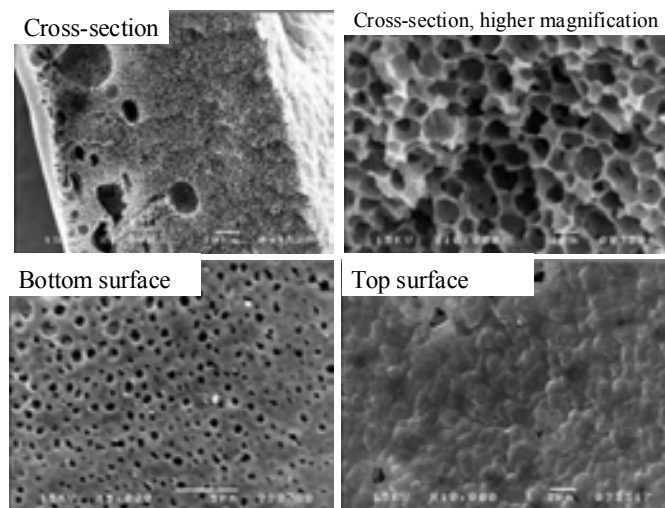


Figure 8.1 SEM photomicrograph for the membranes E/50/O0 prepared from 10% EVAL in DMSO with 50% DMSO in coagulation bath

Figure 8.2 shows the morphology of the E/0/O20 membrane, prepared with 20% 1-octanol in the casting solution and water as coagulation bath. The membrane is free of macrovoids and shows an open porous structure with average pore size around $0.3\ \mu\text{m}$ and high pore interconnectivity. Both, the top and the bottom surface show a more open structure than the E/50/O0 membranes. The E/0/O20 membrane can be dried without pore collapsing, which enlarges the number of possible surface modifications for protein coupling.

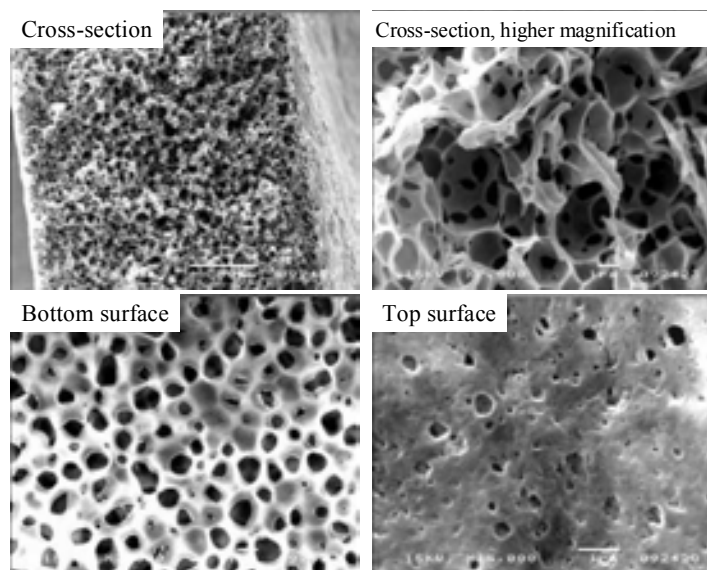


Figure 8.2 SEM photomicrograph for the membranes E/0/O20 prepared from 10% EVAL in DMSO in the presence of 20% 1-octanol.

The structure of the EVAL-based adsorber membranes E/MMA prepared by incorporation of 65% Lewatit 112WS ion exchange resins, with a particle size diameter smaller than $20\ \mu\text{m}$, is presented in Figure 8.3. The membrane possesses an open, interconnected porous structure, without finger-like macrovoids across the entire cross-section. Pores larger than $0.1\ \mu\text{m}$ can be observed on both the glass and the air surfaces. 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.

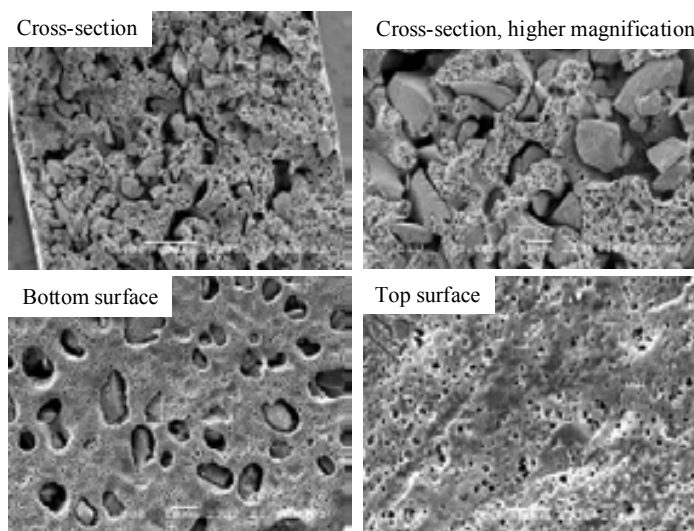


Figure 8.3 SEM photomicrograph for the membranes E/MMA prepared from 10% EVAL in DMSO in the presence of 10% 1-octanol as additive, by incorporation of 65% Lewatit ion exchange particles into the polymeric support.

8.3.2 Membrane modification and ligand coupling

The pre-activated membranes E/50/O0 and E/0/O20 were immersed in a BSA solution and treated as described in Section 8.2.3. Physically adsorbed BSA was removed by desorption in SDS solution and quantified to 2 mg per unit mass of membrane. The remaining covalently coupled BSA per mass of GA-activated E/50/O0 membrane was up to 8 mg/g. The maximum BSA-immobilization values on the sTT activated E/0/O20 membranes were around 18 mg/g of membrane, which is by a factor of 2 higher than for the E/50/O0 membranes.

The protein adsorbed into the E/MMA membranes reaches values of around 150 mg BSA per membrane mass within 24h of incubation. To chemically attach the physically adsorbed protein into the porous matrix, a supplementary crosslinking step with GA was performed. Subsequently, the membranes were washed with solutions of different pH and ionic strength.

The amount of protein released from the membrane into the desorptive solutions decreases with an increasing of crosslinking time (Figure 8.4). Crosslinking reaction performed for more than 6h leads to constant values of protein desorption of approximately 10% of the total BSA adsorbed. Therefore the amount of BSA

chemically coupled into the EVAL polymeric matrix also increases with increasing the crosslinking time. For more than 6h of crosslinking the amount of protein immobilised into the adsorber membrane was about 135 mg BSA/g membrane.

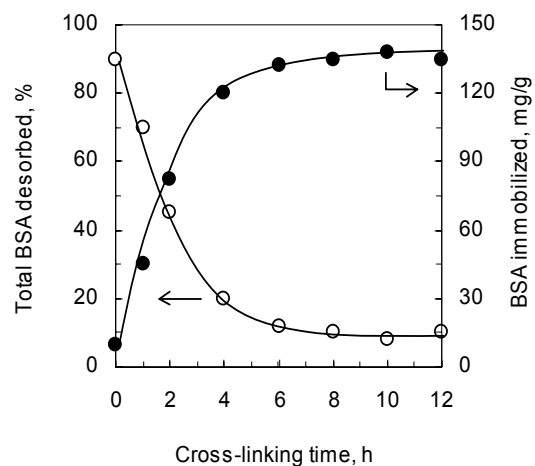


Figure 8.4 The effect of crosslinking time on the BSA the immobilization rate

8.3.3 Bilirubin removal

To minimize the errors cause by the deterioration of BR due to environmental conditions, a study of BR stability at different temperature and light conditions was performed (Figure 8.5).

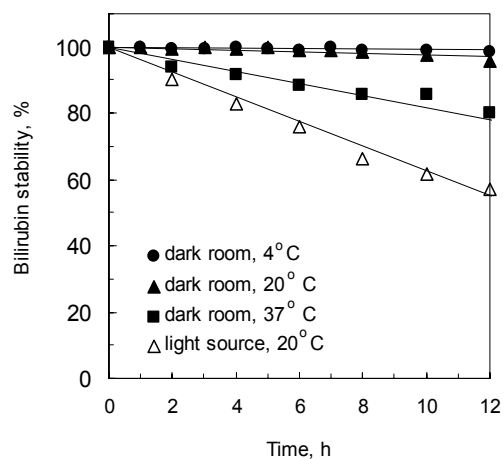


Figure 8.5 Bilirubin stability as a function of temperature and exposure to light.

Almost 50% of the bilirubin was destroyed by direct exposure to sunlight within 12 h of exposure, and about 20% due to an increased temperature. Therefore, all the adsorption experiments were carried out for future experiments in a dark room, at 20°C.

Static bilirubin adsorption capacity

Static BR adsorption capacities of the prepared adsorptive membranes were determined batchwise. Figure 8.6 shows the non-specific and specific adsorption of BR into the unmodified EVAL membranes and the BSA-attached adsorptive membranes respectively, within 12 h of adsorption. The amount of BR adsorbed into the unmodified EVAL adsorptive membranes was lower than 2 mg BR/g membrane. However, much higher adsorption capacities were achieved in the case of BSA-attached EVAL membranes. Note that one of the main requirements in the affinity system is the sorbent specificity. The non-specific interaction between the EVAL polymeric support and the molecules to be removed (bilirubin) should be minimum in order to have a high specificity.

The highest BR adsorption capacities were obtained for the E/MMA membranes (Figure 8.6). Therefore, the adsorption properties for these membranes will be investigated in detail in the following experiments.

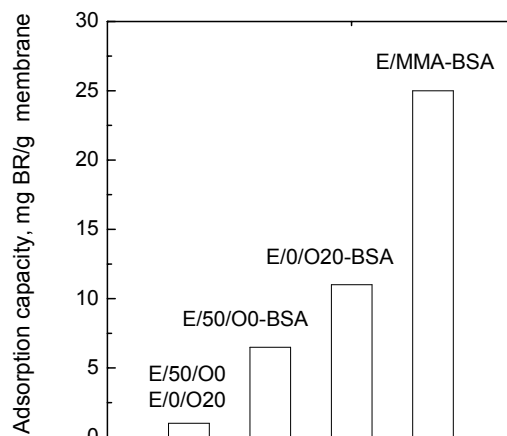


Figure 8.6 Bilirubin adsorption capacities for the prepared adsorptive membranes from an aqueous solution of 0.2 mg BR/ml.

In Figure 8.7 the BR adsorption rate obtained by following the decrease of BR concentration within the bulk solution with time is presented. The curve indicates that the adsorption process is completed within 8 h of incubation, with an equilibrium adsorption value of about 25 ± 2 mg BR/g membrane. We must mention that this time has only little meaning for the treatment time in the actual BR removal process. It is only a measure for the apparent diffusion coefficient of BR into the E/MMA membranes since complex simulations on the chromatographic performances require the diffusion coefficient as an impute parameter.

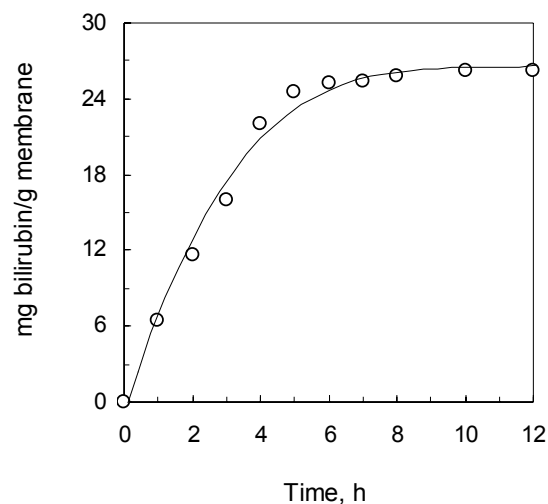


Figure 8.7 Bilirubin adsorption rate from solution of 0.2 mg BR/ml for the membrane E/MMA.

Different thermodynamic models e.g., the linear, the Langmuir, the bi-Langmuir, the Freundlich, the Freundlich-Langmuir isotherms have been proposed to describe the adsorption of solutes from a liquid solution onto a solid surface. The effect of the BR initial concentration on the protein adsorption capacity is presented in Figure 8.8. The measured adsorption equilibrium isotherm of BR on the adsorber membrane E/MMA may be of the Langmuir type, with maximum adsorption capacity of 33 mg BR/g membrane and a dissociation constant of 0.1 mg/ml. These results are comparable with the BR adsorption capacity reported in literature for adsorbers with albumin used as the ligand (1.13-24 mg BR/g adsorber, [16-20, 22, 27]). This allows us to conclude that the mixed matrix adsorber membrane system is a viable alternative for the bilirubin retention from aqueous solution.

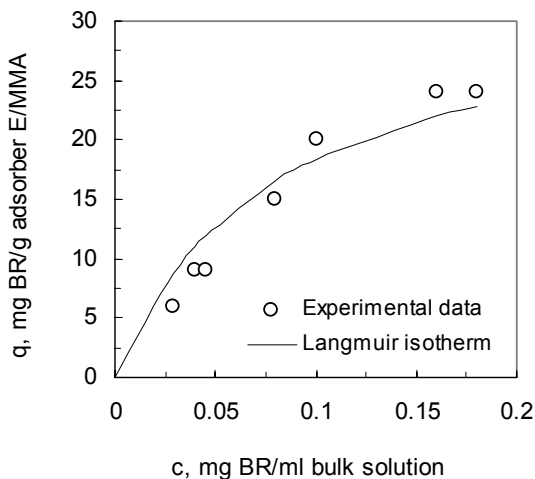


Figure 8.8 The effect of bilirubin concentration on E/MMA membrane adsorption capacity.

Dynamic binding capacity

In dynamic operation, the BR molecules are transported through the adsorptive membrane by the convective flow of the feed solution in a single pass mode. The transport of the BR to the adsorptive sites, is a function of flow conditions, protein diffusion as well as adsorption kinetic parameters. The BR breakthrough curve through a stack of 10 sheets of flat membrane E/MMA at a constant flow rate of 10 l/h/bar/m² is presented in Figure 8.9. The average BR concentration was calculated by numerical integration of the BR concentration in the permeate curve during the filtration run. It can be observed that 80% of the BR was adsorbed into matrix during the permeation (the average BR concentration reached 0.04 mg/ml for a starting feed BR concentration of about 0.2 mg/ml). It is worth to know that in real biomedical applications the filtration process can be stopped in an earlier stage when the average BR concentration in the permeate is below the normal BR level in plasma. Nevertheless, the dynamic BR adsorption capacity is around 2 times lower than the corresponding static capacity. This can be attributed to a non-uniform flow distribution and resistance against mass transport rate processes. Axial and radial diffusion, sorption kinetics and non-uniformities in membrane porosity and thickness can affect affinity membrane performances especially the breakthrough curve sharpness.

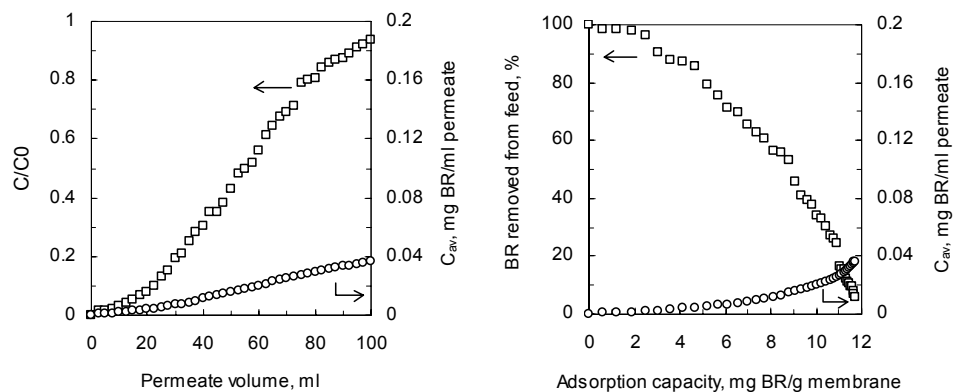


Figure 8.9 Bilirubin removal efficiency for a stack of 10 membranes E/MMA operated at a constant filtration flow rate of 10 l/h/m² with 0.2 mg BR/ml feed concentration.

8.4 CONCLUSION

In this study bilirubin removal by EVAL-based membrane adsorbers was investigated in static as well as in dynamic mode. The amount of bilirubin adsorbed into the unmodified EVAL membranes was lower than 2 mg BR/g polymer, while much higher adsorption values were achieved in the case of BSA-attached EVAL membranes. The highest bilirubin adsorption capacity of 25 mg BR/g membrane was obtained for the E/MMA membranes prepared by incorporation of 65% ion exchange resins into the polymeric matrix. The mixed matrix adsorber membranes show performances equivalent to the highest values reported in the literature. The results allow us to conclude that this system is a viable alternative for the bilirubin retention from human plasma.

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SAMENVATTING

Bio-affiniteitsscheidingen vereisen membranen die een goede compatibiliteit vertonen met de te behandelen biologische vloeistoffen, bindingsplaatsen voor de (bio-)liganden beschikbaar hebben en een hydrofiel oppervlak bezitten om niet-specifieke adsorptie, veroorzaakt door hydrofobe aantrekking, te beperken. Ethyleenvinylalcoholcopolymeer (EVAL) is een membraanmateriaal dat voldoet aan deze eisen en daardoor kansrijk is in biomedische toepassingen. Het werk beschreven in dit proefschrift richt zich op eiwitseparatie door gebruik te maken van affiniteits- of ionenwisselingsinteracties op basis van EVAL membraanadsorbers.

De bereiding van microfiltratie membranen met een open celstructuur middels het ternaire water/DMSO/EVAL systeem is beschreven in **Hoofdstuk 3**. Onderdompeling in water van een 10% EVAL oplossing geeft ogenblikkelijk *l-l* ontmenging. Het gevormde asymmetrische membraan bestaat uit een dichte huid met daaronder een celvormig poreuze steunlaag met daarin grote vingervormige holten, die typisch zijn voor snelle *l-l* ontmengprocessen. Uitstellen van het begin van de fasescheiding door toevoeging van DMSO aan het coagulatiebad leidt tot onderdrukking van de vorming van deze grote holten maar bevordert het kristallisatieproces, een fasescheidingsproces dat wordt beheerst door *s-l* ontmenging. De waterdoorlaatbaarheid van de bereide cellulaire films is laag door een slechte onderlinge verbinding tussen de afzonderlijke poriën. Hierdoor zijn deze structuren ongeschikt om te worden toegepast als affiniteitsmembraan. De membranen waarbij kristallisatie in het vormingsproces een rol speelt vertonen een hogere waterdoorlaatbaarheid maar hebben weinig mechanische sterkte. Door toevoeging van een *n*-alcohol, een zwak oplosmiddel, aan de strijkoplossing, wordt de indiffusie van water geremd. Dit heeft tot gevolg dat de *l-l* ontmenging wordt vertraagd en dat vorming van grote holten wordt onderdrukt. Via deze route worden geen structuren aangetroffen die op kristallisatieprocessen wijzen en behouden de membranen hun mechanische sterkte. Hierdoor bieden membranen bereid uit het quaternaire systeem meer mogelijkheden om op de applicatie toegesneden structuren te ontwerpen. In een homologe reeks *n*-alcoholen wordt door toevoegen van *n*-octanol de maximale onderdrukking van de vorming van grote holten bereikt. Door de juiste verhouding *n*-octanol te kiezen kunnen structuren worden gemaakt die geen grote vingervormige holten bevatten en door een goede onderlinge porieverbinding een hoge waterdoorlaatbaarheid hebben van meer dan 1000 l/h/m²/bar.

Een groot aantal metabolische toxines zoals mercaptanen, vrije vetzuren, ongeconjugeerd bilirubine, endotoxinen van gramnegatieve bacteriën, als ook veel medicaties (nortriptyline, amitriptyline, diazepam, bromazepam) binden aan eiwitten in de bloedsomloop en in het bijzonder aan de in het bloedplasma aanwezige albumine. In **Hoofdstuk 4** is de covalente koppeling van BSA aan cellulaire EVAL microfiltratiemembranen onderzocht. Om de exacte beladingsgraad van het geïmmobiliseerde BSA vast te stellen is middels BET-metingen het interne membraanoppervlak bepaald. De membranen die gemaakt zijn uit het drie componenten systeem water/DMSO/EVAL storten ineen tijdens de droogstap en kunnen derhalve alleen in de natte toestand worden geactiveerd. De activering van de secundaire alcoholgroep van de vinylalcoholsegmenten met glutaraaldehyde en oxiraan levert een BSA-immobilisatie op van $0.2 \mu\text{g}/\text{cm}^2$. Membranen die zijn bereid uit het vier componenten systeem water/1-octanol/DMSO/EVAL vertonen geen ineenstorting van de structuur waardoor het aantal mogelijke activeringsreacties enorm is vergroot omdat de activering nu zowel in organische media als ook middels plasmareacties mogelijk is. Bij gebruik van trichloor-s-triazine en sulfonylchloride is aan het interne oppervlak $0.3\text{--}0.45 \mu\text{g BSA}/\text{cm}^2$ covalent gekoppeld. Bij activering middels een plasma bedroeg de BSA-immobilisatie $0.55 \mu\text{g}/\text{cm}^2$. Het aanbrengen van een BSA-monolaag op het porieoppervlak wordt gezien als maximale belading.

De aantrekkelijkheid van het in **Hoofdstuk 5** beschreven concept is de mogelijkheid om onder milde procescondities deeltjesbevattende membranen te maken, waarbij allerlei typen deeltjes in bijna elk type polymeer kunnen worden ingevangen. Het mengsel van deeltjes en polymeer kan worden gestreken/gesponnen als vlakke sheets/vezels waarna middels het fase-inversieproces membranen kunnen worden gevormd. De bereide adsorptie membranen bevatten 25-75 gew.% ionenwisselingsdeeltjes die door de polymeermatrix stevig worden ingevangen. Hierbij doen het polymeer en de bereidingswijze geen afbreuk aan de activiteit van de deeltjes. De hier voorgestelde membranen kunnen als vlakke films worden gestapeld of als vezels in een module worden ingebracht. De toepassing van dit type membranen is enorm veelzijdig zoals o.a. peptide en eiwit winning uit fermentatie beslagen, eiwit fractionering, ligand immobilisatie voor affiniteitscheidingen, immobilisatie van katalysatoren en enzymen, ontgiften van bloed, productbescherming en systemen voor medicijnafgifte.

In **Hoofdstuk 6** wordt het eiwitadsorptiegedrag van deeltjes bevattende membranen door middel van statische en dynamische adsorptiecapaciteitsmetingen beschreven. De actieve plaatsen in de adsorptiemembranen zijn zeer goed toegankelijk voor eiwitmoleculen, waardoor hoge adsorptiewaarden worden gevonden (30-50 mg BSA/ml membraan).

Daarnaast is het proces makkelijk opschaalbaar. Ook kunnen bij gebruik van geschikte eluens de gewenste componenten tijdens de zuiveringsstap 10-voudig worden geconcentreerd. De adsorptiemembranen kunnen vele malen hergebruikt worden waarbij na een aantal adsorptie-desorptie stappen de hoge adsorptiecapaciteit blijft gewaarborgd. Verder kunnen, in vergelijking met de klassieke chromatografie, kleinere deeltjes worden ingebed waarbij nadelige effecten zoals een hoge drukval, gevoeligheid voor vervuiling en verstopping en een lage doorstromingsnelheid achterwege blijven. Terwijl wel de voordelen van de membraanprocessen, makkelijk opschaalbaar, lage drukval en een hoge volumedoorzet behouden blijven.

In **Hoofdstuk 7** wordt een applicatie beschreven waarbij twee eiwitten van vergelijkbare grootte, BSA en hemoglobine (Hb), van elkaar worden gescheiden door gebruik te maken van het nieuwe concept van deeltjesbevattende adsorptiemembranen. Door het instellen van procescondities (b.v. pH en ionsterkte en filtratiesnelheid) is het mogelijk om één eiwit specifiek te binden terwijl het andere eiwit vrij door de membraanmatrix beweegt. Het effect van de in te stellen parameters op de scheidings eigenschappen is onderzocht voor zowel kationen- als anionenuitwisselingmembraanadsorbers. Selectieve binding van Hb aan kationenuitwisselingsmembranen C_{MMA} bij een ionsterkte van 50 mM en een filtratiesnelheid van 10 l/h/m² geeft op het moment dat de Hb doorslag 10% is, een BSA-Hb scheiding met een scheidingsfactor van meer dan 40. De scheiding van Hb-BSA mengsels met een anionenuitwisselingmembranenadsorber A_{MMA} , verricht onder identieke condities, geeft een scheidingsfactor van bijna 50. Wanneer de filtratiesnelheid wordt verhoogd naar 20 l/h/m² en een ionsterkte van 150 mM wordt gebruikt, werd een iets lagere selectiviteit gemeten. Niettemin worden de met deeltjes geladen membranen scheidingsfactoren behaald die hoger zijn dan de, onder vergelijkbare omstandigheden, in de literatuur gerapporteerde waarden.

De in Hoofdstuk 3 en Hoofdstuk 5 beschreven membranen zijn in **Hoofdstuk 8** gebruikt voor de verwijdering van bilirubine (BR). In hoge concentraties is BR giftig en veroorzaakt geelzucht en onherstelbare hersenbeschadiging. Albumine moleculen hebben twee sterke bindingsplaatsen voor BR moleculen en daarnaast veel zwakke bindingsplaatsen. Daarnaast wordt door immobilisatie van BSA aan het membraanmateriaal de biocompatibiliteit van de gebruikte materialen vergroot. Dit zijn dan ook de redenen dat BSA veel wordt gebruikt als ligand voor de verwijdering van BR. Glutaaraldehyde (GA) of trichloro-triazine (sTT) pre-activering van EVAL membranen, bereid via zowel de ternaire als quaternaire route, staan, via de aminogroepen van het BSA, immobilisatie toe op de gemodificeerde vinylalcoholsegmenten van het membraan. Via deze route wordt 22 mg BSA-

geïmmobiliseerd per gram membraan. Door incorporatie van Lewatit ionenwisselingsdeeltjes in de membraanmatrix kan de BSA-adsorptiecapaciteit worden verhoogd tot 150 mg/g membraan. Na de adsorptiestap wordt BSA door crosslinking met GA chemisch gebonden in de membraanmatrix. De stabiliteit van deze crosslinking is onderzocht als functie van de pH en ionsterkte waarbij minder dan 10% van de initiële concentratie uit het membraan kon worden gespoeld. Dit geeft aan dat crosslinking van BSA in de matrix erg succesvol is. BR verwijdering is zowel in de statische als in de dynamische uitvoeringsvorm gedaan. De maximale BR retentie van EVAL membraanadsorbers bedroeg 25 mg/g membraan, wat vergelijkbaar is met de resultaten die in de literatuur zijn gepresenteerd. Dit betekent dat adsorptiedeeltjesbevattende membranen zeker een alternatief zijn voor de bestaande methoden.

SUMMARY

Bioaffinity separation principally asks for membranes that show a good compatibility to the targeted biological fluids, provide coupling sites for (bio)ligands and possess a hydrophilic surface to reduce non-specific adsorption caused by hydrophobic attraction. Due to its good blood compatibility and very low water solubility, ethylene vinyl alcohol copolymers (EVAL) has become a promising biomedical membrane material, but has so far not been investigated in detail for (bio)affinity separation. The work reported in this thesis focuses on the preparation of EVAL microporous membrane adsorbers for protein separation based on affinity or ion exchange interaction modes.

The use of the ternary water/DMSO/EVAL and the quaternary systems using n-alcohols as additives in the casting solution to prepare open-cellular type EVAL microfiltration membranes that can be used in affinity separation processes is presented in **Chapter 3**. Immersion of 10% EVAL films into water resulted in instantaneous *l-l* demixing. The asymmetric membranes formed consisted of a continuous dense skin layer and a cellular porous support with finger-like macrovoids, typical for fast *l-l* demixing. Delaying the onset of phase separation by adding DMSO to the coagulation bath led to a suppression of macrovoid formation but also favored crystallization and thus a phase separation process that is governed by *s-l* demixing. For the prepared asymmetric cellular membranes the water permeability was, due to the low degree of interconnectivity, too low for application in affinity separation. The particulate membranes displayed higher water permeabilities, but possessed a too low mechanical strength. When alcohols were added as weak non-solvents additives in the casting solution the inflow of water into the film is hindered, resulting in a delay of *l-l* demixing and macrovoid suppression. No particulate structures were observed, which left more possibilities for a directed morphological tailoring. Maximal suppression of macrovoids was obtained using 1-octanol as additives in the homologous n-alcohol series. Adjusting the 1-octanol concentrations resulted in macrovoid-free membranes that possessed high pore interconnectivity with a water permeability of about 1000 l/h/m²/bar.

A large number of metabolic toxins such as mercaptans, free fatty acids, unconjugated bilirubin, endotoxins of gram negative bacteria, as well as many medications (nortriptyline, amitriptyline, diazepam, bromazepam) are bounded to proteins in the bloodstream, especially to the albumin fraction of blood plasma. In **Chapter 4** we investigate covalent coupling of bovine serum albumin (BSA) onto cellular-type EVAL microfiltration membranes. The membranes prepared from the ternary water/DMSO/EVAL system collapse upon drying hence surface activation chemistry is limited to reactions in an aqueous medium. Using glutaraldehyde and oxiran to activate the secondary alcohol groups of the vinylalcohol segments yielded a BSA-immobilisation per internal area up to $0.2 \mu\text{g}/\text{cm}^2$ (8 mg/g per membrane mass). Membranes obtained from the quaternary system water/1-octanol/DMSO/EVAL display a high macroporosity and a large internal and external surface area, which will be beneficiary to the interaction of the matrix-bound ligand with the ligate during the affinity separation step. Furthermore, pore collapse has not been found therefore allowing for surface activation in organic solvents, thus enlarging the possible set of reactions significantly with reactions in organic media as well as surface activation by low-pressure glow discharge treatment. $0.3\text{-}0.45 \mu\text{g}/\text{cm}^2$ BSA per internal area (16-18 mg/g per membrane mass) was covalently coupled onto the porous membranes by applying trichloro-s-triazine and sulfonyl chloride activation reactions, while a BSA-immobilisation of $0.55 \mu\text{g}/\text{cm}^2$ (22 mg/g per membrane mass) was reached via plasma activation. To determine the degree of BSA-immobilisation, the internal surface area of the membranes prepared was measured by BET. The formation of a BSA-monolayer is assumed on the pore surface as maximum immobilisation.

The feature of the concept described in **Chapter 5** is the ability to create particle-loaded membranes under mild process conditions using any type and/or size of entrapped particle and almost any type of polymeric material. A mixture of dissolved polymeric and particulate material was cast/spun into a flat/fiber membrane and then solidified by a phase inversion process. The prepared adsorber membranes contain ion exchange particles tightly held together within a polymeric matrix (25-75% sorbent by weight), the latter not interfering with the activity of the particles. Such membranes can be prepared in different shapes and can be operated either as stacked microporous flat sheet membranes or as modules containing solid or hollow fiber membranes. The preparation method does not influence the integrity of the particulate material during the processing. Heat sensitive particles, which cannot be melt-extruded because of the elevated temperatures required to melt the matrix polymer, can be incorporated into a porous matrix by the phase inversion process without danger of deactivating the particle. Furthermore, since the particles are

functionalized before the incorporation into the polymeric support, extreme conditions can be also applied for the functionalization of the resin particles. The membranes have a wide variety of applications including 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 release systems.

The use of the prepared mixed matrix adsorber membranes as protein adsorbers for single protein (bovine serum albumin, BSA) by mean of static and dynamic protein binding capacities is presented in **Chapter 6**. The heterogeneous mixed matrixes possess a good accessibility for the protein to the adsorptive sides featuring high protein adsorption capacity (30-50 mg BSA/ml membrane) and an easy scale-up for adsorption processes. By choosing the right eluent conditions, the desired components can be concentrated up to 10-fold. This means that beside isolation the mixed matrix adsorber membranes can also act as a concentration medium. The membrane adsorbers can be reused in multiple adsorption/desorption cycles with good adsorption performances. Furthermore, small particles can be embedded into porous polymeric structures without the disadvantages of classical chromatographic columns (high pressure drop, fouling and plugging sensitivity, low flow rate), but with the advantages of membrane technology (easy scale-up, low-pressure drop, high binding capacity).

An extensive characterisation of the new type of mixed matrix adsorber membranes and their application in the separation of two proteins of similar size such as bovine serum albumin (BSA) and bovine hemoglobin (Hb) is reported in **Chapter 7**. 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. The effect of operational parameters such as filtration flow rate, pH, ionic strength, on the protein separation performances was investigated for cation as well as anion exchange adsorber membranes. Selective adsorption of Hb into the cation adsorber membrane C_{MMA} allows separation of BSA and Hb mixture with an average separation factor of 40, calculated over the filtration run until a hemoglobin breakthrough of 10% of the Hb feed concentration, at 50 mM ionic strength and 10 l/h/m² flow rates. The separation of BSA-Hb mixture performed with the anion exchange membranes A_{MMA} in the same conditions offers a separation factor of almost 50. Lower average separation factors were obtained for BSA-Hb fractionation if the filtration flow rate was increased up to 20 l/h/m² or the separation was performed at physiological ionic strength of 150 mM. Nevertheless, the values of the

BSA-Hb separation factor obtained with the mixed matrix adsorber membranes are higher than the ones reported in the literature for similar operating conditions.

Free bilirubin (BR) is toxic and at a high concentration causes hepatic or biliary tract dysfunction and permanent brain damage. It is known that each albumin molecule can bind strongly two bilirubin molecules. There are also numerous other binding sites which bind BR loosely. Furthermore, BSA immobilized onto polymeric supports improves the material biocompatibility. As a consequence, serum albumin has been widely used as the ligand in studies of BR removal. **Chapter 8** presents the preparation of EVAL microporous adsorptive membranes used as affinity supports for BR retention. The first type of adsorptive membranes was prepared directly from hydrophilic ethylene vinyl alcohol copolymers in either ternary or quaternary systems, followed by membrane surface modification and ligand (BSA) coupling. To optimize the BSA-binding into the porous structure, a number of different pre-activation reactions in aqueous media (with glutaraldehyde, GA) as well as in organic environment (with triazine, sTT) have been investigated. The pre-activation allows the BSA coupling onto the modified vinylalcohol segments of the EVAL membranes via the amino groups of the protein with up to 22 mg BSA immobilized per unit of membrane mass. The second class of membranes investigated in this study was prepared by incorporation of Lewatit ion exchange resins as a particulate material into an EVAL porous matrix. Heterogeneous mixed matrix adsorber membranes with high BSA binding capacity (150 mg/g membrane) were formed. In a sequential step the physically adsorbed BSA was chemically attached into the porous matrix by a crosslinking reaction with GA. The membrane stability was investigated by eluents of different pH and ionic strength. Less than 10% from the total adsorbed protein was released in the desorption medium indicating a successful protein crosslinking into the membrane. Bilirubin removal by EVAL-based adsorptive membranes was performed in static as well as in dynamic mode. The maximum BR retention on the prepared EVAL adsorbers was 25 mg/g membrane, comparable with the results presented in the literature for various BSA-immobilised sorbents. This allowed us to conclude that the mixed matrix adsorber membrane system could be an alternative for the retention of other substances such as tryptophan, barbiturates or antidepressant.

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Magda Avramescu