MIXED MATRIX MEMBRANE ADSORBERS

FOR PROTEIN AND BLOOD PURIFICATION

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Mixed Matrix Membrane Adsorbers
for Protein and Blood Purification

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Mixed Matrix Membrane Adsorbers for Protein and Blood Purification

DISSERTATION

to obtain
the doctor's degree at the University of Twente,
on the authority of the rector magnificus,
prof. dr. W.H.M. Zijm,
on account of the decision of the graduation committee,
to be publicly defended
on Thursday 24th May 2007 at 13.15

by

Saiful

born on 22nd September 1969

in Beureugang, NAD-Indonesia

This dissertation has been approved by:

Promotor:	Prof. Dr.–Ing. M. Wessling
Assistant-promotor:	Dr.–Ing. Z. Borneman

Dedicated to my beloved wife and daughter:
Tahiya and Iza

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Chapter 1

General Background and Scope of the Thesis

1.1. General Background

1.1.1. Bioproducts separation

Biotechnology has contributed to and developed new sources of many valuable healthcare and life science products. These bioproducts are produced by gene expression in various biological systems; both prokaryotic and eukaryotic systems[1]. The products include therapeutic proteins and polysaccharides, monoclonals, vaccines, diagnostics, pharmaceutical chemicals and enzymes for the food and consumer markets. The production of bioproducts continue to grow progressively[2]. Current estimates predict that global bio-market is worth over US $48 billion and by the end of this decade global biopharmaceutical products could easily reach over US $100 billion with the annual growth rate of 19%[3].

Various types of bioproducts have to be isolated and purified from mixtures before they can be used in order to ensure the safety and efficacy[4, 5]. The ideal separation process optimizes yield and maintains low manufacturing costs. However, isolation and purification of bioproducts are difficult and expensive. Biologics separation typically involves several consecutive steps to reach the desired purity. It causes products loss, long process times and denaturation of fragile products. The isolation and purification account for 50 to 80 % of the total production costs[6, 7]. Almost all large scale process configurations are based on packed bed chromatographic system because a high capacity and product purity can be obtained. However, these methods have severe major drawbacks and limitations[8, 9] including high pressure drop, low throughput, sensitive to fouling and plugging, channelling, time consuming and bed compression. Innovations and improvements of the existing methods are needed to overcome the shortcomings of the classical technologies and for economical bioproducts recovery.

Adsorptive membrane chromatography has been introduced as integrative approach to reduce the number of steps within a purification process and to bypass the fundamental limitations of packed bed system. It is especially suited for treatment of large volumes of liquid containing low concentrations of target biomolecules. This approach is able to unite the

coarse and the fine purification steps by embedding in microfiltration membrane[8]. Nowadays, adsorptive membrane chromatography can be prepared by two different approaches:

I. By chemically coupled ligands to the internal surface of microporous filtration membranes [8, 10, 11]. The activated membrane has been reported as a potentially advantageous tool to purify proteins at a laboratory, pilot and industrial scale. The main limitation of this adsorptive membrane is the low binding capacity[9, 10, 12].

II. By entrapping functionalized particles (sorbent) into a macroporous polymeric support[13-15]. A polymer solution with dispersed functionalized particles is cast as a flat film or spun into a fiber and then solidified by a phase inversion process to form a so-called Mixed Matrix Membrane (MMM) or Particles Loaded Membrane (PLM). The MMM demonstrates high adsorption capacity, comparable to packed bed systems[15, 16]. By choosing the particles and the supports one can establish various functionalities such as affinity, ion exchange, adsorption, catalysis and reactive chromatography.

The potential for continues progress and tremendous advances in sorbent technologies offer a broad application of the MMM in biotechnology and biomedical fields. As new approach in art chromatographic techniques, there are still many interesting application of the MMM that remain to be investigated with respect to their performance in adsorptive membrane chromatography. In this thesis, the latest development and application of adsorptive chromatographic media based on particles loaded membrane adsorber will be discussed.

1.1.2. Extracorporeal blood purification

Biological fluids purification is nowadays very important in biomedical fields. A growing population of patients with kidney and liver failure, poisoning, sepsis shock, and multi-organs failure by more complex medical problems require technological innovations and improvements to enhance the safety, reliability and efficiency of extracorporeal organs support for removing harmful substances from blood. Kidney and liver failure have become a major threat to public health and expected to rise steeply over the next decade due to ageing and population increase. More than 1.1 million patients throughout the world undergo kidney chronic dialysis[17]. In Germany, approximately 70.000 patients require hospital treatment for severe liver diseases[18] and in the United States, there are more than 360.000 hospitalizations due to chronic liver disease and cirrhosis[19]. Kidney and liver

transplantation can not be routinely provided to this group of patients because of the steadily increasing lack of donor organs[18, 20]. Furthermore, replacing the malfunctioning kidney with a healthy one stimulates the rejection mechanisms of the living body against foreign organ, unless the donor is a near relative[21]. Thus, there is a considerable drive to develop improved therapies for kidney and liver failure with the capacity to replace a wider range of the organ functions, thereby reducing morbidity, mortality and the overall economic impact associated with this condition.

Several techniques have been applied in blood purification (including hemodialysis, peritoneal dialysis, hemodiafiltration, hemofiltration, and plasmapheresis) but such techniques have limitations in removal of harmful substances. In order to achieve higher capacities and mass removal rates, new strategies that combine hemodialysis (hemodiafiltration) with hemoperfusion (sorbent system) and/or plasma-filtration with plasma-adsorption have been studied in both acute and chronic renal failure as well as liver failure[22-24]. Adsorption might in fact be a new form of solute removal, to be used in conjunction with hemodialysis[25]. Historically, charcoal (activated carbon) and resins were used for their adsorptive properties and ability to remove harmful substances from blood. Activated carbon and resins are the most widely used sorbents in commercially available hemoperfusion cartridges due to its huge adsorption capacity and low cost[26]. In clinical practice, they have played a main role in the acute poisoning treatment or drug overdose, in view of their ability to remove protein-bound and lipid-soluble drugs from blood more efficiently than hemodialysis. Sorbent systems have potential applications in different disease states, including sepsis/SIRS, uremia, autoimmune diseases, hyperlipemia, hepatic failure, cardiopulmonary bypass, intoxication of drug over doses and poisonous and multi-organs failure[22, 27, 28]. However, enthusiasm for the use activated carbon and resins in medical applications is often counterbalanced for safety concerns due to the release of small micro-particles, particles coalition and possibly fragmentation, poor homogeneity and bio-incompatibility.

Blood-compatible chromatographic procedures are required to be developed, that do not alter the complex composition of biological fluids. The MMM concept offers opportunities to develop a high adsorption capacity membrane chromatography for the removal of blood harmful substances. Taking the benefits of membrane chromatography can improve blood flow rate in hemoperfusion and improve the safety of sorbents. Therefore, entrapping small sorbent particles into biocompatible porous matrix support can improve surface area and

shorten the diffusional distance of toxic compounds to the active sites. Hence, the MMM can be used for integration of hemofiltration (hemodialysis) with hemoperfusion in which harmful substances are removed in one step.

1.2. Outline of the thesis

This thesis can be divided into two major parts. In the first part (**Chapter 3** and **chapter 4**), the applications of MMM for enzyme capturing from single and binary mixtures are investigated. The adsorptive MMM prepared by dispersing Lewatit CNP80 WS resin (a weak cation exchange resin, carboxyl functional group) in a homogenous polymer solution followed by a wet phase separation process. The newly developed MMMs offer new possibilities, especially for capturing, concentrating and purifying enzymes (proteins) in one step. This separation method combines the specificity of functionalized small particles or adsorbents with the convenience of filtration membranes. The second part (**Chapter 5, 6 and 7**) describes two types of sorbents that are applied in the preparation of the MMM to investigate their performance for blood toxins removal. First, activated carbon based membranes (MMM AC) are prepared by incorporating activated carbon particles into porous cellulose acetate polymeric matrix. Second, an anion exchange resins Lewatit M500 (a strong anion exchange, quaternary amine functional group) particles have been used to form anion exchanger MMM. The developed MMMs are applied to remove creatinine (**Chapter 6**) as well as endotoxins (**Chapter 7**). MMMs improve capacity and safety of hemodialysis membranes. Moreover, entrapping particles in the membrane is an alternative approach to protect micro-particles release and to increase biocompatibility of adsorbent for extracorporeal blood purification.

Chapter 2

An overview in developments and applications of ion exchange membrane chromatography are discussed in this chapter. The discussion focuses on biologics processing with the applications of anion and cation exchange membrane chromatography. The chapter finally reviews with the adsorption and membrane based application in extracorporeal blood purification treatments.

Chapter 3

This chapter describes the use of membrane adsorbers for capturing and concentrating enzymes, one of the greatest challenges in downstream purification. A weak cation exchange resin, Lewatit CNP80 WS, is incorporated as particulate material into a macroporous EVAL matrix to prepare a membrane adsorber with a high particles loading. The performance of the prepared ion exchange MMM as enzyme adsorbers was investigated using Lysozyme (LZ) as a model enzyme. We demonstrated that the adsorptive membrane features both a high static as well as a high dynamic LZ adsorption capacity. Dynamic LZ adsorption capacity of the MMM is significantly higher than the equivalent commercial Sartobind C membrane. The MMM can be reused in multiple adsorption/desorption cycles maintaining the high binding capacity performance. Enzyme activity is maintained after an adsorption and desorption cycle.

Chapter 4

In chapter 4, the MMM concept is proposed as a process with an increased selectivity, capacity and throughput. The application of capturing LZ from BSA-LZ mixtures is studied. The MMM adsorber features a high adsorption capacity in both static and dynamic modes. High separation factors and purities of BSA and LZ are obtained in effluents and elution buffers. LZ, the retained protein, is recovered in the elution buffer in a five-fold increased concentration. At permeate fluxes above 20 $Lm^{-2}h^{-1}$, the adsorption capacity and purification power is independent of the permeate flux. The MMM can be reused in multiple adsorption/desorption cycles thereby maintaining the high binding capacity. This membrane is a mild media for LZ purifier and concentrator.

Chapter 5

In this chapter, we propose the preparation of Mixed Matrix Membrane (MMM) adsorbers for extracorporeal blood purification by incorporating activated carbon into a biocompatible macroporous polymeric support. The MMM adsorbers are prepared by solvent evaporation or by water vapor induced phase separation followed by an immersion precipitation step. Integral MMM double layer membranes with porous sub-structures are obtained by single step co-casting of two polymer solutions on glass plate. The active support layer contains of activated carbon particles that are embedded in cellulose acetate; the separating layer is prepared from a particle free cellulose acetate. Skinned films are obtained by direct

immersion of the polymer solution into the coagulation bath containing non-solvent. Porous membranes are formed when solvent evaporation in humid air takes place preceding the immersion precipitation. The best membranes obtained are the double layer MMM that are formed by solvent evaporation in humid air before immersion precipitation. They show a more porous structure, higher flux and better mechanical strength than other obtained MMM. The co-casting process opens the possibility to improve the mechanical stability, the biocompatibility and prevention of particle loss during preparation and processing.

Chapter 6

Chapter 6 describes the application of the MMM adsorbers as extracorporeal blood purification media for the elimination of model blood uremic toxins such as creatinine. MMMs are prepared by embedding activated carbon particles into macroporous cellulose acetate membranes without decreasing the activity of the activated carbon. To improve biocompatibility and to avoid small particles of activated carbon being mixed into the blood stream, the MMMs are co-cast with a particle-free cellulose acetate. Creatinine (Crt) is used as model uremia toxic compound to investigate the membrane adsorber performance using different process configuration and as function of different process parameters. The MMM features a high static as well as a high dynamic creatinine removal. The MMM is able to integrate hemodialysis as well as hemofiltration with adsorption, in which blood toxins are removed in single process step.

Chapter 7

In this chapter, integration of adsorption and filtration properties is adopted for the removal of endotoxin (Et) from solutions. The aim is to examine and compare the endotoxin adsorption capacity of two different type adsorptive membranes. The first MMM contains embedded activated carbon (AC) particles and the second one contains embedded anion exchange resins in cellulose acetate matrix. The extended application of the MMM for removing large molecular weight harmful substances such as endotoxins are of particular interest in the treatment of sepsis and multi-organ failure. The MMM AC displays a higher adsorption capacity than the MMM M500. The high endotoxin adsorption capacity improves safety of hemodialysis by blocking endotoxin transport across the membrane by adsorption. The developed MMM is able to remove harmful substances by filtration, diffusion, convection and adsorption in single process step.

1.3. List of Symbols and Abbreviations

Symbols

Lewatit CNP80 WS	*A weak cation exchange resin, carboxyl functional group*
Lewatit M500	*A strong cation exchange resin, quartenary amine functional group*
Sartobind C	*A weak cation exchange membrane, carboxyl functional group*

Abbreviations

AC	*Activated carbon*	*Et*	*Endotoxin*
BSA	*Bovine serum albumin*	*MMM*	*Mixed matrix membrane*
CA	*Cellulose acetate*	*LZ*	*Lysozyme*
Crt	*Creatinine*	*PLM*	*Particles loaded membrane*
EVAL	*Ethylene vinyl alcohol*	*SIRS*	*System inflammatory response syndrome*

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Chapter

2 Introduction in Biomolecules Separation and Blood Purification

Biomolecules cover a wide range of products made inside living cells. They have a large, complex and inherently heterogeneous molecular structure and comprise among others drugs, toxins, antitoxins, monoclonals, vaccines, viruses and blood products[1, 2]. Biological products are normally present in low concentrations in complex mixtures. They consist of different proteins with different biological functions, various combinations of cells and cell fragments[3]. Biomolecules that are required for clinical purposes must be ultrapure. The degree of purity of injectable proteins is set by the Food and Drug Administration (F.D.A.). In general, when contaminants can be detected they must be removed or proven to be harmless. Most therapeutic proteins are purified above 99.99 % purity.

Biomolecules can be separated from each other and from other molecules based on differences in size, solubility, charge and binding affinity. Techniques that are applied are precipitation, crystallization, column chromatography and electrophoresis. Most bioseparation processes can be categorized into four main separation classes: (a) removal of insoluble materials (clarification), (b) product isolation, (c) purification and (d) polishing[4]. The overall product yield is reduced by each process step. The key factor for successful and efficient protein purification is to select the most appropriate techniques and to combine them in a logical way to minimize the amount of steps and to maximize the yield[5]. The selection of the applied techniques is based on the properties of the feed stock and the desired product. For intracellular products, the first purification step is cell harvesting. Subsequently, cell lysis takes place to open the host cells and to release the intracellular product. Then the supernatant containing the biomolecules is separated from the cell debris (clarification) by centrifugation or microfiltration.

The isolation and purification of biomolecular targets is difficult. Many purification steps are involved, which cause product loss, require long process times and are expensive. The isolation and purification steps account for 50 to 80 % of the total production costs[6, 7]. Besides purity the biomolecules have to maintain their native structure or biological

activity[3, 8]. Since many biologics, especially proteins (enzymes) are fragile molecules that cannot handle harsh conditions; an effective and reliable separation technique is required to eliminate product denaturation. Minimizing the amount of purification steps and speeding-up the purification process increases the compactness, improves the economics and reduces labor costs and the time to market.

2.1. Membrane and Chromatography

Membrane filtration and chromatography are most widely used and an essential tool in biologics isolation and purification, to obtain the required purity of biotherapeutic and diagnostic products[9]. The advantages and disadvantages of membrane filtration and chromatography are complementary to each other[10]. Membrane filtration is mainly used in downstream processing to remove cell debris, colloidal materials or suspended solids and viruses. Membranes are also applied for the separation of large sized biomolecules from small ones. The major limitation of microfiltration and ultrafiltration is the relatively low sieving resolution[11, 12]. Therefore, this technique is not used for high resolution separations. On the other hand, column chromatography offers high resolution separations in the isolation and purification of biomolecules out of crude mixtures in both capturing and polishing steps. Among the downstream processing chromatographic techniques, ion exchange chromatography is most often used[13, 14]. Chromatography is traditionally carried out using packed beds columns, which have several major limitations[15, 16]. The pressure drop across packed bed is high and tends to increase with time due to combined effects of bed consolidation and column blinding caused by accumulated colloidal materials. Other drawbacks are low throughput, sensitive to fouling and plugging, channelling and bed compression. Moreover, packed bed chromatography is relatively time-consuming due to the dependence on slow intra-particles diffusion transport of biomolecular targets to their binding sites located within the pores of the packing material[17].

Some of the limitations of packed bed chromatography have been overcome using newly developed monodisperse non-porous rigid chromatographic media. However, these media are generally expensive and the solute binding capacity is greatly reduced since binding only takes place at the external surface area. Also with these materials, the problem of high-pressure drop still persists[15]. In parallel with the development of non-porous chromatographic particles, new types of stationary phases including perfusive and super-porous beads were investigated[18, 19]. Perfusive supports are characterized as having large

and interconnected pores with a complement of smaller pores, which contribute significantly to high surface area. Moreover, the binding capacity for perfusive media is higher than that for nonporous supports leading in capturing applications to a more efficient separation process.

During the last decade, membrane chromatography has been introduced as alternative stationary phase to bypass the fundamental limitations of packed-bed chromatography[20-23]. Membrane chromatographic systems (also called membrane adsorbers) are produced by chemically coupling of functional groups to the internal surface of macroporous membranes[16, 23, 24]. The adsorptive membrane is an attractive and competitive alternative technique for a wide variety of separations in biotechnological and medical fields. The membrane adsorber acts as a short wide chromatographic column with minimal operating pressure and maximal throughput (Figure 2.1). Membrane adsorbers can be employed as flat sheets, monolithic discs and hollow fibers. Since the adsorption capacity of a single membrane layer or fiber is limited, and to average out membrane heterogeneities, multiple membranes are stacked or put in series and housed within the filtration modules. The membrane adsorber can be operated either in dead-end or in cross flow mode. Binding selectivity and specificity are based on electrostatic, hydrophobic or affinity interactions between the biomolecule and the ligand. The advantage of adsorptive membranes is the absence of long diffusional path lengths, which are present in packed bed chromatographic systems. By adsorptive membranes, the transport of the dissolved molecules to the active sites in the membrane occurs by convective flow rather than by slow diffusional processes through a stagnant fluid inside the pores of an adsorbent particle. Another major advantage of the membrane adsorber when compared with packed bed systems is the relative ease of scaling up. Further membrane adsorbers offer the possibility to operate sterile with a high reproducibility[24, 25]. The main limitation of membrane adsorbers is the low binding capacity due to low BET area, which makes that membrane adsorbers are especially suitable for the treatment of large volumes with containing low concentrations of target molecules[23, 26, 27].

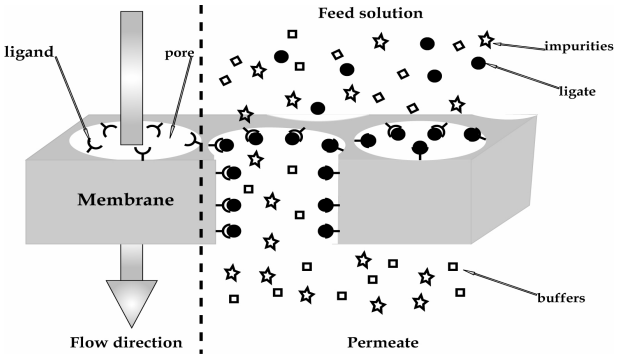

Figure 2.1. *Illustration of membrane chromatography. (Left side of dotted line) Schematic representation of membrane chromatography having ligands and porous structure. (Right side of dotted line) The Principle of membrane chromatography: biomolecules target (ligates) are selectively captured by membrane active sites (ligands) during the process load.*

A second route for the preparation of adsorptive membranes is incorporating functionalized particles into macroporous polymeric structures. Lensmeyer et al.[28] and Lingeman et al.[29] have proposed an analytical separation media, so called solid-phase extraction (SPE) using particles-loaded membranes. The adsorptive membrane can be applied to isolate peptides, proteins, nucleic acids or other organic compounds from complex mixtures. Recently, Avramescu et al.[30] have developed a simple preparation method to produce particles loaded membranes, a robust and high throughput chromatographic media for biomolecules separation. The so called Mixed Matrix Membrane (MMM) posseses a high active area by embedding a high load of small functionalized particles, to capture the molecular targets. The adsorptive MMM show, because of embedding small beads, a higher adsorption capacity than the bigger sized particles containing packed bed systems. The MMM can be prepared in different shapes and can be operated either as flat sheet stack or as module containing fiber membranes. By independent particle and matrix material selection various functions such as ion exchange, adsorption, catalysis or enzymatic activity can be incorporated. Adsorptive MMMs combine the selectivity of chromatography resins with the high throughput of filtration membranes. This results in rapid processing and greatly improves the adsorption, washing, elution and regeneration steps. The particle loaded MMM is commercialized by Mosaic Systems.

An alternative high throughput convective system that might be useful in bioseparation is the monolithic column[31]. The advantages are similar with membrane chromatography, but differ from the classical membrane media in terms of material, preparation and morphology[32, 33]. A monolith is a continuous bed consisting of a single

piece of a highly porous solid material. Similar to membranes, the most important feature of monoliths is that the mobile phase is forced to flow through the big pores of the monolith. The big pores of the monoliths have the advantage of a low flow resistance, however these big pores are accompanied by a low active surface area and thus a low adsorption capacity

2.2. Application of Ion Exchange Membrane Chromatography

This thesis focuses on the development and application of ion exchange membrane chromatography. Ion exchange membrane chromatography is the most widely used chromatographic method for protein separation[15]. The principle of protein separation by ion exchange is based on the electrostatic interaction between the charges of the protein and membrane surface. The target molecule must displace the counter-ion of the exchanger in order to attach to its surface. The protein adsorption capacity of ion exchange membranes can be very high. The protein concentration and the adsorption conditions (pH and ionic strength) determine the binding capacity. Ion exchange membranes can be prepared either by chemically modification of commercially available microfiltration membranes or by embedding of functionalized particles into a macroporous matrix.

Ion exchange membranes can be classified as cation or anion exchange and both classes contain weak and strong varieties. The most common functional group used in anion exchangers membrane are DEAE (diethyl amino ethyl group, a weak anion exchange), EA (ethanol amino group, a weak anion exchange), DEA (diethyl amino, a weak anion exchange), TEAE (Triethyl amino ethyl group, a strong anion exchange), Q (Quaternary ammonium, a strong anion exchange) and QAE (Quaternary amino ethyl, strong anion exchange). Therefore, the functional groups of cation exchangers membrane chromatography often being used are C (carboxy group, a weak anion exchange), CM (carboxy methyl group, a weak cation exchange), S (Methyl sulphonate group, strong cation exchange) and SP (sulphopropyl group, strong cation exchange).

Membrane chromatography has been reported as an advantageous tool to purify proteins at laboratory, pilot and industrial scale[34-37]. It is applicable at several stages in a purification train: clarification, isolation, purification and polishing. Depending on the membrane geometry and module design, adsorptive membranes can accommodate any of these tasks.

Table 2.1. *Applications of anion exchange membrane chromatography with different type of ligands*

Matrix support	Manufacture	Functional group	Biomolecule target	Geometry	Ref.
Chitosan- EGDCE	Non-commercial	A	Cytochrome c, LZ, ovalbumin, human serum albumin and soybean trypsin inhibitor	FS	[38]
Cellulose	Millipore	DEAE	Ovalbumin and myoglobin	FS	[39]
	Millipore		Phospodiesterase	FS	[40]
	Millipore		LZ, cytochrome c, conalbumin chymotrypsinogen, lactalbumin and ovalbumin	FS	[41]
	Whatman		LZ, BSA and γ-globulin	FS	[42]
PE	Non-commercial		BSA	HF	[43]
Modified cellulose	Sartorius		Aedes aegypti densonucleosis virus	FS	[44]
PE-GMA	Asahi		Gelsolin	HF	[45]
GMA-EDMA	Non-commercial		Soybean trypsin inhibitor, myoglobin and conalbumin	FS	[46]
GMA-EDMA	Non-commercial	DEA	Myoglobin, conalbumin, ovalbumin and soyabean trysin inhibitor	FS	[25]
Cellulose	Millipore & sartorius		Milk protein	FS	[47]
PE	Non-commercial	DEA & EA	BSA	HF	[48]
Cellulose	Whatman	DEAE & QA	BSA	FS	[49]
Nylon 66	Non-commercial	DEAE, PEI, DAH:DOC	Endotoxin	FS	[50]
Nylon 66	Non-commercial	DEAE, PEI, AH:DOC, PMB,PLL	Endotoxin	FS	[51]
EVAL	Non-commercial	Q	BSA and Hemoglobin	FS	[52]
Modified cellulose	Sartorius		Antisense oligonucleotide	FS	[53]
	Sartorius		Aedes aegypti, lysozyme, BSA and thyroglobulin	FS	[54]
	Sartorius		BSA, IgM	FS	[55]
	Sartorius		Plasma protein	FS	[56]
	Sartorius		Monoclonal antibody	FS	[34]
	Sartorius		LZ, chymotrypsinogen and soybean trypsin inhibitor	FS	[57]
	Millipore		Whey protein	FS	[58]
	Sartorius		Aedes aegypti densonucleosis virus	FS	[44]
	Viva science		B-lactoglobulin	FS	[59]
	Pall		DNA	FS	[60]
	Pall		Hemoglobin	FS	[61]
PES	Pall		DNA and LZ	FS	[62]
PES	Pall		DNA and RNA	FS	[63]
GMA	Saulentechnik		Human tumor necrosis	FS	[64]
CA	Kinetek	QAE	B-galactosidase	FS	[65]

Table 2.2. *Applications of cation exchange membrane chromatography with different types of ligands*

Matrix support	Manufacture	Functional group	Biomolecule target	Geometry	Ref.
EVAL	Non-commercial	C	BSA	FS, FB	[66]
	Non-commercial		Lysozyme	FS	[67]
Modified cellulose	Sartorius		Aedes aegypti densonucleosis virus	FS	[68]
Cellulose	Millipore	CM	Lysozyme, cytochrome c, chymotrpsinogen, lactalbumin, conalbumin and ovalbumin	FS	[41]
	Millipore		Immunotoxin and monoclonal antibody	FS	[69]
	Whatman	P	Lyzoyme, BSA and γ-globulin	FS	[42]
Modified cellulose	Sartorius	S	Aedes aegypti, lysozyme, BSA and thyroglobulin	FS	[54]
	Sartorius		Aedes aegypti densonucleosis virus	FS	[68]
	Sartorius		Bovine lactoferrin, lactoperoxidase and bovine lactoferricin	FS	[70]
	Sartorius		Hemoglobin and lysozyme	FS	[71]
	Sartorius		Lysozyme, ovalbumin	FS	[55]
	Sartorius		Plasma protein	FS	[56]
	Sartorius		Monoclonal antibody	FS	[34]
	Sartorius		Lysozyme, chymotrypsinogen A and soybean trypsin inhibitor	FS	[57]
	Sartorius		Monoclonal antibody	FS	[72]
	Sartorius		Monoclonal antibody and antithrombin III	FS	[73]
	Sartorius		Lysozyme and BSA	FS	[74]
Cellulose	Millipore		Whey protein	FS	[75]
	Millipore and Sartorius		Milk protein	FS	[47]
	Sartorius		Lactoglobulin, lysozyme, conalbumin, cytochrome c and chymotripsinogen	FS	[76]
	Metachem		BSA	FS	[77]
	Millipore		Lactoferrin and lactoperoxidase	FS	[78]
PES	Pall		Lysozyme and thyroglobulin	FS	[79]
EVAL	Non-commercial		BSA and Hb	FS	[52]
PE-GMA	Non-commercial		Lysozyme		[80]
EVAL	Non-commercial	S -BSA	Bilirubin	FS	[81]
PE-GMA	Non-commercial	SP	Lysozyme	FS	[82]
Cellulose	Millipore		Lactalbumin and BSA	FS	[83]

In table 2.1 and 2.2 ion exchange membrane adsorbers are presented with various functionalities and configurations, i.e. flat sheets, hollow fibers, full fibers and spiral wounds. Within the class of ion exchange membranes, anion exchange membrane chromatography (Table 2.1), is most widely applied. The main fields of interest are antibodies separation, isolation of food proteins and enzymes recovery and plasma proteins separation. Quaternary anion exchange membranes (strong basic) are applied in viral vaccine production and DNA purification for gene therapeutic agent production[60]. Furthermore, anion exchange chromatography has been successfully applied for endotoxin (LPS) removal from biological products[50, 51].

Cation exchange membranes (Table 2.2), have been used to separate single molecules from complex mixtures such as isolation of whey proteins, hemoglobin from blood, enzymes from fermentation liquors and monoclonal antibodies recovery from biological fluids. Monoclonal antibodies and human recombinant antithrombin III were subjected to preparative purification on Sepharose® FF packed bed columns and Sartobind S units[73]. The adsorptive membrane yielded higher throughputs (13-fold for monoclonal antibodies) and comparable or higher recoveries than the Sepharose columns. However, higher concentration factors were achieved on the Sepharose columns. The separation of the similar sized proteins serum albumin and hemoglobin using cation or anion exchange resins loaded membrane adsorbers was recently reported[84]. A comparing process for the isolation of HSA employing stacks of flat-sheet membranes and ion exchange porous beads was reported by Gebauer et al.[56]. At the laboratory scale, the productivity of the membrane based systems was up to eight folds higher than the traditional packed bed systems.

2.3. Extracorporeal Blood Purification

Extracorporeal blood purification is an essential technique used in medicine for the treatment of patients with acute or chronic kidney insufficiency, liver failure, detoxification, septic shock and multiple organ failure (MOF)[85-87]. The techniques used include hemodialysis, hemodiafiltration, hemofiltration, plasmapheresis and hemoperfusion. In extracorporeal blood purification hemodialysis and hemoperfusion are the most widely applied therapeutic treatments[88, 89].

A hemodialysis treatment replaces the function of the kidneys, which normally serve as the body's natural filtration system. Through the use of a blood filter and a chemical

solution known as dialysate, the treatment removes waste products and excess fluid from the bloodstream, while maintaining the proper chemical balance of the blood.

The disadvantage of hemodialysis is that the removal depends only on the toxins molecular weight with a lack of specificity; without any chemical selectivity of normal kidney or liver function. Middle-sized molecules in the range of 300 to 2000 Dalton, which include polypeptides that are suspects of causing uremic symptoms, diffuse poorly across hemodialysis membranes. Larger molecules are even unable to pass through the membranes. The failure of these therapies has stimulated the development of other treatments for blood purification[90]. Hemodialysis is beneficial for correcting acute renal failure, pulmonary edema, fluid and electrolyte disturbances that may accompany poisoning.

In the last three decades, sorbent technology (hemoperfusion) has been applied in the treatment of severe intoxication and to increase the efficiency of hemodialysis, or replace it, in renal replacement therapy and fulminant hepatic failure[87]. Hemoperfusion is a treatment technique in which large volumes of the patient's blood are passed over an adsorbent in order to remove toxic substances from the blood.

Sorbent hemoperfusion is gaining ground as a valuable adjunct to dialysis, especially in regeneration of dialysate. There are two kinds of sorbents used in medical treatments, i.e. natural sorbents (e.g. activated carbon) and the synthetic sorbents (e.g. ion exchange resins). Activated carbon is an excellent sorbent for removing organic metabolic wastes, drugs and other undesirable components from the blood. Resins have been applied in severe intoxication and in removing endotoxin, $ß_2$-microglobulin, leptin, retinol, angiogenin, IL-1β, TNF-α, bilirubin and another harmful subtances. Activated carbons and ion exchange resins are the most widely used sorbent and are as cartridges commercially available (Table 2.3). Other sorbents, for example various immunosorbents and more complex sorbent systems; incorporated with bio-functional agents (e.g. antigens, antibodies, enzymes) are utilized for clinical applications[91]. The pioneering work of Yatzidis[92] reported an effective removal of creatinine, uric acid, phenols, organic acids and barbiraturates by direct hemoperfusion through uncoated activated charcoal. Ever since, there has been growing interest in the perfusion of blood through sorbents to remove toxic substances. Recently, Winchester[93] reported detoxication by hemoperfusion for the removal of hypnotics and sedatives (ethchlorvynol, gluthetimide, meprobamate); analgetics (aspirin and paracetamol);

Table 2.3. *Applications of sorbent hemoperfusion*

Product name/System	Manufacture	Functional group or sorbent type	Application / molecules target	Ref.
Adsorba 300C	Gambro	Charcoal (coated with cellulose)	Non-specific	[94]
Amberlite®, Amberchrome®	Belco SpA	Amberlite XAD, Amberchrome	Non-specific	[95]
BetaSorb	Renal Tech. Int.	Polystyrene resin (coated with PVP)	Non-specific	[87]
Biocompatible System	Clark R&D, Inc.	Charcoal (coated with heparin)	Non-specific	[96]
Biologic DT, DTPF	Hemocleanse	Charcoal and cation exchange (uncoated)	Non-specific	[97]
CytoSorb	Renal Tech. Int.	Polystyrene	ß$_2$-microglobulin, leptin, retinol, angiogenin, IL-1β and TNF-α	[98]
DALI System	Fresenius	Anti-Apo antibodies	Lipoprotein	[99]
Hemosorba	Asahi Med. Co.Ltd.	Charcoal (coated with poly-HEMA)	Non-specific	[100]
HELP system	B. Braun	Heparin	LDL cholesterol, lipoprotein and fibrinogen	[101]
Hemapur 260	Organon-Teknika	Norit extruded charcoal (coated with cellulose acetate)	Non-specific	[102]
Immunosorba	Fresenius	Staphylococcal protein A (SPA)	nt-BNP and nt-ANP, and FVIII antibodies	[103]
Liposorba	Kaneka	Dextran sulfate	Apolipoprotein B, LDL cholesterol and lipoprotein	[104]
Lixelle	Kaneka	Hexadecyl alkil	β$_2$-microglobulin	[105]
MARS	Teraklin	Active carbon and anion exchange resin (uncoated)	Non-specific	[106]
MATISSE	Fresenius	Albumin	Endotoxins, cytokines and chemokines	[107]
Medisorba MG 50	Kuraray Med. Inc.	Anti-acetylocholine	Myasthenia gravis	[108]
Medisorba BL-300	Kararay Med. Inc.	Anion resin coated PHEMA	Bilirubin	[109]
Prosorba	Kaneka	Staphylococcal protein A (SPA)	IgG and low-density lipoproteins-cholesterol	[110]
PH-350	Asahi Med. Co.Ltd.	Phenylalanine	Anti-DNA Ab and immune components	[111]
Plasorba BR-350	Asahi Med. Co.Ltd.	Anion exchange resin (uncoated)	Endotoxin and bilirubin	[112]
PMX-20R	Toray	Polymixin B	Endotoxins, cytokines,chemokines	[113]
REDY system	Renal solution	Charcoal and ion exchange (uncoated)	Non-specific	[114]
Rheosorb	PlasmaSelect	Fibrinogen-binding pentapeptide	Fibrinogen, fibrin and fibrinogen	[115]
Selesorb	Kaneka	Dextran sulfate	Antibodies and immune complexes	[116]
Therasorb	Baxter	Anti-IgG antibodies	FVIII inhibitors	[117]
TR-350	Asahi Med. Co.Ltd.	Trypthophan	myastenia, autoimmune polineuropathy, rheumatoid arthritis	[118]
Detoxyl3	Belco SpA	Charcoal (uncoated)	Non-specific	[119]
MDS	Univ. Krems	Neutral resin, charcoal and anion exchange (uncoated)	Non-specific	[120]

agricultural chemicals (parathion, paraquat, polychlorinated and organophosphorus compounds) and cardiovascular agents (digoxin). Many of these blood toxins are lipid-soluble or protein-bound and are not or poorly dialyzable. Figure 2.2 demonstrates that all harmful uremic toxins present in ultrafiltrate can be removed by 5 h perfusion through an activated carbon column.

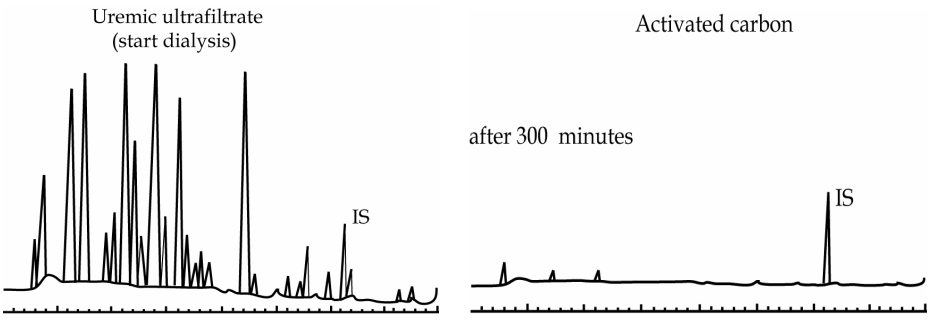

Figure 2.2. Chromatograms uremic toxins. HPLC chromatogram of uremic ultrafiltrates before(left) and after(right) perfusion through an activated carbon containing column, replotted from [121]. IS is internal standard.

Hemoperfusion can not fully substitute hemodialysis because it limited in urea sorption and cannot control the fluid balance since it is not able to remove excess water[87]. Davankov et al.[122] suggested that a more efficient artificial kidney might be produced by combining the strengths of dialysis membranes with the adsorption power of high surface area sorbents. The reason behind this idea is that filtration through semipermeable membranes should remove excess water together with urea and other small toxins, whereas hemoperfusion should remove larger molecules such as β_2-microglobulin and pro-inflammatory cytokines. Hemoperfusion is recently applied for the treatment of chronic uremia in adjunction to hemodialysis or hemofiltration[123] (Figure 2.3). The regular use of charcoal hemoperfusion as an adjunct to hemodialysis in chronic uremia is capable to improve the patient's clinical and laboratory condition as well as reducing the weekly treatment time [124]. However, the hemodialysis-hemoperfusion treatment is expensive and more complex then a separate hemodialysis treatment.

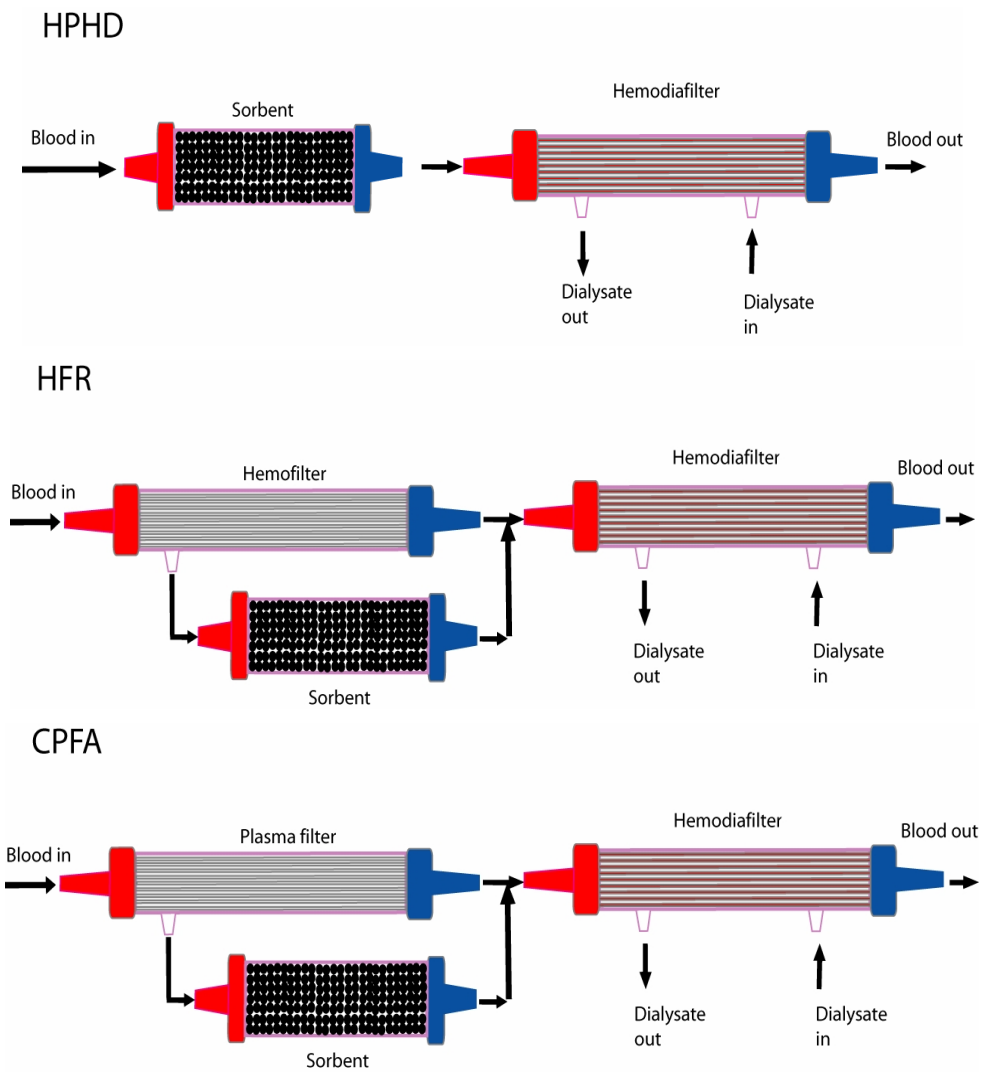

Figure 2.3. *Possible application modes of sorbent,s replotted from[123]. Top: Hemoperfusion-hemodialysis (HPHD), the sorbent unit is placed in series before the hemofilter. Blood comes into direct contact with sorbent and high biocompatibility is required. Middle: Hemodiafiltration with endogenous ultrafiltrate regeneration (HFR), the sorbent unit is placed on-line in the ultrafiltrate produce from a hemofilter. The system is used for on-line hemodiafiltration. Bottom: Couple plasma filtration adsorption (CPFA), the sorbent unit is placed online in the plasma filtrate, produced by the plasma filter. The plasma filter is placed in series with the hemodiafilter.*

Apart from undesirable side effects of sorbent hemoperfusion such as removal of essential plasma components and the relatively poor blood compatibility presents a major challenge for the material scientists. The high affinity of activated carbon to blood

components in combination with the fragmentation of activated carbon results in emboli formation. This means that direct blood contact with activated carbon should be avoided. These harmful effects stimulate the need for more biocompatible-activated carbon adsorbents. Many attempts have been made to overcome these problems by sorbent coating with a polymer solution and by encapsulating the activated carbon particles into a polymeric hull. This approach was already initiated by Yatzidis[92], the "father" of carbon hemoperfusion. The sorbent encapsulation has been further developed by Chang et al.[125], who used cellulose nitrate (collodion) and demonstrated that encapsulation minimizes the generation of microparticles and improves the blood compatibility. However, the additional layer reduces the hemoperfusion efficiency and the coated sorbents still may be involved in micro emboli formation due to not complete coverage of the coating, mechanical abrasion of the naked carbon surfaces prior casting and the fragility of the capsule. Applying a double coating solves this problem[126].

2.4. Mixed Matrix Membranes

Membrane characteristics can be tailored by embedding of functionalized particles. These membranes are called Mixed Matrix Membranes (MMMs). The matrix material has to confine the functionalized particles without interfering with the activity of the particles. These membranes are expected to combine the selectivity of the filler materials with the low costs, manufacturing ease and flow behaviour of polymer membranes[127]. Particularly, to achieve high permeability and selectivity values for membrane separation processes. Hybrid materials have been proved to be very useful in many separation areas. Most of the MMM work described in the literature deals with the pervaporation of aqueous mixtures or with the gases separation. The studies have been extended to porous membranes for ultrafiltration and microfiltration application. Based on type of MMMs matrix support and filler, they can be classified into 3 classes:

(a) Dense polymeric matrix

The first type of zeolite filled mixed-matrix membrane relies on relative sorption of different permeants to obtain an improved pervaporation separation. Early work by te Hennepe et al.[128] showed an improvement in alcohol selectivity and permeability by embedding silicalite, a hydrophobic zeolite, as alcohol-selective adsorbent in silicone rubber. In the second type, the adsorptive properties of molecular sieve are used to enhance selectivity for given gas mixtures by increasing the sorption of the desired gas component

within the mixed matrix membrane. Relatively permeation occurs by a combination of diffusion through the polymer phase and by diffusion through the permeable molecular sieve particles. Initial success in incorporating molecular sieves was achieved by embedding zeolites in highly flexible rubbers such as polydimethylsiloxane and ethylene–propylene diene rubber (EPDM)[129], and in glassy flexible polymers, such as cellulose acetate[130] and polyvinyl acetate (PVAc)[131].

(b) Dense inorganic matrix

Introducing molecular sieving functions into inorganic thin films (membranes) is another strategy to utilize chemical selectivity. Inorganic-zeolite membranes, where the zeolite is the dispersed phase, were introduced to amorphous silica or to carbon molecular sieve matrices[132]. If co-polyimide membranes are subjected to temperatures over 350 °C in an inert atmosphere, decomposition of carbon takes place resulting Carbon Molecular Sieve (CMS) membranes[133]. These materials combine high permeabilities with molecular sieve properties and have shown to be promising membrane materials. To enhance the separation properties of these CMS membranes the carbon matrix can be functionalized with materials that show a high affinity towards one of the permeating gas species. For example adding Ag-nanoclusters to increase the O_2 selectivity over N_2[134].

(c) Porous matrix support

In membrane applications, adsorbents like ion exchange particles[30], carbon[135], and catalyst [136] have been dispersed within micro- and macroporous polymeric structures, resulting in enhanced filtering capabilities. These membranes are available as alternative stationary chromatographic phases as flat disks and fibers with a high cross-sectional surface area[137]. The porous mixed matrix adsorber membranes have, depending upon the particle selection, a wide variety of applications (Table 2.1 and Table 2.2). For example, applications include peptide and protein isolation from fermentation broths, protein fractionation, ligand immobilisation for affinity-based separations, immobilised enzymes for reactions, detoxification.

Nowadays, Mixed Matrix Membranes can simply be prepared by phase inversion methods[138]. Dispersions of dissolved polymer and particulate material can be solidified by a phase inversion process into flat films or fibers. The phase inversion process can be established by a change in temperature (thermal induced phase separation, TIPS) or by a change in composition. The composition change can be induced by solvent evaporation, immersion into a non-solvent coagulation bath (non-solvent induced phase separation, NIPS)

or by exposure to non-solvent vapor phase (non-solvent vapor induced phase separation, VIPS)[139]. Among these techniques, NIPS and VIPS are the most common methods.

The MMM fiber concept is nowadays commercialized by Mosaic Systems. The adsorber membranes contain particles tightly held together within a polymeric matrix of different shapes. An important parameter for the performance of adsorptive membranes is the amount of embedded resins. By increasing the resin load, the adsorptive sites density increases resulting in higher adsorption capacities. The polymer used should not interfere with the activity of the particles. The polymer function is just to control the hydrodynamics and to confine the particles within the three dimensional MMM structure. The developed concept is extremely flexible, the geometry, the adsorption capacity, as well as the selectivity of the adsorber membrane can easily be tailored.

2.5. Conclusions

The main advantage of membrane chromatographic systems is the absence of pore diffusion, which is the main transport resistance in conventional column chromatography using porous particles. As a result of the convective flow the mass transfer resistance is tremendously reduced. This results in chromatographic systems that are characterized by rapid processing, which greatly improves the adsorption, washing, elution, and regeneration steps and decreases the probability of inactivation/denaturation of fragile components like proteins.

Although many ligands and applications have already been introduced, there are still many interesting applications that remain to be investigated with respect to their performance in membrane chromatography. This is especially valid for mixed matrix membranes as new alternative stationary phase in medical treatments. The application of adsorptive membranes in extracorporeal medical therapies such as detoxification during kidney and liver failure improves the quality and shortens the time of the medical treatment. The dialysis membranes have reached their limit for transport of middle and large molecular weight substances. The addition of sorbents to dialysis is a major advance in the removal of middle and large sized molecules. By embedding the sorbents into hemofiltration (hemodiafiltration) membranes, hemofiltration and sorbent technology are combined in one single treatment step.

2.6. Acknowledgements

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2.7. List of Abbreviations

A	*Amino*
AC	*Activated carbon*
BSA	*Bovine serum albumin*
C	*Carboxy*
CA	*Cellulose acetate*
CM	*Carboxy methyl*
CMS	*Carbon Molecular Sieve*
CPFA	*Couple plasma filtration adsorption*
DAH	*1,6-diaminohexane*
DNA	*Deoxyribonucleic acid*
DTPF	*Detoxifier and plasma filter*
DEA	*Diethylamino*
DEAE	*Diethylaminoethyl*
DOC	*deoxycholate*
EA	*(Hydroxyethyl)amino*
EDMA	*Ethylene dimethacrylate*
EGDCE	*Ethylene glycol diglycidyl ether*
EVAL	*Ethylene vinyl alcohol*
EPDM	*ethylene–propylene diene rubber*
FB	*Full fiber*
FDA	*Food and drug administration*
FS	*Flat sheet*
IgG	*Immunoglobulin*
IL	*Interleukin*
FV	*Factor*
GMA	*Glycidyl methacrylate*
HFR	*Hemofiltration filtrate regeneration*
HF	*Hollow fiber*
LDL	*Low density lipoprotein*
LZ	*Lysozyme*
LPS	*Lipopolysaccharide*
β_2M	*Microglobulin*
MARS	*Molecular absorbent recirculating system*
MMM	*Mixed matrix membrane*
MOF	*Multi organs failure*
NA	*Not available*
NIPS	*Non-solvent induced phase separation*
Nt-BNP	*N-terminal brain natriuretic peptide*
Nt-ANP	*N-terminal atrial natriuretic peptide*
PDMS	*polydimethylsiloxane*
PE	*Polyethylene*
PLL	*Poly-l-lysin*
PLM	*Particle loaded membrane,*
PVAc	*polyvinyl acetate*
PMB	*Polymyxin B*
QAE	*Quaternary amino ethyl*
R	*Radial*
REDY	*Recirculating dialysis*
RNA	*Ribonucleic acid*
SP	*Sulfopropyl*
S	*Sulphonic acid*
TIPS	*Thermal induced phase separation*
TNF	*Tumor necrosis factor*
VIPS	*Vapor-induced phase separation*

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Chapter

3 Enzyme Capturing and Concentration With MMM Adsorbers*)

Abstract

This study reports the use of membrane adsorbers for Lysozyme (LZ) capturing and concentration: the membrane adsorbers are prepared by incorporation of ion exchange resins into an EVAL porous matrix. The Mixed Matrix Membrane (MMM) adsorber possesses an open and interconnected porous structure with a large ion exchange surface available for enzyme adsorption. The adsorptive membrane features both a high static as well as a high dynamic LZ adsorption capacity. The measured LZ adsorption isotherm is of the Langmuir type, with a maximum adsorption capacity of 147 mg LZ/mL membrane. Dynamic LZ adsorption capacity at a flux of 25 $Lm^{-2}h^{-1}$ was 63 mg LZ/mL membrane, which is significantly higher than the equivalent commercial membrane Sartobind C. Since the kinetics of desorption processes are faster than the kinetics of adsorption processes, the performance can be improved by exerting the desorption processes at higher fluxes than the adsorption processes. The MMM can be reused in multiple adsorption/desorption cycles maintaining the high binding capacity performance. Fluorescence spectra of the LZ after adsorption and elution were similar to native LZ. This is confirmed by activity tests showing that the activity of LZ was maintained after an adsorption and desorption cycle.

Keywords: *Mixed Matrix Membrane (MMM), Sartobind C, adsorption, Lysozyme (LZ) and activity*

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3.1. Introduction

Chromatography is the method most widely used to obtain the required purity for biotherapeutics and diagnostics[1]. Among all the chromatographic methods, ion exchange is one of the most often used techniques in downstream processing. It is employed for recovery and purification of proteins, polypeptides, nucleic acids, polynucleotides and other biomolecules. Ion exchange is advantageous in terms of broad applicability, high resolution and large adsorption capacity in large-scale protein purification processes[2]. Another advantage of this technique is that the elution takes place under mild conditions so that the proteins maintain their native conformation during the chromatographic processing. Protein separation in ion-exchange chromatography is mainly determined by the electrostatic interaction between the solute and the oppositely charged of the surface stationary phase[3]. Many types ion exchange resins are nowadays commercial available and have been applied in protein capturing, purifying and polishing steps[4, 5].

The developments in bio downstream processes are promoted by reliable and efficient separation and purification methods, for the many diverse biomolecules that are produced and studied in biopharmaceutical and bioproduct industries. Large-scale separation and purification processes must be cost efficient because they contribute for a large extend to the total product manufacturing costs. In a typical protein production process from fermentation to final product, isolation and purification account for 50 to 80% of the total production costs[6, 7]. The desirable properties in protein purification are among others a high throughput, a high capacity and the ability of the adsorbent to be used repeatedly[8].

In the recent years there is a considerable and increasing interest in developing membrane adsorbers. The membrane adsorber acts as a short and wide chromatographic column. Membrane adsorbers can exist in a variety of configurations[9-12] with a variety of adsorptive mechanisms (e.g. ion exchange, hydrophobic, reversed-phase or affinity interactions). The advantage of adsorptive membranes is the absence of a long diffusion path length, which often occur in fixed bed chromatography. In adsorptive membranes, the transport of the dissolved molecules to the active sites in the membrane occurs by convective flow rather than by diffusion through a stagnant fluid inside the pores of an adsorbent particle. Another major advantage of the membrane adsorbers is the relative ease of scaling up when compared with packed bed systems[13]. Membranes with adsorptive chromatography properties do exhibit technological progress in both membrane filtration and

fixed-bed liquid chromatography. Membrane adsorbers combine the selectivity of chromatography resins with the high productivity of filtration membranes[10, 11, 13, 14].

Avramescu et al.[13] described a new chromatographic concept for a single-step process for the preparation of ion exchange Mixed Matrix Membrane (MMM) adsorbers having particulate material entrapped in a porous matrix. A mixture of dissolved polymers and additives in which particulate material was dispersed, was cast/spun and solidified by a phase inversion process into a flat/fibrous membrane. The prepared MMM contain ion exchange particles tightly held together within a porous polymeric matrix, 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 fibrous membranes. By independent selection of particles and matrix material various functions such as ion exchange, adsorption, catalysis or enzymatic activity can be incorporated. Such membrane adsorbers may also serve as a platform to which an end-user can couple a specific ligate[10].

MMM adsorbers have been applied for separation and recovery of BSA, Hb and bilirubin[13, 15, 16]. Although several applications have been introduced to membrane adsorbers already, there are still many interesting applications that remain to be explored with respect to their performance in membrane chromatography. One of the greatest challenges in downstream purification processing is the capturing and concentration of enzymes. Many biomolecules are difficult to purify in a native and biological active form. An effective and reliable separation technique is needed to achieve this requirement. Due to their low pressure drop and mild process conditions, a MMM adsorbers is a good candidate to maintain the native conformation and the biological activity of proteins or enzymes during the isolation and purification processes. In this study we provide an extensive characterisation of the MMM adsorbers in static and dynamic mode for enzyme recovery. A weak cation exchange resin, Lewatit CNP80 WS, is incorporated as particulate material into a porous EVAL matrix to prepare adsorber membranes with a high particle loading. The performance of the prepared ion exchange MMM as enzyme adsorbers was investigated using Lysozyme (LZ) as a model enzyme. System parameters such as membrane porosity, swelling and permeability and process conditions like pH, ionic strength and membrane flux were varied to study their effect on the performances of mixed membrane adsorber during Lysozyme recovery.

3.2. Experimental

3.2.1. Materials

EVAL (a random copolymer of ethylene and vinyl alcohol) with an average ethylene content of 44 mol % was purchased from Aldrich and was used as membrane material without further modification. Dimethylsulfoxide (DMSO, Merck) was employed as solvent and 1-octanol (Fluka) as non-solvent-additive in the casting solution. Water was used as non-solvent in the coagulation bath. Lewatit ion exchange resins CNP80 WS (Bayer) were used as adsorbent particle. The particles were grinded and fractionated down to a fraction with an average size of 10 μm. This size was chosen because of the high surface over volume ratio while the pores in the membrane are still sufficient to maintain a high flux and to avoid particle loss out of the matrix. Hydrochloric acid (HCl) is used for the regeneration of MMM adsorbers. The enzyme used for the adsorption measurements was Lysozyme (LZ). LZ was obtained by Fluka and has a Mw of 14600 g/mol and a pI around 11. Freeze-dried *Micrococcus lysodeikticus* cells (ATCC strain # 4698) were used as enzyme substrate, purchased from Fluka biochemical. Sartobind C membranes, kindly supplied by Sartorius, were used as a representative of commercial membrane adsorbers.

Buffer solutions were freshly prepared in ultra pure water. Ultra pure water was prepared using a Millipore purification unit Milli-Q plus. The buffers used for adsorption were an acetate buffer for the pH 4-5 regime, a phosphate buffer for pH 3, 6-8 and 12 regime, a Tris buffer for pH value 9 and a Carbonate buffer for pH values between 10 to11. The ionic strength of the adsorption buffers was kept constant at 17 mM, which is close to physiological conditions, by addition of NaCl. This low ionic strength results in maximum adsorption[17]. The elution buffer was prepared by increasing the ionic strength of the adsorption buffer pH 7.0 to 0.5 M using NaCl.

3.2.2. Membrane preparation and characterisation

3.2.2.1. Membrane preparation

To obtain membranes with protein adsorptive properties, Lewatit ion exchange particles with an average diameter of 10 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. The mixtures were stirred over night at 50 °C to break down clusters of particles. The MMM were prepared by immersion precipitation. For this the polymeric mixture was cast (casting knife 0.475 mm) on a glass plate and immediately

immersed into a water coagulation bath at 40-45 °C and the membranes were formed a few moments after immersion. The membranes were washed with tap water at room temperature to remove residual solvent and 1-octanol. After washing, the membranes were dried in the air overnight and afterwards dried in a conventional oven at 50 °C.

An important parameter for the MMM adsorbers preparation is the *resin loading*, i.e. the amount of resins contained in a membrane. The resin loading is calculated by equation (1), where W_r is the amount of ion exchange resins (g) and W_p is the amount of EVAL polymer in the casting solution (g).

$$R_{loading} = \left(\frac{W_r}{W_p + W_r} \right) \times 100 \qquad \textbf{Eq . (3.1)}$$

3.2.2.2. Scanning electron microscopy

The membranes were characterized by Scanning Electron Microscopy (SEM) using a JEOL TM 220 A. The cross-section was scanned as well as the top and bottom surfaces of the membrane. For the cross-section the membranes are fractured in liquid nitrogen. Before examination the samples were gold-coated using Balzers Union SCD 040 sputter coater.

3.2.2.3. Membrane porosity and swelling degree

The membrane porosity was determined by the water uptake of a calibrated volume of membrane. Average values were obtained from three different samples. The membrane porosity, ε, and swelling degree, sd, were determined from the swelling experiments. A micrometer of Mitutoyo was used to measure the thickness of the membrane. Average values were obtained from three different samples. Porosity was calculated by the following equation:

$$\varepsilon(\%) = \frac{V_{membrane,wet} - V_{membrane,dry}}{V_{membrane,wet}} \cdot 100 \qquad \textbf{Eq. (3.2)}$$

The swelling degree is determined by the volume of water compared to the volume of the dry membrane.

$$sd(\%) = \frac{V_{membrane,wet} - V_{membrane,dry}}{V_{membrane,dry}} \cdot 100 \qquad \textbf{Eq. (3.3)}$$

Where $V_{membrane,\mathrm{dry}}$ and $V_{membrane,\mathrm{wet}}$ are respectively the volume of the dry membrane and the volume of the swollen membrane after 24 hours immersing in a water bath at room

temperature. Before weighing the attached water was removed by dry padding the membrane with filter paper.

3.2.2.4. Clean water flux

Ultra pure water fluxes of the membranes are determined at room temperature by using a nitrogen pressurized 'Amicon type' dead-end filtration cell. The applied pressure is 1 x 10^4 Pa (0.1 bar). The reported flux values are measured at steady state conditions, under 3.33 Hz (200 rpm). An average flux is calculated from several experiments using different pieces of membrane.

3.2.3. Adsorption experiments

3.2.3.1. Measurement of zeta potential of Lysozyme

The net charge of Lysozyme as a function of pH is determined with a dynamic light scattering method using a Malvern Zetasizer 2000. Acetate, phosphate and carbonate buffers, all 17mM, are used to cover pH values ranging from 3 to 12. Lysozyme solutions were prepared in the buffers in concentrations of 1 mg/mL. The sample is measured with the Zetasizer for 20 seconds at least 5 times. All measurements were carried out at room temperature (25 ^{o}C).

3.2.3.2. Batch adsorption experiments

The adsorption capacities of the prepared cation exchange membranes have been determined by batch experiments. Protein concentrations of 1-2 mg/mL are employed in the adsorption experiments. A known weight of membrane is immersed overnight in ultra-pure water to ensure a steady inner structure during the experiments and afterwards transferred in the protein solution. The protein solutions are prepared using different buffer solutions with pH's ranging from 3-12. The ionic strength of all buffer solutions applied for adsorption experiments is kept constant at 17mM by adding NaCl. The samples containing a known concentration of LZ are incubated with an exact amount of membranes in a sealed container under continuous shaking at 25°C. The protein adsorbs on the membrane adsorbers thereby reducing the LZ concentration in the bulk until equilibrium is reached. The equilibrium protein concentration is determined by spectrophotometric analysis after 24 hours. Protein absorption was measured at 280 nm and 2mm quartz cuvettes are used for measuring the

absorbance. The adsorbed protein amount, q_{eq} [mg protein /g membrane], at equilibrium is calculated by

$$q_{eq} = \frac{(C_0 - C_{eq}) \cdot V}{W_{membrane}} \qquad \textbf{Eq. (3.4)}$$

Where C_0 is the initial protein concentration [mg/mL], C_{eq} is the protein concentration at equilibrium [mg/mL], V is the volume of the solution [mL] and $W_{membrane}$ is the weight of the dry membrane [g].

3.2.3.3. Adsorption kinetics

Approximately 0.6 g of membrane adsorber was incubated in a LZ solution of pH 11 at 25°C in a shaking bath. The LZ concentration in the solution was monitored in time with a Philips analytical PU 8720 UV-VIS Spectrophotometer. For the measurement on the pure resins the solutions were filtered using a Spartan 30 filter (regenerated Cellulose) with a pore size of 0.45µm to avoid possible disturbing of the UV-signal by resin particles.

3.2.3.4. Adsorption isotherm

In order to determine the adsorption isotherm batch adsorption experiments were carried out with different initial protein concentration. The same amount of membrane was used in the experiments to end up with different equilibrium concentrations. From literature is known that the Langmuir adsorption model can be used for protein adsorption. The Langmuir equation can be rewritten as:

$$\frac{1}{q_{eq}} = \frac{1}{q_m} + \frac{K_d}{q_m} \times \frac{1}{C_{eq}} \qquad \textbf{Eq. (3.5)}$$

The parameters q_{eq} (adsorbed protein concentration into the membrane adsorbers) and C_{eq} (the equilibrium protein concentration in the bulk solution) are experimental data. A plot of $1/C_{eq}$ versus $1/q_{eq}$ allows determining the maximum adsorption capacity (q_m) and the dissociation constant (K_d), which have been estimated by fitting the equation to experimental results using the least-square regression.

3.2.3.5. Desorption

After an adsorption and washing step the membranes are transferred into the desorption buffer. Desorption was accomplished in static as well as in dynamic measurements by using a phosphate buffer of pH=7, I= 0.5 M NaCl for static as well as

dynamic experiments. The desorption was carried out for 24 hours in a shaking bath at 25 °C. The recovery is defined as the percentage of desorbed protein, the amount desorbed protein ($q_{desorbed}$) divided by the amount of adsorbed protein ($q_{adsorbed}$).

3.2.3.6. Regeneration

After an adsorption, washing and desorption cycle the membrane adsorbers were washed using 0.50 M NaCl. Then 10 % HCl was used to guarantee complete removal of residual LZ that might have been attached to the membrane by non-specific interactions and cannot be eluted by a high ionic strength NaCl solution. The contact time between the membranes and the HCl solution was about 24 h in total at room temperature. Before the next adsorption/desorption cycle could start the membranes were conditioned by washing out the remaining HCl with ultra pure water until neutral pH was obtained. The capacity of the regenerated membrane was then re-measured. Since Sartobind C membranes are ready to use (according to the producer) we did not test the effect of regeneration of these membranes.

3.2.3.7. Dynamic adsorption experiments

The dynamic adsorption performance of the flat sheet MMM was measured at constant permeation rate using a continuous feeding stirred dead-end filtration cell. To overcome the limited adsorption capacity and to average out inhomogeneities[18], 10 sheets of membranes were stacked. 2 mg/mL LZ solution in Carbonate buffer pH 11 was loaded through the stack of 10 membranes with a flux of 25 $Lm^{-2}h^{-1}$. The permeate was collected and fractionated in samples of 2 mL using a fraction collector and the LZ concentration in the permeate samples was followed spectrophotometrically at 280 nm. After reaching the breakthrough point the experiment was stopped and the membrane stack was washed with fresh buffer to release the unbounded proteins and subsequently a phosphate buffer containing 0.5 M sodium chloride were permeated through the membranes for elution of the adsorbed proteins. The protein mass adsorbed per unit of membrane bed was calculated by numerical integration over the filtration run up to a breakthrough concentration 10% of the feed concentration. The effect of the flux on both the loading and the elution were investigated at five different fluxes (25, 50, 100, 300, 500 $Lm^{-2}h^{-1}$). A comparison of the effect of the flux was also obtained for MMM and commercial membrane adsorbers. Sartobind C membranes were used as representative commercial membrane.

3.2.3.8. Fluorescence measurements

In order to evaluate the effect of the process conditions on the LZ activity, fluorescence spectra of the native LZ, heat denaturated LZ and desorbed LZ were measured. LZ fluorescence emission spectra were recorded from 300 to 400 nm after excitation at 284 nm using an Edinburgh Analytical Instruments F900. All measurements were carried out at 25 °C at a LZ concentration of 2 mg/mL in the presence of the desired salts concentration and pH 7. Native LZ aqueous solution (2 mg/mL, pH 11) was denaturated at 85 °C for 2 hours. LZ fluorescence emission mainly stems from two buried Tryptophan (Trp) residues, which can be selectively excited at 284 nm[19-21].

3.2.3.9. Enzymatic activity

The enzyme activity of LZ was determined by a spectrometric method using *Micrococcus lysodiekticus* as substrate [22-24]. This assay is based on the decrease in Abs (450 nm) of a *M. lysodeikticus* cell suspension when the cells are digested by LZ. The amount of LZ containing sample should be adjusted so that a decrease in absorbance of 0.02 to 0.04 per minute is obtained (LZ concentration is about 0.0125 mg/mL). LZ was first dissolved either in pure water or phosphate buffer (0.025 M) and kept on ice. 3 mg of lyophilized *M. lysodeikticus* cells were dissolved in 10 mL 0.1 M phosphate buffer (pH 7), kept on ice. Optical density of the cell suspension was measured at 450 nm. To obtain an optical density between 0.5 and 0.7 lyophilized cells or phosphate buffer was added to a cell suspension, final substrate concentration about 0.3 mg/mL. Thereafter, 100 µl of the enzyme solution was added to 1.4 mL cell suspension of *M. lysodeikticus* and the change in the A_{450} was recorded every 15 seconds for 2 minutes. Temperature was kept constant at 25 °C. One activity unit (U.A.) is equivalent to a decrease of 0.001 absorbance unit per minutes. Specific activity is the ratio of enzyme activity to mass of protein in the sample, usually expressed as units of activity per mg protein (U/mg).

3.3. Results and Discussion

Experimental results are presented and discussed in this section. First the charge density of LZ as function of pH is described. Thereafter, a detailed membrane characterization in terms of morphology, porosity and clean water flux is presented, followed by LZ adsorption capacity in both static and dynamic mode. Most relevant is the dynamic capacity, which represents the functional capacity at defined fluxes. The influences in both

adsorption and desorption processes will demonstrate the applicability of MMM in the isolation LZ maintaining its biological activity.

3.3.1. Net Charge of Lysozyme

The pH of a polypeptide (enzyme in our case) influences the behavior and amount of adsorption in ion exchange processes. Identifying the electrostatic properties of a protein surface is useful for the interpretation of biochemical data and for a better understanding the

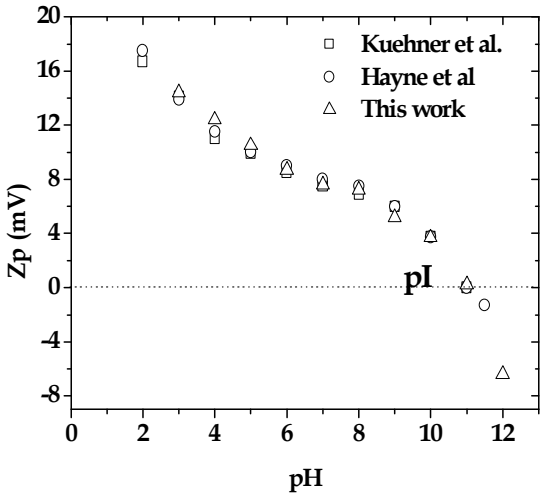

Figure 3.1. *Zeta potential of LZ at different pH's in buffered 17 mM ionic strength solutions. Experimental data were compare with data reported from literature.*

ion exchange adsorption behavior. Figure 3.1 shows the net charge of LZ at different pH's. The values are in agreement with literature data[25-28]. The pI, the pH where the LZ net charge is zero, is 11.0.

3.3.2. Mixed matrix membrane adsorbers characterization

Lewatit CNP80 WS, a weak cation exchange resin, was employed in the preparation of mixed matrix membranes (MMM). The membrane containing Lewatit CNP80 WS has carboxylic functional groups as active binding sites. An important parameter in the preparation of MMM is the amount of resins incorporated into the polymeric matrix. Both a high loading and the application of small particles favor a high adsorption capacity of the MMM.

The viscosity of the casting solution increases with increasing resin loading. The maximum resin loading that could be obtained was 65%; above this loading the viscosity of the casting solution was too high for a proper casting of the polymer solution. The membrane with a 65 % resin loading has a desired set of properties. The membrane posses an open and interconnected porous structure (Figure 3.2). There is no evidence of finger-like macrovoids and the resin particles are uniformly distributed in the membrane matrix across the entire cross-section (Figures 3.2A and 3.2B). The ion exchange particles are tightly held together within the porous polymeric matrix and were uniformly distributed. No significant loss of particles was observed during the membrane formation process on the glass plate and the membrane possesses sufficient mechanical strength. The porosity of these membranes was 70% and swelling degree was 22 %. Clean water flux was 100 $Lm^{-2}h^{-1}$ at a trans-membrane pressure of 1 x 10^4 Pa (0.1 bar).

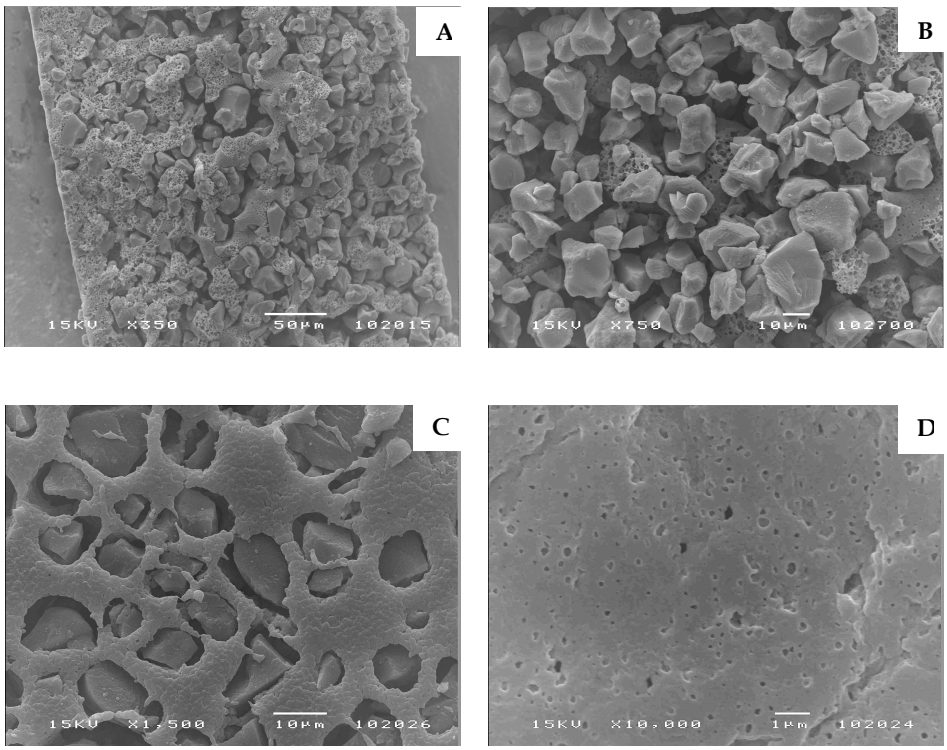

Figure 3.2. *SEM micrograph of the membrane prepared out of a 10% EVAL, 10% 1-octanol, in DMSO solution containing 65% loading Lew CNP80 WS. Temperature of the coagulation bath 40-45 °C and the membranes were dried by at room temperature and conventional oven at 50 °C. A). Cross section, magnification x350, the size bar indicates 50µm; B) Cross section, magnification x750, the size bar indicates 10 µm; C) Bottom surface, magnification x1500, the size bar indicates 10 µm; D) Top surface, magnification 10000x, the size bar indicates 1 µm.*

Ideal membrane adsorbers possess a low non-selective protein adsorption onto the polymer surface and a high adsorption onto resin particles. By incorporating resin particle in the matrix polymer the non-specific adsorption will decrease. Since EVAL is a hydrophilic polymer the protein adsorption on the particle free membrane is 5.5 mg/mL membrane, which is less than 4 % of the lowest obtained total adsorption capacity at pH=9 in Figure 3.4. The adsorption of LZ on particle-free membranes was mainly attributed to hydrophobic interaction and dipol-dipol interaction.

The adsorption of LZ was measured in time for suspensions of resins and MMM to investigate the accessibility of the active sites (Figure 3.3). The adsorption measurements were performed at pH 11. We found that the adsorption capacity increased in time during 12 hours where after equilibrium was reached. The obtained equilibrium capacity after 24 hours was 305 mg LZ /g resin and for the MMM 205 mg/g membrane which is equal to 315 mg/g resin in the membrane. The equivalent values for the LZ adsorption capacity of pure resins and resins incorporated into the polymeric structure proved that the active sites in the membrane are well accessible for LZ.

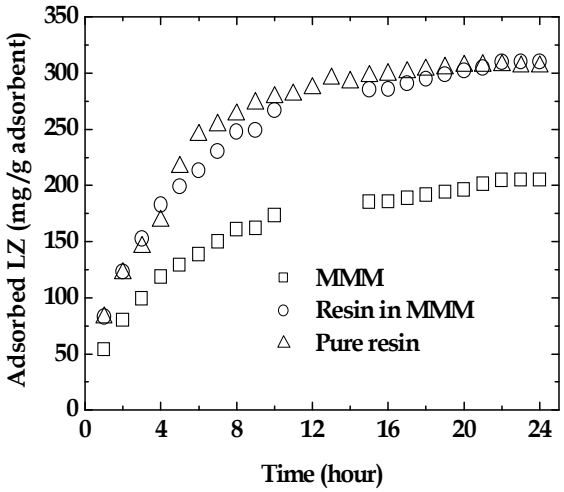

Figure 3.3. *Static adsorption of LZ onto suspension resin Lew CNP80 WS and MMM Lew CNP80 WS in time at pH 11.0 and temperature 25°C. LZ initial concentration was 2 mg/mL at ionic strength 17 mM and equilibrium time 24 hours.*

The total protein adsorption depends on the accessibility of the adsorptive resins in the membrane. Figure 3.4 compares the LZ adsorption capacity of the pure resins, the mixed

matrix membrane in total and the resin as estimated to be in the MMM at different pH's. First of all, this graph clearly shows that all particles are well accessible in the MMM adsorber. It also can be seen that LZ adsorption declines up to a pH=9 where after a maximum adsorption is obtained at pH=11. Above pH=11 the sorption of LZ drops in capacity, which can be related to repulsive charges and instability of the protein. At pH=3 the adsorption capacity of the pure resins was 260 mg LZ per gram of adsorbent: this number decreased gradually to 220 at pH=9 and after that it increased again and reached a maximum at pH=11 of 305 mg LZ per gram resin where after the capacity drops till 190 at pH=12. The adsorption profile of LZ in MMM adsorbers has a similar tendency.

Proteins have no net charge at their isoelectric points and therefore the maximum adsorption from aqueous solutions is usually observed at their isoelectric point [29]. This nonlinear dependence is inconsistent with an adsorption mechanism dominated by net electrostatic forces between enzyme and adsorbent: otherwise the adsorption capacity would

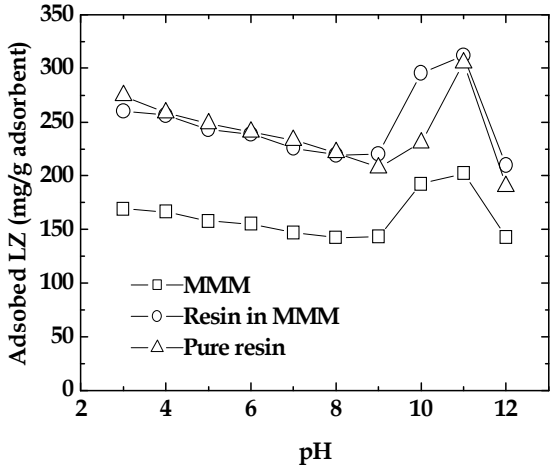

Figure 3.4. *LZ adsorption on suspended Lewatit CNP80 WS resins and MMM Lewatit 80 WS adsorbers at different pH's and temperature 25 °C. LZ initial concentration was 2 mg/mL at ionic strength 17 mM.*

be highest at extreme acid pH and decrease monotonically with increasing pH. Around the isoelectric point where the protein carries as many positively charged group as negatively charged ones, the net intramolecular coulombic interaction is attractive and dispersive (or London-van der Waals) interaction favors a compact structure. At pI, there exists no net

electrostatic repulsion between the proteins in the adsorption layer. Therefore the proteins can be tightly packed on the surface[30] and this leads to increased adsorption at pI. Additional contribution may be related to interaction of the hydrogen bonding between the charged resin and neutral protein. Haynes et al.[27] also found that LZ adsorption on a negatively ($-SO_3^-$ group) charged polystyrene latex shows a maximum at or near the pI of the protein. Shen et al.[31] developed the stoichiometric displacement model, which accounts for the effects of hydrogen ion Donnan equilibrium (or protein charge regulation) during the ion exchange adsorption particularly for case of adsorption near the pI of the protein. The net charge of a protein depends not only on pH and ionic strength of the surrounding fluid, but also on the proximity of protein to other charge surfaces, resulted from electrostatic interaction between the charged surface and the ionogenic functional groups on the protein. When the pH is near the protein pI, protein charge regulation is an important factor, which causes discrepancy between the characteristic binding charge and the electrical charge on the protein in free solution. The adsorption of protein near pI of protein contributes from hydrogen ion Donnan equilibrium effect. The ion Donnan equilibrium also explains the ability of proteins to adsorb onto a surface with the same charge sign because the protein in the fluid and adsorbed phase can have an opposite charge signs due to hydrogen Donnan equilibrium effect. Kang et al.[32] reported that adsorption near the pI could improve resolution of proteins when using ion-exchange chromatography focusing with salt gradient elution. The resolution of protein in IEC is highest when the pH of liquid phase is near the pI of protein since this condition again enhances relative adsorption affinity. The investigator suggests that the adsorption of protein near the pI controlled by charge distribution on the surface. This argument is also reasonable to rationalize adsorption of LZ near the pI. A LZ molecule has 20 positively charged groups, 1 is histidine, 8 lysine and 11 arginine groups. Besides these positively charged groups LZ has also 10 negatively charged groups namely 2 glutamic acid and 8 aspartic acid[33]. Rasmol molecular-graphic visualization of Lysozyme shows that most residue groups reside on or near the molecular surface[25]. The present of positively charged lysine and arginine groups on the surface favor positive charge around pI of LZ, which can interact with a cation exchange surface. An additional explanation can be found in the solubility of lysozyme, which is dependant on the pH and the buffers used. Since the chaotropic effect of phosphate and acetate buffers used in this research is similar (same position in the Hofmeister series) the difference can only be explained by the pH dependant solubility. Ladisch [34] reported data about the solubility of lysozyme in his book

Bioseparations Engineering, Table 4.5. The maximum solubility is between pH=6 and 7 (almost 50 % higher than at pH 5 and 8). Expected is that the adsorption follows this behavior.

The measured LZ adsorption isotherm on MMM is used to characterize the interaction with the adsorbent. This provides a relationship between the concentration of the protein in solution and the amount of protein adsorbed on the solid phase when the two phases are in equilibrium. Adsorption isotherm data were obtained for LZ at pH 11 and 17 mM ionic strength, as shown in Figure 3.5. Equilibrium data were fit with the Langmuir model by nonlinear least-squares regression analysis. The solid line in the figure is the Langmuir isotherm that best fit the data. The maximum adsorption capacity (q_m) is 147 mg LZ/mL membrane and the dissociation constant (K_d) is 4.4x10^{-2} mg/mL. This value prove that the mixed matrix membrane adsorbers posses a good accessibility of LZ to the adsorptive site. The adsorption capacities are high compared with published data in literature. Tejeda-Mansir et al.[35] investigated adsorption of LZ on dye cellulosics. Their Langmuir isotherm values

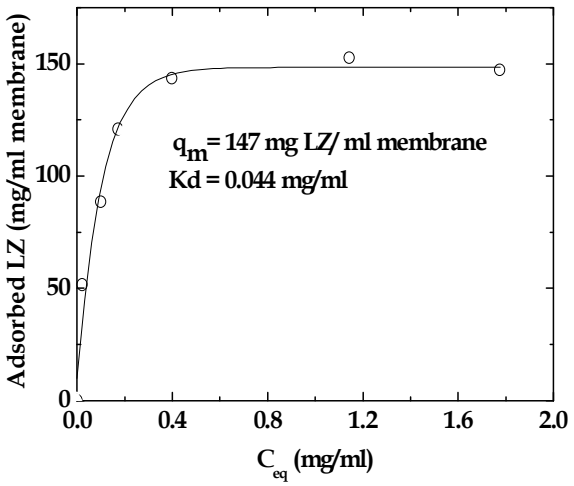

Figure 3.5. *Correlation between Experimental data and Langmuir model of the LZ adsorption isotherm at ionic strength 17 mM, equilibrium time 24 hours*

were q_m=2.0 mg/mL and K_d=5.9x10^{-2} mg/mL. LZ adsorption capacities of Zn(II), Cu(II) and Ni(II)-chelated hollow fibers were different. The maximum capacities of Zn(II), Cu(II) or Ni(II)-chelated hollow fibers were 144, 75 and 69 mg/g, respectively [26]. LZ adsorption capacity of a dye-immobilized membrane was 13.3 mg/mL [36]. The high capacity of the

described MMM adsorbers can be attributed to the fact that the surface over volume is high when small sized ion exchange particles in high loadings are embedded. Lysozyme adsorption takes place mainly at the outer surface of the particles: when instead of small particles big porous particles are used the area available for adsorption is much lower.

3.3.3. Effect of ionic strength on LZ desorption

A complete separation sequence should include an adsorption, desorption, washing and regeneration step. Desorption (elution) of ion exchange adsorbent is commonly performed by increasing of the salt concentration or by changing the pH of the elution buffer. A phosphate buffer at pH=7 with varying concentration of NaCl was used in desorption experiments. The desorption of the adsorbed LZ from MMM was studied in a batch system. Figure 3.6 gives the amount of LZ desorbed using different salt concentrations. Desorption of LZ from the MMM can be improved by increasing the ionic strength of the desorption buffer. In operations, this results in a higher productivity.

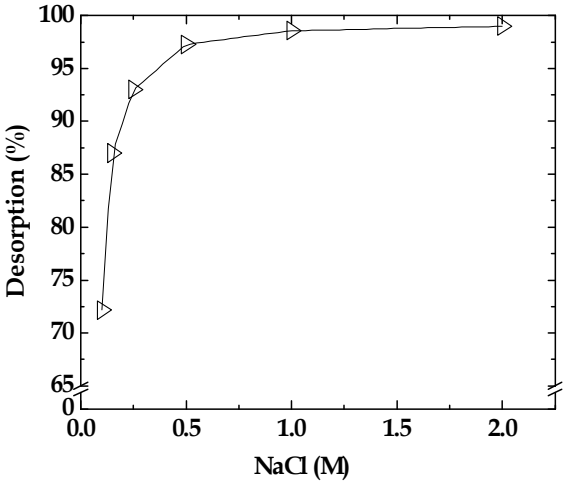

Figure 3.6. *Effect of salt on static LZ desorption from membrane Lew CNP80 WS. Desorption experiments were perform at pH 7 and in buffered 0.5 M NaC, equilibrium time 24 hours at temperature 25 ^{o}C*

Elution involves displacement of the protein from fixed charges by other counterions with a greater binding affinity for the fixed charge than the protein and which then becomes the new counter ion. The ability of counter ions (salts) to displace the protein bound to fixed

charge is a function of the difference in affinities between the fixed charges and non-fixed charges of both the protein and the salt. Affinities in turn are affected by several variables, including of magnitude of the net charge of protein and the concentration and type of salt used for displacement [37]. As the salt concentration is stepped up, salt diffuses faster to the particle creating very large driving forces for desorption. By these conditions a fraction of the adsorbed protein becomes rapidly unbound and diffuses away from the particle surface[38].

Dickman and Proctor[39] indicated that the LZ activity increases by increasing the NaCl concentration from 0 till 0.5 M but declines rapidly when the NaCl concentration is higher than 0.5 M. For our system, desorption of LZ increases with the ionic strength. At 0.5 M NaCl more than 98 % of the adsorbed LZ was recovered and recovery stays high. In all desorption or elution experiments 0.5 M NaCl solutions were applied.

3.3.4. Regeneration and reuse the membrane Lew CNP80 WS

It is important to characterize the adsorption and desorption capacity as function of sequence of adsorption and desorption cycles. After an adsorption and desorption step, regeneration was accomplished by washing the membranes with 10% HCl to guarantee thorough removal of non-specific bound components which were not eluted by the desorption buffer. The contact time between the membrane and the HCl solution was about

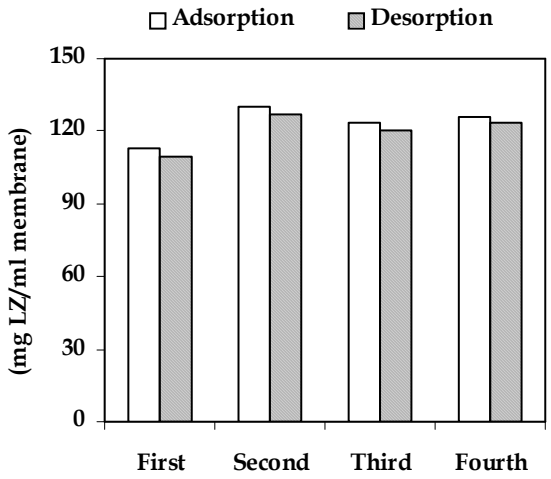

Figure 3.7. *Repeated static adsorption-desorption LZ cycles onto MMM Lew CNP80 WS. Adsorption at pH 11 and temperature 25°C. Initial LZ concentration was 2 mg/mL at ionic strength 17 mM. Desorption at pH 7 in buffered 0.5 M NaCl. The adsorption capacity was measured after 24 hours*

24 hour in total at room temperature. After the regeneration step the HCl was washed out with ultra pure water until neutral pH. During the regeneration the enzyme was completely removed from the resin. Regenerating the membrane for three times showed no loss in adsorption capacity as shown in Figure 3.7. The adsorption after the first regeneration is slightly higher than in the first adsorption cycle. An explanation for this phenomenon may be contamination or partly inactivation of the unconditioned resins. After the first regeneration step the particles are clean and the intrinsic adsorption capacity can be obtained.

3.3.5. Dynamic adsorption of LZ on membrane Lew CNP80 WS

The breakthrough and elution curves of the cation-exchange porous membrane adsorbers for LZ are shown in Fig. 3.8 as a function of permeate volume of the protein solution. A LZ feed solution (2mg/mL) was loaded at pH 11. In the desorption step a buffer of pH 7 with ionic strength 0.5 M NaCl was applied. The dynamic binding capacity is defined as the amount of protein bound when the effluent concentration reaches 10% of the feed concentration. The dynamic LZ adsorption capacity at 10% breakthrough was 58 mg LZ/mL

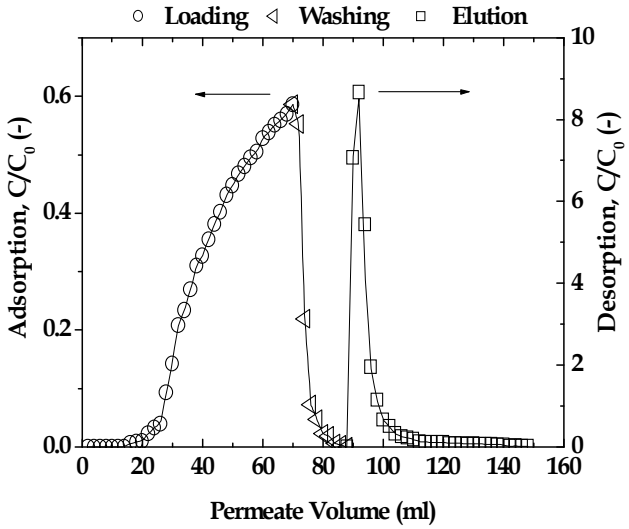

Figure 3.8. *Typical breakthrough and elution curves of LZ for a stack of 10 MMM Lew CNP80 WS operated at a feed concentration of 2 mg/mL LZ and a constant filtration flux of 50 $Lm^{-2}h^{-1}$.*

membrane at a flux 50 $Lm^{-2}h^{-1}$. This is about 2.5 times lower than the maximum static binding capacity. Elution of the adsorbed protein-MMM complex with phosphate buffer pH 7 at a flux

of 50 $Lm^{-2}h^{-1}$, leads to LZ recovery values up to 98 %. The protein was concentrated up to 8 fold into the desorptive buffer, as indicated on right-hand y-axis of Figure 3.8.

The obtained dynamic LZ adsorption capacity was lower than the corresponding static capacity because convective contribution to mass transport dominates over the diffusive contribution. Liquid containing the protein molecules flows preferentially through the large pores. Adsorption in smaller pores can only occur by slower diffusional transport, hence these sites are less likely to be accessed. Bypassing and uneven flow distributions are others sources, which lead to lower dynamic capacities. Full adsorption capacity can only be achieved if the protein residence time in the MMM adsorber is long enough to allow each adsorbate molecules to diffuse to the adsorptive site and to rearrange/unfold their structure at the adsorptive interface.

3.3.6. Effect of the flux on dynamic adsorption

A lysozyme solution (2 mg LZ/mL) prepared in a binding buffer pH 11 was used to study the effect of flux on the adsorption capacity. By changing the flux from 25 up to 500 $Lm^{-2}h^{-1}$ a capacity loss of a factor 3 was found. We can conclude from Figure 3.9 that the dynamic adsorption capacity is constant using fluxes above 300 $Lm^{-2}h^{-1}$. Although the loss in capacity between 25 and 300 $Lm^{-2}h^{-1}$ is significant, this drop in capacity is acceptable, considering the 20-fold increase in throughput. Nevertheless, the independent ratio of flux and adsorption in the high flux regimes indicates that in this convective controlled regime LZ had sufficient time to bind to the binding sites in the mixed matrix membranes. Furthermore, we can conclude from the breakthrough curves that the effect of the flux is much bigger in the adsorption mode than in the elution mode (Figure 3.10), especially when fluxes above 100 $Lm^{-2}h^{-1}$ were applied. Adsorption of proteins on solid surfaces is a complex process and is influenced by many factors such as re-arrangement and conformational changes of the protein where as desorption is strongly dependant on the ionic strength and the kinetics the salt ions.

Dynamic adsorption capacity is strongly correlated with the accessible area and is influenced to a large extent by mean pore size, pore size distribution, pore interconnectivity and the protein diffusion coefficient and residence time in the adsorber membrane. Sartobind C membrane adsorbers that posses the same carboxylic functionality as the CNP80 containing MMM are selected as a commercial reference. The morphology of Sartobind membranes is very different: the cation exchange functionality is grafted onto the surface of the starting membrane. The MMM adsorber obtain their functionality through the mixing of cation

exchange particles into the bare membrane. Hence it is difficult to relate any difference in adsorption performance to morphological characteristics. Rather, we like to compare the two different membranes in order benchmark the MMM adsorbers to an industrial reference.

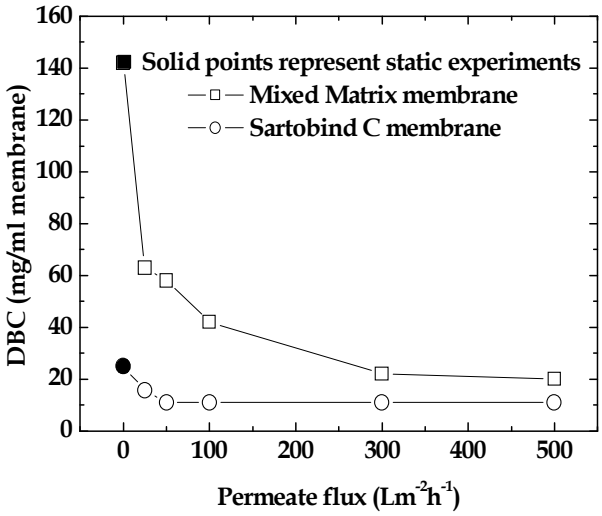

Figure 3.9. *Dynamic binding capacity of LZ on MMM Lew CNP80 WS and Sartobind C membrane at different flux and room temperature. LZ loading concentration was 2 mg/mL at ionic strength 17 mM. (DBC=dynamic binding capacity)*

The dynamic binding capacity of the Sartobind membranes is almost independent of the flux (Figure 3.9 and Table 3.1). Convection controls the adsorption and only at low fluxes there is an additional adsorption due to diffusion of LZ to the active sites in diffusional pores. This result is in agreement with the data published by Santarelli et al.[40]. They studied the dynamic capacity of strong cation exchange Sartobind S membranes at different permeate rates. They found that by increasing the permeate flux from 120 $Lm^{-2}h^{-1}$ to 1200 $Lm^{-2}h^{-1}$ a slight decrease in adsorption from 1.33 till 1.20 mg/cm^2 membrane. Our MMM adsorbers show a bigger flux dependent adsorption behavior. The MMM adsorbers start with an up to 7 times higher adsorption capacity compared to the Sartobind C membranes. The Sartobind membranes show an almost flux independent capacity, but the capacity of the MMM adsorbers is flux sensitive. Nevertheless, the dynamic capacity is twice as large at a flux of 500 $Lm^{-2}h^{-1}$.

Table 3.1. *Dynamic binding capacities of LZ on membrane Lew CNP80 WS and Sartobind C at 10 % breakthrough point*

Flux ($Lh^{-1}m^{-2}$)	Dynamic binding (mg LZ/mL membrane)		Concentrated		Recovery (%)	
	MMM	SC	MMM	SC	MMM	SC
25	63	16	9 fold	3.5 fold	98	99
50	58	11	8 fold	3.5 fold	98	99
100	42	11	7 fold	3.5 fold	98	99
300	22	11	6.5 fold	3.5 fold	98	99
500	20	11	6 fold	3.5 fold	98	99

MMM= mixed matrix membrane, SC = sartobind C

The throughput during elution is an important parameter for the efficient productivity of LZ from the membranes. Fig. 3.10 presents the elution profiles of LZ at different fluxes. A 0.025 M sodium phosphate buffer containing 0.5 M NaCl could elute LZ bound to the membrane quantitatively. Sharp elution peaks were obtained at all fluxes. The capacities at different fluxes of the CNP80 WS containing adsorbers are summarized in Table 1. From this we can conclude that highest productivity can be obtained by adsorbing at moderate fluxes followed by desorption at high fluxes. The MMM adsorber shows much higher concentration factors, 9 fold, during elution compared to the Sartobind membrane, 3

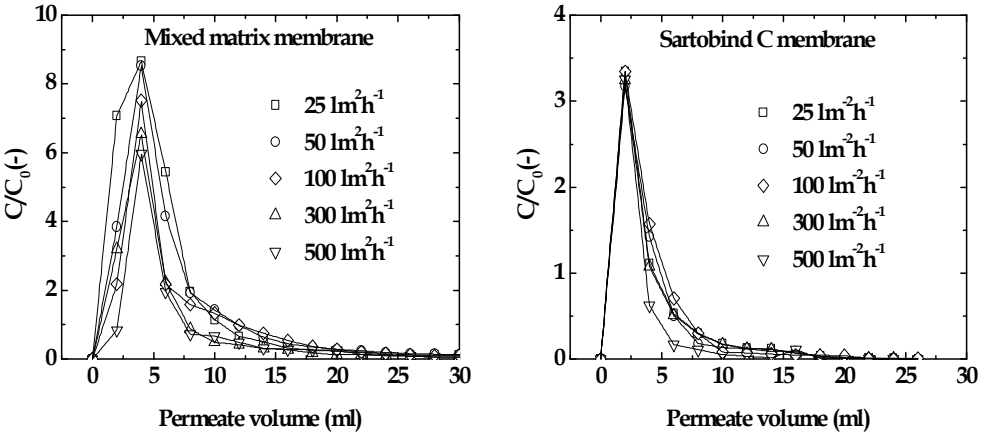

Figure 3.10. *Typical elution curve of LZ at different flux on MMM Lew CNP80 WS and Sartobind C membrane. Phosphate buffer pH=7 containing 0.5 M NaCl was used as elution buffer*

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fold, This can be contributed to the higher adsorption capacity of the MMM adsorber where small embedded IEX-particles are responsible for a high active surface area per volume of

membrane where as the Sartobind membranes possess a low active area because of the presence of big go through pores. And since almost all the active sites in the Sartobind membranes are located on the pore wall of these convective pores, the Sartobind membranes are less sensitive to the rate of the permeate flux.

3.3.7. Lysozyme stability and biological activity

Stability and activity of enzymes are essential during their production, storage and use to promote their industrial applicability. However, environmental conditions such as high temperature, shear, extreme pH and strong salinity can cause protein denaturation, leading to protein aggregation[23]. Native and denatured states of a protein can be investigated by fluorescence spectroscopy[20-22, 41-43]. LZ is a protein that contains six tryptophan residues, but only two of them, Trp62 and Trp108, appear to dominate the fluorescence spectrum. Trp62 and Trp108 are arranged close to the substrate-binding site (the protein split). They play an important role in substrate binding to an inhibitor and in the protein stabilization. Fluorescence analyses of these tryptophan residues have provided information on the LZ-ligand interaction and ligand-induced conformational change around the binding site[44, 45].

The fluorescence spectra of native LZ, recovered from elution step and denaturated LZ were recorded (Figure 3.11). A significant change can be observed with respect to the maximum wavelength of 339 nm: the denaturated LZ shows a much broader maximum as compared to native one. In contrast, the fluorescence spectra of the LZ obtained from the elution step resemble the one of the native LZ very well and no significant shift of maximum wavelength was detected in the spectra of these samples relative to the native protein. This indicates that no detectable conformational change of LZ in mixed membrane adsorbers separation processes.

Specific activity and total activity are critical parameters in enzyme purification. The influence adsorption and elution process on enzymatic activity was also evaluated. A suspension of *M. luteus* bacteria is subjected to LZ fraction, the cell walls are broken down and the optical density of this suspension decreases with time. This occurs because the LZ induces bacterial cell lysis by hydrolyzing β-1,4 linked glycosidic bond of cell wall's peptidoglycan. The rate of this reaction depends on the amount of active enzyme present, so the rate of decrease in optical density can be used to measure enzyme activity[23, 24, 46]. LZ obtained by MMM was used to compare the activity properties with native one.

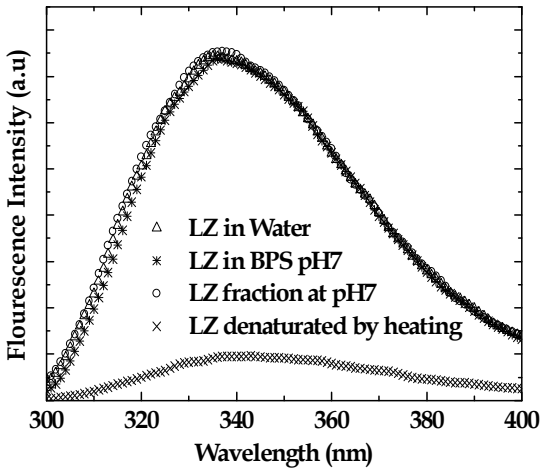

Figure 3.11. *Fluorescence spectra obtained for LZ before adsorption, after desorption and denaturated. All measurement were carried out at 25 °C and LZ concentration 2 mg/mL. (BPS=buffer phosphate)*

The adsorption and elution process did not significantly affect to the activity of LZ. The specific activity of native LZ was 79000 unit/mg and the specific activity of recovered LZ was 73000 unit/mg. This activity test demonstrates that fraction of LZ was maintained above 90 % activity after the separation process. This confirms that the MMM process is a very gentle process; the active LZ was maintained above 90% of the native Lysozyme.

3.4. Conclusions

The performance of MMM adsorbers for enzyme capturing and concentration was studied. The MMM adsorbers are prepared by a phase inversion method. The capacity is established by embedding of small particles into a microporous polymeric membrane. A membrane with higher resin loading is preferable for providing a higher adsorption capacity. The MMM adsorbers demonstrate a high static and dynamic protein adsorption capacity and an easy scale up for the separation of LZ. The dynamic binding was found to be dependent on the flux. Where the dynamic elution of LZ is independent of the flux and leads to several fold concentration and LZ recovery values up to 98 %. The fluorescence spectra of the LZ from the elution step indicate that no obvious conformational change of LZ took place. Activity test with *Micrococcus lysodeikticus* as the substrate demonstrate that the activity of isolated LZ was

maintained for more than 90 % after separation processes. This indicates that the MMM adsorbers process is a mild capturing and concentration process for LZ recovery. A comparison between MMM and commercial membranes demonstrates that MMM posses a higher dynamic adsorption capacity. The MMM adsorbers concept combines robustness, with high capacities and high throughput and mild process conditions.

3.5. Acknowledgements

We want to acknowledge the financial support for this work through the Membrane Technology Group of the University of Twente and the Ministry of Education of the Republic of Indonesia. We thank Sartorius kindly for supplying the Sartobind C membranes.

3.6. List of Symbols and Abbreviations

Symbols

C_0	*Initial protein concentration*	*mg/mL*
C_{eq}	*Protein concentration at equilibrium*	*mg/mL*
I	*Ionic strength*	*mol/l*
Kd	*Dissociation constant*	*mg/mL*
Mw	*Molecular weight*	*g/mol*
Q	*Adsorption capacity*	*mg/g membrane*
$q_{desorbed}$	*Amount of protein desorbed*	*mg/g membrane*
q_{eq}	*Adsorption capacity at equilibrium*	*mg/g membrane*
q_m	*Maximum adsorption capacity*	*mg/g membrane*
R	*Recovery*	*%*
sd	*Swelling degree*	*%*
V	*Volume*	*mL*
W	*Weight*	*g*
Wr	*Amount of ion exchange resin*	*g*
Wp	*Amount of polymer*	*g*
z_i	*Charge number*	-
ε	*Porosity*	*%*
#	*Number*	-

Abbreviations

ATCC	*American type culture collection*
BSA	*Bovine serum albumin*
CER	*Cation exchange resin*
DMSO	*Dimethylsulfoxide*

EVAL	*Ethylene-vinyl copolymer*
HCl	*Hydrochloric acid*
LZ	*Lysozyme*
Lew	*Lewatit*
MMM	*Mixed matrix membrane*
pI	*Isoelectric point*
U.A	*Unit activity*
Trp	*Tryptophan*

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Chapter

4 Enzyme Capturing and Concentration from Binary Mixtures

Abstract

Developments in bioseparations are necessary to fulfill the demands of higher throughputs, higher selectivity's and higher capacities. The Mixed Matrix Membranes (MMM) concept is proposed as a moderate process with an increased throughput and dynamic adsorption capacity. This study presents the application of EVAL-based adsorptive MMM containing weak cation exchange resins for the separation and concentration of protein mixtures. The MMM are prepared by a wet phase inversion method. The adsorptive membranes feature a high adsorption capacity in both static and dynamic modes. High separation factors and purities of Bovine Serum Albumin (BSA) and Lysozyme (LZ) are obtained in effluent and elution buffers. LZ, the retained protein, is recovered with a high purity in the elution buffer in a five fold increased concentration. At fluxes above 20 $Lm^{-2}h^{-1}$, the adsorption capacity and purification power are independent of the flux. The MMM can be reused in multiple adsorption/desorption cycles thereby maintaining the high binding capacity. The operational conditions using MMM adsorbers are mild and do not affect the three-dimensional structure of the LZ molecules, which is proven by fluorescence spectroscopy and biological activity tests. The MMM act in protein separations both as protein purifier and concentrator and is an attractive alternative for packed bed systems because of its high capacity, high throughput, robustness, ease of scaling up and mild separation conditions.

Keywords: *Membrane adsorbers, Bovine Serum Albumin (BSA), Lysozyme (LZ) and adsorption*

4.1. Introduction

Bio-downstream processing by means of protein isolation and purification plays an important role in the formulation of biopharmaceutical products and contributes significantly to overall production costs[1, 2]. An improvement of existing methods or the development of a new technology is necessary to overcome the shortcomings of the classical bio-downstream processes.

In the early days of protein purification, protein separation from complex mixtures was based on protein precipitation by water miscible organic solvents[3]. Alternative processes including adsorption, gel filtration, liquid phase partitioning, electrophoretic methods and membrane technology have been developed for protein purification. Membrane filtration is used primarily for purification and concentration of biomolecules. Membrane filtration offers a fast route for removal of cell debris, colloidal or suspended solids and viruses from homogenized suspensions of bacterial cells[4-6]. One of the major limitation membranes based on sieving properties is their relatively low resolution[7, 8]. Therefore, this technique is not used for fine separation. The another drawbacks of membrane filtration in protein filtration is concentration polarization and membrane fouling[9-11]. Adsorption techniques often result in purification steps with greatest increase in protein purity. Therefore, these techniques have become widely used especially when adopted in combination with chromatographic [12-14] and membrane processes[15-19].

Several authors have described the fractionation of Bovine serum albumin (BSA) and Lysozyme (LZ) mixtures by means of membrane filtration and adsorptive membrane chromatography. Iritani et al.[20] reported the fractionation of BSA and LZ by membrane filtration using 30 kDa polysulfone membranes. The membranes were assumed to be permeable for LZ and almost completely retentive for BSA. The LZ permeation was found to be very strongly depending on the BSA concentration. An increase in BSA concentration resulted in a decrease in the LZ flux. Millesime et al.[21] reported the fractionation of BSA and LZ using both modified and unmodified inorganic membranes. Since protein fractionation is affected by protein - membrane surface interactions, the separation selectivity may be improved by modification of the membrane surface. Several authors[22-24] tried to improve the membrane performance by optimizing the process parameters such as membrane pretreatments, and adjusting the pH and ionic strength of the protein solution. Kanani et al.[25] reported a favorable protein-protein interaction by adding BSA in the UF process for the separation of LZ from myoglobin. A binary mixture of LZ and myoglobin was

ultrafiltered using a 30 kDa MWCO polyethersulfone membrane. LZ was permeated significantly faster than myoglobin. However, when BSA was added to the feed solution, LZ was largely retained while the permeation of myoglobin was significantly increased. The decrease in LZ permeation rate was explained by the electrostatic BSA–LZ interaction, while the simultaneous increase in myoglobin permeation was contributed to the Donnan effect in combination with size exclusion.

Fang et al.[26] investigated the separation of BSA and LZ by using polysulfone-based cation-exchange membranes. In batch experiments at pH 7.4, the adsorption capacity of LZ was 15.6 mg/mL membrane, where BSA showed no adsorption at all. In a dynamic adsorption process using a 10 layered membrane stack, BSA and LZ were effectively separated with a LZ recovery of about 93 %. Lin et al.[27] investigated the separation of BSA and LZ using ion-exchange membranes in plate-and-frame modules. In batch mode experiments they found that using cation-exchange membranes the maximum LZ adsorption capacity was 46.5±17.8 mg/mL membrane and the maximum BSA adsorption capacity onto anion-exchange membrane was 47.5±20.0 mg/mL membrane. In a dynamic filtration process, the separation performance is for large extend influenced by the operating conditions. Furthermore a critical factor that limits the application of adsorptive membrane systems in protein fractionation is a low binding capacity[26, 28].

In bioseparation processes, chromatography is another essential technique for obtaining the required purity of biotherapeutic and diagnostic products. By using chromatographic methods, very good separations and high purities can be achieved. Chromatographic processes are traditionally carried out using packed beds. However, these methods have some severe major drawbacks[19, 29]. An approach to overcome the limitations associated with packed beds is to use synthetic microporous or macroporous membranes as alternative chromatographic stationary phase[15, 19]. Avramescu et al.[30] proposed a simple method to produce macroporous adsorptive membranes, the so called Mixed Matrix Membranes (MMM) adsorbers concept. The MMM adsorbers concept is an effective approach to increase the selectivity and capacity of membrane chromatographic systems. Functionalization of the macroporous membranes takes place by entrapping small affinity particles, it results in an uniform distribution of ligands through the whole matrix and a capacity comparable with that of packed bed systems[31, 32]. The MMM adsorber captures selectively proteins of interest using the affinity properties of the adsorbent. Unbound materials and interferences are washed out where after the bound species are eluted in high

concentration and purity. An additional advantage of the MMM adsorbers is the robustness. This makes that an intensive pretreatment, such as commonly used in packed bed chromatography, is unnecessary. The MMM-technique combines the selectivity and capacity of chromatographic resins (particles) with the low flow resistance of macroporous membranes[33]. Important is that the nature and activity of the proteins (enzymes) is not affected by the purification process[31].

Applications of the MMM have been investigated for the separation and recovery of single components, *i.e.* BSA, Hb, LZ, bilirubin[30, 31, 34, 35]. Recently, the use of MMM adsorbers was reported for fractionation of BSA-Hb mixtures[33]. In the present work, new applications of the MMM for fractionation of BSA and LZ mixtures are studied. BSA and LZ are representing two extremes with respect to their physicochemical properties[36, 37]. At physiological pH LZ is positively charged whereas BSA is a negatively charged.

In this chapter, we present an extensive performance study of the MMM application for the fractionation of BSA and LZ in both static and dynamic mode. To prepare weak cation exchange MMM adsorbers, Lewatit CNP80 WS particles are incorporated into a porous EVAL matrix. System parameters such as membrane properties and the adsorption conditions in chromatographic separations of proteins are investigated. Adsorption selectivity of BSA and LZ from mixtures is evaluated and compared with literature data.

4.2. Experiments

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 a membrane material without further modification. Dimethylsulfoxide (DMSO, Merck) was employed as solvent and 1-octanol (Fluka) as non-solvent additive in the casting solution. Water was used as a non-solvent in the coagulation bath. Ion exchange functionality was introduced by embedding Lewatit CNP80 WS (a weak acidic macroporous acrylic-based cationic exchange resin), kindly supply by Caldic, Belgium. The particles were milled and air classified to obtain a fraction with an average particles diameter of 10 micron. Hydrochloric acid (HCl) was used for the regeneration of the MMM adsorbers. Bovine Serum Albumin (BSA) and Lysozyme (LZ) obtained from Fluka were used for the adsorption measurements.

Buffer solutions were freshly prepared in ultra-pure water. Ultra-pure water was prepared using a Millipore purification unit Milli-Q plus. The 17 mM ionic strength of buffers

used for zeta potential measurements were an acetate buffer for the pH 4-5 regime, a phosphate buffer for pH 3, 6-8 and 12 regimes, a tris buffer for pH value 9 and a carbonate buffer for pH values between 10 to 11. The ionic strength of the adsorption buffers (7.5 mM phosphate buffer pH 7) was kept constant at 17 mM by the addition of NaCl. pH 7 was chosen as the operational pH for adsorption, which is close to the physiological pH. The elution buffer was prepared by increasing the ionic strength of the adsorption buffer to 0.5 M using NaCl.

4.2.2. Membrane preparation

The membranes used in this study are prepared by the phase inversion method. The membranes are prepared out of a DMSO solution, containing 10 % EVAL, 10 % 1-octanol and 65 wt% of resin loading. The 1-octanol was added to the casting solution in order to improve the membrane morphology. The mixture was stirred over night at 50 °C to break down possible particles clusters. The MMM were prepared by immersion precipitation. For this, the polymeric mixture was cast (casting knife 0.475 mm) on a glass plate and immediately immersed into a water coagulation bath at 40-45 °C and the membranes were formed a few moments after immersion. Then the membranes were washed with tap water at room temperature to remove residual solvent. After washing, the membranes were overnight air dried and afterwards dried in a conventional oven at 50 °C.

4.2.3. Membrane Characterization

4.2.3.1. Scanning electron microscopy

The cross sections, as well as the top and bottom surfaces of the membrane were characterized by Scanning Electron Microscopy (SEM, JEOL TM 220 A). For the characterization of the top and bottom surfaces, pieces of membranes are air dried and glued with double side adhesive tape on sample stubs. For the cross-section the wet membranes are fractured in liquid nitrogen. After preparation the samples are dried at 30 ^{o}C in a vacuum oven and before examination gold-coated using a Balzers Union SCD 040 sputter coater.

4.2.3.2. Membrane porosity and swelling degree

The membrane porosity (ε) and swelling degree (sd) were determined from swelling experiments by calculating the weight and the volume of both dry and wet membranes. Their porosity was determined from the water uptake of a calibrated volume of dry membrane. The

membranes were submersed for 24 hour at room temperature in water and the change in weight was recorded. Before weighing the attached water was removed from the surface of the swollen membrane by padding it dry using a filter paper. A digital micrometer (Mitutoyo) was used to measure the membrane thickness. The reported values are averages of three different samples.

4.2.3.3. Clean water flux

Water fluxes of the membranes are determined at room temperature by using a nitrogen pressurized stirred dead-end filtration cell. The applied pressure is $1x10^4$ Pa (0.1 bar). The reported flux values are measured at steady state conditions, at 3.33 Hz (200 rpm). The average flux was calculated from numerous experiments using different pieces of membrane.

4.2.4. Adsorption experiments

4.2.4.1. Zeta potential measurement of pure BSA and LZ

The net charges of BSA and LZ as a function of pH were determined using a Malvern Zetasizer 2000. Acetate, phosphate and carbonate buffers, all 17 mM ionic strength, were used to cover pH values ranging from 3 to 12. Protein solutions are prepared in the buffers in concentrations of 1 mg/mL. The Zeta potential was measured during 20 seconds for at least five times. All measurements were carried out at room temperature (25 ^oC).

4.2.4.2. Adsorption isotherm

Protein adsorption onto ion exchange resins follows the Langmuir adsorption isotherm[17, 38]. To determine the adsorption isotherm, batch adsorption experiments were carried out using different initial protein concentrations. After 24 hours, the protein depletion was determined spectrophotometrically at 280 nm in 2mm quartz cuvettes. The equilibrium data are plotted and fitted with the linear form of the Langmuir model by the using least-squares regression analysis. From this plot the maximum adsorption capacity (q_m) and the dissociation constant (K_d) can be calculated.

4.2.4.3. Dynamic adsorption experiment

The dynamic adsorption performance of flat sheet EVAL adsorber membranes is measured at a constant permeation rate using a nitrogen-pressurized stirred dead-end

filtration cell. To average out membrane inhomogeneities, 6 membrane sheets were stacked. A solution containing BSA and LZ in 17 mM ionic strength of phosphate buffer at pH 7 was permeated with a flux of 50 $Lm^{-2}h^{-1}$ through the membrane stack. Permeate was collected and fractionated in samples of 2 mL. After the breakthrough point was reached, the experiment was stopped and the stack was washed with binding buffer to rinse out the unbounded proteins. Subsequently, a phosphate buffer containing 0.5 M sodium chloride was permeated through the membrane stack to elute the adsorbed proteins. The BSA and LZ concentrations in the feed and permeate samples were analyzed by HPLC (Waters 717 plus) attached with a Waters 2487 dual λ absorbance detector. An analytical protocol based on gradient chromatography using a cation exchange column (Water HPLC Column, Biosuite™ CM 10 μm CXC) is used. As binding buffer 20 mM phosphate buffer pH 6.1 and as eluting buffer 20 mM phosphate buffer pH 6.1 containing 0.5 M NaCl is used. The protein mass adsorbed per unit of membrane volume is calculated by numerical integration over the filtration run until a breakthrough concentration of 10 % was reached. The effect of permeation rate of both loading and elution was investigated at 3 different permeation rates (25, 50, 100 $Lm^{-2}h^{-1}$). For the 50 $Lm^{-2}h^{-1}$ the effect of the ionic strength on the adsorber performance is also investigated.

4.2.4.4. Regeneration

After an adsorption, washing and desorption cycle, the membrane adsorbers were washed with 10 % HCl to guarantee complete removal of residual BSA and LZ that might have been attached to the membrane surface by non-specific interactions and was not eluted by the high ionic strength NaCl solution in the desorption step. Before the next adsorption/desorption cycle could start, the membranes were pre-conditioned by washing out the remaining HCl with ultra pure water until neutral pH. The adsorption capacity of the regenerated membrane was then re-measured.

4.2.4.5. LZ stability and enzymatic activity

Effect of adsorption and desorption conditions on LZ stability and enzymatic activity were determined. The LZ stability was obtained by measuring the fluorescence spectra of native LZ, heat-denaturated LZ and desorbed LZ using Edinburgh Analytical Instruments F900. The LZ fluorescence emission spectra were recorded from 300 to 400 nm at 284 nm excitation wavelength. The enzyme activity of LZ was determined by a spectrometric

method using *Micrococcus lysodiekticus* as substrate. This assay is based on the decrease in absorbance (450 nm) of a *M. lysodeikticus* cell suspension when LZ digests the cells.

3 mg of lyophilized *M. lysodeikticus* cells were dissolved in 10 mL 0.1 M phosphate buffer (pH 7), kept on ice. LZ was dissolved in 0.025 M phosphate buffer and kept on ice. The amount of LZ in the sample had to be adjusted so that a decrease in absorbance of 0.02 to 0.04 per minute was obtained (LZ concentration is about 0.0125 mg/mL). Optical density of the cell suspension was measured at 450 nm. To obtain an optical density between 0.5 and 0.7, lyophilized cells or phosphate buffer were added to a cell suspension to reach a final substrate concentration about 0.3 mg/mL. Thereafter, 100 µl of the enzyme solution was added to 1.4 mL cell suspension of *M. lysodeikticus* and the change in the A_{450} was recorded every 15 seconds for 2 minutes. Temperature was kept constant at 25 ^{o}C. One activity unit (U.A.) is equivalent to a decrease of 0.001 absorbance unit per minutes. Specific activity is the ratio of enzyme activity to mass of protein in the sample, usually expressed in units of activity per mg protein (U/mg).

4.3. Results and Discussions

Experimental results are presented and discussed in this section. First, charge densities of pure BSA and LZ as function of pH are described. Followed by a detailed membrane characterization in terms of morphology, porosity and clean water flux. Then, the protein adsorption and fractionation using the MMM adsorbers is evaluated in both static and dynamic mode. Finally, the protein separation efficiency was evaluated by determining the separation factor and purity of the isolated product.

4.3.1. Zeta potentials BSA and LZ

The zeta potential gives information about the overall surface charges of components in relation to its environment. Insight in the protein surface charge improves the understanding of the ion exchange adsorption behavior. The density of charges of single BSA and LZ were determined between pH 3 until 12 in 1 mg/mL solutions. Figure 4.1 presents the net charge of BSA and LZ at different pH values. The figure illustrates that the electrostatic properties differ significantly as function of pH. The IEP for which the net charges are zero can be deduced from the graph as 4.7 and 11.0 for BSA and LZ respectively. These values are in agreement with literature data [10, 39]. The graph further shows that at pH 7, close to physiological pH, the BSA has a net negative charge where LZ has a net positive charge. This

discrepancy in electrostatic properties can be exploited in adsorptive separation using cation exchange materials. Consequently, pH 7 was chosen as operational pH in the fractionation of BSA and LZ process.

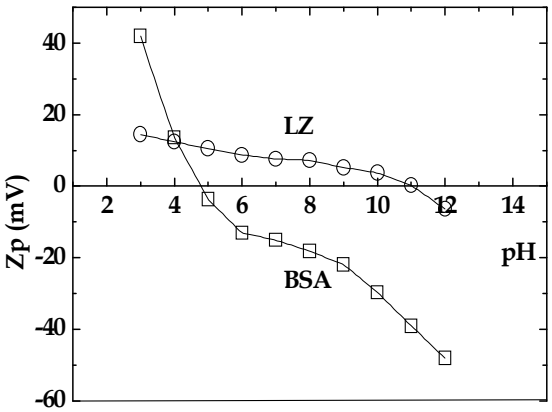

Figure 4.1. *Zeta potentials of pure BSA and LZ at difference pH's, measured in buffered 17 mM ionic strength solutions.*

4.3.2. Membrane characterization

Figure 4.2 presents SEM images of Mixed Matrix Membrane (MMM) Lewatit CNP 80WS prepared by direct immersion precipitation in a water containing coagulation bath. An important parameter for the preparation of membrane adsorbers is the amount of active groups that can be incorporated into the polymeric matrix. The adsorption capacity increases proportionally with IEX resin loading[30]. The maximum resin load applied in this research was 65 wt%. Above this level, the viscosity of the casting solution is too high for a proper casting. In addition, a very high particles loading reduces the mechanical strength of the membrane, which can lead to mechanical failure under pressure. The membranes containing a 65 wt% IEX resin loading are well structured and posses an open and interconnected porous structure.

There are no evident finger-like macrovoids present in the membrane and the resins are uniformly distributed over the entire cross-section. The absence of macrovoids is required in membrane chromatography because macrovoids create mal-distribution, leading to lower dynamic adsorption capacities. The ion exchange particles are tightly held together within the porous polymeric matrix and no significant loss of the particles was observed during the membrane formation. The membranes mechanical strength is sufficient, the porosity is 70 %

and the swelling degree 20 %. The membranes demonstrate high clean water fluxes due to a high pore interconnectivity and porosity. The clean water fluxes are on average 1000 $Lm^{-2}h^{-1}bar^{-1}$. This high permeability allows MMM adsorbers stacking to increase the module adsorption capacity and to minimize flow mal-distribution.

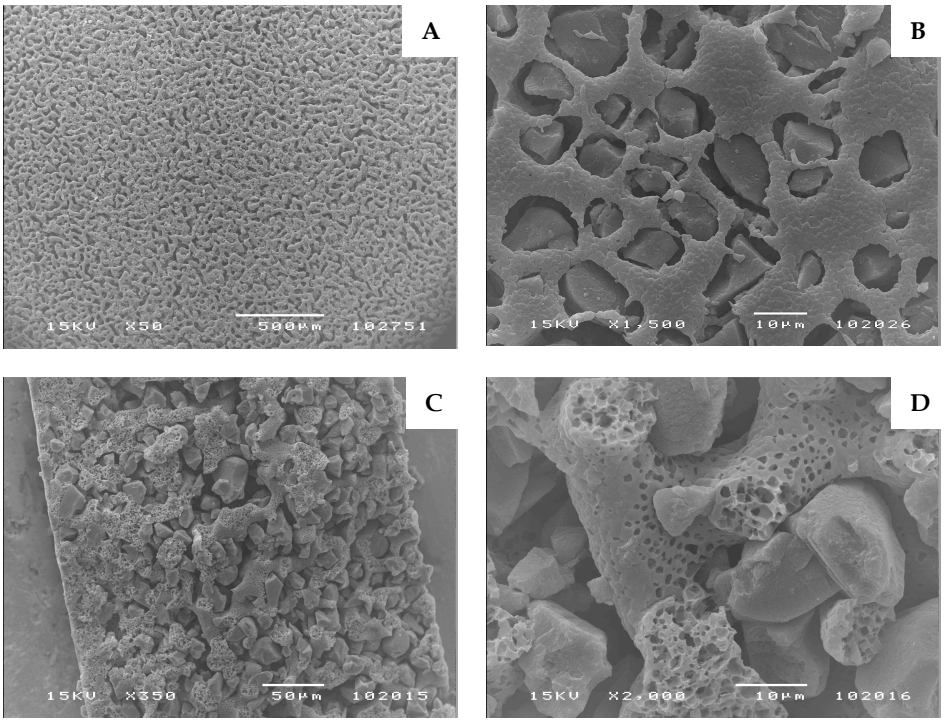

Figure 4.2. *SEM micrographs of an EVAL based membrane adsorber containing 65wt% Lewatit CNP80 particles: A) and B) Bottom-surface, magnification x50 and x1500; C) and D) Cross section, magnification x350 and x2000. The membranes were prepared by direct immersion precipitation in water containing coagulation batch at room temperature.*

4.3.3. Protein adsorption isotherm

The protein adsorption isotherms are measured to characterize the interaction of each protein with the adsorbent at a certain pH and ionic strength. The outcome provides the relationship between the equilibrium concentration of the protein in the bulk solution and the amount of protein adsorbed on to the solid phase. The adsorption isotherm for single BSA and LZ are determined in batch experiments at pH 7 and 17 mM ionic strength. The measured adsorption isotherms appear to be Langmuir type for both proteins. The obtained adsorption isotherms are presented in Figure 4.3. The maximum adsorption capacities (q_m) of

BSA and LZ are 8 and 91 mg/mL membrane respectively (equal to 11.70 and 133.10 mg/g membrane for BSA and LZ respectively). The dissociation constants (K_d) are 3.2 10^{-1} mg/mL for BSA and 4.5 10^{-2} mg/mL for LZ, which confirms a stronger LZ than BSA interaction with the membrane adsorber. The obtained static capacities of the adsorptive membranes for BSA and LZ are higher than the reported values in literatures that range from 6 to 40 mg per mL membrane[17]. Fang et al.[26] reported 15.6 mg LZ/mL membrane for the adsorption capacity with a cation-exchange activated polysulfone-based membranes, the membranes did not show any measurable BSA adsorption.

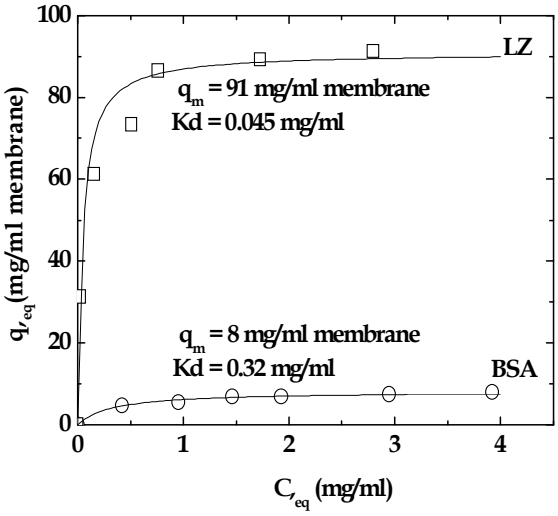

Figure 4.3. *Adsorption isotherms for pure BSA and LZ on mixed matrix membrane adsorbers at pH7 and ionic strength 17 mM, equilibrium time 24 hours. Experimental data are correlated with the Langmuir adsorption isotherm model. The solid line figure is best fit with the Langmuir isotherm.*

Based on the assumption that the MMM and proteins mainly interact through electrostatic interactions, the adsorption behavior of the different mixtures can be anticipated. At an operational pH (pH=7), the MMM demonstrates higher adsorption for LZ than for BSA. Since at pH 7 the MMM is negatively charged, the effective interaction takes place predominantly with the positively charged LZ molecules and not with the negatively charged BSA molecules. BSA adsorption mainly takes place at positive domains in the overall negative charged BSA molecules and by non-specific interaction. When the pH is above the isoelectric point BSA changes into a more extended conformation with more hydrophobic

groups exposed to the outer part of the molecule leading to higher adsorption values on solid interfaces[40]. This nonspecific protein adsorption also plays a role in the protein separation process.

3.4. Breakthrough curves for BSA and LZ at flux 50 $lm^{-2}h^{-1}$

The typical breakthrough and elution curves of BSA and LZ mixtures using cation-exchange porous membrane adsorbers as a function of permeate volume of the protein solution are shown in Figure 4.4. In the dynamic adsorption experiments, a stack of 6 membranes was used to increase the adsorption capacity and to minimize flow mal-distribution by averaging out membrane heterogeneities. The dynamic binding capacity,

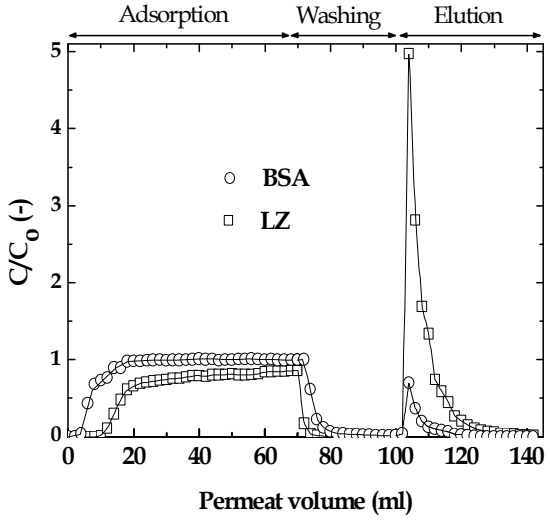

Figure 4.4. *Typical breakthrough and elution curves of BSA and LZ mixtures through a 6 membranes stack at pH 7 and ionic strength 17 mM and a constant filtration flux of 50 $Lm^{-2}h^{-1}$. Feed concentration was 1 mg/mL for both BSA and LZ. Elution steps were performed at pH7 with 7.5 mM phosphate buffer containing 0.5 M sodium chloride.*

DBC 10 %, defined as the amount of bound protein per volume membrane when the effluent concentration reaches 10 % of the feed concentration, is 7 mg/mL membrane for BSA and 20 mg/mL membrane for LZ. The breakthrough curve also confirms that most adsorbed BSA elutes during the washing step, which increases the LZ purity in the elution step. The amount of eluted LZ, calculated by numerical integration over the elution curve, shows that 98 % of the loaded LZ is recovered. Besides a higher purification grade, the LZ is also more than five fold

concentrated into the desorptive buffer. Biomolecular targets are generally present in low concentrations in complex mixtures, which mean that often-large volumes have to be treated. The adsorptive MMM technology is because of the low flow resistance and the robustness is very suitable for handling large volumes containing low concentrations of target proteins. The adsorptive MMM acts both as purifier and concentrator in this LZ recovery process.

The obtained dynamic LZ adsorption capacity (at DBC 10 %) is lower than the corresponding static adsorption capacity. This can be explained by the fact that in the static mode slow diffusional processes control the adsorption where as in dynamic mode there is a balance between convection and diffusional processes. Since not all the adsorptive sites are located in big convective go-through-pores the adsorption capacity of the system is to a certain extend flow rate dependent. Besides this also membrane stack flow mal-distribution contributes to a lower DBC 10 % compared to the static adsorption. These lower dynamic adsorption capacities are in agreement with data published by Avramescu et al. [30] and Roper et al [17],who also found lower dynamic than static adsorption capacities.

4.3.5. Purity BSA and LZ at flux 50 $Lm^{-2}h^{-1}$

The separation efficiency of BSA and LZ is evaluated by the separation factor. The separation factor $S_{BSA/LZ}$ is defined as:

$$S_{BSA/LZ} = \frac{C_{p,BSA} / C_{p,LZ}}{C_{f,BSA} / C_{f,LZ}} \qquad \textbf{(4.1)}$$

where $C_{f,BSA}$ and $C_{f,LZ}$ are the protein concentrations in the feed solution and $C_{p,BSA}$ and $C_{p,LZ}$ are the protein concentrations in the collected permeate fractions during filtration, calculated by numerical integration of the permeation curve over the filtration run until the 10 % breakthrough. Figure 4.5 presents the purity of the individual proteins during permeation of BSA – LZ mixtures in the adsorption, washing and elution step.

At the operating pH (pH=7), the membrane adsorbs preferential LZ due to opposite protein/membrane charges and allows BSA to freely pass the membrane due to repulsive charge forces. During the first part of filtration run, the LZ concentration in permeate is below the HPLC detection limit (40 µg/mL). Hypothetical LZ concentrations, being the detection limit of the HPLC, are used to calculate a minimal separation factor. In the first filtration fractions this separation factor, $S_{BSA/LZ}$, is above 1100, which indicates a BSA purity of at least 99.9 % (Figure 4.5). In the wash step weakly and unbound BSA is rinsed out in low

concentration with high purities. Finally, the bound products (LZ) are dissociated from the adsorptive IEX resins and eluted. The eluted LZ fractions have a high purity of more than 95% and are initially more than 5 times concentrated when compared with the feed solution. The overall LZ process recovery is more than 98 %.

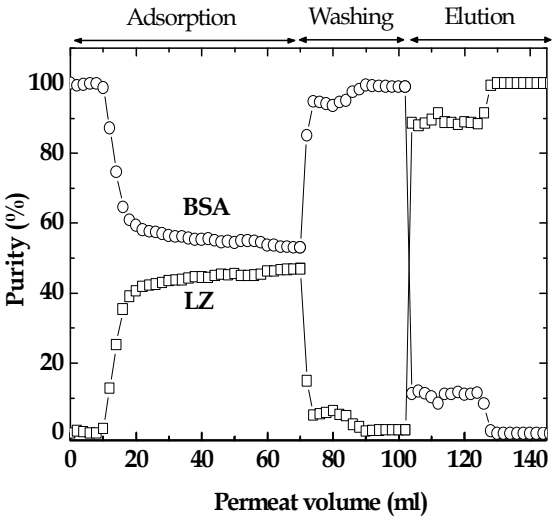

Figure 4.5. *The protein purity in the different process steps. The feed contains equal amounts of BSA and LZ. In the adsorption and washing steps, the BSA purity is high and the LZ purity is high in the elution step. Initial BSA and LZ concentrations were 1 mg/mL, ionic strength 17 mM and the filtration flow rate 50 $lm^{-2}h^{-1}$.*

Separations of BSA and LZ mixtures by ultrafiltration and adsorptive membranes have been investigated in several studies. The BSA and LZ purity obtained by ultrafiltration strongly depends on the membrane solute rejection and process parameters. Bellare et al.[41] reported an almost complete BSA retention where the LZ retention varies with the amount of treated volume. The obtained membrane selectivity was 20 and the LZ purity in the permeate was above 95 %. Fang et al.[26] reported BSA and LZ separation with a cation exchange polysulfone based membrane, ten membrane layers were stacked in a plate and frame module. The BSA and LZ were effectively separated at permeate fluxes of 74 $Lm^{-2}h^{-1}$ with a LZ recovery above 93 %. The main advantages of MMM systems in the fractionation of proteins compared to ultrafiltration and another adsorptive membranes are the combination of both a high static and dynamic adsorption capacity. Furthermore, the MMM can operate at

higher flow rates than ultrafiltration membranes without loosing their capacity. Moreover, higher purities and higher concentrations can be obtained during elution.

4.3.6. Effect of the permeation rate

The influence of the permeation rate, which is proportional to the protein residence time in the adsorber, on the dynamic LZ binding capacity and the separation factor $S_{BSA/LZ}$ are examined by increasing the flux from 25–100 $Lm^{-2}h^{-1}$. Figure 4.6 shows that the LZ dynamic binding capacity slightly depends on the LZ residence time in the membrane. When the flux increased from 25 to 50 $Lm^{-2}h^{-1}$ the dynamic LZ binding capacity decreases only 5 %. A further decrease in the LZ residence time does not result in any further decline in the dynamic adsorption capacity.

Figure 4.6 also shows a slight initial dependency of the separation factor $S_{BSA/LZ}$ on the flux. At a flux of 25 $Lm^{-2}h^{-1}$, the separation factor $S_{BSA/LZ}$ is 1200 and decreases by only 8 % when the filtration flux increased by 100 % (50 $Lm^{-2}h^{-1}$). A further flux increase has no impact on the separation factor $S_{BSA/LZ}$.

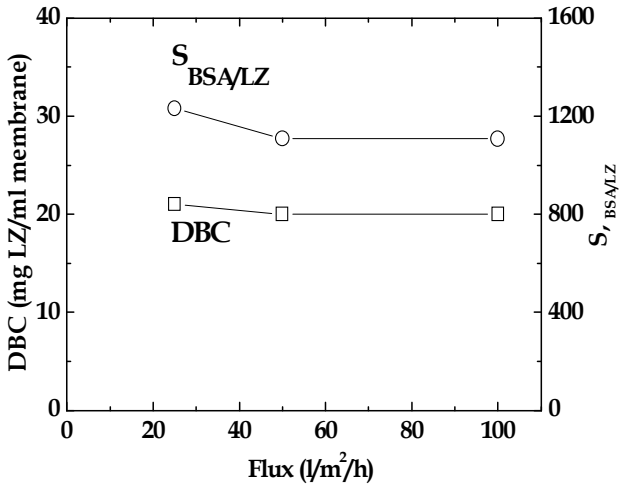

Figure 4.6. *The influence of the flux on the dynamic LZ binding capacity and BSA-LZ separation factor. Feed concentration for LZ and BSA 1 mg/mL, ionic strength 17 mM.*

The adsorption process involves the transport of proteins from the feed solution to the interface, their binding to the surface usually via electrostatic and hydrophobic interactions and their refolding on the surface via conformational changes[42]. Full utilization

of the adsorption capacity can be achieved only if the protein residence time in the membrane adsorbers is sufficient to allow the protein molecules to diffuse to the adsorptive sites. In traditional used packed bed systems containing gel type beads the adsorption process is for a big extend diffusive controlled, which means flow rate dependent. The advantage of the described MMM adsorbers is that almost all the active sites are reached by convection, which means that at high flow rates the MMM adsorbers are more effective than packed bed systems.

4.3.7. Effect of ionic strength

Electrostatic interactions are the dominating driving forces for protein adsorption onto ion exchange surfaces. The protein adsorption behavior can be controlled by varying the ionic strength of the protein solution. The effect of the ionic strength on the dynamic adsorption capacity and the BSA purity in the effluent fractions is represented in Figure 4.7. The LZ adsorption capacity slowly decreases with increasing ionic strength and the separation factor follows the same tendency.

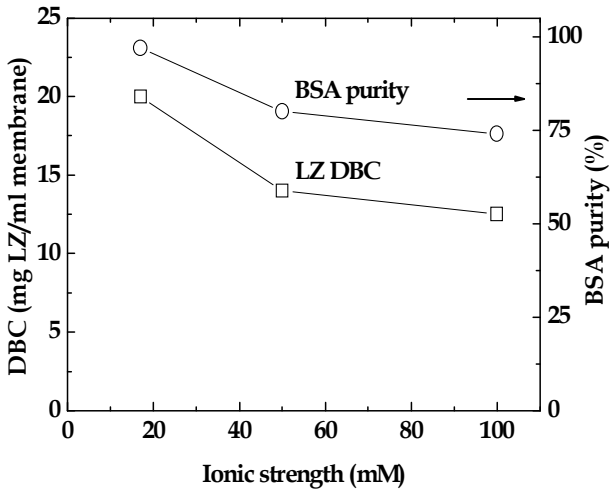

Figure 4.7. *The effect of the ionic strength on the BSA purification. The experiments are performed at room temperature, pH 7, ionic strength 17 mM, permeate flow rate 50l/m²/h with initial. BSA and LZ concentrations of 1 mg/mL*

This means that the BSA adsorption capacity is dependent of the ionic strength. When the ionic strength increases from 17 to 50 mM the adsorption capacity of LZ and purity

of BSA decreases by 20 %. The effect of ionic strength on the protein adsorption onto ion exchange resins is a very complex phenomenon and can be considered from different aspects[43]. Charged proteins and surfaces are always attracting counter ions to their surfaces to form an electrical double layer. As the ionic strength increases the screening of the surface charges is more effective and the attractive interaction between the positively charged protein and the negatively charged functional groups weakens. The increase of the ionic strength causes a decrease in the ligand accessibility by the target proteins[44] and reduces the porosity of the resin due to shrinkage network of the resin beads[43]. Typically, high salt concentrations are used to desorb and elute the adsorbed proteins from ion exchange surfaces[44]. This effect is confirmed by Avramescu et al.[33], they reported that high salt concentrations cause a decrease in the BSA and Hb adsorption capacity on the MMM containing weak cation exchange resins. Their obtained separation factors also follow the same trend. Several other researchers have reported similar phenomena for different protein adsorbent pairs[45, 46].

4.3.8. Reuse of membrane

Repetitive use of membrane adsorbers that maintain their adsorption performance is of significant concern in adsorption technology and membrane chromatography. After adsorption and desorption steps, regeneration is accomplished by washing the membranes with 10% HCl to guarantee thorough removal of remaining protein residues, which are bound to the membrane adsorber by other then electrostatic interaction forces and cannot be eluted by a salt gradient. Then, the HCl was rinsed out with ultra pure water until neutral pH. Where after the membranes were used in the next adsorption /desorption cycle. The capacity of regenerated membrane was measured and no loss in adsorption capacity was observed. Figure 4.8 shows that the dynamic LZ binding capacity after regeneration is even slightly increased. This can be explained by the fact that fresh resins/membrane are partly inactivated by contamination and are supplied in sodium form, which is less active compared to the hydrogen form. The binding interaction of a hydrogen ion is lower than the binding of a sodium ion[47, 48]. Adsorption on ion exchange resins involves a displacement of counter ions from fixed charges by proteins.

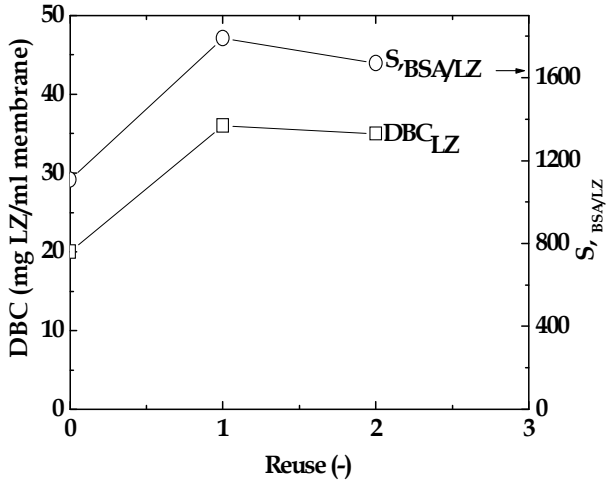

Figure 4.8. *Repeated dynamic binding capacity and separation factor of BSA and LZ onto membrane Lew CNP80 WS. Adsorption at pH 7 and room temperature. Initial BSA and LZ concentrations were 1 mg/mL and ionic strength 17 mM.*

4.3.9. LZ stability and activity

In order to evaluate the effect of the separation processes on the LZ stability and activity, fluorescence spectra and enzymatic activities were determined. Figure 4.9 presents spectra from native, eluted and denaturated LZ. The fluorescence spectra of the LZ from the elution step are very close to those of native LZ and no shift in the maximum wavelength is visible. The fluorescence spectra showed that there is no denaturation or conformational change the during separation processes.

These findings are confirmed with activity tests using *Micrococcus lysodiekticus* as substrate. The activity tests proved that more than 90 % of the LZ activity is preserved, indicating that the LZ is not denaturated in the purification and concentration steps. This proves that the operational conditions using MMM adsorbers are mild and do not affect the three dimensional structure of the LZ molecules.

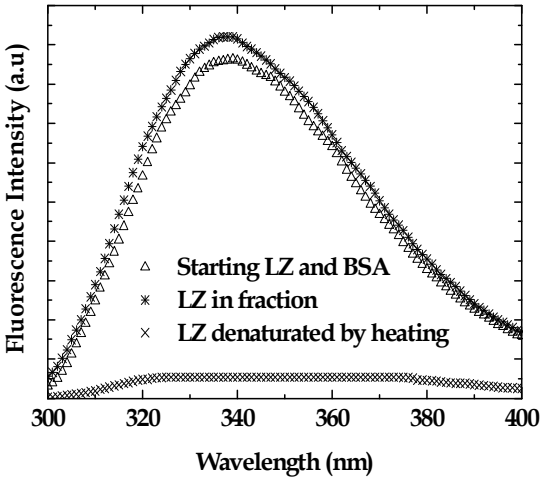

Figure 4.9. *Fluorescence spectra obtained for LZ in different condition*

4.4. Conclusions

The MMM adsorbers containing weak cation exchange resins are prepared using a phase inversion method. The developed MMM adsorbers demonstrate both a high static and dynamic protein adsorption capacity and selectivity in the fractionation and purification of BSA - LZ mixtures. High separation factors and high degrees in purity for both BSA and LZ are obtained in both effluents and elution buffers. At permeate fluxes above 20 $Lm^{-2}h^{-1}$, the adsorption capacity and purification power becomes independent of the permeate flux. The operational conditions using MMM adsorbers are mild and are not affecting the three dimensional structure of the LZ molecules, which is proven by fluorescence spectroscopy and biological activity tests.

The MMM concept is flexible and can be applied in many applications by entrapping different type of particles or by adjusting the process conditions. The membranes can be applied in both protein capturing and polishing steps. The MMM are attractive in protein separation because of its high capacity, high throughput, robustness, and ease of scaling up.

4.5. Acknowledgements

We want to acknowledge the Membrane Technology Group of the University of Twente and the Ministry of Education of the Republic of Indonesia for the financial support.

4.6. List of Symbols and Abbreviations

Symbols

C_0	*Initial protein concentration*	*mg/mL*
C_{eq}	*Protein concentration at equilibrium*	*mg/mL*
I	*Ionic strength*	*mol/l*
Kd	*Dissociation constant*	*mg/mL*
Mw	*Molecular weight*	*g/mol*
Q	*Adsorption capacity*	*mg/g membrane*
$q_{desorbed}$	*Amount of protein desorbed*	*mg/g membrane*
q_{eq}	*Adsorption capacity at equilibrium*	*mg/g membrane*
q_m	*Maximum adsorption capacity*	*mg/g membrane*
R	*Recovery*	*%*
sd	*Swelling degree*	*%*
V	*Volume*	*mL*
W	*Weight*	*g*
Wr	*Amount of ion exchange resin*	*g*
Wp	*Amount of polymer*	*g*
z_i	*Charge number*	-
ε	*Porosity*	*%*
#	*Number*	-

Abbreviations

TCC	*American type culture collection*
BSA	*Bovine serum albumin*
CER	*Cation exchange resin*
DMSO	*Dimethylsulfoxide*
EVAL	*Ethylene-vinyl copolymer*
HCl	*Hydrochloric acid*
LZ	*Lysozyme*
Lew	*Lewatit*
MMM	*Mixed matrix membrane*
pI	*Isoelectric point*
U.A	*Unit activity*
Trp	*Tryptophan*

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Chapter

5 Preparation of Double Layer Mixed Matrix Membrane

Abstract

In this chapter, we propose the preparation of Mixed Matrix Membrane (MMM) adsorbers for extracorporeal blood purification by incorporating activated carbon into a biocompatible porous polymeric support. The MMM adsorbers are prepared by solvent evaporation or by water vapor induced phase separation followed by an immersion precipitation step. Integral mixed matrix double layer membranes consisting of an active support and a separating layer are prepared by co-casting two polymer solutions onto a glass plate. The active support layer consists of activated carbon particles embedded in macroporous cellulose acetate; the separating layer consists of particle free cellulose acetate. Dense or open porous structures can be prepared depending on the applied process parameters. Dense films are obtained by direct immersion of the polymer solution into a water containing coagulation bath. Porous membranes are formed when solvent evaporation in humid air takes place preceding the immersion precipitation. The co-casting process opens the possibility to improve the mechanical stability and the biocompatibility of the membrane adsorber while preventing particle loss during preparation and processing.

Keywords: *Mixed Matrix Membrane (MMM), activated carbon, phase inversion, double layers and flux.*

5.1. Introduction

Porous Mixed-Matrix Membrane (MMM) adsorbers offer a wide variety of applications, depending on the particle selection. Applications include peptide and protein isolation from fermentation broths, protein fractionation, ligand immobilization for affinity-based separations, immobilization of catalysts and enzymes, blood detoxification, product protection and drug release systems[1]. In particular, for blood purification several types of sorbent particles with various affinities are nowadays available. The affinity of adsorptive particles for specific molecules can be defined in terms of hydrophobic, hydrophilic, electrostatic, molecular recognition, or other specific interactions. Historically in extracorporeal sorbent hemoperfusion, activated carbon and resin are used for their adsorptive capacity and ability to remove harmful substances[2].

In the previous chapter we demonstrated that the MMM concept is a promising alternative approach to chemical surface activation. By incorporating small particles, high binding capacity membrane adsorbers can be obtained comparable or better than packed bed systems[1, 3]. We propose that the MMM concept can be applied in blood purification by incorporating hemoperfusion sorbents into a hemocompatible porous polymeric supports. It is important that the polymeric matrix does not interfere with the particles accessibility. The developed membranes can be applied in multiple therapeutic modalities (hemodialysis, hemodiafiltration, hemofiltration and hemoperfusion) by tailoring the pore size or flux.

Nowadays, non-solvent induced phase separation (wet casting) and non-solvent vapor induced phase separation (dry wet casting) are used to produce polymeric membranes with different characteristics. In wet casting, a polymer solution is brought into contact with a non-solvent. By exchange of the solvent and non-solvent, the solution becomes unstable and phase separates in a polymer rich phase and a polymer lean phase. The polymer rich phase solidifies and forms the polymer network of the membrane where as the polymer lean phase forms the pores. Dry wet casting involves the residence of an initially homogeneous polymer solution film in a non-solvent vapor environment before immersion into a non-solvent bath to induce phase separation. When the polymer solution is exposed to dry air, the volatile components evaporate from the polymer solution, which results in an increase in the polymer concentration leading to the development of a dense skin, i.e., barrier layer. Vapor-induced phase separation (VIPS) occurs when the cast polymer solution is exposed to a non-solvent vapor. The non-solvent molecules are diffusing into the polymer solution bringing the solution closer to demixing, thereby enhancing the speed of phase separation. Evaporation of

solvent from the polymer solution in combination with the penetration of non-solvent vapor into the polymer solution yields a variety of membrane structures including asymmetric or symmetric, anisotropic or isotropic, with a dense or porous surface.

Selectivity, mechanical stability and surface chemistry are very important membrane parameters. These characteristics can be tailored by producing double layer (multilayer) membranes. Double layer membranes are basically composed of two main components, i.e. outer layer (surface layer) and inner layer (support layer). The surface layer is a thin layer that controls the high permeability and high selectivity membrane. The function of support layer is to provide maximum membrane mechanical strength. Kools et al.[4] and He et al.[5] used a simple method to produce integral multilayer porous membranes by simultaneously co-casting a plurality of polymer solutions (different polymer types or different polymer concentration) onto a support and then immersing the total assembly into a coagulation bath. The term "co-casting" means that the individual polymer solution layers are cast simultaneously, minimizing the delay time between the casting of the layers. By this way, membranes with a continuous change in structure can be formed without a discontinuity in between the layers. Holzki et al.[6] reported the production of multilayered membranes from polymer solutions with different viscosities. The viscosity of the lower layer should be higher than the viscosity of the upper layer. By varying the viscosity of the polymer solution and the casting thickness, selective layers with different characteristics can be formed.

The double layer membrane concept can be combined with the concept of adsorptive particle-loaded membranes[7, 8]. The particle containing layer can be co-cast with a particle free polymer solution to obtain the desired performances in blood purifications. The new membrane structure then has good blood-compatibility, high flux, and good mechanical strength can be obtained by co-casting the MMM layer with a blood-compatible polymer solution. Ideally, the co-casting layer should be highly porous in order to facilitate high molecule transport to active sites in the underlying adsorption layer. The particle free layer also acts as a selective barrier to prevent adsorption of large proteins and to minimize activation blood complement system by the particles. Another advantage of developing double layer MMM is preventing micro-particles loss during membrane preparation and processing. The issue of small particles being mixed into the blood stream is inherent in the use of sorbent hemoperfusion[2, 9]. The MMM that has surface and porous structure modified could be prevented the particles release into blood stream during treatment without affecting the accessibility the active sites of particles in the MMM.

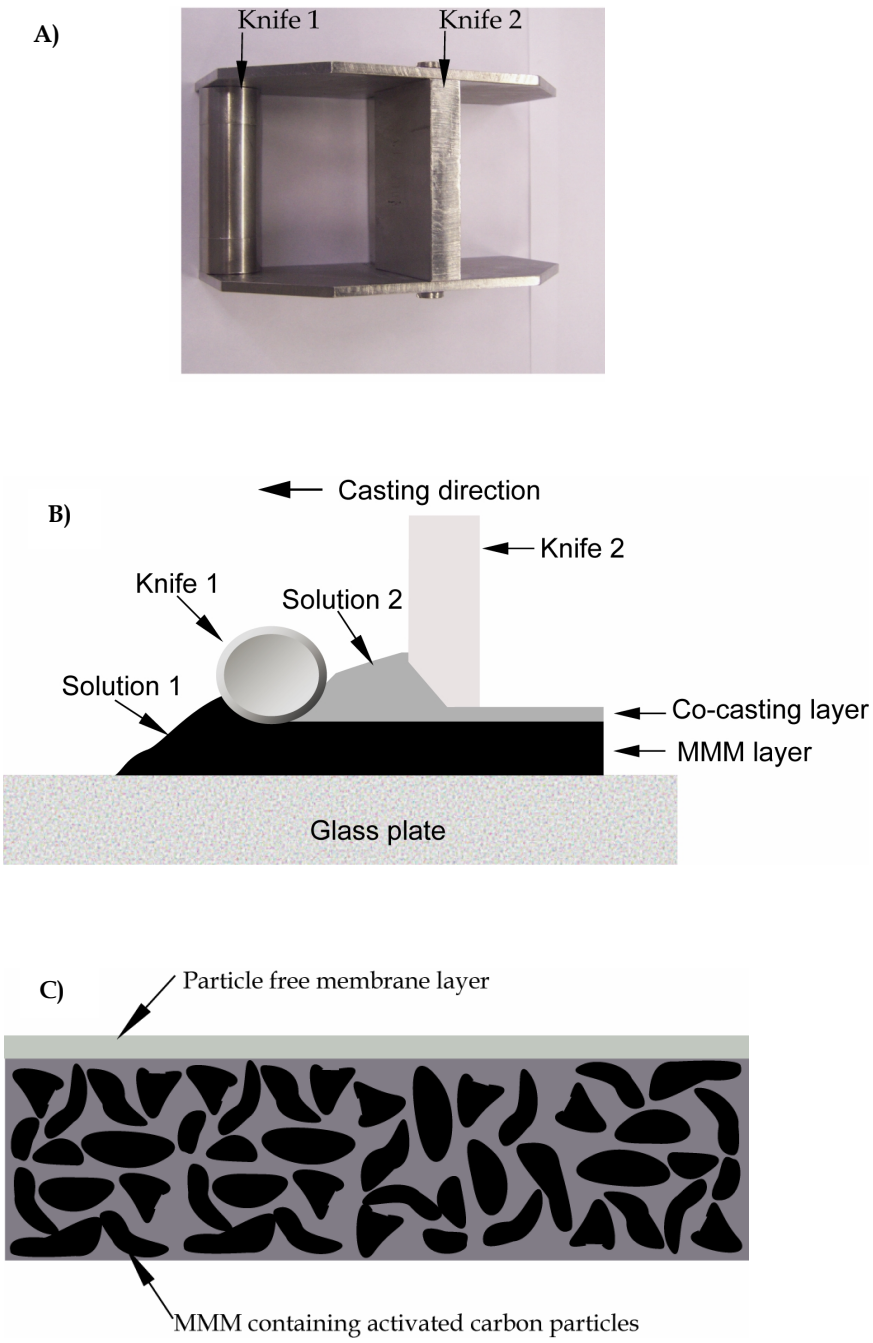

Figure 5.1. *A) Co-casting knife; B) Schematic representation of the co-casting process and C) Resulting membrane structure comprising a porous surface layer and a layer containing activated carbon particles.*

In this chapter we study the preparation of double layer Mixed Matrix Membrane (MMM) adsorbers with activated carbon particles incorporated into a porous cellulose acetate structure. The membranes are prepared by phase inversion. A double casting knife is used to cast two different polymer solution layers simultaneously, as shown in Figure 5.1. The first slit of the casting knife is used to cast the polymer solution containing the adsorptive particles and the second slit is used to cast the particle free polymer solution on top of the first solution.

The membranes are prepared after a residence time in air with different water activities or by direct immersion in a water bath. Cellulose acetate was selected as biocompatible polymer for the backbone structure. The resulting membrane structures are characterized in terms of morphology, clean water flux, pore size, swelling degree and porosity. The performance of this membrane for blood toxic substances removal will be presented in next chapters (**chapters 6 and 7**).

5.2. Experimental

5.2.1. Materials

Cellulose acetate (MW ≅ 30.000) with an average acetate content of 38.8 mol% was purchased from Aldrich and used as membrane material without further modification. Acetone (Merck) was employed as solvent. Ultra pure water (Milli-Q water is prepared using a Milli-Q plus (Millipore) purification unit) was used as both additive and non-solvent in the coagulation bath. Activated carbon (Norit A Supra EUR kindly supplied by Norit Netherlands B.V.) was used as adsorbent particles. The adsorbent particles were fractionated down to a particle size below 40 μm with average size of 20 μm.

5.2.2. Membrane preparation and characterization

5.2.2.1. Membrane preparation

To obtain membranes with adsorptive properties, activated carbon was dispersed into a homogenous solution containing 12 wt% cellulose acetate (CA) and 20 wt% water in acetone. The water, being a non-solvent additive was added to the casting solution in order to facilitate instantaneous demixing, which improves the membrane morphology. The dispersion was stirred over night at room temperature to break down possible particles clusters. The MMM were prepared by immersion precipitation, air drying, water vapor induced phase separation or by a combination of these methods. Figure 5.2 illustrates the

schematic process. The polymer solutions were co-cast on a glass plate using a double casting knife with adjustable height. The height of the first and the second slit was 475 and 550 μm

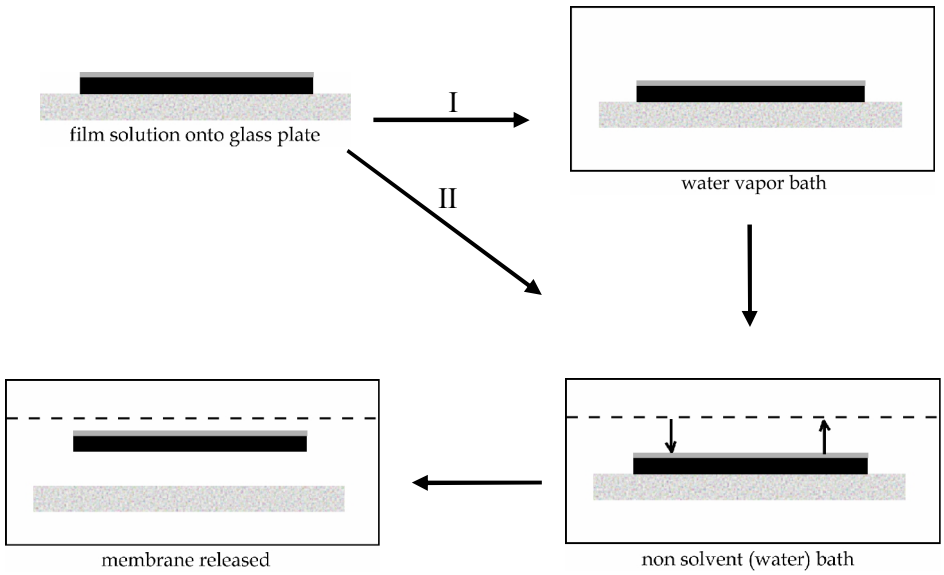

Figure 5.2. *Schematic processes of MMM preparation. I) Membrane is prepared after a certain residence time in water vapor before immersing into a water coagulation bath. II) Membrane is prepared by direct immersion into the water containing coagulation bath.*

respectively. In the direct immersion process the polymeric mixture immediately after casting immersed into a water containing coagulation bath of 22-23 °C. In the air dry process, the cast polymer solution was allowed to solvent evaporate before immersing into a water coagulation bath. In the water vapor induced phase separation process, the cast polymer solution was exposed to water vapor (temperature 30-33 °C and relative humidity of 98 %) prior to immersion in a water containing coagulation bath. After the formation process, the membranes were washed with milli-Q water at room temperature to remove the residual solvent traces. The membranes were overnight dried in air and stored in a vacuum oven at 30 °C.

To study the influence of the casting thickness and polymer concentration of the co-casting layer on the membrane forming process, polymer solutions containing different CA concentrations 10, 12 and 14 wt% were cast at two different co-casting thicknesses (75 μm and 125 μm).

5.2.2.2. Scanning electron microscopy

The membrane structures were characterized by Scanning Electron Microscopy (SEM) using a JEOL TM 220 A. The membrane top surfaces with and without co-casting layer as well as the cross sections and bottom surfaces were examined. Cross-section samples were prepared by cryogenic breaking fresh samples in liquid nitrogen. The samples were allowed to dry overnight under vacuum at room temperature and then gold-coated using a Balzers Union SCD 040 sputter coater.

5.2.2.3. Membrane porosity and swelling degree

The membrane porosity was determined from the water uptake of a calibrated volume of dry membrane. Average values were obtained from three different samples. The membrane porosity, ε, and swelling degree, sd, were determined from swelling experiments. A digital micrometer (Mitutoyo) was used to measure the thickness of the membrane. Average values were obtained from three different samples (refer to equation 3.2 and 3.3).

5.2.2.4. Clean water flux

Clean water fluxes of the membranes were determined using a nitrogen pressurized stirred dead-end ultra filtration cell. The applied pressure was $1x10^5$ Pa (1 bar). The reported flux values were measured at steady state conditions, under 3,33 Hz (200 rpm). The average flux was calculated from several experiments using different membrane pieces of 3.14 cm^2 surface area.

5.2.2.5. Pore size measurements

The membrane pore size distribution was determined using a Coulter Porometer II. The membranes were impregnated with Porofil®, a commercial wetting agent (Aldrich). By applying gas pressure on the membrane, the wetting agent can be pushed out the pores, leading to a gas flow through the membrane. The required pressure depends on the pore radius (Laplace law). By plotting gas flow rate versus the pressure and applying Darcy's law, the pore size distribution (minimum and maximum pore size as well as mean flow pore size) can be calculated.

5.3. Results and Discussion

Experimental results are presented and discussed in this section. First, the preparation and characterization of single layer membranes using the direct immersion method is discussed followed by dry air and water vapor induced preparation methods. The membrane characterization in terms of morphology, clean water flux, pore size, swelling degree and porosity is presented. In the last part of the results and discussion section, the co-casting process will be discussed.

5.3.1. Mixed matrix membrane adsorbers preparation

A proper casting of a particle dispersed polymer solution depends on the amount of polymer and sorbent particles added to the solution. A high particle load is preferred to obtain a high density of active sites in the Mixed Matrix Membrane adsorber. On the other hand, the particle load and polymer matrix are dictating the mechanical strength and morphology of the MMM. Based on previous knowledge[1, 3], the optimal polymer concentration to prepare MMM ranges from 10 to 14 (wt%). Above this amount, the polymer solution becomes too viscous, which limits the dispersing of high amounts of particles. The maximum allowable particle load is limited to approximately 65 wt%. Above this loading, the viscosity of the casting solution is too high for proper casting. Furthermore, the final structure of the MMM exhibits mechanical instabilities and becomes brittle. Therefore, for the preparation of activated carbon based mixed matrix membranes (MMM AC), we used a casting solution containing 12 wt% of cellulose acetate to incorporate 60 wt% of active carbon particles.

The challenge of embedding activated carbon in mixed matrix membranes application is related to the activity of the activated carbon. Many possible organic solvents and additives are adsorbing so strongly to the activated carbon, that desorption can only be accomplished in very low yields[11]. This means that the remaining adsorption capacity is strongly reduced. Therefore, Milli-Q water rather than tap water was used as pore former to maintain the activity of the activated carbon as much as possible. Acetone was used as solvent since it can be very easily eliminated from activated carbon due to its high volatility. Furthermore, Milli-Q water was added to the casting solution to force the instantaneous onset of liquid-liquid demixing which forms the ''fine'' porous structure. Membranes with a water-acetone ratio above of 12-% (v/v) in the casting solution showed that phase separation occurs instantaneously[12]. According to Zeman et al.[13], binary systems of CA/Acetone yield

dense films, while the ternary systems with water as non-solvent additive in casting solution, yield skinned membranes with very low water permeabilities.

5.3.2. Membrane morphology

Images of cross sections and surface sections of prepared membranes are presented in Figure 5.3 and Figure 5.4 respectively. The results demonstrate that the particles show a good dispersion and are held tightly together within the porous polymeric structure (Figure 5.3). No particle loss is observed during membrane formation. The particles are uniformly dispersed throughout the porous substructure. The addition of the particles increases the viscosity of the polymer solution and thereby restricting the growth of the polymer lean domains, which results in the formation of a porous layer containing a small pore network[10]. No macrovoids are observed in the particle containing substructure for all membranes types. The MMM without co-casting layer, which are formed by direct immersion and air drying show a dense top surface. A more open top surface is obtained by exposing the casting solution to water vapor (Figure 5.4). The membrane morphology of the co-casting layer varies from asymmetric to symmetric, with either a dense or porous skin layer, supported by an interconnected porous layer with an open porous structure at the bottom surface.

Figure 5.3 and 5.4 show the SEM pictures of MMM prepared by co-casting with a particle-free cellulose acetate solution followed by: A) direct immersion into a water containing coagulation bath, B) 1 minute residence time in dry air before immersing into a water containing coagulation bath, C) 6 minutes residence time in water vapor before immersing into a water containing coagulation bath. In the case that the MMM are prepared merely by applying a direct immersion precipitation process, a dense top layer is formed covering the macrovoids containing co-casting layer. The finger-like macrovoids are formed only in solutions that start to demix instantaneously[12]. It would be desirable to improve the mechanical strength and the flow profile through the membrane by preparing integral multilayer MMM with a microporous structure without macrovoids. SEM analysis revealed that no macrovoids were present in any MMM prepared by air casting or water vapor induces phase separation. Exposure to highly humid nitrogen causes phase separation of a polymer solution, which leads to the formation of porous films. The MMM AC spongy structure was obtained in an environment with 98 % humidity (Figure 5.4 C_2).

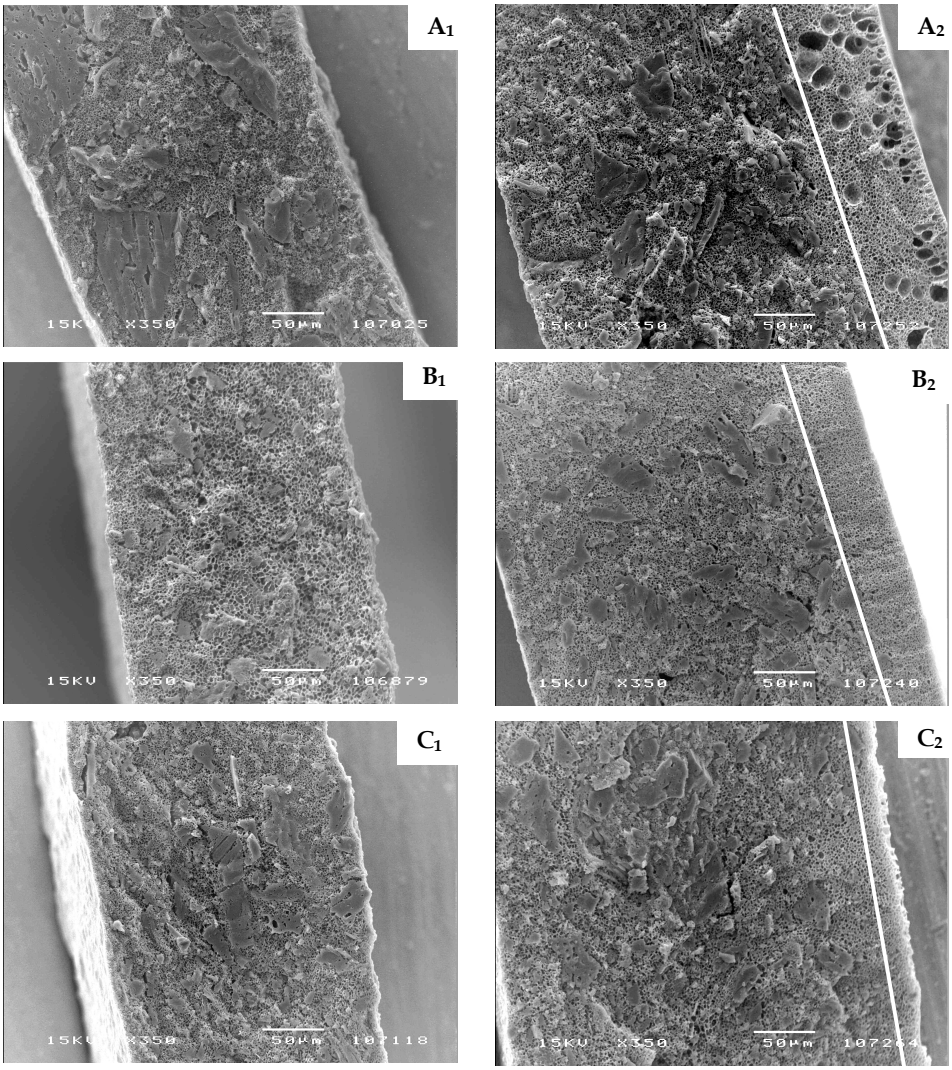

Figure 5.3. *Effect of different induced phase separation methods on the resulting membrane morphology. The membranes are prepared with a 12 % CA, 20 % water and 60 % loading of AC in acetone;* ***A)*** *MMM prepared by direct immersion precipitation in a water coagulation bath.* ***B)*** *MMM prepared after 1 minute evaporation step in dry air at ambient temperature (25°C) before immersing in a water coagulation bath.* ***C)*** *MMM prepared by water vapor induced phase separation, 6 minutes, before immersing in a water coagulation bath.* ***1)*** *Single layer membranes, cross sections, magnification x350, the size bar indicates 50 µm;* ***2)*** *Dual layer membranes, cross section, magnification x350, the size bar indicates 50 µm. Co-casting solution 10 wt% CA, 20wt% water in acetone. Temperature of the coagulation bath: 22-24 °C. Vapor bath temperature 30 to 33 °C. Casting thicknesses for sub layer and top layer were 475 and 75 µm respectively. White lines in images (1) indicate the transition between MMM layer and co-casting layer.*

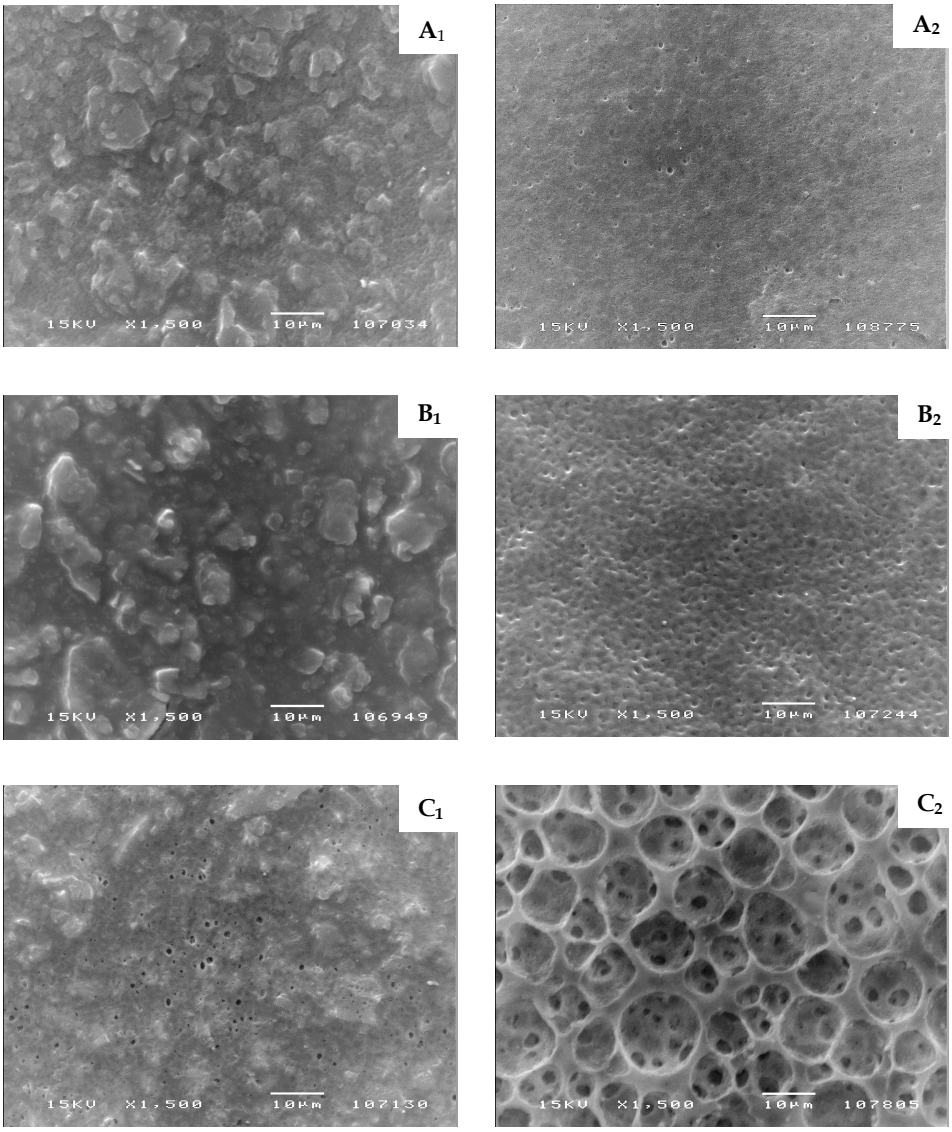

Figure 5.4. *Effect of different induced phase separation methods on the resulting membrane morphology. The membranes are prepared with a 12 % CA, 20 % water and 60 % loading of AC in acetone;* ***A)*** *MMM prepared by direct immersion precipitation in a water coagulation bath.* ***B)*** *MMM prepared after a 1 minute evaporation step in dry air at ambient temperature (25°C) before immersing in water coagulation bath.* ***C)*** *MMM prepared by water vapor induced phase separation, 6 minutes, before immersing in a water coagulation bath.* ***1)*** *Single layer membranes top surfaces, magnification x1500, the size bar indicates 10µm;* ***2)*** *Dual layer top surfaces, magnification x1500, the size bar indicates 10 µm. Co-casting solution containing 10 wt% CA, 20 wt% water in acetone.*

Matsuyama et al. [14, 15] demonstrate that an increase in relative humidity can change the morphology of PVDF membranes from dense to porous structures. The water vapor influences the solvent and non-solvent mass transfer in the polymer solution, resulting in a variety of different polymeric structures. Park et al.[16] reported that by using non-volatile solvents, porous polysulfone membrane morphologies can be obtained after 3 h exposure by using relative humidities ranging from 65 % to 100 %. On the other hand, phase separation in VIPS with volatile solvent takes places in shorter times. Yip et al. [17] reported precipitation times of 207 s and 151 s for relative humidities of 68 % and 98.5 %, respectively. In the MMM preparation, a volatile solvent (acetone) is used. During evaporation the polymer concentration of the solution increases, resulting in solidification of the polymer solution. A high relative humidity enables a high in diffusion rate of water vapor into the polymer solution, thereby enhancing the speed of the phase separation resulting in the formation of larger sized pores. Coarsening phenomena play an important role in the formation of larger pore sizes. Coalescence of the polymer-lean phases is the main coarsening process and leads to the formation of open porous structures[16]. The co-cast MMM prepared by water vapor induced phase separation, possess higher clean water fluxes as well as better mechanical properties than membranes formed by air drying or by direct immersion into a water coagulation bath. The VIPS membranes combine large pores at the top surface with good interconnected pores throughout the entire cross section.

5.3.3. Membrane characteristics

Membranes with a highly interconnected porous sub-layer can be obtained by water addition to the casting solution, which clearly shows the pore former strength of water. An increase of the water content in the dope solution may affect pore sizes and clean water fluxes. We observed that the maximal amount of water that can be added to the casting solution is 20 wt%, using higher water concentrations the casting solution becomes unstable and starts to demix.

Even with water additions as pore former, the MMM prepared by solvent evaporation (air drying) or by direct immersion, yield membranes with more dense top layer and low clean water fluxes. However, both water addition to the polymer solution and 6 minutes residence time in water vapor before immersion in a water containing coagulation bath results in membranes with high fluxes. The exposure of the cast polymer solution to humid nitrogen has the most pronounced effect on the membrane performance. Pore size and

clean water flux were significantly increased under high humidity (Table 5.1). Furthermore, membranes prepared by vapor induced phase separation in combination with immersion precipitation also display a very good mechanical strength. These membranes have a swelling degree of 6% which is a half of swelling degree of bare CA membranes.

Table 5.1. *Porosities, pore sizes and clean water fluxes of prepared cellulose acetate membranes and with activated carbon embedded MMM.*

Membrane Type	Water in co-casting solution (%)	Porosity (%)		Mean Pore size(μm)			Clean water flux ($Lm^{-2}h^{-1}$)*)		
		WB	WV	WB	DA	WV	WB	DA (1 min)	WV (6 min)
CA (bare)	-	68	73	0.000	0.000	0.262	2	0	1200
MMM	-	55	55	0.076	0.037	0.114	20	10	500
MMM co-casting	10	52	52	0.000	0.000	0.115	0	0	300
MMM co-casting	15	52	52	0.022	0.000	0.152	8	0	500
MMM co-casting	20	53	53	0.083	0.091	0.234	40	160	800

*WB= water bath, WV= water vapor, DA= dry air and *) applied pressure 1bar.*

The maximum pore size in the top layer of a membrane strongly depends on the water vapor activity and the residence time of the polymer solution in the vapor environment. Figure 5.5 presents the clean water fluxes of both bare cellulose acetate membranes and the MMM AC, prepared with different water vapor residence times.

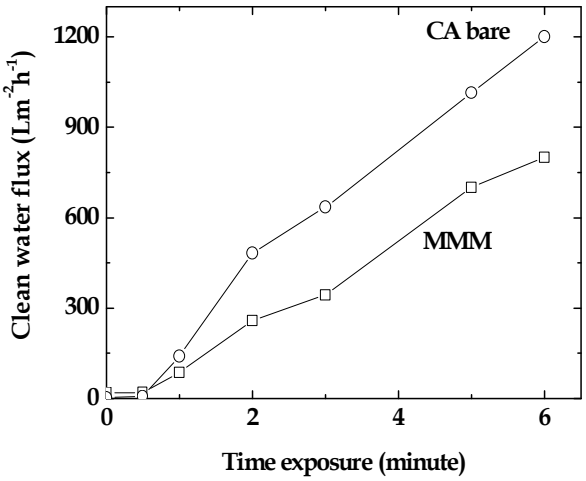

Figure 5.5. *The influence of the water vapor residence time on the clean water flux. Temperature of the water vapor varies between 30 and 33 ^{0}C. The temperature of the coagulation bath was 22-24 ^{o}C.*

Membranes with dense top layers with macrovoids are formed by direct immersion of polymer solution in a water containing coagulation bath where instantaneously demixing takes place. The resulted membranes show very low clean water fluxes. When the water vapor residence time increases from 0 to 2 min, the membrane structure changes from an asymmetrical structure with a dense layer and finger-like macrovoids, to a structure with a porous top layer without macrovoids in the cross section. The clean water permeability increases with the surface porosity. By increasing the water vapor residence even further coarse porous structures are formed due to coarsening of the polymer-lean phase. This pore size enlargement results in higher clean water fluxes.

The water vapor residence time and the vapor temperature have a pronounced effect on the membrane formation. After casting the polymer solution on a glass plate, the solvent starts to evaporate and non-solvent starts to penetrate into the polymer solution. The originally transparent polymer solution becomes turbid. We observed that the change from a clear film solution to a turbid solution starts after 25 s. When the water vapor residence time proceeds for a longer period, an opaque film is formed. The water vapor residence time at 30 ^{0}C necessary to obtain a very open top surface was six minutes. By solvent evaporation, the polymer solution volume decreases, which results in a formation of a thinner MMM. We also observed that increasing the water vapor temperature results in the formation of membranes with larger pores. When membranes formed at 35-40 °C some pinholes appear at the surface, indicating that under these evaporation conditions, the polymer lean domains grow and merge to pores that comprise the entire cross section of the membrane.

5.3.4. Effect of the polymer concentration in the co-casting layer

The co-casting layer of the MMM appears to be a key factor to tailor the clean water flux of the membranes. Adjustment of the MMM flow resistance can be achieved by varying the polymer concentration of the co-casting layer. Experiments are conducted using co-casting solutions with different initial polymer concentrations, ranging from 10-14 wt% CA. The main morphology differences that can be observed with SEM are related to the top surface, in terms of porosity and pore size distribution (Figure 5.6). The membrane porosity was decreased by 6 % when increasing CA concentration from 10 to 14 wt%. The clean water fluxes are significantly reduced by increasing the polymer concentration of the co-casting layer. The obtained clean water flux at 14 wt% CA was 535 $Lm^{-2}h^{-1}bar^{-1}$ which was correspond to flux decline by 33 %.

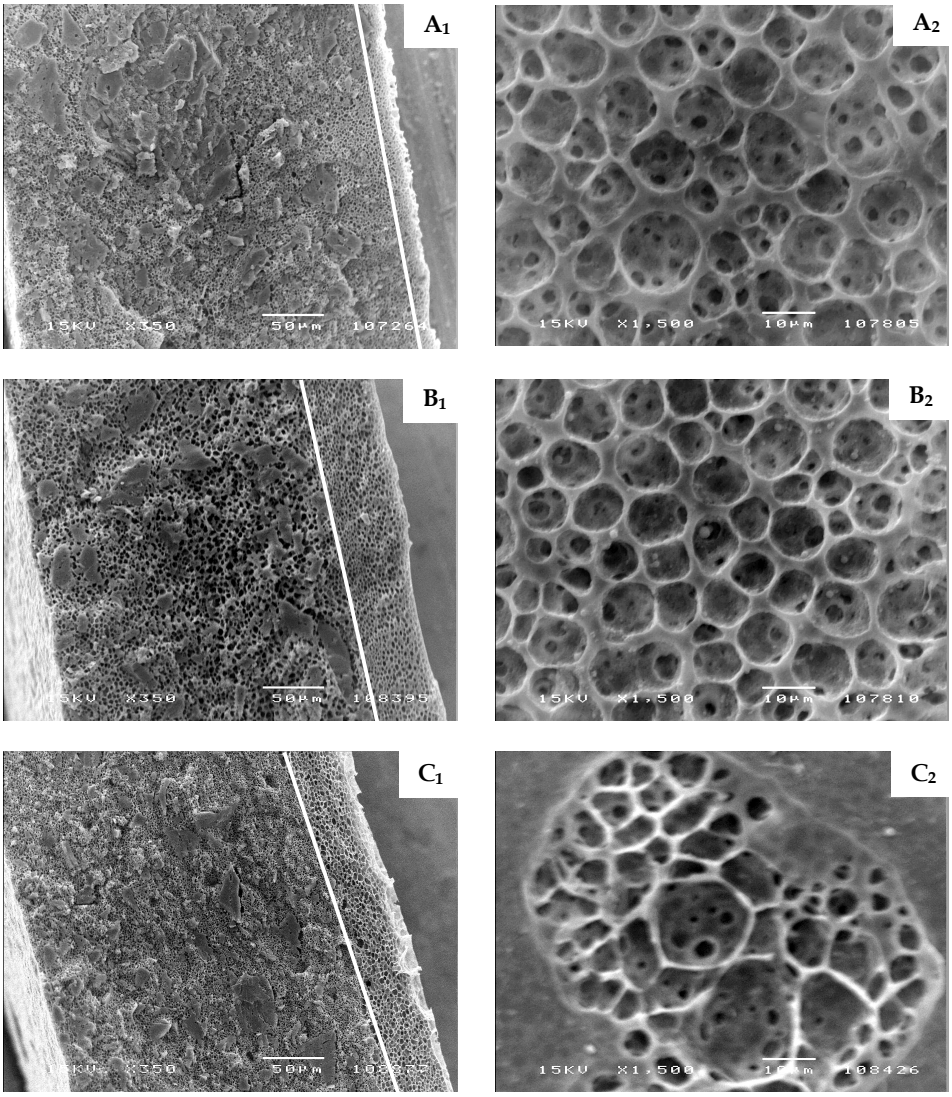

Figure 5.6. *The effect of the polymer concentration in the co-casting layer on the surface porosity. MMM are co-cast with different cellulose acetate concentrations in 20 % water in acetone.* ***A)*** *10 %,* ***B)*** *12 % and* ***C)*** *14 % CA.* ***1)*** *Cross sections, magnification x350, the size bar indicates 50 µm; 2) Top surfaces, magnification x1500, the size bar indicates 10 µm. Temperature in vapor bath 30 to 33 °C. The sub-layer of all membranes is prepared out solution containing 12 % CA, 20 % water, 60 wt% loading of AC, with acetone as a solvent; 6 minutes residence in water vapor before immersing into a water containing coagulation bath. Casting thicknesses for sub layer and top layer were 475 and 75 µm respectively. White lines in images (1) indicate the transition between MMM layer and co-casting layer.*

SEM characterization showed that an increase in CA concentration lead to a decrease of the pore size of the top surface. The bigger pores on the top surface from membranes cast from 10 wt% CA solution resulted from the lower viscosity, higher mobility of molecules and faster in diffusion of water vapor. Irregular pore sizes and denser surface structures are observed when membranes are cast from a 14 wt% CA solution.

Co-cast membranes from dilute polymer solutions possess thin and porous skin layers with high clean water fluxes. Furthermore, membranes co-cast with low polymer concentrations show no significant transition layer between the membrane co-cast layer and the adsorption layer. On the other hand, membranes prepared from casting solutions with a high polymer concentration exhibited a discrete thicker skin layer. By increasing the polymer concentration in the co-casting layer fewer pores are formed. This phenomenon was also observed by Matsuyama[14].

The thickness of the co-casting layer is hardly affecting the MMM permeability. However, to enhance the active sites density in the whole MMM, a thin co-casting layer is preferred. The main task of the co-casting layer is to prevent particle loss, improve the biocompatibility and control transport of molecules to the active sites in the MMM.

MMM co-casting can be useful for any application field where membranes with adsorptive properties are concerned. Changing the surface pore size opens the possibility to develop MMM in which transport of solute by diffusion, convection and combination of diffusion and convection can be controlled. Altering the surface chemistry of the MMM by polymer co-casting, opens the possibility to obtain MMM with different hydrophilic and hydrophobic moieties. The presence of hydrophilic and hydrophobic domains improves the biocompatibility of the adsorber, which is desirable in blood purification applications[18, 19]. We report the use and performance of these membranes for blood toxins removal in the next chapters (chapter 6 and 7).

5.4. Conclusions

Various types of the MMMs AC have been prepared by direct immersion precipitation, and by a combination of immersion precipitation preceding with evaporation or vapor induced phase separation. We have demonstrated that the resulting membrane morphology can be tailored from dense asymmetric to porous symmetric. Dense films are obtained by direct immersion of the polymer solution into a water containing coagulation bath or by dry air solvent evaporation. When solvent evaporation takes place in humid air,

membranes containing porous structures without macrovoids are formed. MMM AC with open top surfaces and high clean water fluxes are obtained by co-casting the MMM layer with a particles-free cellulose acetate solution. Co-casting with a particle free polymer solution is especially useful to tailor the membrane permeability independent of the composition and particles load of the active layer. Besides this particle loss during production and direct particles - blood contact during processing can be avoided, which enormously increase the blood compatibility of the Mixed Matrix Membrane. The best membranes obtained are the double layer MMMs that are formed by solvent evaporation in humid air before immersion precipitation. They demonstrate more porous structure, higher flux and better mechanical strength than other obtained MMMs.

5.5. Acknowledgements

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5.6. List of Symbols and Abbreviations

Symbols

Mw	*Molecular weight*	*g/mol*
sd	*Swelling degree*	*%*
Wt	*Weight*	*g*
ε	*Porosity*	*%*

Abbreviations

AC	*Activated carbon*	*WV*	*Water vapor*
CA	*Cellulose acetate*	*MMM*	*Mixed Matrix Membrane(s)*
DA	*Dry air*	*PVDF*	*Polyvinylidene fluoride*
NIPS	*Non-solvent induce phase separation*	*VIPS*	*Vapor induce phase separation*
WB	*Water batch*		

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Chapter

6 Carbon Based Mixed Matrix Membranes for Blood Toxin Removal

Abstract

The new platform technology, adsorptive Mixed Matrix Membranes (MMM), combines high throughput, high capacity, and robust operation. Any sorbent or functionalized particle can be incorporated into a macroporous membrane structure. In this study, MMM adsorbers are developed for blood toxin removal by incorporation of activated carbon into cellulose acetate macroporous membranes. The membranes are prepared by water vapor induced phase separation followed by an immersion precipitation step. To improve biocompatibility and to avoid small activated carbon particles being in direct contact with the blood stream, the MMM are co-cast with a particle-free cellulose acetate solution. The MMM possess an open and interconnected macroporous structure with a high loading of activated carbon available for blood toxins adsorption. The prepared membranes show a high static as well as dynamic Creatinine (Crt) adsorption capacity. The Crt adsorption isotherm is of the Langmuir type, with a maximum adsorption capacity of 15 mg Crt/g membrane (equal to 27 mg Crt/g embedded activated carbon). The dynamic adsorption capacity is evaluated in different process configurations with various process parameters. High dynamic Crt removal is obtained in dead-end, cross flow filtration, as well as in dialysis mode. The MMM AC has characteristics of hemodialysis as well as hemofiltration integrated with adsorption, in which blood toxins are removed in one step.

Keywords: *Mixed Matrix Membrane (MMM), activated carbon, creatinine, adsorption, hemodialysis and hemoperfusion*

6.1. Introduction

A hemoperfusion system, extracorporeal blood purification based on sorbent adsorption, has been developed with the use of activated carbon (charcoal) as adsorbent. Activated carbon has recently been applied to increase the efficiency of hemodialysis, to remove toxic compounds during kidney or liver failure, autoimmune diseases and encephalopathy[1, 2]. Activated carbon is also effective in regeneration of dialysate[3-5]. Furthermore, the use of activated carbon hemoperfusion columns are introduced to improve protein-bound and water soluble toxins removal, detoxification by drugs overdoses and poisonous and endotoxin removal[6-10]. Activated carbon based hemoperfusion is attractive, since it provides large adsorptive surface areas (up to 500 m^2/g of adsorbent) for low costs[11].

Because sorbent systems are not able to the control fluid balance (no removal of excess water) and due to its limited urea sorption, hemoperfusion cannot fully substitute hemodialysis[2]. Hemodialysis on the other hand purifies only based on molecular weight with a lack of molecular recognition. Dialysis has a high removal rate of low molecular weight substances, a poor removal rate of middle molecular weight substances and is unable to remove high molecular weight substances. The idea of developing a therapy that combines two or more techniques to achieve separations that can not be achieved with one single technique is desired. Davankov et al.[12] suggest that by combining the strengths of dialysis membranes with the adsorption power of high surface area adsorbents, a more efficient artificial kidney might be produced. Furthermore, plasma filtration could be coupled with adsorption, as in CPFA (coupled plasma filtration adsorption)[13]. CPFA is a newly developed technique that uses a plasma filter membrane in series with a charcoal and resin cartridge. The plasma filtrate is circulated through the adsorptive cartridges and re-infused in the extracorporeal circuit. Ash et al. [14] have developed the Biologic-DTPF (detoxifier plasma filter) system. This device combines hemodiadsorption (dialysis of blood against powdered sorbents, the biologic-DT system) with push-pull sorbent pheresis (plasma filter with a separated sorbent system, the PF add-on module). The DT system is capable in removing low molecular weight compounds while the PF system is able to remove large molecules by allowing a direct contact between plasma and sorbent. By this method protein-bound toxins and endotoxins can be removed from the blood stream. The DTPF system was tested clinically with a limited number of patients with liver failure, sepsis and multi-organ failure[7]. Mitzner et al.[15] have developed the so called MARS (*Molecular Adsorbent*

Recirculating System) membrane, which has been clinically used as extracorporeal support during liver and kidney failures. It relies on dialysis across an albumin-impregnated high flux polysulfone membrane against an albumin-rich (20 %) dialysate. The albumin dialysate is in turn regenerated by a combination of a sorbent system and a hemodialysis membrane. The MARS membrane system aims to eliminate bilirubin, bile acids, aromatic amino acids, medium chain fatty acids and cytokines. The MARS membrane system was also clinically used by brain dysfunction and multiple organ failure[16].

The use of adsorbents in medical applications are often excluded for safety concerns, such as release of small micro-particles, poor homogeneity and biocompatibility, as evidenced by thrombocytopenia and neutropenia[13, 17-19]. Therefore, direct contact of activated carbon with blood components in a hemoperfusion circuit should be prevented. Many attempts have been made to overcome these problems by coating the particles with a polymer or by encapsulating the activated carbon particles in a polymeric shell. However, the additional layer reduces the efficiency of the hemoperfusion treatment[7, 20] and the coated sorbents still may be involved in microemboli formation due to non-uniformity, incomplete coating, mechanical abrasion of the naked carbon surfaces prior to casting and the fragility of the capsule. Applying double coatings could solve these problems[21]. The hemoperfusion treatment uses large palletized granular sorbents, which are at least 500 μm till 1000 μm in diameter[22-24]. The large particle size of sorbent granules in hemoperfusion columns limits the available active surface area and are therefore rapidly exhausted[13]. To increase the effective active surface area hemoperfusion should be carried out with smaller sized particles. However, small particles are not applicable in hemoperfusion because of several drawbacks and limitations including column plugging, channeling, column packing and high pressure drop[7]. Moreover, smaller particles are more difficult to effectively deal with in circuits and difficult to encapsulate.

In clinical practice of blood purification therapies, membrane separations are combined with adsorption processes. Therefore, it is essential to develop or modify a novel hemodialysis (hemofiltration) membrane which integrates filtration and adsorption. Most of the extracorporeal blood detoxification treatments including kidney and liver support do not work without adsorptive purification systems[7, 13, 20, 25, 26].

A new approach to obtain adsorptive filtration membranes is integration of small functionalized particles (sorbents) with membrane filtration. This concept is reliable and efficient in biologics separation processes. During past few years the Mixed Matrix

Membranes (MMM) technology is developed to capture proteins, enzymes and bilirubin[27-31]. As described in the previous chapters, these membranes are established by embedding adsorbents (functionalized particles) in polymeric macroporous structures, formed by a phase inversion method. The MMM concept can be applied for small particles, thereby increasing the active surface area. This approach prevents mechanical particle stress by coalition and agitation, which might result in fragmentation of the activated carbon particles. The advantage of particle loaded membrane chromatography is the high blood flow rate, since the molecular transport is predominantly controlled by convection. It overcomes the limitations in conventional hemoperfusion, including inhomogeneous blood flow distribution and stagnation phenomena within the unit[24, 32]. Moreover, the pressure drop across the MMM is lower than in similar particle sized packed bed systems. The MMM adsorbers concept offers possibilities to integrate hemoadsorption and hemofiltration in one unit operation.

In this chapter, we are applying the MMM adsorbers concept, which offers an alternative approach for blood toxins removal by blood purification membranes meanwhile increasing the biocompatibility of hemoperfusion sorbents (activated carbon). The activated membranes are prepared by incorporating activated carbon into a biocompatible macroporous polymeric matrix without affecting the activity. The MMM adsorbers are prepared by phase inversion methods. To improve the blood-compatibility, and to avoid mixing of small activated carbon particles with the blood stream, the MMM is co-cast with a particle-free cellulose acetate solution with a particle free top layer preventing blood components getting in contact with adsorbent particles. Creatinine (Crt) is used as model blood toxin to characterize the membrane adsorber performance. The MMM are evaluated in different configurations as a function of various process parameters, in both static and dynamic mode.

6.2. Experimental

6.2.1. Materials

Cellulose acetate with an average acetate content of 38.8 mol % was purchased from Aldrich (M.W≅30.000) and used as membrane material without further modification. Acetone (Merck) was employed as solvent. Ultra pure water, prepared using a Millipore Milli-Q plus purification unit, was used as additive in the polymer solution and as non-solvent in the coagulation bath. Activated carbon Norit A Supra EUR, kindly supplied by Norit Netherlands B.V. was used as adsorbent particles. Creatinine was used as a model blood

uremic-toxin[33, 34] to investigate the adsorption capacity of the MMM. BSA obtained from Fluka was used to evaluate non specific protein adsorption.

The buffer solutions were freshly prepared in ultrapure water. Tyrode buffer was chosen as representing adequately the ionic solutes, pH and glucose concentrations of the blood plasma. The Tyrode buffer composed of 137 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11,9 mM $NaHCO_3$, 0.47 mM NaH_2PO4, 5.5 mM and glucose (pH=7.4). The dialysate buffer contained of 140 mM NaCl, 2 mM KCl, 1.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 35 mM $NaHCO_3$, 4 mM acetate and 5.5 mM glucose (pH=7.4).

6.2.2. Membrane preparation and characterization

The Mixed matrix membranes containing activated carbon (MMM AC) were prepared by water vapor induced phase separation followed by immersion in a water containing coagulation bath as described in **chapter 5 (section 5.2.2.)**. The membranes were characterized in terms of morphology, porosity, clean water fluxes, pore sizes and Crt adsorption capacity.

6.2.3. Adsorption experiments

6.2.3.1. Batch experiments

The creatinine adsorption capacity of the prepared MMM AC was determined by batch experiments. Creatinine (Crt) concentrations of 0.10-0.12 mg/mL were employed in the adsorption experiments. Known membrane weights were 24 hours equilibrated in a phosphate buffer to ensure a steady inner structure during the adsorption experiments and afterwards transferred in the Crt solution. The Crt solutions were prepared in the Tyrode buffer as described above. The samples containing a known Crt concentration were incubated with an exact amount of membranes in sealed containers under continuous shaking at 25 °C. The MMM AC adsorbs the Crt thereby reducing the Crt concentration in the bulk. The equilibrium Crt concentration after 24 hours was determined by spectrophotometric (Philips analytical PU 8720 UV-VIS spectrophotometer) analysis. The Crt depletion was measured at 230 nm with 2 mm quartz cuvettes. The amount of adsorbed Crt, q_{eq} [mg Crt /g membrane], at equilibrium is calculated by:

$$q_{eq} = \frac{(C_0 - C_{eq}) \cdot V}{W_{membrane}} \qquad \textbf{Eq. (6.1)}$$

Where C_0 is the initial Crt concentration [mg/mL], C_{eq} is the Crt concentration at equilibrium [mg/mL], V is the volume of the Crt solution [mL] and $W_{membrane}$ is the weight of the dry membrane [g]. For the Crt absorbance on pure activated carbon particles experiments, the Crt solutions were pre-filtered before the UV-measurements to avoid possible light scattering by dispersed carbon particles using 0.45µm Spartan 30 (regenerated cellulose) filters.

6.2.3.2. Adsorption isotherm

In order to determine the Crt adsorption isotherm, batch adsorption experiments were carried out with different amounts of the MMM AC using the same initial Crt concentration to end up with different equilibrium concentrations. From literature, it is well known that the Langmuir adsorption model can be applied to determine the adsorption isotherm of solutes on activated carbon[35-37]. The Langmuir equation can be written in linear form as:

$$\frac{1}{q_{eq}} = \frac{1}{q_m} + \frac{K_d}{q_m} \times \frac{1}{C_{eq}} \qquad \textbf{Eq. (6.2)}$$

The parameters q_{eq}, the adsorbed Crt concentration into the membrane adsorbers [mg/g] and C_{eq}, the equilibrium Crt concentration in the bulk solution [mg/mL] are experimental data. A plot of $1/C_{eq}$ versus $1/q_{eq}$ allows determining q_m, the maximum adsorption capacity [mg/g] and K_d, the dissociation constant [mg/mL], which can be calculated from a linear curve fit.

6.2.3.3. Non-specific BSA adsorption

BSA adsorption of suspended AC particles, the MMM AC, the particles-free CA membrane was investigated to determine effect of non-specific adsorption of the blood plasma protein which is of prime importance for the biocompatibility of the materials. The adsorption of bovine serum albumin (BSA) was performed by incubation an exact amount of the activated carbon, the particle-free cellulose acetate membrane and the MMM AC in a protein solution. BSA concentrations of 2 mg/mL in a Tyrode buffer pH=7.4 are employed in the adsorption experiments. A known weight of sorbent is transferred in the protein solution. The samples were kept under continues shaking at 25 ^oC for 24 hours. After 24 hours the absorbance of the protein solutions were measured spectrophotometrically at 280 nm. For pure activated carbon experiments the solution was pre-filtered with 0.45 µm Spartan 30 filters before measuring the UV absorbance.

6.2.3.4. Dynamic adsorption experiments

The dynamic adsorption experiments were investigated in three different modes: a) dead-end filtration, b) cross flow filtration and c) dialysis. Figure 6.1 presents the schematics of the used dynamic experimental set-ups.

a. Dead-end filtration

The dynamic adsorption performance of flat sheet MMM was measured at constant permeation rate using a stirred dead-end filtration cell. To overcome the limited adsorption capacity and to average out membrane inhomogeneities, 57.7 cm^2 (6 layers of 0.787 g) of adsorptive membranes were stacked. 125 mL Crt solution (0.1 mg/mL in Tyrode buffer pH 7.4) was recirculated through the membrane stack. During the experiment, samples from the feed reservoir were taken to determine the Crt depletion as function of time. After the UV-absorption measurement the samples were returned to the feed reservoir. The experiments were evaluated at three different fluxes (42, 85, 212 $Lm^{-2}h^{-1}$) during six hours operation.

b. Cross flow filtration

The cross flow filtration was performed at constant permeate flux at (22-23 ^oC). Both retentate and permeated were recirculated back to the feed reservoir. The membrane area used was 58.8 cm^2 (single layer of 0.802 gram). The cross flow filtrations were performed using a Minitan-S cross flow module (Millipore) with an effective membrane cross flow area of 30 cm^2. Before starting the experiment, the membranes were wetted with phosphate buffer pH 7.4. Crt solutions, 125 mL of 0.1 mg/mL in Tyrode buffer, were used as feed solution. During the experiments, samples of the feed solution (reservoir) were taken to determine the Crt depletion as function of time by measuring the UV-absorption at 230 nm. After measuring the UV-absorption, the samples were returned to the feed reservoir. The cross flow filtration experiments were evaluated for six hours before the process was stopped. The effect of the permeate rate on the Crt adsorption was investigated with permeate fluxes ranging from 30 to 100 $Lm^{-2}h^{-1}$.

c. Dialysis test

The diffusive transport of Crt was investigated using a two-compartment dialysis cell at room temperature (Figure 6.1C). The first compartment was filled with Crt in the Tyrode buffer and the second compartment was filled with dialysate buffer. The compartments were separated by the MMM AC. The volume of each compartment was 105 mL and the active membrane area was 19.6 cm^2 (0.25 gram). Both solutions were recirculated at flow rate of 3 mL/minute using a peristaltic pump. During the experiment,

1 mL samples were taken as function of time to determine the Crt concentration in both compartments. Immediately after measuring the UV absorbance, at 230 nm the samples were returned to the compartments. The dialysis experiments were evaluated for six hours before the process were stopped.

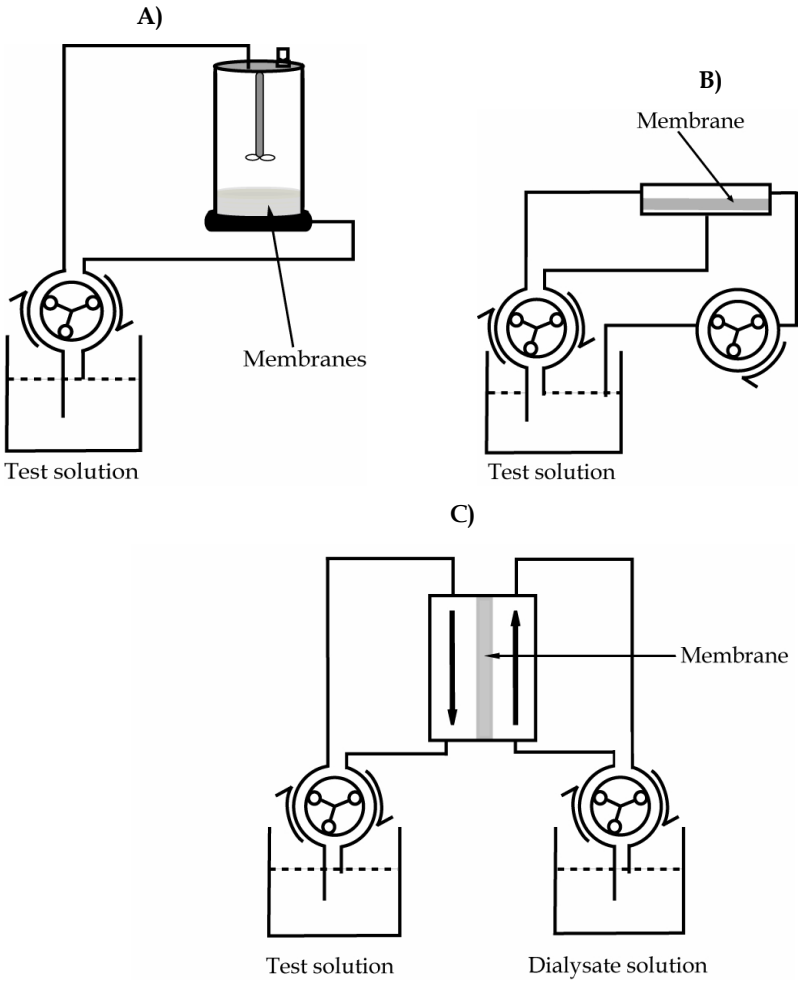

Figure 6.1. *Schematic representation of the experimental set-ups used in the dynamic adsorption experiments. A) Dead-end filtration. B) Cross flow filtration. C) Dialysis. All systems have complete retentate and permeate recirculation.*

6.3. Results and Discussions

The results and discussions section firstly present the membrane performance, which is evaluates in both static and dynamic modes as function of different process parameters and different process configurations. Followed by the feasibility of these membranes in extracorporeal blood purification applications.

6.3.1. Static adsorption of creatinine

Static creatinine (Crt) adsorption capacities for AC particles, AC particles embedded in cellulose acetate membranes and bare cellulose acetate membranes are studied batch wise. For this the sorbents are immersed for 24 h into a 0.1 mg Crt/mL Tyrode buffer solution, pH 7.4 at room temperature. The adsorbed Crt amount is presented in Table 6.1. Bare CA does not adsorb any creatinine. The activated carbon particles adsorb 26 mg Crt/g adsorbent. The activated carbon load for the membranes is 60wt% for the membranes without a co-cast layer; the membranes with a co-cast layer have because of the additional layer an AC load of 55wt%. The for AC weight normalized adsorption capacity of activated carbon embedded in the membranes is similar to the adsorption capacity of free AC particles, being 26 mg/g sorbent.

Table 6.1 Static Crt adsorption onto pure cellulose acetate membranes, activated carbon (AC) particles, and different type of MMM AC membranes.

Preparation method		**Adsorbed creatinine**	
		(mg/g membrane)	**(mg/g adsorbent)**
Pure CA		0.0	0.0
AC particles		26.0	26.0
MMM without co-casting	Water vapor	14.5	24.2
MMM without co-casting	Water bath	14.0	23.3
MMM co-casting	Water vapor	13.5	24.5
MMM co-casting	Water bath	13.0	23.6

Overall, the entrapped activated carbon particles are very accessible for Crt adsorption. The high porosities and the good pore interconnectivity in entire cross section of the MMM AC contributes to entire accessibility of the activated carbon particles in the MMM.

The additional co-cast layer prevents particle loss and possesses an open structure that acts as selective barrier for large molecules, but does not show any retention for small sized molecules like Crt. The slightly lower Crt adsorption capacity of the co-cast layer MMM AC is related to the contribution of the non adsorbing co-cast layer to the total adsorbent weight. Table 6.1 confirms that the cellulose acetate matrix does not contribute to the Crt adsorption capacity of the MMM AC.

The unrestricted accessibility of activated carbon particles in the MMM can also be visualized by measuring the Crt adsorption in time of suspended activated carbon particles and activated carbon particles entrapped in the MMM Figure 6.3. The Crt adsorption capacities are measured during 24 hours. The figure clearly shows the fast adsorption kinetics within the first 3 hours, which cover approximately 50 % of the total adsorbed Crt. The Crt adsorption kinetics of embedded activated carbon particles in the MMM is close to that of suspended activated carbon particles. Indicating that the macroporous polymer matrix hardly contributes to the Crt diffusion resistance. The obtained equilibrium capacity after 24 hours is 13.3 mg Crt/g MMM, which is equal to 24.2 mg Crt/ g activated carbon in the membrane (co-cast layer contributes to 5 % of the total MMM AC weight).

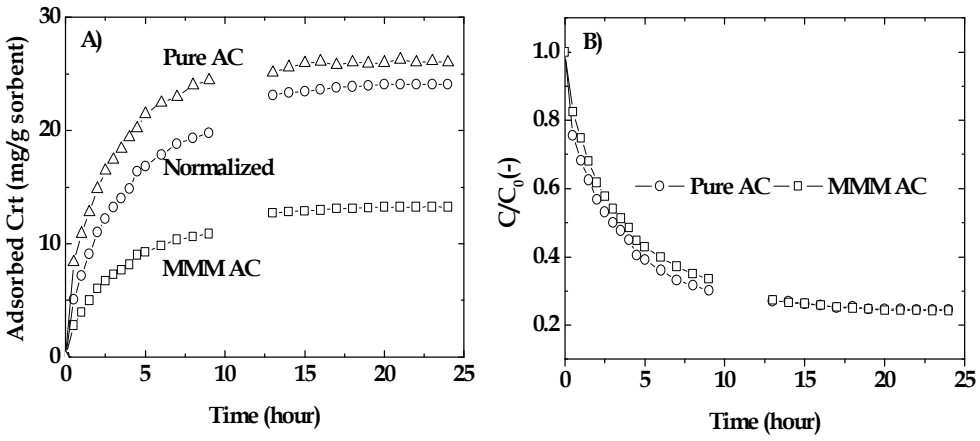

Figure 6.3. *A) Kinetic Crt adsorption onto the MMM AC co-cast with a particle-free CA layer, the membranes are prepared by vapor induce phase separation preceding the immersion in a water coagulation bath,. B) Reduction of creatinine concentration in time during incubation of the MMM AC. Starting concentration of 0.1 mg Crt/mL in Tyrode buffers.*

6.3.2. Crt adsorption isotherm and adsorbent effectiveness

The Crt adsorption isotherm is determined to appoint the maximum Crt adsorption capacity of MMM AC. The solid line in figure 6.4 presents the best Crt adsorption Langmuir isotherm curve fit. The maximum adsorption capacity (q_m) is 15 mg Crt/gram MMM AC, which is equal to 27 mg Crt/g AC in the MMM. The dissociation constant (K_d) value is 0.04 mg/mL. The obtained maximum Crt adsorption capacity is comparable with highest literature data. Lee et al. [37] reported a maximum Crt adsorption capacity being 30 mg Crt/g activated carbon in a phosphate buffer of 37 °C. Tsai et al. [38] used molecular imprinted (MIP) for Crt adsorption. Using the Langmuir isotherm adsorption model he found a maximum adsorption of 5 mg Crt/g MIP. Malchesky et al.[39] reported total Crt removal using extracorporeal implantable blood interchange devices with activated carbon as adsorption material. Using a 0.94 mg Crt /mL starting concentration they found a total adsorption capacity of 26.0 mg Crt/g charcoal. This value is in agreement with the data reported by Lee et al.[37] and our own mixed MMM AC data.

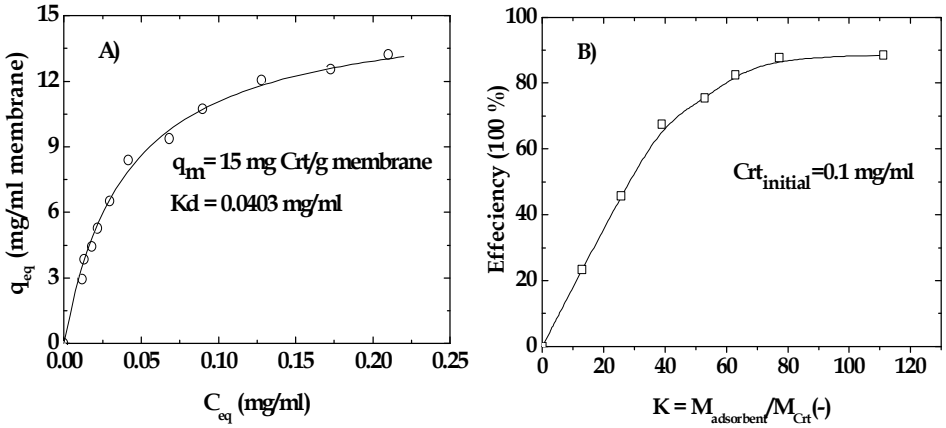

Figure 6.4. *A) Crt adsorption isotherm of MMM AC. The solid line indicates that there is a good agreement between the experimental data and the best curve fit using the Langmuir equation. B) Efficiency of Crt adsorption versus the mass ratio of the MMM AC over creatinine. The MMM AC are prepared by water vapor induced co-casting, temperature 30-33 °C.*

The Crt adsorption efficiency can be reported as the percentage removed Crt as function of the adsorbent weight divided by the initial Crt concentration. These studies are executed in batch experiments with initial Crt concentrations of 0.1 mg/mL in the Tyrode

buffer. Figure 6.4B proves that the Crt removal efficiency improves using higher adsorbent - Crt concentration ratios. By increasing the sorbent - Crt ratio up 80, the Crt removal efficiency reaches 90 %.

The high Crt adsorptive capacity of activated carbon is not only determined by its porosity (physical nature) but also by its chemical composition (functional groups on the surface), both factors are contributing to the adsorption performance. Therefore, large activated carbon surface areas are needed to obtain high Crt adsorption. Since Crt is a rather small molecule, 0.5 nm, there is hardly any steric hindrance effect that limits intraparticle Crt pore penetration in activated carbon. Functional groups, especially oxygen containing functionalities and delocalized electrons in the structure determine the chemical behaviour of activated carbon surfaces[40]. Since Crt molecules are hydrophilic, hydrogen bonding and dipole-dipole interactions are assumed to be a major interaction forces between Crt and activated carbon[38, 41].

6.3.3. Non specific BSA adsorption

Protein adsorption at blood-biomaterial interfaces is of prime importance for the biocompatibility of the material. The main factor promoting protein adsorption on surfaces is the hydrophobic interaction force between the sorbent surface and the protein molecule. BSA adsorption on particle-free CA membranes, MMM AC and AC particle suspensions is investigated to determine non-specific BSA (a bovine blood plasma protein) adsorption on entrapped active carbon particles. We have chosen BSA rather than HSA in order to compare the adsorption behavior with previous developed membranes (sorbents). Table 6.2 presents the BSA adsorption at the different substrates. Activated carbon particles show a high non specific BSA adsorption. The BSA adsorption capacity from a 2 mg BSA/mL solution is up to 50 mg/g activated carbon particles. Contrary, the BSA adsorption capacity of entrapped activated carbon particles in cellulose acetate membrane, drops by a factor 50 down about 1 mg/ sorbent, which is equal to the pure cellulose acetate BSA adsorption capacity. This value is in agreement with data reported by Liu et al.[42], who measured 0.3 mg BSA/g membrane onto pure CA membranes, feed concentration 0.5 mg BSA/mL. The low BSA adsorption of the MMM AC demonstrates that entrapped activated carbon particles in the MMM are not accessible for high molecular weight BSA molecules where low molecular weight Crt molecules have free access. This makes that the MMM AC is very suitable for removal of toxic Crt out BSA containing (plasma) solutions.

The improved blood compatibility of active carbon after embedding into porous cellulose acetate can be explained by formation of a cellulose acetate shell (coating) around the entrapped particles. SEM micrographs (Figure 5.4 and Figure 6.5) confirmed that activated carbon particles in the MMM are coated by cellulose acetate. The formation of a CA coating is a major additional advantage of entrapping activated carbon particles in a CA support, particularly in blood purification processes where the blood biocompatibility of sorbents is a major concern.

Table 6.2 Non-specific BSA adsorption onto pure CA membranes, activated carbon particles and MMM AC

Material	Adsorbed BSA (mg/g adsorbent)
Pure cellulose acetate membranes	1
Activated carbon particles	50
MMM AC	1

Covering particles with cellulose acetate also increases the hydrophilic nature of carbon particles. It is well known that cellulose acetate has been used as polymeric support for activated carbon coatings (and another sorbents) in order to improve the biocompatibility[43-46]. Meanwhile, the membrane possesses a porous structure and a very good pore interconnectivity so that harmful substances can utilize all active sites. The effects

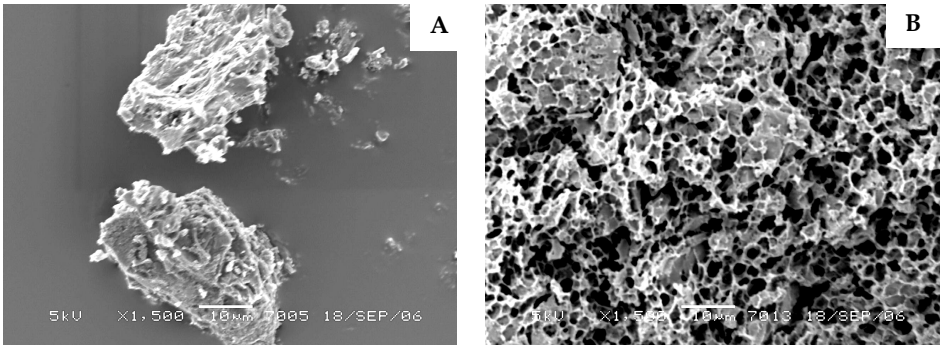

Figure 6.5. *The effect of embedded particles on surface coating activated carbon particles. SEM micrographs of pure activated carbon particles (A) and activated carbon embedded in a CA support, cross section (B), magnification 1500x. Because of particle coating and the co-cast top layer there is no direct blood contact with the embedded activated carbon particles in the MMM AC.*

of a cellulose acetate coating layer has also been demonstrated by Tijssen et al.[47]. The BSA adsorption of coated particles was approximately 0.8 mg BSA/g sorbent which is close to the non-specific adsorption value of the MMM AC. These results proof that the biocompatibility problems of sorbent hemoperfusion processes can be solved with the MMM approach. Furthermore, the biocompatibility of the blood side of the MMM adsorber can even be further improved by co-casting the MMM with a biocompatible layer.

6.3.4. Dynamic creatinine adsorption

a) Dead-end filtration

Dynamic adsorption of Crt from a plasma mimicking solution (in absence of BSA) was examined. A tyrode buffer containing 0.1 mg Crt /mL is perfused through a dead-end ultrafiltration module containing a 6 stack of MMM AC. The adsorptive membranes are stacked to average out membrane heterogeneities, porosity and thickness variations. Figure 6.6 presents the Crt decrease in solution and the amount of adsorbed Crt onto the MMM AC during 6 hours filtration at different fluxes: 42, 85 and 212 $Lm^{-2}h^{-1}$ as function of permeate volume.

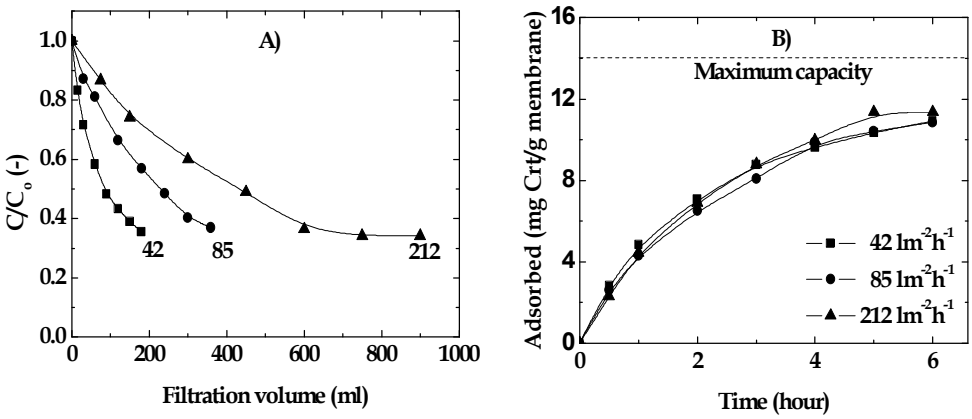

Figure 6.6. ***A).*** *Crt removal rate using different filtration fluxes.* ***B)*** *Crt adsorption as function of the filtrated volume using different fluxes. The initial Crt concentration is approximately 0.1 mg/mL. Total feed volume 125 mL. Experiments are performed at 25 ^oC. Duration of all filtration runs is 6 hours.*

Total blood toxin removal is a very important demand in hemoperfusion (adsorption). The total Crt removal in a recirculation mode can be controlled by the flux or by recirculation of the plasma. The Figure 6.6 demonstrates that the dynamic Crt depletion

(removal) from the feed solution depends on the permeate flux. By changing the filtration flux the Crt residence time in the membrane structure also changes. At low fluxes, Crt molecules have more time to reach active sites that are located in diffusive pores, resulting in more a pronounced decrease in Crt concentration. On the other hand the low fluxes restrict the volume of plasma treated. By increasing the flux the treated volume increases and the membrane residence time is shorter. The results depicted in figure 6.8B indicate that total amount Crt removal in six hours recirculation is almost independent of the filtration flux.

b) Cross flow filtration

Dynamic Crt removal rate by MMM AC is investigated in a complete recycle cross flow mode by filtrating a Tyrode buffer containing 0.1 mg Crt /mL for six hours. The influence of the permeate flux on the adsorption behavior is investigated from 30 to 100 $Lm^{-2}h^{-1}$ at cross flow velocity of $1.5x10^{-2}$ m/s to $3.7x10^{-2}$ m/s (stage cut was lower than 0.05). Figure 6.7A) presents the Crt depletion in the feed solution as function of the filtrated volume.

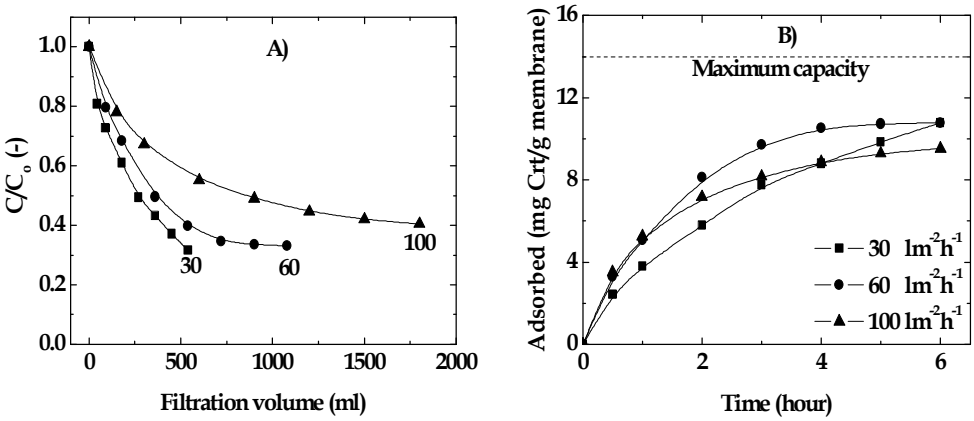

Figure 6.7. *Crt removal by MMM AC adsorption during cross flow filtration.* ***A)*** *The influence of the permeation on the Crt removal rate.* ***B)*** *Crt adsorption as function of the filtrated volume for different fluxs. The initial Crt concentration is 0.1 mg/mL and the total feed volume is 125 mL. The experiments were performed at 25 ^oC for six hours.*

We observed that changing the flux from 30 to 100 $Lm^{-2}h^{-1}$ influences the kinetic Crt adsorption. By increasing the flux up to 100 $Lm^{-2}h^{-1}$ the decrease in Crt adsorption capacity is significantly. This can be contributed to flow maldistribution influenced by the MMM AC morphology, i.e. inhomogeneity within a single membrane layer. The toxins containing feed permeates preferentially through the large pores. Adsorption in smaller pores only occurs by

slow diffusional processes, for which the membrane residence time is not sufficient, hence these sites are less likely accessed by Crt. Using a complete recirculation mode for 6 hours the Crt adsorption capacity for all filtration fluxes is almost completely utilized. However the filtration flux of 60 $Lm^{-2}h^{-1}$ is most effective, since with this flux the Crt adsorption capacity is utilized within 4 hours of filtration (figure 6.7B)).

For a successful application in blood purification, an adsorbent should be capable of removing Crt in the presence of serum albumin. To investigate the Crt adsorption of MMM AC in the presence of BSA a Tyrode buffer containing 0.1 mg Crt /mL and 2 mg BSA /mL was filtered in cross flow mode through a single layer of the MMM AC. Figure 6.8 presents the Crt adsorption capacity in presence of BSA during six hours of cross flow filtration. The results prove that the MMM AC is indeed capable to selectively capture Crt out of a BSA containing solution. Since Crt is an un-bounded uremia blood toxin, its adsorption capacity is only slightly influenced by presence of BSA[48].

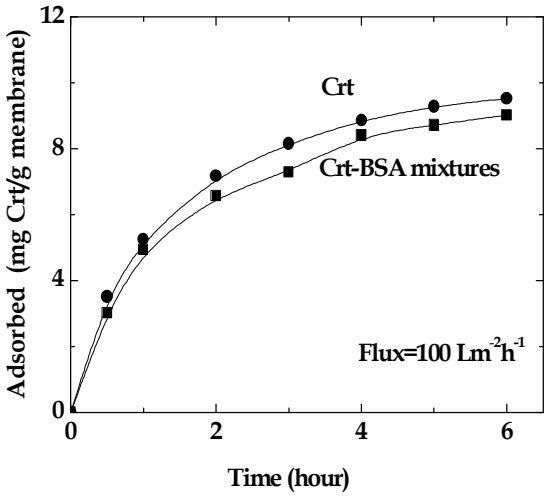

Figure 6.8. *Creatinine adsorption onto MMM AC in the presence of BSA. Concentration BSA 2 mg/mL and creatinine concentration 0.1 mg/mL.*

c) Dialysis

The MMM AC offers the possibility to integrate adsorption and hemodialysis treatments in one step. We investigated the performance of the MMM AC for this purpose. The adsorptive membrane was placed in a dialysis cell, which separates two compartments:

one containing the Crt solution and the other containing the dialysate. The total Crt removal from the sample solution and the Crt concentration in the dialysate side were determined in time. The amount of Crt removed by adsorption onto the MMM AC can be calculated by subtraction the increase in Crt in the dialysate cell from the Crt that is removed from the sample side. Figure 6.11 presents the Crt removal during six hours of dialysis. Crt is significantly removed during the dialysis process by a combination of adsorption in the MMM AC and diffusion across the membrane.

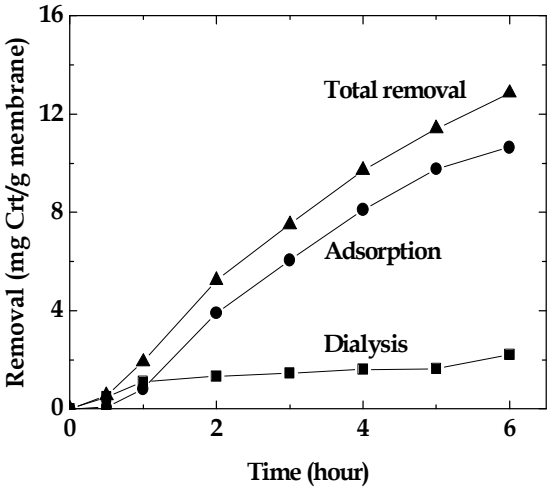

Figure 6.9. *Creatinine removal during adsorptive dialysis. The MMM AC was prepared by vapor induced phase separation, 6 minutes, before immersion into water coagulation bath. Starting concentration of Crt was approximately 0.1 mg/mL.*

The relative contribution of adsorption and dialysis to the total removal of Crt can clearly be observed (Figure 6.9). This indicates that most of Crt is captured by active carbon when diffusion through the membrane. The Crt removal by adsorption contributes to amore than 83 % of the total removal. Even after 6 dialyzing hours, the adsorption capacity of the MMM AC is not fully utilized yet, which means that Crt cross over to the other compartment still is minimized. An increase in Crt removal by diffusion (dialysis) across the membrane can be expected when all of the active sites in the MMM AC are occupied (breakthrough).

These results proved that the integration of adsorption functions in dialysis membranes significantly improves the performance of a dialysis treatment by preventing the diffusion of toxins between the two fluid streams (compartments). The adsorptive MMM can be adapted

to different extracorporeal blood purification treatments, i.e. hemoperfusion, hemofiltration and hemodialysis. The total amount Crt removed during a six hour treatment depends solely on the MMM adsorption capacity. The MMM concept is ensure as safe treatment since only parts of the blood perfuses through the adsorbent and blood cellular component do not interact with the embedded sorbents.

6.4. Conclusion

Mixed matrix membrane (MMM) adsorbers are studied for removal of blood toxins. The MMM adsorbers are prepared by incorporating activated carbon into macroporous cellulose acetate membranes. The membranes prepared by direct immersion precipitation in a water containing coagulation bath contain a skin layer covering a macroporous substructure. Membranes prepared after a vapor residence time prior to immersion into a water containing coagulation bath contain a porous top layer covering a macroporous substructure. The biocompatibility of the membrane adsorber can be improved by co-casting with a particle-free cellulose acetate solution. The prepared membranes show a high static as well as dynamic Creatinine (Crt) adsorption capacity. The measured Crt adsorption isotherm is of the Langmuir type, with a maximum adsorption capacity of 15 mg Crt/g membrane, equivalent to 27 mg Crt/g embedded activated carbon. High dynamic Crt removal rates are obtained in dead-end and cross flow filtration, as well as in dialysis mode. The Crt removal is for all techniques more dependant on the Crt residence time in the MM AC than on the filtration flux. After six hours of adsorptive dialysis using a 0.1 mg Crt /mL solution the adsorption capacity is not fully utilized yet, which means a minimal Crt cross over between the dialysis compartment. The MMM AC technology is suitable for integration of hemofiltration and dialysis with adsorption.

6.5. Acknowledgements

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6.6. List of Symbols and Abbreviations

Symbols

Mw	*Molecular weight*	*g/mol*
$q_{desorbed}$	*Amount of protein desorbed*	*mg/g membrane*

q_{eq}	*Adsorption capacity at equilibrium*	*mg/g membrane*
q_m	*Maximum adsorption capacity*	*mg/g membrane*
R	*Loading*	*%*
V	*Volume*	*mL*
W	*Weight*	*g*
Wr	*Amount of adsorbent*	*g*
Wp	*Amount of polymer*	*g*

Abbreviations

AC	*Activated carbon*	*MMM*	*Mixed matrix membrane*
CA	*Cellulose acetate*	*NA*	*Not available*
BSA	*Bovine serum albumin*	*NIPS*	*Non-solvent induce phase separation*
Crt	*Creatinine*	*VIPS*	*Vapor-induced phase separation*
Kd	*Dissociation constant*	*WB*	*Water bath*
MIP	*Molecular imprinted polymer*	*WV*	*Water vapor*

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Chapter

7 Mixed Matrix Membrane Adsorbers for Endotoxin adsorption

Abstract

This chapter presents Mixed Matrix Membrane (MMM) adsorbers for endotoxin removal. The MMM are prepared by water vapor induced phase separation followed by immersion precipitation. The adsorptive capacity is obtained by embedding activated carbon (AC) particles and anion exchange resins (Lewatit M500) into porous cellulose acetate membranes. The MMMs were co-cast with particles-free cellulose acetate solutions to improve the biocompatibility and mechanical strength. The co-casting layer acts as selective layer that avoids particle loss and controls the transport of toxic compound to the active sites. The optimized MMM adsorber possesses a porous structure and contains a high particle load available for endotoxin adsorption. The membranes feature both high static as well as high dynamic endotoxin removal capacities. MMM AC displays a higher adsorption capacity than MMM M500. The high endotoxin adsorption capacity of MMM improves safety and modality of hemodialysis by blocking endotoxin membrane passage. The developed MMM is able to remove harmful substances by filtration, diffusion, convection and adsorption in one step during a hemodialysis or hemofiltration treatment.

Keywords: *Mixed Matrix Membrane (MMM), activated carbon, anion exchange, endotoxin, adsorption, hemodialysis, hemofiltration, hemoperfusion*

7.1. Introduction

Endotoxins or lipopolysaccharides (LPS) are components of the outer cell wall of gram negative bacteria. LPS consists of a lipid A anchor, a polysaccharide core and chains of repetitive carbohydrates. The repeating oligosaccharides are strain-specific for diverse gram-negative species and appear to be highly variable[1]. This leads to a wide range of molecular weights, 2 to 20 kDa, with an average molecular weight of 10-12 kDa. It is known that the lipid A component is responsible for the endotoxic action such as induction of fever, chill, hypotension, nausea, adult respiratory distress syndrome, vasodilatation, diarrhoea, disseminated intravascular coagulation and multi-organ failure[2, 3]. LPS can activate several pathways in the humoral and cellular immune defence resulting in the activation of blood complement and contact system of plasmatic coagulation. An initial inflammatory response syndrome (SIRS) is followed by three stages: sepsis, severe sepsis and septic shock. Sepsis (infection in the bloodstream) is a major cause of morbidity and mortality among hospitalized patients or in intensive care units[4]. With the increase of the stages from sepsis to septic shock the organ failure and mortality rates increase[5]. Many patients with endotoxic infections have progressive deterioration of organ functions which result in multiple organ dysfunction syndrome (MODS) and finally in multiple organ failure (MOF), which can eventually lead to death[4, 6]. Furthermore, LPS directly effect the activation of monocytes, neutrophils, endothelial cells and lymphocytes, which leads to the release of several cytokines, among them TNF-α, IL-1α, IL-1 β and IL-6[7] that appear to contribute to the onset of SIRS, sepsis and mortality of patient. Therefore, IL-1 (α and β) and TNF-α are the major contributing factors in enhancing the production of β_2-microglobulin which is the main factor in the complication of long-term hemodialysis[7, 8].

7.1.1. Endotoxin removal

High morbidity and mortality rates upon infection with endotoxins require suitable treatments to detoxify endotoxin (Et) contained in infuse solutions. Different approaches are possible to decrease the morbidity and mortality rates upon infection with endotoxin. When endotoxins are neutralized the activation of the cytokines is stopped and the effects of the endotoxin infections are minimized. The effects of endotoxin infections can be minimized when patients are treated with antibodies, inhibition agents or neutralizing proteins[9]. However these approaches have been clinically applied with little success[10, 11]. Endotoxin can also be detoxified by the administration of polymixin B[12], a widespread antibiotic active

against most strains of gram-negative bacteria. A disadvantage of polymixin B (PMB) is that it is neurotoxic, nephrotoxic and stimulates monocytes to release interleukin-1[13]. In view of the fact that drug therapy has failed to provide convincing results as yet, another approach has been gaining in treatment of sepsis[14].

Endotoxin, several pro- and anti-inflammatory mediators can be directly removed from the bloodstream of the patients by extracorporeal blood purification, i.e. hemofiltration (plasmafiltration) and hemoperfusion[15, 16]. The hemofilters used in these blood treatment systems have molecular weight cutoffs of 30-50 kDa. Convection of fluid across these membranes should carry molecules up to the cutoff weight. However, some endotoxins (cytokines) which aggregate or bind to much larger plasma proteins have shown limited removal[17]. In order to improve the efficacy of a blood purification system with septic patients, unselective adsorption on a cartridge (hemoperfusion) was coupled to plasmafiltration and conventional hemodiafiltration in a newly designed extracorporeal device, so called coupled plasmafiltration-adsorption (CPFA). Studies of this technique recently reported improved hemodynamics and survival in animal and human sepsis[18]. Recently, the in vitro study of the heparin induced extracorporeal lipoprotein fibrinogen precipitation (HELP) system[11] has shown to be efficient in removing endotoxins and TNF-α from plasma. Ash et al.[19] have introduced the BioLogic-DTPF (detoxification plasma filtration) detoxification system, which is able to remove excess cytokines and endotoxins from bovine plasma. Lately, Nakamura et al.[20] have reported the use of polymyxin B immobilized fiber (PMX-F) as a specific sorbents to remove endotoxins by direct hemoperfusion. However, the patient has to accept the risk that if polymyxin B, which is nephrotoxic and neurotoxic, leaches out the fiber kidney damage occurs[15, 21]. Furthermore, PMB has quite poor hemocompatibility for thrombocytes[22].

7.1.2. Back transport in hemodialysis

Therapy during renal failure requires the ongoing treatment of patients with hemodialysis. In the hemodialysis treatment, the blood stream containing uremic toxic compounds is dialyzed against a countercurrent flow of dialysate separated by a semipermeable membrane. The dialysate is proportioned in each hemodialysis unit and is inevitably contaminated with bacterial substances and endotoxins[23]. The issue of bacterial contamination of dialysate still represents a challenge for the attending nephrologists[24] despite the availability of elaborate water treatment facilities for many countries and centers

including the introduction of reverse osmosis, dialysate ultrafiltration, single-pass hydraulic circuit in dialysis machine. Investigations in dialysis centers revealed that a high percentage of the centers tested were using dialysis water and dialysis fluid exhibiting microbial contamination at levels above the standards set by the Association for the Advancement of Medical Instrumentation (AAMI). The main contributors to contamination were proven to be the water used for preparing the dialysis fluid, the bicarbonate concentrate, which promotes the growth of water-born microorganisms commonly detected in water and dialysate including several *Pseudomonas* species as well as other gram-negative bacteria such as *Alcaligenes species, Moraxella and Corynebacteri*[25]. Furthermore, biofilm may be developed in the dead spaces in the fluid distribution system[26]. Hence, inadequate maintenance of tanks, improper design and inadequate microbiological monitoring of water treatment systems promotes biofilm growth on various conduits of the hemodialysis machines and the water treatment systems[23], resulting on high level contamination of dialysate. Even when dialysis machines are disinfected daily, biofilm contamination may not be completely eradicated[27].

Several studies have shown that both in low- and high-flux hemodialysis membranes back-transport phenomenon are unavoidable, i.e. Endotoxin transfer from contaminated dialysate fluids to the blood stream are leading to potential risks[24, 25, 28-31]. Production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-2 and -6 (IL-2, IL-6) and interleukin-1 beta (IL-1β) play a pivotal role in inflammatory complications in HD-patients. The consequence is a state of micro-inflammation that may contribute to progressive inflammatory diseases in chronic renal failure such as β_2-microglobulin amyloidosis, protein catabolism and atherosclerosis[25, 32]. Long-term hemodialysis often has complicating conditions such as dialysis-related amyloidosis as consequences of middle molecule substances like β_2-microglobulin not being removed from the blood in patients by low flux hemodialysis membrane[33, 34]. Therefore, high flux dialysis membranes (larger pore size) are needed to enable these proteins be successfully removed during hemodialysis[33, 35]. Due to greater efficiency and the reduced dialysis treatment time high-flux membranes are increasingly used. An accompanying disadvantage of high-flux membranes is the increased contribution of back transport.

Consequently, the high-flux dialysis membrane should offer additional properties, more definitive protection against endotoxins[23]. Various kinds of Et-blocking filtration membranes are presently used to remove Et from the dialysate[35]. However, not all present hemodialysis membranes are able to prevent endotoxin passage or show a limited endotoxin

adsorption capacity. Et-blocking membranes remove Et and their fragments by both retention and adsorption. Adsorption is most pronounced for hemodialysis membranes made of polymethyl methacrylate and acrylonitrile69[36]. Recently, polysulfone and polyether sulfone hemodialysis membranes prepared with different skin layers properties are able to trap endotoxins[35, 37]. Due to the wide molecular weight range distribution of endotoxins, they are difficult to remove (block) by size exclusion, i.e. by membrane filtration. Nowadays, the hemodialysis membrane concept focuses not only on the diffusion process and mass transfer across a semipermeable membrane but also on adsorption properties[24, 38, 39]. The demand for newly developed hemodialysis membranes is that they combine a high Et blocking while maintaining a high diffusive or convective permeability for low and middle molecular weight substances. These highly functional membranes are made of materials that show physical or chemical endotoxin interactions. The pores of the membrane are allowed to be bigger because the Et and other harmful compounds such as interleukin-1, tumor necrosis factor, peptides, interleukin-6, β-2-microglobulin will not pass the membrane but adsorb to its surface. Dialysis membranes that contain such adsorptive properties contribute to a decrease in morbidity and mortality of patients undergoing both acute and chronic dialysis[38]. High adsorption capacities can only be achieved when the membranes combine large surface areas with a high active site density[40]. The development or improvement of dialysis membrane for this application is of continuing interest.

In this chapter, integration of adsorption properties and high-flux filtration are deployed for endotoxin removal. The aim of the present study is to develop and characterize the efficiency of two different tailor made endotoxin adsorbing membranes. The adsorptive functionality of the membranes is established by embedding activated carbon (AC) particles or anion exchange resins Lewatit M500 into macroporous cellulose acetate membranes. The efficiency in endotoxin removal and the prevention of endotoxin back transport of high-flux MMM AC and MMM M500 is esteemed in both static and dynamic experiments.

7.2. Experimental

7. 2.1. Materials

Cellulose acetate with average acetate content 38.8 % was purchased from Aldrich (MW≅30.000) and used as membrane material without further modification. Acetone (Merck) was employed as solvent. Water was used as pore former and non-solvent in the coagulation bath. Activated carbon Norit A Supra EUR (particle size < 40 μm, kindly supplied by Norit)

and strong anion exchange resin M500 (particles size < 50 µm, kindly supplied by Caldic BV. Belgium) were used as adsorbent particles.

Buffer solutions were freshly prepared in ultra-pure water. Ultrapure water was prepared using a Millipore purification unit Milli-Q plus. Endotoxin assays were performed using the *Limulus Amebocyte* Lysate (LAL) Chromogenic end-point method from Cambrex Bio Science Walkersville. LAL reagent and control standard endotoxin (CSE) used to generate standard curves were supplied by Cambrex. The endotoxin bulk source used for the feedstock was *Escherichia coli* (0111:b4) lipopolysaccharide (LPS), purchased from Sigma-Aldrich. Pyrogen-free water used for buffer preparations was obtained from Cambrex, England. All materials that were used to collect water samples, to dilute standard and sample solutions and to run assays procedure were endotoxin-free and disposable (Cambrex).

7.2.2. Removing endotoxin contamination

Endotoxin can strongly adhere to glassware and is hard to remove by conventional washing. To remove endotoxin from the surfaces, all glassware used was washed in a cycle wash of solutions: in NaOH (1 N), HCl (1 N) and then 70 % ethanol, in an ultrasonic bath. Rinsing with ultrapure water was performed between each solution and after the last ethanol washing step. Afterwards the cleaned glassware was dried and stored endotoxin-free by heating at 180 °C for more than 3 hours[41]. All depyrogenated glassware was store in closed containers to protect it for environmental contamination. Before measuring, the samples were stored at 2-8 °C and used within 24 hours. Samples that could not be measured within 24 hours were frozen until used.

7.2.3. Assay endotoxin concentration

Purified LPS originating from *E. coli* O 111:B4 was used as the endotoxin-containing samples. In most studies, the bacterial concentration used was around 100-fold higher than those encountered in the majority of clinical situations[34, 42, 43]. In this study, endotoxin challenge concentrations were approximately 500 EU/mL (equivalent to 50 ng/mL). The endotoxin solutions were prepared in 10 mM phosphate buffer pH 7.4. The ionic strength of the buffer solutions was adjusted to 150 mM by adding NaCl. Endotoxin-free water was used as control.

The endotoxin content was determined by the quantitative chromogenic *Limulus amebocyte* lysate assay (QCL-1000; Cambrex Bio Science Walkersville, Inc.) according to the

manufacturer's instructions. The released amount of *p*-nitroaniline (*p*NA) was measured spectrophotometrically at λ_{max} = 405 nm and endotoxin from *E. coli* O111:B4 was used as standard. The method is sensitive (0.0005 EU/mL) and give a linear correlation between the *p*NA release and endotoxin concentration in the 0.1 to 1.0 EU/mL range. All samples were measured in duplicate. If the deviation exceeded 10%, the measurement was repeated. To increase the sensitivity of the QCL-1000 reagent kit, the LAL assay time was extended to 30 min. For calibration curves, the standard endotoxin solution was diluted to 0.1, 0.25, 0.5 and 1.0 EU/mL. The samples out of range of the calibration curve were diluted using pyrogen-free water (Cambrex). The temperature of incubator was adjusted to 37 °C for the LAL assay. Spectrophotometric detection was performed using disposable micro-volume cuvettes (500 µL).

7.2.4. Membrane preparation and characterization

The MMM preparation and characterization is described in **chapter 5 (section 5.2.2.)**. Both MMM AC and MMM M500 are prepared after 6 minutes water vapor induced phase separation followed by immersion precipitation in a water containing coagulation bath. The MMM AC contains 60wt% activated carbon and the MMM M500 contains 50wt% strong anion exchange resins. The membranes are characterized in terms of morphology, porosity, clean water fluxes and pore sizes.

7.2.5. Adsorption experiments

7.2.5.1. Batch experiments

The endotoxin adsorption capacity of the prepared MMM AC and MMM M500 was determined by batch experiments. The membranes were cycle washed with NaOH 0.1 N, HCl 0.1 N and ethanol 70 % before used. Afterwards, the membranes were rinsed with pyrogen-free water. The membranes were dried in a vacuum oven. All membranes used in the endotoxin experiments were treated in same manner. Known membranes weight were equilibrated with equilibrium buffers and afterwards transferred into the endotoxin solution, in sealed container under continuous shaking at 25 °C. The endotoxin adsorbs on the adsorptive membrane thereby reducing the endotoxin concentration in the bulk until equilibrium was reached. The equilibrium endotoxin concentration was determined by LAL test as described in **section 7.2.3.**

Static endotoxin adsorption was also investigated in time. This experiment was similar to the batch process as described above, except that the measurement was followed in

time. For pure activated carbon and ion exchange particles experiments the endotoxin solution was filtered before LAL test using Spartan 30 (regenerated cellulose membrane). In order to determine the adsorption isotherm batch adsorption experiments were carried out with different initial endotoxin concentration as described in **chapter 3**.

7.2.5.2. Dialysis experiment

In the dialysis mode, the experiments were carried out in a cell which has two identical compartments; the dialysate compartment and the endotoxin solution compartment. The compartments were separated by the MMM membrane. The volumes of each solution were 65 mL and the membrane area was 19.6 cm^2. The solutions were gently stirred. During the experiment, 10 µl samples of the solution undergoing dialysis were taken to determine the concentration of endotoxin from both compartments as a function of time. The concentration of endotoxin was determined by the LAL test as described in **section 7.2.3.** The dialysis process was evaluated for six hours.

7.2.5.3. Dead-end filtration

In dead-end filtration, the dynamic adsorption performance of the flat sheet MMM was measured at constant permeation rate. To overcome the limited adsorption capacity and to average out membrane inhomogeneities, three sheets of adsorptive membranes were stacked. The membrane areas used were 14.73 cm^2 (the membrane thicknesses are 0.32 mm and 0.27 mm for MMM AC and MMM M500 respectively). The filtrations are performed using a plastic syringe filter holder of diameter 25 mm (Sartorius). 30 mL endotoxin solution of 500 EU/mL in phosphate buffer pH 7.4 was permeated with complete recirculation through the membranes module with 55 $Lm^{-2}h^{-1}$. During 6 hours 10 µl samples of the solution undergoing filtration were taken to determine the concentration of endotoxin as a function of time. The Et concentration was determined by LAL test as described in **section 7.2.3.**

7.2.6. Regeneration of MMM M500

Regeneration use of MMM M500 is investigated in order to check its repetitive performance, which is a significant concern in non-clinical membrane chromatographic applications and in adsorption technology. After an adsorption, washing and desorption cycle, the MMM 500 were washed in a cycle wash of solutions: in NaOH (0.1 N), HCl (0.1 N) and then 70 % ethanol, in continues shaking bath to guarantee complete removal of residual

endotoxin. Before the next adsorption/desorption cycle could start, the membranes were pre-conditioned by rinsing with endotoxin free water. The adsorption capacity of the regenerated membrane was then re-measured.

7.3. Result and Discussion

Experimental results are presented and discussed in this section. Firstly, membrane formation and a detailed membrane characterization in terms of morphology, porosity and clean water flux are presented. Then, the endotoxin adsorption capacity of MMM AC and MMM M500 are evaluated and compared in both static and dynamic mode. Finally, an important characteristic: endotoxin adsorption by dialysis membrane is discussed.

7.3.1. Membrane preparation and characterization

Figure 7.1 shows SEM images of the MMM, which have been prepared with two different types of sorbent, i.e. activated carbon (AC) and strong anion exchange resins M500. The MMM AC was prepared from a solution of 12 % CA, 20% water in acetone containing 60 wt% loading activated carbon, under exposure to water vapor environment. On other hand, MMM M500 was prepared from a solution of 14 % CA, 20 % water, in acetone containing 50 wt% loading M500. Regular membrane structures were obtained by means of these compositions. The prepared MMM AC and MMM M500 show good mechanical properties and no particle loss is observed during membrane formation. All MMM types possess open and interconnected macroporous structures. The membranes display open structures both at bottom and top surface.

The top layer of the membranes consists of a highly interconnected macroporous structure, which forms a continuous structure with the sub-layer. The average pore size of the membranes as determined by bubble point measurement is 0.234 and 0.212 μm for MMM AC and MMM M500, respectively. The MMM AC has a swelling degree of 6.5 %, porosity of 53 % and clean water flux of 800 $Lm^{-2}h^{-1}bar^{-1}$. On the other hand, MMM M500 has a swelling degree of 22 %, porosity of 70 % and a clean water flux of 750 $Lm^{-2}h^{-1}bar^{-1}$. The higher swelling degree and porosity of the MMM M500 compared to the MMM AC is due to the high water uptake of the embedded M500 particles. SEM pictures confirm this by showing the particle shrinkage upon drying by SEM sample preparation.

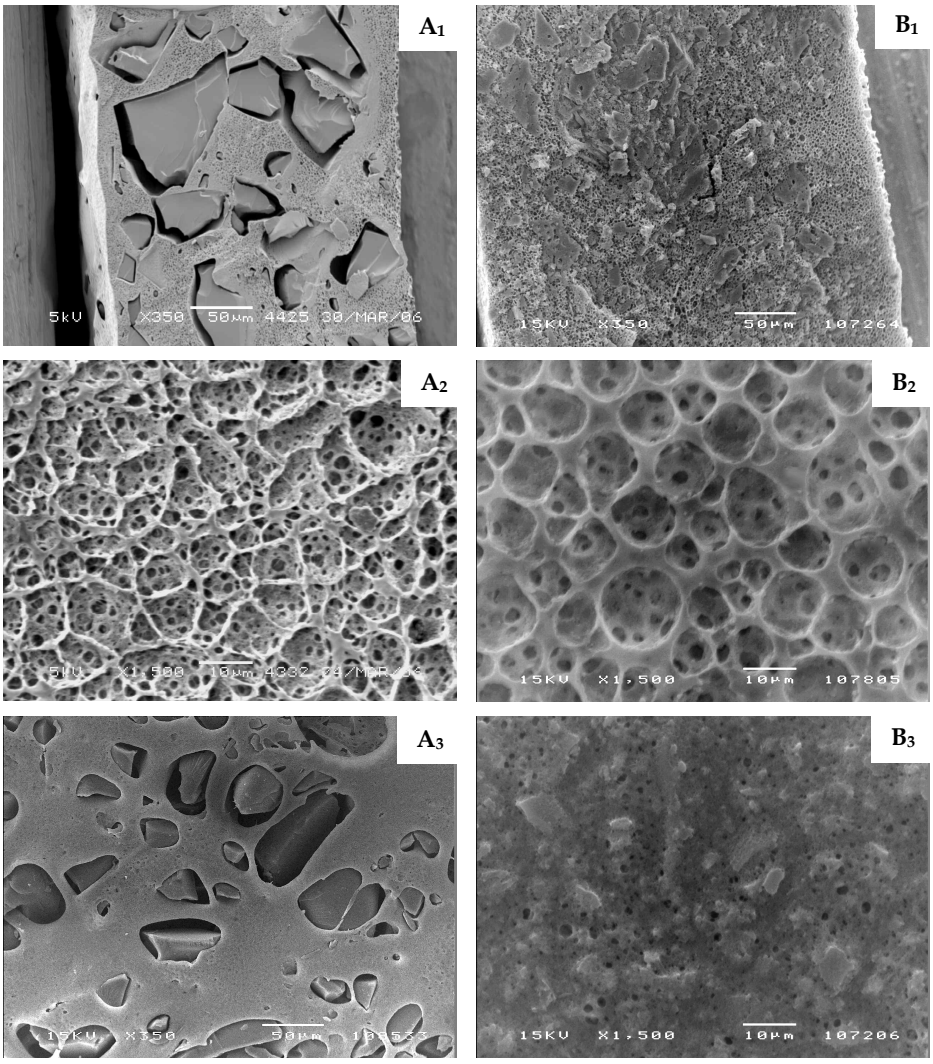

Figure 7.1. *SEM micrographs of co-cast MMM prepared by water vapor induced phase separation and immersion precipitation.* ***A)*** *MMM with embedded activated carbon particles.* ***B)*** *MMM with embedded M500 particles.* ***1)*** *Cross section, magnification x350, the size bar indicates 50µm;* ***2)*** *Top surface, magnification x1500, the size bar indicates 10 µm;* ***3)*** *Left side, bottom surface, magnification x350, the size bar indicates 50 µm. Right side, bottom surface, magnification x1500, the size bar indicates 10 µm.*

7.3.2. Endotoxin adsorption by different loading

Endotoxins are amphiphilic; they bear both hydrophobic (the fatty acids part in lipid A) and hydrophilic (the phosphoric acid groups and sugar part) properties[44]. The amphiphilic nature of endotoxin initiates both hydrophobic and electrostatic interactions

between endotoxin molecules and adsorbent materials[3]. Accordingly, endotoxin adsorption even improves by making use of both interactions types simultaneously using different functionalized particles. By comparing two different adsorbents, activated carbon and strong anion exchange resins, we intend to see the adsorption behavior and particles accessibility in the membrane. The effect of the particle load on the endotoxin adsorption capacity of prepared MMM AC and MMM M500 is presented in Figure 7.2. It shows that the endotoxin adsorption capacity scales with the particle load. Pure Cellulose acetate membranes possess an adsorption capacity of 750 EU/g membrane, which is less than 2.3 % and 3.4 % of the total adsorption capacity of MMM AC and MMM M500 respectively. The endotoxin adsorption capacity of the embedded activated carbon and M500 particles is close to the adsorption capacity particles suspensions. This leads to the conclusion that the embedded sorbent particles are very well accessible and determine the endotoxin adsorption capacity of the prepared MMM without interference of the matrix polymer.

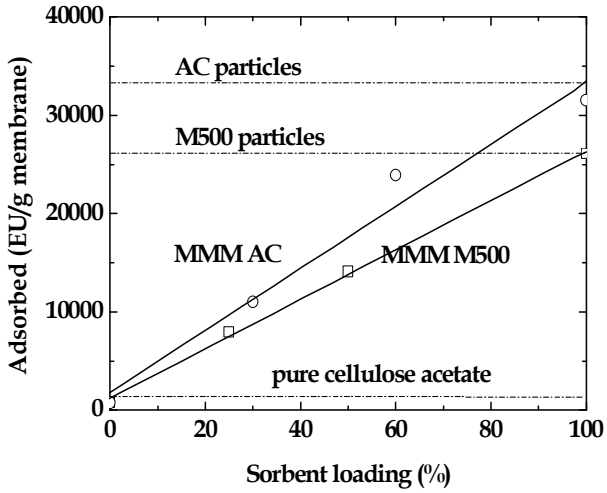

Figure 7.2. *The effect of particle load, incorporated into a cellulose acetate matrix, on the endotoxin adsorption capacity of prepared MMM AC and MMM M500. Experiments were carried out at pH 7.4, I 150 mM and temperature 25 °C. Initial endotoxin concentration 500 EU/mL.*

In aqueous solvents, hydrophobic patches on endotoxin will preferentially interact with the hydrophobic surface of the sorbent, where the negatively part of endotoxin mainly interacts with the hydrophilic part of the sorbent. Hydrophobic interaction predominantly takes place between endotoxin and activated carbon. Since at pH 7.4 the endotoxin is

negatively charged and the anion exchange M500 resin is positively charged, electrostatic attractive forces are favoring endotoxin adsorption on M500 resins. Figure 7.2 demonstrates that MMM AC membranes display a slightly higher endotoxin adsorption capacity than MMM M500. This difference can be contributed to the particle size (different outer surface), the density difference of the particles ($d_{active\ carbon}$=0.48 g/mL and d_{M500}=1.08 g/mL) and the particles porosity. Both the hydrophobic interaction and heterogeneity in surface chemistry lead to a high endotoxin adsorption capacity of MMM AC. Activated carbon contains oxygen functionalities and delocalized electrons in their structure, which determine the chemical behaviour of the carbon surface[45]. The hydrophilic sites of endotoxin are able to interact by electrostatic or hydrogen binding with the hydrophilic sites of activated carbon. Hayama et al.[35] reported that hydrophobic properties are more pronounced for endotoxin adsorption than hydrophilic properties.

7.3.3. Adsorption in time

The endotoxin removal is measured for particle suspensions and MMM during six hours, which is longer than a normal dialysis treatment. Figure 7.3 presents endotoxin adsorption by free and embedded particles by six hours static adsorption. Within this time interval, the amount endotoxin adsorption increases and reaches equilibrium. During the first

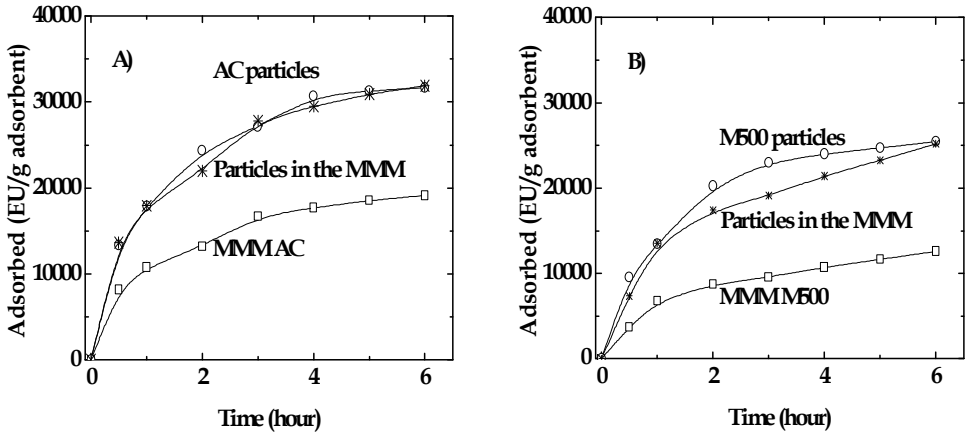

Figure 7.3. *Endotoxin adsorption by free and embedded sorbents by six hours static adsorption. A)Free ACparticles and AC embedded in the membrane. B) Free M500 resin particles and M500 embedded in the membrane. Endotoxin initial concentration was 500 EU /mL. Experiment were performed at pH 7.4 and temperature 25 °C.*

three hours the free particles and the MMM adsorb more than 90 % of the total amount endotoxin that is removed after six hour. The total endotoxin removal after six hours are 19.131 and 12.593 EU/g membrane for MMM AC and MMM M500 respectively or equivalent to 32.000 and 25000 EU/gram particles in the MMM for AC and M500 correspondingly. The equivalent values for the endotoxin adsorption capacity of pure particles and entrapped particles into the membrane demonstrated that the adsorptive sites are very accessible for endotoxin adsorption.The endotoxin adsorption kinetics are faster on MMM AC than on MMM M500, which may be contributed to the smaller size of AC particles, which accompanies with a larger external particle surface area.

7.3.4. Adsorption isotherm of endotoxin

The endotoxin adsorption isotherm for MMM AC and MMM M500 is determined batch wise by varying the concentration of endotoxin (E. coli 0lll: B4 LPS) in a phosphate buffer at physiological conditions (pH 7.4, I = 150 mM). The endotoxin adsorption isotherm is obtained by plotting the equilibrium endotoxin concentration versus the amount endotoxin adsorbed on the sorbent. Figure 7.4 illustrates the endotoxin adsorption isotherm onto both MMM structures. The solid line is the Langmuir adsorption isotherm that best fit the data.

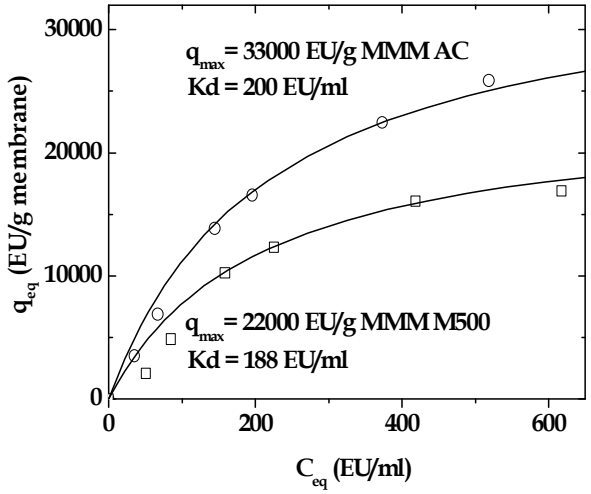

Figure 7. 4. *Correlation between experimental endotoxin adsorption data and the Langmuir isotherm model onto MMM AC and MMM M500. At pH 7.4 and 25 °C.*

The plot of $1/C_{eq}$ versus $1/q_{eq}$ allows determining q_m, the maximum endotoxin adsorption capacity. The maximum endotoxin adsorption capacity is 33000EU/g MMM AC (≅ 3.3 µg endotoxin/g MMM AC) and 22000 EU/g MMM M500 (≅ 2.2 µg endotoxin/g MMM M500). The resulting dissociation constants (Kd) are 200 EU/mL and 188 EU/mL for MMM AC and MMM M500, respectively.

Endotoxin adsorption isotherms display a high adsorption capacity for both types of membrane. It proves that the mixed matrix membrane adsorbers posses a good endotoxin accessibility. The endotoxin adsorptive capacity onto activated carbon was 50 % higher than that of the MMM M500 although both membranes showed similar K_d values, which indicates that the binding kinetics between the two MMM's are comparable.

The MMM Et adsorption capacities are comparable with published data. Anspach et al.[46] reported a maximum endotoxin adsorption capacity on immobilized DEAE being 4.05 mg/mL membrane at initial concentration of 8000 EU/mL. Recently, the endotoxin adsorption capacity of Lixelle® was reported[47]. Lixelle® adsorbs endotoxin by hydrophobic interaction; it is comprised of beads with a ligand of hexadecyl alkyl chain bound on the surface of porous cellulose beads. At initial Et concentration of 504.8 EU/mL, the Et adsorption capacity was 1.18 µg Et/mL sorbent. Minobe et al.[48] applied immobilized histamine for endotoxin adsorption. The Et adsorption capacity was 0.74 mg LPS/g sorbent. LPS adsorption on carbon sieves Carboxen 1003 and Carboxen 1010 has been studied by Gun'Ko et al.[49]. The LPS adsorption capacity of Carboxen 1003 is higher (9 µg/g sorbent) than that of Carboxen 1010 (6 µg/g sorbent). This difference originates from the bigger Carboxen 1003 pores that also accommodate large LPS molecules and their agglomerates. Because of the small pore size Carboxen 1010 adsorbs LPS mainly on the external surface. Hence, we could improve the performance of the MMM by using the carbons in the membrane matrix.

7.3.5. Effect of the ionic strength

The influence of the ionic strength on the endotoxin adsorption by both MMM adsorber types is examined. The endotoxin adsorption capacity onto MMM AC increases significantly with increasing ionic strength. This proves that hydrophobic interaction dominates the endotoxin adsorption mechanism onto AC. Higher ionic strengths decrease the solubility of endotoxin thereby favoring the endotoxin adsorption process. Indeed, figure 7.5 demonstrates that by increasing the ionic strength to physiological conditions the endotoxin

adsorption capacity increases almost by a factor 3. The endotoxin adsorption involves the interaction between the LPS lipid-A group and the hydrophobic activated carbon surface. This mechanism is confirmed by many data published in refereed journals[35, 50-53].

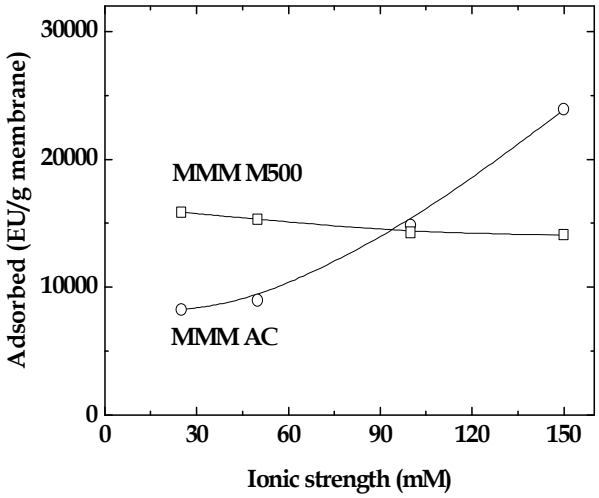

Figure 7.5. *Effect of salt on static endotoxin adsorption desorption from membrane Lew CNP80 WS. Desorption experiments were performed at pH 7 and in buffered 0.5 M NaC, equilibrium time 24 hours at temperature 25 °C.*

Figure 7.5 also illustrates that the endotoxin adsorption capacity onto MMM M500 decreases slightly with increasing ionic strength. The principle of ion exchange is the interaction between the charges of the endotoxin and the adsorbate surface. The endotoxin molecules are displacing the counter ions of the ion exchanger and become attached on the sorbent surface. At higher ionic strength solutions, there is more competition between the negative groups located on the endotoxin surface and the counter ions. This results in a lower endotoxin adsorption capacity. The primary interaction between endotoxin and strong basic anion exchange groups from the Lewatit M500 particles are electrostatic interaction forces. The interactions take place between the anionic phosphate groups of the endotoxin molecule and the cationic ligands on the sorbents. Generally, a higher ionic strength decreases the binding efficiency of ion exchange materials as is observed for almost all affinity ligands[3, 54, 55]. When an endotoxin molecule in addition to its hydrophobic character is negatively charged, the adsorption of endotoxin can be also due to the simultaneous effects of the cationic properties originating from the ligands and the hydrophobic properties originating

from the MMM[35, 50, 56]. This means that in a mixed mode the endotoxin adsorption takes place both by electrostatic as well as hydrophobic interaction.

7.3.6. Endotoxin adsorption during dialysis

The ability of MMM to increase modality and safety of hemodialysis by preventing endotoxin back transport from dialysate side to the blood stream is very attractive. This feasibility is evaluated by simulating a dialysis treatment. In this experiment, the starting dialysate endotoxin concentration and the endotoxin in the sample solution was 500 and 3 EU/mL respectively. The adsorption side of the membrane was oriented towards the low endotoxin concentration liquid (blood side). However, screening experiments show that orientation not affects the endotoxin adsorption capacity. In hemodialysis treatment, facing co-casting layer to the blood side is preferred in order to increase the blood compatibility. The dialysis adsorption process was monitored in time for both pool solutions. Figure 7.6 demonstrates the decrease in endotoxin concentration in both the dialysate and blood side of the dialysis membrane. This indicates that during the dialysis treatment no endotoxins cross over takes place from the high endotoxin concentration side to the low endotoxin concentration side.

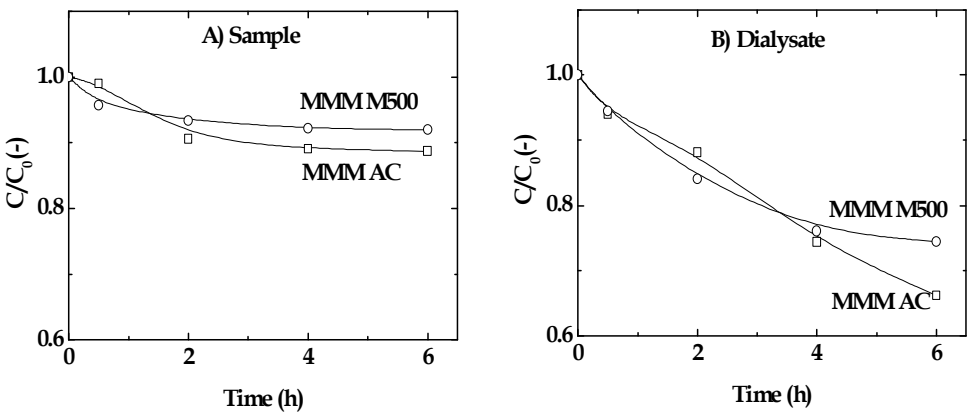

Figure 7.6. *Endotoxin adsorption during dialysis tests onto MMM AC and M500. Starting concentration of endotoxin in dialysate and sample (blood side) is 3 EU and 500 EU respectively.*

Both co-cast MMM have a high endotoxin adsorption capacity during the dialysis treatment. Yamamoto[37] and Hayama[35] reported that double layer hemodialysis

membranes which comprised of a hydrophilic inner skin layer and completely hydrophobic void and outer skin layers preventing endotoxin passage from the dialysate. Endotoxin is adsorbed at the outer skin facing the dialysate. The MMM presented here show endotoxin adsorption occuring from both the dialysate and the blood side. This gives raise to additional advantages of the MMM since harmful substances from the blood side can be removed by diffusion, convection and adsorption in one step. A number of studies report that endotoxin adsorption properties improves the outcome of the patient in both acute and chronic kidney and liver failure[18, 19, 38, 57].

7.3.7. Dead-end filtration of endotoxin

The effect of endotoxin removal by permeation through a MMM AC and MMM M500 is presented in Figure 7.8. The endotoxin solution was permeated with a flow rate 55 $Lm^{-2}h^{-1}$ in complete recirculation mode through a dead-end module containing a 3 stack of MMM. Endotoxin adsorption during the filtration onto MMM significantly lowers the

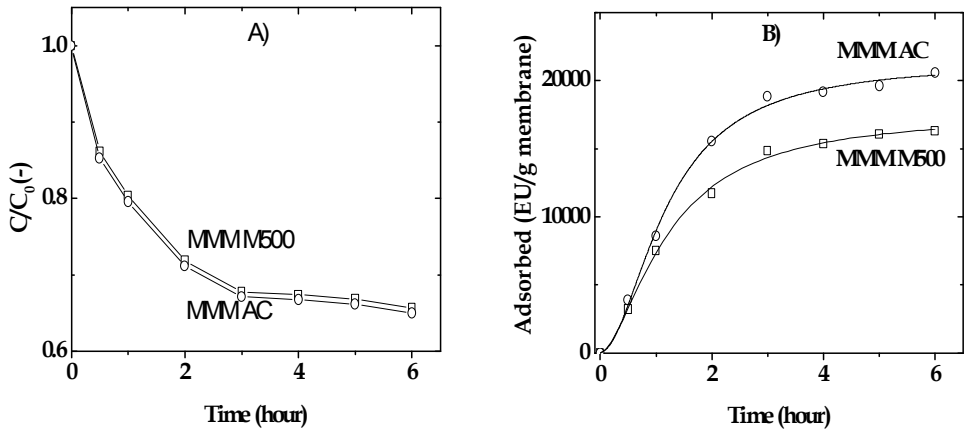

Figure 7.7. *Endotoxin removal and adsorption by dead-end filtration.* ***A)*** *Reduction of endotoxin concentration in reservoir as function of treatment time.* ***B)*** *The complementary adsorption curve. The solution was recirculated at permeate flux 50 $l/m^2/h$. The initial concentration of endotoxin was approximately 500 EU/mL. Experiment was performed at 25 ^oC for six hours.*

endotoxin concentration in the feed solution. The results show a fast adsorption of endotoxin during the first two filtration hours, which is amply within the four till six hours of a normal clinical hemodialysis treatment. In the perspective of a clinical hemoperfusion treatment, fully

endotoxin removal should be completed as quickly as possible. Figure 7.7 shows that in six filtration hours the endotoxin removal per gram of adsorbent is 17.000 EU and 20.000 EU for the MMM M500 and MMM AC respectively. The difference between the two MMM can be contributed to difference in particle loading, particle density, pore size, pore size distribution, particle size, and adsorption mechanism.

7.3.8. Reuse MMM M500

Most adsorbents and membranes applied in blood purification systems are single use. Lowrie et al.[58] proposed for economic reasons the reprocessing of (disposable) dialysers. Wolff et al. [67] and Lacson et al. [68] however, identified several potential problems with reuse including the possibility of exposure to toxic reprocessing chemicals, alterations in solute clearance because of membrane modification, the presence of residual plasma proteins and endotoxin on or within the membrane and changes in biocompatibility. In this research, we are investigating the regeneration of MMM M500. In medical treatments, single use is the current standard. However, in adsorption technology and non-clinical membrane chromatographic applications sequential use is necessary to be competitive with alternative methods like packed bed chromatography and adsorber columns. Therefore, the MMM M500 performance in sequential adsorption and desorption cycles is determined.

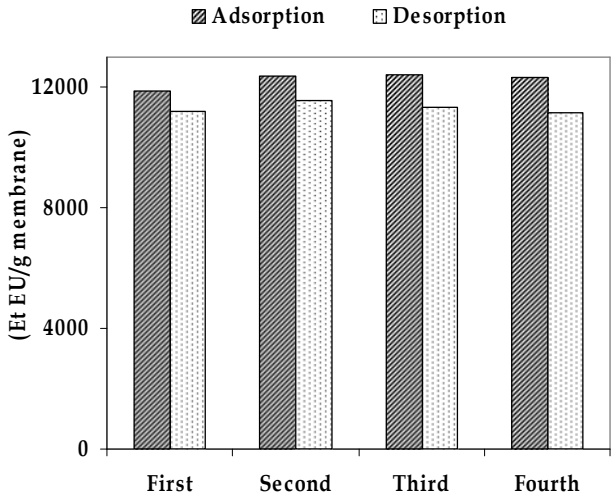

Figure 7.8. *Repeated static endotoxin adsorption-desorption-regeneration cycles onto MMM M500. Adsorption performed at pH 7.4, ionic strength 150 mM and 25°C. Initial endotoxin concentration 500 EU/mL. Desorption at pH 7.4 in buffered 0.5 M NaCl. The adsorption capacity was measured after 24 hours.*

Electrostatic interaction forces are dominating endotoxin adsorption onto strong basic anion exchange MMM M500. Increasing the ionic strength of the phosphate binding buffer weakens the electrostatic interactions between the endotoxins and MMM M500, as presented already in figure 7.6, and desorption takes place. After the desorption step complete cleaning is accomplished by a cycle wash with NaOH (0.1 N), HCl (0.1 N) followed by 70 % ethanol to guarantee thorough removal of non-specific bound components which do not elute by the desorption buffer. Figure 7.9 proves that regenerated membranes maintain their endotoxin adsorption capacity even after several cleaning treatments.

In contrast, with MMM M500 regeneration of MMM AC by increasing the salt concentration or by using organic solvent is not possible. Endotoxin adsorption onto MMM AC takes place predominantly by non specific interaction, i.e. Van der Waals and hydrophobic interaction. Cycle wash with NaOH, HCl and ethanol restores less than 20 % of the endotoxin adsorption capacity. Therefore, MMM AC membranes are contrary to MMM M500 not suitable for multiple uses.

7.4. Conclusions

We proposed a concept where small particles with endotoxin adsorptive properties are embedded in hemodialysis membranes: the so called Mixed Matrix Membranes (MMM) adsorbers. The MMM adsorbers concept offers the possibility to capture endotoxin (LPS) molecules during a hemodialyis or hemofiltration treatment. The adsorptive functions are established by embedding high particle loads, activated carbon particles or strong anion exchange resins into macroporous cellulose acetate membranes.

MMMs prepared with a particle free cellulose acetate co-cast offer additional advantages. The most important ones are preventing particles loss, increase in biocompatibility and an additional tool to tailor the adsorber porosity. The adsorptive MMMs feature both a high static as well as a high dynamic endotoxin adsorption capacity. The high endotoxin adsorption capacity makes that MMMs are extremely suitable for the integration of hemofiltration and hemodialysis with hemoperfusion. Integration of these techniques by applying the MMM concept improves the quality of the treatment since it removes both small and middle sized toxins in a single treatment step. MMM hemodialysis is effective in blocking endotoxin back transfer from dialysate to the blood stream. The membranes can be applied in extracorporeal blood purification devices for the elimination of toxic substances from the blood during kidney or liver failure.

7.5. Acknowledgements

We want to acknowledge the Membrane Technology Group of the University of Twente and the Ministry of Education of the Republic of Indonesia for the financial support.

7.6. List of Symbols and Abbreviations

Symbols

I	*Ionic strength*	*mol/l*
Kd	*Dissociation constant*	*mg/mL*
Mw	*Molecular weight*	*g/mol*
q_{max}	*Maximum adsorption capacity*	*EU/g*

Abbreviations

AC	*Activated carbon*	*LAL*	*Limulus Amebocyte lysate*
CA	*Cellulose acetate*	*LPS*	*lipopolysacharides*
CPFA	*Couple plasma filtration adsorption*	*MMM*	*Mixed matrix membrane*
CSE	*Control standard endotoxin*	*MOF*	*Multi organ failure*
DTPF	*Detoxification plasma filtration*	*MODS*	*Multiple organ dysfunction syndrome*
Et	*Endotoxin*	*NIPS*	*non-solvent induce phase separation*
EU	*Endotoxin unit*	*PMP*	*Polymixin B*
HD	*Hemodialysis*	*PMX-F*	*Polymixin B immobilized fiber*
HELP	*Heparin induced extracorporeal lipoprotein fibrinogen precipitation*	*SIRS*	*System inflammatory response syndrome*
IL	*interleukin*	*TNF*	*tumor necrosis factor*
Kd	*Dissociation constant*		

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SUMMARY

Biotechnology and bio-manufacturing markets are continuously growing, generating new sources of many valuable healthcare and life science products including therapeutic proteins and polysaccharides, monoclonals, vaccines, diagnostics, pharmaceutical chemicals and enzymes. These bioproducts have to be isolated and purified from complex mixtures before they can be used to ensure safety and efficacy. Nowadays, there are different techniques available for biomolecules isolation and purification. Membrane filtration and chromatography methods are the most widely techniques used to obtain the required purity for biotherapeutic and diagnostic products. The advantages and disadvantages of these two methods are complementary to each others. Therefore, if these methods can be integrated into a one single unit operation, maximal yield can be combined with minimal product loss. Furthermore, the integration of different separation steps into one single unit operation increases the compactness, economics, and reduces the time to market.

Biological fluids purification is very important in food as well in the biomedical field. A growing population of patients with kidney and liver failure, poisoning, and septic shock by more complex medical problems require technological innovations to improve the safety, reliability and efficiency of the treatment. Standard hemodialysis, although supportive, has not provided a treatment modality which completely replaces kidney function to remove all harmful substances. Hemodialysis membrane contains pores that allow small molecules, including water to pass easily, the passage of middle molecule is more restricted, where large molecules are completely rejected by the membrane. Lately, it has been demonstrated that hemoperfusion over variety of sorbents might remove toxic waste products, which are poorly or not removed by standard hemodialysis membranes. However, the use of sorbents in medical applications is often counterbalanced for safety concerns due to the release of small micro-particles, poor homogeneity and bio-incompatibility. Clearly, there is a room for innovations and improvements in capacity and efficacy of hemodialysis membrane as well as in the safety of sorbent systems.

In this thesis we developed an alternative biological separation process: instead of using sorbent packed beds we integrate small sized sorbents and membrane filtration, being the so called Mixed Matrix Membranes (MMMs). This technique combines the chromatographic advantages being a high adsorption capacity and selectivity with the high throughput of macroporous membranes. The MMM concept offers the opportunity to remove blood toxins by embedding hemoperfusion sorbents into a blood-compatible porous polymeric membranes. This background and the scope of the research are described in ***chapter 1***.

The recent developments and applications of membrane chromatography in biological separations are reviewed in ***chapter* 2.** The discussion focuses mainly on ion exchange membrane chromatography in biotechnology and medical fields. The chapter finalizes with an overview of adsorptive and membrane based extracorporeal blood purification treatments.

The performance of MMM adsorbers for enzyme capturing and concentrating has been described in ***chapter 3***. The MMM adsorbers are prepared by a phase inversion method. The adsorption capacity is established by embedding small Lewatit CNP80 WS (a weak cation exchange, carboxyl functional group) particles into a macroporous polymeric membrane. The MMM adsorber demonstrates a high static and dynamic Lysozyme (LZ) adsorption capacity and an easy scale up. The dynamic binding is dependent on the flux, residence time in the membrane, where the elution of LZ is flux independent and leads to a multiple increase in LZ concentration with recovery values up to 98 %. Fluorescence spectra of eluted LZ indicate that there are no obvious conformational changes. Activity tests with *Micrococcus lysodeikticus* as substrate demonstrate that the activity of the isolated LZ is maintained for more than 90 % after the separation processes, which indicate that capturing and concentration with MMM adsorbers is a mild process. A comparison between MMMs and commercial Sartobind Smembranes demonstrates that MMM posses a higher adsorption capacity.

In ***chapter 4,*** an extensive characterisation of a new mixed matrix membrane adsorber application, the separation of bovine serum albumin (BSA) and LZ is studied. The developed MMM adsorbers demonstrate a high static and dynamic protein adsorption capacity and selectivity in the fractionation of BSA and LZ. The MMMs allow the adsorptive membranes to function both as protein purifier and concentrator. High values for the separation factors and purities of BSA and LZ are obtained in effluents and elution buffers.

A major advantage of the membrane adsorbers concept is the ability to create particle-loaded membranes using any type of particles and almost any type of polymeric material. In ***chapter 5*** we propose that the MMM concept can be applied in blood purification by embedding activated carbon into a biocompatible porous polymeric support. Integral double layers MMM with porous sub-structures are obtained by single step co-casting of two polymer solutions with different compositions on a glass plate. Double layer mixed matrix membranes consist out of an active support and a separating layer. The active support layer consists of activated carbon particles that are embedded in cellulose acetate; the separating layer consists of particle free cellulose acetate. The porosity of the MMM surface layer depends on the applied process parameters. Skinned films are obtained by direct immersion of the polymer solution into water containing coagulation bath. When preceding the immersion precipitation solvent evaporation takes place in humid air, membranes with porous surfaces are formed. The highest clean water fluxes are obtained by membranes prepared via water vapor induced phase separation. The co-casting process opens the possibility to improve the mechanical stability, the biocompatibility and prevents particles loss during membrane preparation and processing.

The adsorption of creatinine (Crt) as a model of uremia blood toxin for MMM AC has been investigated in ***chapter 6.*** The MMM adsorber captures toxic molecules during hemodialysis or hemofiltration processes. The prepared membranes show a high static as well as dynamic Crt adsorption capacity. The measured Crt adsorption isotherm is of the Langmuir type, with a maximum adsorption capacity of 15 mg Crt/g membrane. High dynamic Crt removals are obtained in dead-end and cross flow filtration, as well as in dialysis mode. The dynamic binding capacity depends on the permeate flux. However, six hours recirculation is sufficient to utilize the available MMM adsorption capacity in all adsorption modes. In Crt dialysis process, Crt cross transport can be avoided by using Crt adsorptive MMM. The MMM presents an alternative route to increase the biocompatiblity of activated carbon. The MMM AC has characteristics of hemodialysis as well as hemofiltration integrated with adsorption, in which blood toxins are removed in one step.

Proof-of-concept that the MMMs can improve the safety and capacity of extracorporeal blood purification membrane are presented in ***chapter 7***. The adsorptive membranes are prepared by embedding activated carbon particles and anion exchange resins Lewatit M500 (a strong anion exchange, quaternary amine functional group) into porous cellulose acetate membranes. MMM dialysis is effective in blocking endotoxin transfer from

dialysate to the blood stream by adsorption. Integration of hemofiltration and/or hemodialysis with hemoperfusion by applying the MMM concept improves the quality of the treatment since it removes small, middle and large sized toxins in a single treatment step. The membranes can be applied in extracorporeal blood purification devices for the elimination of toxic substances from blood during kidney or liver failure, poisoning and sepsis shock.

SAMENVATTING

De biotechnologie zorgt voor economische groei door het genereren van nieuwe bronnen voor de productie van waardevolle biowetenschappelijke en verzorgings-producten zoals: therapeutische eiwitten en polysachariden, monoklonalen, vaccins, diagnostica, farmaceutische chemicaliën en enzymen voor de voedings- en consumentenmarkt. Al deze producten moeten uit complexe mengsels worden geïsoleerd en gezuiverd om de productveiligheid en werkzaamheid te garanderen. Momenteel zijn er voor de zuivering en isolering van biomoleculen meerdere technieken beschikbaar, met elk haar eigen voor- en nadelen. De meest toegepaste technieken om de vereiste zuiverheid van biotherapeutica en diagnostische producten te krijgen zijn membraanfiltratie en chromatografie. De voor- en nadelen van deze technieken zijn complementair aan elkaar. Het integreren van deze technieken in een processtap is daarom een logische keuze om de productopbrengst te maximaliseren. Daarnaast zorgt de integratie voor compactheid, betere economie, kortere marktintroductietijd en lagere loonkosten.

De zuivering van biologische vloeistoffen is met name belangrijk in voedings- en biomedische toepassingen. Het groeiend aantal patiënten met nier- en leveraandoeningen, vergiftigingsverschijnselen en septische shock vragen om technologische innovaties om de veiligheid, betrouwbaarheid en efficiëntie van medische behandelingen te verhogen. Standaard hemodialyse is slechts ondersteunend en is geen behandeling die de nierfunctie volledig kan vervangen bij de verwijdering van schadelijke stoffen. Hemodialyse membranen bevatten poriën die kleine moleculen, inclusief water, doorlaten. De doorlaatbaarheid van middelgrote moleculen is meer beperkt waar grote moleculen in het geheel niet worden doorgelaten. Recentelijk is aangetoond dat hemodialyse door een grotere variëteit aan sorbentia in staat is toxische afvalproducten te verwijderen die slechts gedeeltelijk of in het geheel niet door standaard hemodialyse-membranen worden verwijderd. Daar tegenover staan echter enkele veiligheidsnadelen zoals: mogelijke afgifte van kleine microdeeltjes, een slechte homogeniteit en biocompatibiliteit. Het staat daarom

onomstotelijk vast dat er ruimte is voor innovaties om de doelmatigheid van hemodialyse membranen, als ook de veiligheid van sorbentsystemen te verhogen.

In dit proefschrift staan alternatieve biologische scheidingsprocessen beschreven die in plaats van sorbentia in gepakte bedden gebruik maken van kleine gefunctionaliseerde deeltjes die zijn ingebed in poreuze membraanstructuren, de zogenoemde "Mixed Matrix Membranes" (MMMs). Deze techniek combineert de voordelen van chromatografie, een hoge adsorptiecapaciteit, met de voordelen van membraansystemen, een hoge doorzet. Het MMMs-concept biedt de mogelijkheid om giftige stoffen uit bloed te verwijderen door het inbouwen van hemoperfusiesorbentia in bloedcompatibele poreuze membranen. Het doel en de achtergrond van dit onderzoek staan beschreven in **hoofdstuk 1** van dit proefschrift.
De recente ontwikkelingen en toepassingen van membraanchromatografie in biologische scheidingen zijn beschreven in **hoofdstuk 2**. De discussie richt zich met name op ionenwisselingsmembraanchromatografie in biologische en medische toepassingen. Het hoofdstuk eindigt met een overzicht van adsorptieve membranen de gebruikt worden bij bloedzuiveringsbehandelingstechnieken.

De prestaties van MMMs adsorptiesystemen voor het afvangen en concentreren van enzymen is beschreven in **hoofdstuk 3**. De MMMs adsorptiesystemen zijn gemaakt door middel van een faseinversieproces. De adsorptiecapaciteit wordt verkregen door het inbouwen van kleine Lewatit CNP80 WS (een zwakke kationwisselaar) deeltjes in macroporeuze polymeermembranen. De adsorptieve MMMs bezitten een hoge statische en dynamische lysozyme (LZ) adsorptiecapaciteit en zijn gemakkelijk op te schalen. De dynamische LZ-adsorptiecapaciteit is afhankelijk van de permeatiesnelheid, de verblijftijd in het membraan. De LZ-elutie daar in tegen is onafhankelijk is van de permeatiesnelheid en een levert een veelvoudige toename in de LZ concentratie op met een rendement van meer dan 98 %. Fluorescentiemicroscopie toont aan dat er tijdens het zuiveren en concentreren geen LZ-conformatieverandering optreedt. Dit wordt bevestigd door activiteitstesten met *Micrococcus Lysodeikticus* als substraat, waarmee wordt aangetoond dat de activiteit van geïsoleerd LZ voor meer dan 90 % blijft behouden. Dit betekent dat het afvangen en concentreren met adsorptieve MMMs systemen een mild proces is. Een vergelijk tussen de eigen MMMs en de commercieel beschikbare Sartobind S membranen laat zien dat MMMs systemen een hogere statische adsorptiecapaciteit bezitten.

In **hoofdstuk 4** worden de nieuwe MMMs uitvoerig onderzocht op de geschiktheid om runderalbumine (BSA) en lysozyme (LZ) van elkaar te scheiden. Het ontwikkelde adsorptieve MMM heeft een hoge statische en dynamische adsorptiecapaciteit als ook een hoge selectiviteit in de BSA - LZ fractionering. De MMMs hebben in dit proces tegelijkertijd de functie van zuiveraar en concentrator, waarbij hoge scheidingsfactoren en zuiverheden worden behaald in effluent- en elutiebuffers.

Een groot voordeel van het MMM-concept is de mogelijkheid om verschillende typen deeltjes met bijna alle beschikbare polymeren te combineren. In **hoofdstuk 5** toetsen we het MMMs-concept op haar geschiktheid in bloedzuiveringstoepassingen door het inbouwen van actiefkooldeeltjes in een biocompatibele poreuze polymere drager. Integrale MMM-dubbellagen worden verkregen door twee polymeeroplossingen met verschillende samenstellingen tegelijkertijd te strijken op een glasplaat. Het dubbellaags-MMM bestaat uit een actieve steunlaag met daarop een scheidingslaag. De actieve steunlaag bevat actiefkooldeeltjes; de scheidingslaag bestaat uit een deeltjesvrije celluloseacetaat. De porositeit van de MMM-scheidingslaag wordt bepaald door de gebruikte procescondities. Films met een dichtere scheidingslaag worden verkregen door directe onderdompeling van de polymeeroplossing in een water bevattend coagulatiebad. Wanneer de onderdompeling vooraf wordt gegaan door een verblijftijd in waterdamp wordt een meer open laag, met hogere schoonwaterfluxen, verkregen. Het co-casting proces biedt daarnaast de mogelijkheid om de mechanische stabiliteit en de biocompatibiliteit te verhogen, en voorkomt tevens deeltjesuitval tijdens de bereiding en procesvoering.

De creatinine (Crt)-adsorptie, een modelstof voor bloedgifstoffen, aan MMMs is beschreven in **hoofdstuk 6**. De MMMs-adsorber vangt toxische moleculen af tijdens hemodialyse en hemofiltratie. De bereide membranen hebben een hoge statische en dynamische Crt-adsorptiecapaciteit. Het adsorptiegedrag is te beschrijven met een Langmuir isotherm, waarbij de maximale adsorptiewaarde 15 mg Crt/g membraan bedraagt. De hoge adsorptiewaarden worden verkregen in zowel "dead-end" en "cross-flow" als ook in de dialyse uitvoering. De dynamische bindingscapaciteit hangt af van de permeatiesnelheid. Het blijkt dat 6 uur recirculatie van de voedingsvloeistof voor alle uitvoeringsvormen voldoende is om de adsorptiecapaciteit van de membranen volledig te benutten. In het dialyseproces kan Crt-terugtransport worden voorkomen door gebruik te maken van MMMs met adsorptiefuncties. Tijdens inbouwen in een MMM krijgt het

actiefkooldeeltje een deklaag waardoor de biocompatibiliteit wordt verhoogd. Het actieve kooldeeltjes bevattende MMM heeft de karakteristieken van hemodialyse en hemofiltratie in combinatie met adsorptie, verenigd in een processtap.

Bewijs dat dit MMMs-concept de veiligheid en capaciteit van extracorporale bloedzuivering verhoogd wordt gepresenteerd in **hoofdstuk 7**. Het bereide adsorptieve membraan bevat zowel actiefkool als ook Lewatit M500 anionenwisselingsdeeltjes in poreus celluloseacetaat. Hierdoor wordt tijdens de dialysebehandeling endotoxine transport van de dialysaat- naar de bloedzijde door adsorptie effectief geblokkeerd. Integratie van hemofiltratie en/of hemodialyse met hemoperfusie door gebruik te maken van het MMMs-concept verbetert de kwaliteit van de medische behandeling omdat het zowel kleine, middelgrote als ook grote toxische moleculen verwijdert in een enkelvoudige processtap. De MMMs kunnen worden ingezet voor het verwijderen van toxische stoffen uit bloed tijdens nier- of leverfunctieverlies, vergiftiging en tijdens het verkeren in een septische shock waardoor de patiënt sneller zal herstellen.

RINGKASAN

Kemajuan pesat dalam bidang bioteknologi dan bioindustri telah menghasilkan banyak senyawa dan produk yang sangat berguna dalam bidang kesehatan dan life science seperti senyawa obat-obatan dari protein dan karbohidrat, antibodi, hormon, vaksin, senyawa diagnosis, bahan kimia farmasi, dan enzim untuk industri makanan and minuman. Untuk dapat digunakan bagi keperluan tertentu, semua senyawa produk bioteknologi ini harus dipisahkan dan dimurnikan terlebih dahulu dari campuran dan pengotornya agar terjamin keamanan dan khasiatnya. Ada berbagai macam teknik yang dapat digunakan untuk pemisahan molekul-molekul tersebut dimana setiap teknik memiliki keunggulan dan kelemahan masing-masing. Metode membrane filtrasi dan kromatografi merupakan dua teknik yang sangat penting dalam pemisahan dan pemurnian produk bioteknologi. Kelebihan dan kekurangan kedua metode ini saling melengkapi satu sama lainnya. Jika kedua metode ini bisa disatukan maka akan sangat berguna sekali karena bisa mempersingkat proses pemisahan dan pemurnian, maksimal hasilnya, lebih sederhana, dan ekonomis.

Metode pemisahan senyawa-senyawa produk biologi sangat penting pula dalam bidang medis (pengobatan) yaitu untuk memisahkan dan membuang racun dan bahan pengotor yang terdapat dalam aliran darah. Seiring dengan meningkatnya jumlah pasien yang menderita penyakit ginjal, hati, keracunan, dan sepsis serta dengan permasalahan yang sangat komplek diperlukan suatu inovasi dan pengembangan teknik pemisahan agar terjamin keamanan, tahan uji, dan efesien dalam terapi. Metode hemodialisis, meskipun dapat membantu pasien tetapi tidak dapat berfungsi sebagaimana kerjanya ginjal atau hati dalam membuang semua bahan kotoran dan racun dari dalam tubuh. Pemisahan dengan membran hemodialisis hanya bergantung pada ukuran pori. Membran ini hanya mampu membuang air dan racun dalam ukuran kecil, kurang mampu membuang racun yang ukuran sedang dan tidak mampu membuang racun dalam ukuran besar. Untuk dapat membuang semua bahan racun dalam tubuh maka metode hemodialisis harus digabungkan dengan sistem adsorben (bahan penyerap), dimana adsorben mampu menyerap semua racun yang tidak bisa dibuang dengan metode hemodialisis. Namun demikian, adsorben juga

mempunyai kekurangan yaitu tidak cukup aman dalam aplikasi karena mempunyai efek samping (kurang biocompatibel) dan adsorben dapat pecah menjadi partikel kecil dan bisa masuk kedalam darah. Untuk itu sangat perlu untuk dipelajari dan dikembangkan lebih lanjut agar dapat dihasilkan membran hemodialisis yang mempunyai kemampuan handal dan sistem adsorben yang aman dalam aplikasinya.

Dalam tesis ini telah dipelajari suatu metode alternatif untuk pemisahan dan pemurnian senyawa-senyawa produk biologi dengan menyatukan metode membran dan adsorben menjadi satu metode yang disebut *Mixed Matrix Membrane* (MMM, disebut juga membran kromatografi). Membran ini mempunyai dua kinerja sekaligus yaitu menyaring dan menyerap dalam satu tahap. Membran ini mampu menyaring senyawa dengan baik dan sekaligus mampu menyerap senyawa-senyawa yang ditargetkan. Untuk aplikasi pada bidang medis maka membran dan adsorben yang digunakan harus aman dan tidak menimbulkan efek samping bagi komponen-komponen penting yang ada dalam darah. Latar belakang dan batasan masalah dalam tesis ini telah digambarkan dengan jelas dalam **bab 1.**

Gambaran mengenai perkembangan dan aplikasi membran kromatografi dalam pemisahan dan pemurnian senyawa-senyawa biologi seperti protein, enzim dan senyawa beracun dijelaskan dalam **bab 2**. Dalam tesis ini, pembahasan hanya difokuskan pada membran kromatografi penukar ion dalam bidang bioteknologi dan medis. Kemudian beberapa metode yang telah digunakan dalam pemurnian darah beserta kelemahannya juga dijelaskan pada bab ini.

Kinerja MMM dalam menyerap dan mengkonsentrasikan suatu enzim telah dipelajari dalam **bab 3**. MMM dibuat dengan metode pembalikan fasa. Kemampuan adsorpsi membran ini diperoleh dengan menempatkan partikel adsorben Lewatit CNP80 WS (suatu resin penukar kation) didalam membran yang berpori. Membran yang diinginkan adalah membran dengan kandungan partikel resin yang tinggi agar mempunyai kemampuan adsorpsi yang maksimum. Hasil uji menunjukkan bahwa membran ini mempunyai daya adsorpsi yang tinggi untuk senyawa Lysozyme (LZ) dan mudah dalam penanganan proses pemisahannya. Kemampuan membran ini dalam menyerap senyawa LZ dipengaruhi oleh kecepatan alir dari larutan tetapi kecepatan alir tidak mempengaruhi dalam recoveri LZ. Konsentrasi LZ yang diperoleh 5 kali lebih tinggi dari konsentrasi awalnya dengan recoveri mencapai 98%. Berdasarkan analisa spektrum floresen, molekul LZ hasil pemisahan stabil dan hal ini dibuktikan dengan hasil uji aktivitas menggunakan *Micrococcus lysodeikticus* sebagai subtrat, lebih dari 90% molekul LZ masih aktiv. Ini menunjukkan bahwa proses

pemisahan dengan MMM dapat mempertahankan stabilitas dan aktivitas dari senyawa sejenis LZ. Membran ini juga mempunyai kemampuan adsorpsi yang lebih baik dibandingkan dengan membran sejenis yang sudah dijual di pasaran.

Dalam **bab 4**, suatu aplikasi baru MMM untuk pemisahan campuran protein bovine serum albumin (BSA) dan Lysozyme (LZ) telah dipelajari secara detil. Membran yang telah dikembang menunjukkan kemampuan adsorpsi and selektivitas yang tinggi dalam fraksionasi BSA dan LZ. Membran ini dapat berfungsi sebagai media pemisah dan sekaligus sebagai pemekat dari protein. Nilai faktor pemisahan dan kemurnian yang dicapai sangat tinggi. Dengan cara yang fleksible, membran adsorpsi dapat diterapkan untuk berbagai aplikasi yaitu dengan cara memilih adsorben yang cocok atau mengontrol kondisi proses pemisahan.

Manfaat yang sangat utama dari konsep membran adsorpsi yang telah dikembangkan yaitu kemudahan untuk membuat membran adsorpsi dengan berbagai sifat fisika dan kimia yang berbeda: partikel adsorben jenis apapun dapat ditempatkan dalam berbagi jenis membran polimer yang berbeda. Dalam **bab 5** konsep MMM telah dikembangkan untuk aplikasi dalam penghilangan senyawa racun darah dengan menempatkan karbon aktiv dalam membran polimer yang *hemocompatible* (aman bagi komponen darah). Untuk lebih aman dan kuat, membran dengan sistem dua lapis telah dikembangkan dalam bab ini. Membran dua lapis dicetak sekaligus secara bersamaan dengan komposisi larutan polimer yang berbeda; lapisan pertama yaitu lapisan aktiv yang mengandung aktiv karbon dan polimer pendukung sedangkan yang satu lagi adalah lapisan penyaring tanpa karbon aktiv. Membran berpori dan tidak berpori dapat dibuat tergantung kondisi dan parameter dalam pencetakan. Membran jenis dua lapis ini dapat meningkat daya tahan dari membran, lebih baik biocompatibiliti, dan melindungi partikel untuk tidak keluar dari dalam membran.

Adsorpsi suatu model senyawa racun dalam darah, pada kasus ini adsorpsi creatinine (Crt) pada MMM AC telah dipelajari dalam **bab 6**. Membran ini bisa menangkap racun selama proses hemodialisis dan hemofiltrasi. Dia mampu mengadsorpsi molekul Crt baik dalam kondisi statis atau dinamik dengan kemampuang yang tinggi. Kemampuan membran yang tinggi dalam menghilangkan racun secara dinamis dibuktikan dalam *dead-end* filtrasi, *cross-flow* filtrasi dan dialisis. Dalam kondisi dinamis, kemampuan membran untuk menangkap racun dipengaruhi oleh kecepatan alir dari larutan. Tetapi karena prosesnya sirkulasi secara kontinu maka jumlah racun yang diserap oleh membran adalah sama. Dalam dialisis, sifat adsorpsi dari

membran lebih utama perannya dalam penghilangan racun Crt daripada difusi. Secara umum MMM AC mempunyai sifat yang khas yaitu mampu melakukan dua kinerja yaitu adsorpsi dan filtrasi secara bersamaan dalam satu tahap.

Pembuktian bahwa MMM lebih aman dan memiliki kemampuan lebih tinggi dalam pemisahan senyawa beracun telah dipelajari dalam **bab 7**. Untuk ini telah dibuat dua jenis membran dengan menempatkan aktiv karbon dan resin penukar anion Lewatit M500 kedalam cellulose asetat membran. Membran ini menunjukkan kemampuan yang tinggi dalam mengadsorpsi endotoxin baik dalam keadaan statis atau dinamis. Membran ini sangat efektiv dalam mengadsorpsi endotoxin baik dari bagian dialisat maupun dari bagian cairan darah. Kemampuannya ini menunjukkan bahwa MMM adalah cocok dalam menyatukan membran hemofiltrasi dan hemodialisis dengan hemoperfusi (adsorpsi). Penyatuan teknik ini memungkinkan untuk meningkatkan kualitas dari terapi karena membran mempunyai kapasitas yang tinggi untuk menghilang baik racun ukuran kecil, sedang, maupun besar dari dalam darah. Membrane ini juga mampu memblok endotoxin dari dialisat untuk tidak masuk ke dalam cairan darah. Dengan kemampuannya ini, akan mempunyai prospek yang sangat baik untuk diaplikasikan pada pasien yang menderita penyakit ginjal, hati, keracunan, dan sepsis dalam membantu pemisahan dan penghilangan semua bahan beracun dan pengotor dari dalam darah.

About the Author

Curriculum Vitae

Saiful was born on 22 September 1969 in Beureugang-Meulaboh-Nangroe Aceh Darussalam, a city located in Northern of Sumatra Island, Indonesia. He graduated with bachelor degree in Chemistry at the Institute Technology Sepuluh Nopember Surabaya, Indonesia. In 1999, he obtained his Master of Science in Analytical Chemistry in the Institute Technology of Bandung, Indonesia. After graduation he became a junior lecturer at Chemistry Department, Syiah Kuala University Banda Aceh. In February 2003 he started as a PhD student at Membrane Technology Group of the University of Twente, the Netherlands. His project was focusing mainly on developing Mixed Matrix Membrane for Biologics separation, specifically for protein (enzyme) capturing and concentration and blood toxins removal. The project was supported by the Membrane Technology Group of the University of Twente and the Ministry of Education of the Republic Indonesia. He will back to Syiah Kuala University Banda Aceh, to continue his carrier in research and education.

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