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Journal of Chromatography B, 803 (2004) 215–223
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Adsorptive membranes for bilirubin removal

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Received 4 August 2003; received in revised form 23 December 2003; accepted 29 December 2003

Abstract

In this study, we employed ethylene vinyl alcohol (EVAL) adsorptive membranes with bovine serum albumin (BSA) as bioligand for affinity supports for bilirubin (BR) retention. Microfiltration membranes were prepared from ternary or quaternary water/(1-octanol)/DMSO/EVAL systems. To obtain active binding sites for BSA, the EVAL membranes were either chemically functionalized in aqueous and organic medium and by plasma dischargement or physically activated by entrapping of active particles. Static BR removal was determined for all EVAL-BSA membranes. BR retentions relevant for human plasma were gained for the mixed adsorber membranes and additionally investigated in the dynamic mode.

Keywords: Adsorptive membranes; Bilirubin

1. Introduction

In recent years, (*bio*)*affinity adsorption* has developed into a powerful tool in the biomedical or biochemical field, especially for the removal of toxins from human plasma. Beside ongoing research in protein chromatographic systems, hydrophilic microfiltration membranes with functional sites that can be used for binding (bio)affinity ligands are increasingly investigated as alternative adsorbents [1,2].

The advantages that microporous adsorber membranes offer compared to fixed bed chromatography are the large surface areas, short diffusion paths and low-pressure drops. Transport through the porous structure is controlled by convective instead of diffusive transport leading to a tremendous reduction in the mass transfer resistance. The adsorption processes are therefore dominated by the binding kinetics, resulting in shorter process times and minimal denaturation of product.

An ideal membrane for biomedical application requires high hydrophilicity and thus low nonspecific protein adsorption, fairly large pores with a narrow pore size distribution, high biological, chemical and mechanical resistance and a high density of reactive groups that can bind ligands with a specific binding capacity for the targeted ligate [3,4]. Next to surface modified microfiltration membranes, also particle-loaded membranes and particle-embedded glass fiber disks have widely been used to isolate and concentrate selected analytes prior to chromatographic analyses [5,6].

In this work we focus on the removal of bilirubin (BR) from aqueous solution by microporous BSA-immobilized ethylene vinyl alcohol (EVAL) adsorptive membranes, whose preparation, functionalization and ability to bind bovine serum albumin (BSA) has been investigated in several articles [7–9]. Functionalization of the EVAL membranes has been carried out either by direct coupling of BSA to pre-activated OH-groups of the vinyl alcohol units (*polymer functionalized EVAL membranes*) or by incorporation of ion-exchange particles (*particle loaded EVAL membranes*) with a high BSA adsorption capacity. Determination of the selective BR adsorption capacity allows us also to verify whether the original biological activity of BSA has been retained after applying the different BSA immobilization routes. BSA is one of the natural carrier proteins for bilirubin. Even though the exact locations of the binding sites on serum albumins have not yet been clearly elucidated, it is generally accepted that each albumin molecule has two strong bilirubin binding sites and numerous binding sites which bind BR loosely [10].

Bilirubin, a bile pigment, is formed as a result of the catabolism of hemoglobin from aged red blood cells in all mammals [11]. Free BR is toxic, and is therefore transported

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doi:10.1016/j.jchromb.2003.12.020

as water-soluble complex with albumin to the liver where it is normally conjugated and excreted into the bile. Although the physiological function of BR in the human body is not yet fully understood, it has been suggested that bilirubin can serve as a chain-breaking antioxidant. Disorders in the metabolism of BR, especially common among newborn infants, may cause jaundice, a yellow discoloration of the skin and other tissue. High concentrations of free BR can evoke hepatic or biliary tract dysfunction and permanent brain damage [12]. Neurological dysfunctions as kernicterus or bilirubin encephalophaty may develop if the BR concentration in the plasma rises above 0.03 mg/ml.

Although methods are known that help the body to eliminate the excess of bilirubin, the main treatments carried out, such as hemodialysis, phototherapy and hemoperfusion, remove BR directly from the plasma of patients suffering from hyperbilirubinemia. Under these, hemoperfusion, i.e., circulation of blood through an extracorporeal unit containing an adsorbent system for BR, has become the most promising technique [13–25]. In most cases basic ion exchange materials have been used, but uncharged polymers can also adsorb bilirubin from aqueous media. Numerous natural polymers like agarose, agar and charcoal as well as synthetic polymers, such as polystyrene divinyl benzene, ethylene glycol dimethacrylate, hydroxyethyl methacrylate, acrylamide have recently been investigated as sorbents for the removal of BR directly from the plasma of patients suffering from hyperbilirubinemia. The applicability of albumin in BR removal is limited by the large amount of bound BSA required to remove significant amounts of bilirubin. Its high BR specificity and the fact that albumin immobilization onto natural or synthetic polymeric supports improves their biocompatibility [26], however, led to an extensive use of BSA as bioligand in studies of BR removal [27–30].

This paper is organized in the way that we will first summarize the results on the preparation and functionalization of the EVAL membranes tailored as affinity adsorbents for BR removal. In the results and discussion section optimal conditions for the preparation of the BSA-immobilized particle loaded EVAL membranes are described in detail. All BSA-immobilized EVAL membranes are investigated for static BR removal and the mixed matrix adsorber membranes, which gained the highest retentions additionally in the dynamic mode.

1.1. EVAL adsorptive membranes

Ethylene vinyl alcohol, a semi-crystalline random copolymer consisting of *hydrophobic* ethylene and *hydrophilic* vinyl alcohol segments, has become a promising biomedical material since it is water insoluble and possesses a good blood compatibility. Recently, EVAL membranes have been investigated for possible application in blood purification and plasma protein separation [31,32], microfiltration [33,34], islets for artificial pancreas [35], and as support for growth of neuronal cells [36]. The presence of the hydrophilic vinyl alcohol segments on the membrane surface increases the water-wettability allowing a lower operating pressure gradient and reduces the hydrophobic interaction with biological compounds (ligates). The latter is especially in protein separations responsible for non-selective adsorption and membrane fouling. Despite the usability of the secondary alcohol groups for covalent binding of affinity ligands, only few studies have been performed on the surface activation and functionalization of commercial EVAL membranes and their application in affinity based separation processes [37–40].

In previous articles, we have investigated the preparation and functionalization of cellular-type EVAL microfiltration membranes with respect to their applications in affinity separation and in protein recovery [7–9]. To prepare membranes with suitable morphologies and properties, the membrane forming phase separation process has been studied in detail for the ternary and quaternary water/(*n*-alcohol)/DMSO/EVAL systems [7]. In the ternary system delayed demixing conditions have been induced by addition of DMSO to the coagulation bath. The best cellular-type membranes obtained (E/50/O0) were not completely free of macrovoids, displayed a nearly dense top surface and a substructure with a poor pore-interconnectivity, which could not be completely dried without pore collapse. All details concerning the preparation, morphology and performance are given in Fig. 1 and Table 1. Addition of 1-octanol as nonsolvent additive to the casting solution, leads to an open, high interconnected porous structure without macrovoids. The high pore interconnectivity is beneficiary for the interaction of the matrix-bound ligand with the ligate during the affinity binding step.

1.2. Polymer functionalized EVAL membranes

We investigated direct functionalization of the EVAL membranes by coupling of BSA as model ligand via the amino groups of the protein after activation of the secondary alcohol groups [8]. The E/50/O0 membranes were activated in aqueous solution with glutaraldehyde according to the reaction scheme presented in Fig. 2. Optimal conditions for activation and BSA coupling reactions are presented in Table 1 together with the obtained BSA loading. Activation of the collapse-resistant E/0/O20 membranes was examined using trichloro-*s*-triazine (sTT) in organic medium (see scheme in Fig. 2) and by low-pressure glow dischargement. The latter introduces more reactive oxygen-containing functional groups, such as hydroperoxide, epoxide, carbonyl, and carboxylic acid groups at the membrane surface. During the plasma treatment the surface roughness and porosity of the top layer increases dramatically leading to higher water fluxes, but leaves the morphology of the cross-section nearly unchanged (Fig. 1). The highest BSA-immobilizations were obtained for the sTT or CO_2-plasma activated E/0/O20 membranes, whereby the latter reaches values as reported in literature for a complete BSA monolayer formation.

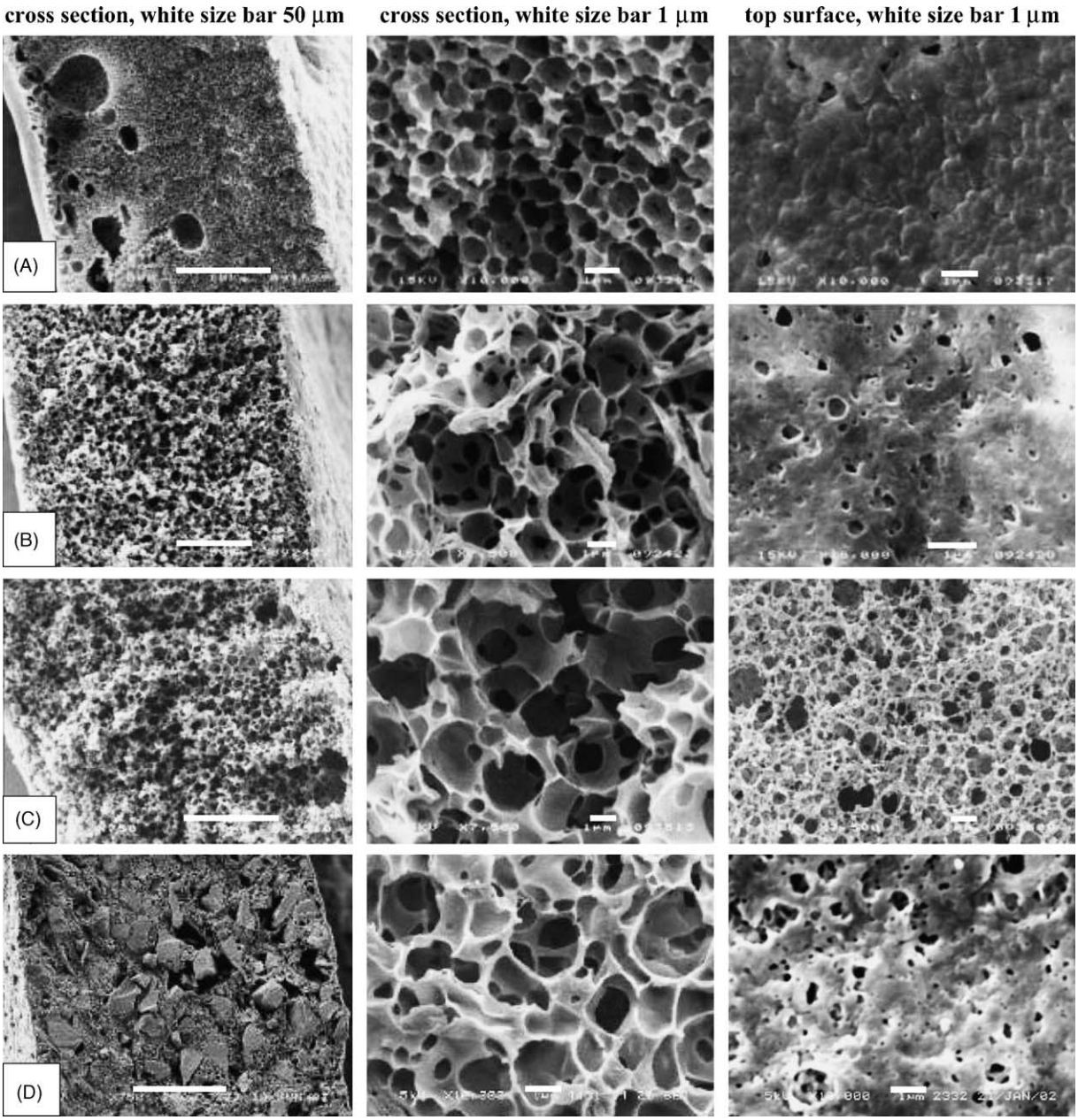


Fig. 1. SEM micrographs for adsorptive EVAL membranes: (A) E/50/O0 prepared by immersing a cast film of 10% EVAL in DMSO into a coagulation bath containing 50% DMSO at 50 °C; (B) E/0/O20 prepared from 10% EVAL in DMSO in the presence of 20% 1-octanol as non-solvent additive by immersing the cast film into water at 50 °C; (C) E/0/O20 prepared from 10% EVAL in DMSO in the presence of 20% 1-octanol after plasma treatment; (D) E/MMA prepared from 10% EVAL in DMSO in the presence of 10% 1-octanol as non-solvent additive, by incorporation of 65% Lewatit ion exchange particles into the polymeric support.

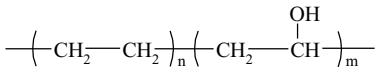
1.3. Particle loaded EVAL membranes

Despite direct functionalization using the secondary alcohol groups of the vinyl alcohol segments, we also employed the microporous EVAL membranes for the preparation of mixed matrix adsorber membranes by incorporation of ion exchange particles. The mixed adsorber systems combine the advantages of membrane technology (easy scale up, low pressure drop and high throughputs) with those of column chromatography (high selectivity and high binding capacity). The good accessibility for the protein to adsorptive sites was reflected in the high static and dynamic protein capacities obtained that allow for the employment of the particle loaded EVAL membranes for protein recovery, as protein concentration medium [9] and for the separation of similarly sized proteins with different isoelectric points [41].

The E/MMA membranes, which are suitable for later application in BR removal, contain 65 wt.% cation exchange resins (CER) and were prepared by incorporation of Lewatit type particles in the casting solution before immersing into

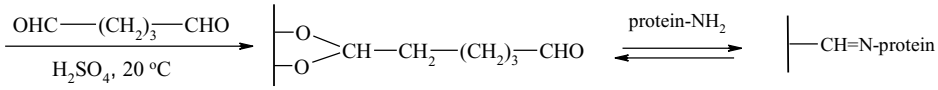
Table 1
Details in membrane preparation, characterization and modification as well as the obtained BSA-loading for the polymer-functionalized and particle loaded EVAL adsorptive membranes applied in BR removal

Membrane	E/50/O0	E/0/O20		E/MMA
Preparation conditions				
Casting solution	10% EVAL in DMSO	10% EVAL, 20% 1-octanol in DMSO		8.5% EVAL, 8.5% octanol, 15.66% CER (Lewatit 112WS, particles <20 μm) in DMSO
Coagulation bath	50% DMSO at 50 °C	Water at 50 °C		Water at 40°C
Drying conditions	By solvent exchange, not completely dryable	In air, completely dryable		In air, completely dryable
Characterization		Inactivated membrane	After CO_2 plasma treatment	
Morphology	Asymmetric, macrovoids, dense top surface, cellular microporous support, low interconnectivity	Macrovoid free, more open top surface, high pore interconnectivity	Macrovoid free, very open surfaces, open porous structure, high pore interconnectivity	Macrovoids free, open surfaces, particles tightly held together within the porous matrix, high pore interconnectivity, no significant loss of particles on the glass surface
Average pore size	0.2 μm	0.3 μm	0.5 μm	0.3–0.5 μm
Water flux	300 l/(h m^2 bar)	1200 l/(h m^2 bar)	5500 l/(h m^2 bar)	3000 l/(h m^2 bar)
Modification				
Activation reaction	Glutaraldehyde method	Trichloro-*s*-triazine method	CO_2-plasma	Unnecessary since the membranes bear an adsorptive function with high protein binding capacities
Reaction medium	Aqueous media	Organic solvents	Dry state	
Activation conditions	1% GA at pH 3, 24 h at room temperature	10% sTT in dry dioxane, 30 min at room temperature	0.15 mbar, 75 W, 10 cm^3/min CO_2, 15 min	
BSA loading				
Loading conditions	1 mg/ml BSA at pH 7, 24 h at 4 °C	1 mg/ml BSA at pH 5, 24 h at 4 °C	1 mg/ml BSA at pH 4.5, 24 h at 4 °C	5 mg/ml BSA at pH 4.5, 24 h at 4 °C
Maximal BSA loading	8 mg BSA/g membrane	18 mg BSA/g membrane	22 mg BSA/g membrane	135 mg BSA/g membrane



EVAL

1. protein coupling via glutaraldehyde activation



2. protein coupling via trichloro-s-triazine activation

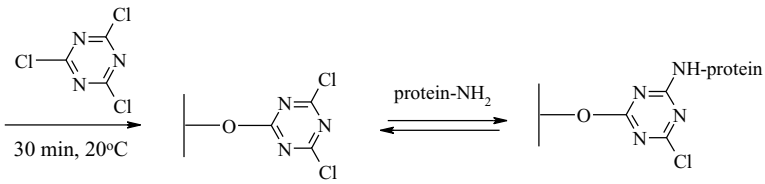


Fig. 2. Reaction scheme for activation and BSA coupling on the E/50/O0 and E/0/O20 membranes using glutaraldehyde (GA) in aqueous media and trichloro-*s*-triazine (sTT) in organic medium, respectively.

a water coagulation bath at 40 °C [9]. The ion exchange particles are tightly held together within the interconnected open porous polymeric matrix (Fig. 1). No significant loss of particles was observed during the membrane formation process. At pH values lower than the BSA isoelectric point ($pI_{BSA} = 4.8$), BSA adsorbs onto the resin particles whereby BSA loadings were obtained which were about 7–18 times higher than those obtained for the covalently bound BSA membranes (see Table 1).

2. Experimental

2.1. Materials and membrane formation

All basic EVAL membranes were prepared from casting solutions containing EVAL with an average ethylene content of 44 mol% (Aldrich), dimethylsulfoxide (DMSO, Merck) as solvent and for the quaternary systems 1-octanol (Fluka) as nonsolvent-additive by immersion precipitation. As coagulation bath either mixtures of 50% DMSO in water (ternary system) or pure water (quaternary system) were used. The exact preparation conditions for the three membranes investigated (E/50/O0, E/0/O20, and E/MMA) are given in Table 1, more information can be found in [7,9].

Surface modification of the secondary alcohol-groups of the vinyl alcohol segments to obtain the polymerfunctionlized EVAL adsorptive membranes, was carried out with glutaraldehyde (GA, Sigma) in aqueous solution as well as trichloro-*s*-triazine (sTT, Aldrich) in dioxane (Merck). GA has been purified over activated carbon until a single absorption peak was observed at 280 nm. Dioxane was dried before use over molecular sieves with 4 Å pores (25 g per liter of reagent). Lewatit ion exchange resins 112WS, kindly supplied by Caldic, Belgium were incorporated into the basic EVAL polymeric membranes to prepare the particle loaded adsorptive membranes [9]. The natural bilirubin carrier BSA (fraction V, Sigma) was used as bioligand for the EVAL based affinity membranes. Bradford reagent (Sigma) was employed to determine protein concentrations. Sodium dodecyl sulfate (SDS, Acros) was used to remove physically adsorbed BSA from the membrane surface. Solid bilirubin (Fluka) was immediately before use in the affinity separation experiments dissolved in 10 mM NaOH solution followed by dilution with ultrapure water. Water was purified with a Millipore purification unit Milli-Q plus and all other chemicals were used as received.

2.2. Ligand coupling onto EVAL based adsorptive membranes

The functionalized EVAL E/50/O0 and E/0/O20 membranes (see Table 1) were immersed in a protein solution (1 mg/ml BSA) at pH values varying between pH 4–8, depending on the functional group induced in activating the membrane, and incubated at 4 °C for 24 h. After BSA-coupling, the membranes were washed with phosphate buffer and deionized water in order to remove unreacted proteins. Where after the membrane was immersed for 24 h in a 3% SDS solution in order to remove the physically adsorbed proteins. The amount of protein covalently bound onto the EVAL-membranes was determined by a depletion method. The protein concentration was measured using the Bradford reagent method [42] at 595 nm with a PU 8720 UV-Vis spectrophotometer. The amount of protein desorbed in SDS was subtracted to obtain the values for covalent protein coupling.

For immobilization of albumin onto the particle loaded EVAL E/MMA membranes a known amount of cation exchange membrane was equilibrated with a concentrated solution of BSA dissolved in 50 mM acetate buffer at pH 4.5. At this pH, which is lower than the BSA isoelectric point ($pI_{BSA} = 4.8$), the protein is positively charged while the entrapped sorptive particles are negatively charged. The protein is adsorbed by electrostatic interactions onto the resins embedded in the mixed matrix adsorber membranes. Hence the BSA concentration in the bulk solution decreases until equilibrium is reached. The protein uptake per membrane mass was determined using a depletion method by measuring the absorbance at 280 nm.

Since non-covalent coupling of BSA onto the adsorptive membrane can result in significant ligand losses from the polymeric support, covalent coupling is preferable. Therefore, in a sequential step, the adsorbed BSA-E/MMA membrane complex was treated with 1% GA solution at pH 4 to crosslink the BSA molecules adsorbed on the ion exchange particles and thus to minimize protein leaching out due to changes of environmental conditions such as pH or ionic strength. The reaction time was varied from 1 to 12 h. For testing the stability of the coupled BSA, the membranes were subsequently exposed to desorptive solutions such as 1 M NaCl and 50 mM borate buffer at pH 10 for 4 and 12 h, respectively. The amount of BSA immobilized by cross-linking was calculated by subtracting the amount of protein desorbed into the desorptive buffers from the total amount of BSA adsorbed into the membrane.

2.3. Bilirubin removal

Bilirubin removal by the BSA-immobilized adsorptive EVAL membranes was carried out both in the static and the dynamic mode. The BR solution employed in the experiments was prepared by dissolving solid BR in 10 ml of a 10 mM NaOH solution. Just before use, the solution was diluted with ultrapure water to 0.2 mg/ml, which equals 10 times the normal bilirubin concentration in the plasma. For *static adsorption experiments*, a known amount of membrane was equilibrated with the BR solution. The amount of BR removed per unit of membrane mass (q) was calculated applying a depletion method by measuring the absorbance at 438 nm. The adsorption isotherm for the BSA-immobilized E/MMA membrane was obtained by incubating the same

amount of membrane with different initial amounts of BR and followed in time to obtain the equilibrium adsorption as function of the equilibrium concentration.

The *dynamic adsorption performance* of the BSA-immobilized particle loaded EVAL membrane adsorbers was evaluated at constant flow rate of 10 l/(h m^2) using a compressed nitrogen stirred dead-end filtration cell. Because the capacity of a single adsorptive membrane is limited, 10 sheets were stacked in an ultrafiltration device to achieve the necessary accuracy in the measurements. This configuration permits rapid, low-pressure adsorption of BR in either batch or continuous recycle modes, whereby the targeted product can substantially be concentrated. A solution containing 0.2 mg BR/ml was permeated through the membrane stack. The permeate was collected and split into fractions as time proceeds. BR concentrations in the feed and the collected permeate fractions were determined spectrophotometrically by measuring the absorbance at 438 nm. The protein mass adsorbed per unit of membrane bed (q) was calculated by numerical integration over the filtration run as mentioned above.

3. Results and discussion

In the present study, we applied the three differently chemically activated EVAL membranes and the particle loaded EVAL membrane after BSA loading for the removal of bilirubin from aqueous solutions. Their structural properties are summarized in Table 1. The E/50/O0 and E/0/O20 membranes could be applied without post treatments since the BSA binding was covalent attached to the membrane surface. In case of the E/MMA membranes the BSA is only adsorbed by electrostatic interactions and therefore needed to be fixated on the surface before employment in BR removal.

3.1. Ligand coupling onto pre-activated membrane

Physically adsorbed BSA onto the pre-activated membranes E/50/O0 and E/0/O20 was removed by washing out using a SDS solution. The amount of physically bound BSA was quantified to about 2 mg BSA per unit mass of membrane. The remaining covalently coupled BSA per mass of GA-activated E/50/O0 membrane amounted to 8 mg BSA/g membrane (see Table 1). For the sTT activated E/0/O20 membranes a maximum BSA-immobilization of 18 mg/g of membrane was determined. This value is by a factor of 2 higher than for the E/5O/O0 membranes. The highest degree of BSA-immobilization, 22 mg/g per membrane mass, was obtained after pre-activation in the dry state by a CO_2-plasma of the E/0/O20 membranes.

BSA adsorption onto the E/MMA membranes reaches values of around 150 mg BSA per membrane mass within 24 h of incubation. To fixate the physically adsorbed BSA onto the porous matrix cross-linking with GA has been investigated. Fig. 3 shows the amount of BSA released from

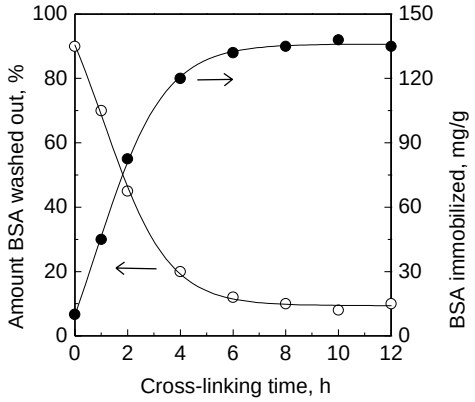


Fig. 3. The effect of cross-linking time on the BSA immobilization rate onto the particle loaded EVAL membranes.

the membrane into a washing solution as a function of the exposure time of the BSA loaded membrane in the GA-solution. For the washing, solutions with different pH and ionic strength were used than in the BSA adsorption step. The amount of uncrosslinked BSA decreases with the cross-linking time and reaches a constant value of approximately 10% of the total BSA adsorbed, when cross-linking was carried out for more than 6 h. The amount of chemically attached BSA onto the EVAL polymeric matrix increases thus with increasing cross-linking time. After 6 h the amount of protein immobilized onto the membrane adsorber amounted to about 135 mg BSA/g membrane. This value is six times higher than the maximum albumin immobilization by covalent coupling onto the E/O/O20 membranes.

3.2. Static bilirubin adsorption capacity

To minimize the errors caused by the deterioration of BR due to environmental conditions, a study of BR stability at different temperature and light conditions was performed (Fig. 4). Within 12 h almost 40% of the bilirubin decomposed by direct exposure to sunlight and about 20% by increasing the temperature from room to body temperature. Therefore, all further adsorption experiments were carried out in the dark maintaining the temperature at 20 °C.

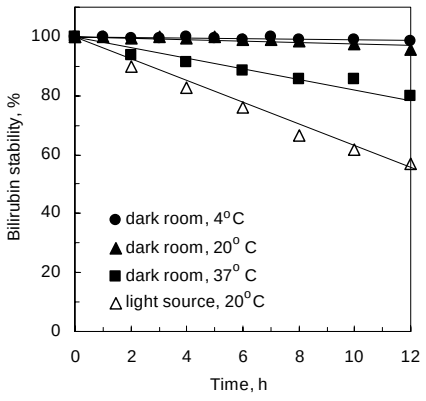


Fig. 4. Bilirubin stability as a function of temperature and light exposure.

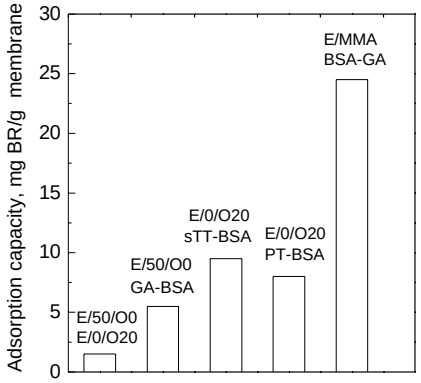


Fig. 5. Bilirubin adsorption capacities for the BSA-immobilized adsorptive EVAL membranes from aqueous solution containing 0.2 mg BR/ml.

Static BR adsorption capacities of the prepared adsorptive membranes were determined batch wise. Fig. 5 shows the non-specific adsorption of BR onto the bare E/50/O0 and E/0/O20 EVAL membranes and the specific BR adsorption onto the BSA-functionalized membranes within 12 h of adsorption. One should keep in mind, that the sorbent specificity presents a major requirement in affinity separation. The non-specific interaction between the EVAL polymeric support and the targeted ligate (BR) should be minimal in order to display a high specificity. The amount of BR adsorbed onto the bare EVAL membranes was below 2 mg BR/g membrane. By a factor of 2.5–12.5 higher adsorption capacities (ranging from 5 to 25 mg BR/g adsorptive membrane) were obtained in the case of BSA-functionalized adsorptive EVAL membranes. The results demonstrate an increase in BR retention with the rate of BSA immobilization, indicating that the biological activity of BSA is retained during the different immobilization procedures employed.

The more precise comparison reveals, that although the albumin immobilization onto the E/MMA membranes is by a factor of 6 higher than for the E/O/O20 membranes, the amount of bilirubin removed by the particle-loaded E/MMA adsorber is just three times higher than the one obtained for the BSA loaded E/O/O20 membranes (Fig. 5). This can be explained by the involvement and using up of the BR active sites of BSA in the cross-linking reaction. Furthermore, some of the binding sites on the albumin molecule may after immobilization be less accessible for bilirubin. This assumption is supported by a factor of 10 decrease in the permeation rate through the E/MMA membranes after cross-linking.

For the BSA-immobilized E/MMA membranes, which revealed the highest BR retention, the time dependence of the BR adsorption was also investigated. Fig. 6 shows the BR adsorption rate obtained by following the decrease of BR concentration within the bulk solution as a function of time. The graph indicates that the adsorption process is completed within 6 h of incubation, reaching an equilibrium adsorption value of about 25 mg BR/g membrane. At this point, it is worthwhile mentioning that the time found has only little meaning for the time of treatment in the actual BR removal

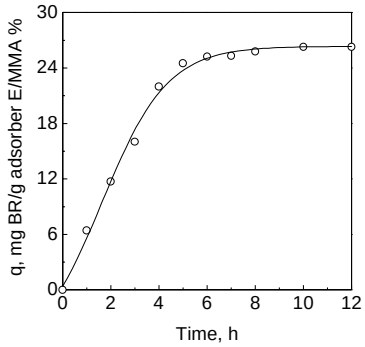


Fig. 6. Bilirubin adsorption rate from solution of 0.2 mg BR/ml for the membrane E/MMA.

process. It presents only a measure for the apparent diffusion coefficient of BR into the E/MMA membranes. However, to perform more complex simulations for chromatographic performances the diffusion coefficient is required as an input parameter.

To obtain the adsorption isotherm, the dependence of the BR adsorption on the bilirubin concentration has been measured for the BSA-immobilized E/MMA adsorber membrane. Fig. 7 displays the equilibrium BR adsorption capacity as a function of the equilibrium bulk concentration. The maximum adsorption capacity is reached for BR concentrations higher than 0.15 mg BR/ml bulk solution. Adsorption of proteins is a semi-reversible process and a complete model, which describes both adsorption/desorption and exchange of proteins, has not yet been developed. A description of the protein adsorption onto solid surfaces requires solving of the kinetic and transport equations coupled by the boundary conditions. The measured BR adsorption isotherm can be reasonably described by a Langmuir adsorption isotherm, with a maximum adsorption capacity of 30 mg BR/g membrane and a dissociation constant of 0.08 mg/ml (dashed curve in Fig. 7). The Langmuir adsorption isotherm is most commonly used to describe protein adsorption equilibrium onto ion exchangers. It assumes that the adsorbate molecule adsorbs only at one adsorption site and implies that the adsorption process is completely reversible and that no permanent interactions take place between adsorbed molecules.

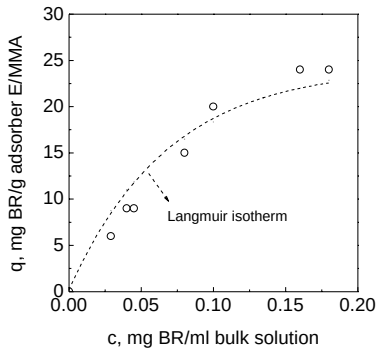


Fig. 7. The effect of bilirubin concentration on the BR adsorption capacity of the BSA-immobilized E/MMA membrane.

The BR adsorption capacities obtained for the BSA-immobilized EVAL adsorber membranes are comparable with literature data for adsorbers with serum albumin as ligand. The high molar ratio of BR/Albumin can be explained by the fact that albumin attached to polymers show a higher binding capacity for albumin-bound toxins than free albumin [43]. It may also be reasonable to expect that the free bilirubin in bulk solution aggregate onto adsorbed bilirubin [24,44–46]. The use of epoxy-activated gels such as modified cross-linked polybutadiene hydroxyethyl methacrylate for albumin immobilization and their performances as supports in affinity chromatography to retain BR was investigated by Alvarez et al. [30] giving maximum bilirubin retentions of 3.1 mg BR/g beads. Sideman et al. [13,26] suggested the application of hemoperfusion for the removal of the BR from jaundiced newborn babies by using albumin-deposited macroreticular resins with bilirubin adsorption capacities ranging from 2 to 24 mg BR/g resin. In this perspective, the results obtained allow us to conclude that the prepared EVAL-based adsorptive membrane systems represent a viable alternative for BR retention from aqueous solutions.

Recently, research interest focused on preparation of bilirubin adsorbents with other functional ligands including different peptide sequences as pendant group that corresponds to the active sites on albumin as well as dye-ligands. Polypeptide functionalized polyacrylamide beads prepared by Zhu et al. were used as an affinity sorbent system for BR removal with adsorptions of 0.2–75 mg BR/g resin [10]. Denizli et al. [21–23] have produced bioaffinity sorbents with poly(HEMA) or poly(EGDMA-HEMA) microbeads as basic carrier for bilirubin removal from aqueous media, including plasma, with a yield of 6.8–32.5 mg/g sorbent. Senel et al. [25] reported that up to 48.9 mg BR were retained per gram of modified polyamide hollow fibers with Cibacron Blue F3GA attached as affinity ligand on the membrane surface. Nonetheless their application is limited mainly by the adsorbent poor biocompatibility and lack of specificity. By employing synthetic “bio” ligands, the gain in adsorption capacity is often paired with a loss in biocompatibility.

3.3. Dynamic binding capacity

Based on the promising BR retentions obtained for the BSA-immobilized particle loaded membranes, we carried out dynamic BR binding capacity measurements through a stack of membranes. In dynamic operations, ligate molecules are transported through the adsorptive membrane by the convective flow of the feed solution in a single pass mode. Transport of ligates to the adsorptive sites is a function of flow conditions, protein diffusion and kinetic adsorption parameters. To obtain the BR breakthrough curve through a stack of 10 flat sheets E/MMA membranes, a bilirubin solution of 0.2 mg/ml was permeated through the stack in a stirred dead-end filtration cell at a constant flow rate of 10 l/(h m^2). Fig. 8A presents the ratio between permeate and feed concentrations $C/C0$ as function of the permeate volume. The average BR concentration C_{av} was calculated by numerical integration of the BR concentration in the permeate curve during the filtration run. In Fig. 8B both C_{av} and the percentage of BR removed from the feed solution are shown as a function of the BR adsorption capacity. It can be seen that 80% of BR was adsorbed into the matrix during the permeation (the average bilirubin concentration in the permeate reached 0.04 mg/ml for a starting feed BR concentration of about 0.2 mg BR/ml). In real biomedical applications the filtration process can be stopped in an earlier stage when the average BR concentration in the permeate is below the normal BR level in the plasma.

The dynamic BR adsorption capacity, however, is about two times lower than the corresponding static capacity. This can be attributed to a non-uniform flow distribution and to resistance against mass transport processes. Axial and radial diffusion, sorption kinetics and non-uniformities in membrane porosity and thickness can affect affinity membrane performance characteristics, especially the sharpness of the breakthrough curve. Full adsorption capacities can only be achieved if the flow rate through the adsorber membrane is slow enough to allow each adsorbate molecules to diffuse to the adsorptive site and to rearrange and/or unfold their structure to its most favorable state before the interstitial volume continues to move through the membrane.

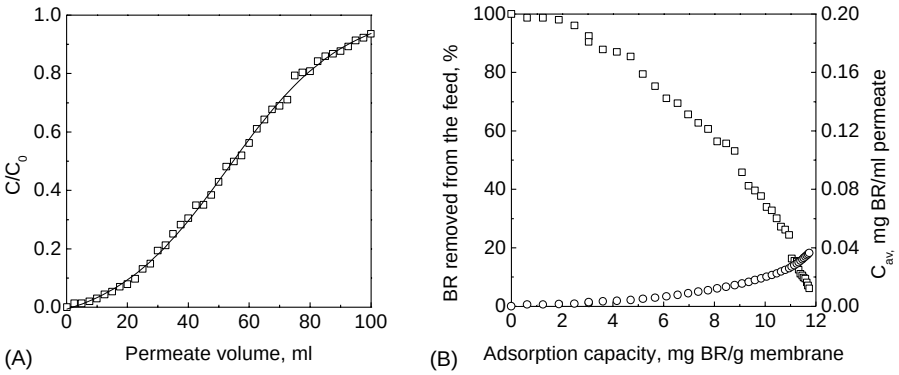


Fig. 8. Efficiency of bilirubin removal for a stack of 10 BSA-immobilized E/MMA membranes operated at a constant filtration flow rate of 10 l/(h m^2) with 0.2 mg BR/ml feed concentration.

4. Conclusions

In this affinity separation study, we investigated the static and dynamic removal of bilirubin from aqueous solutions. BSA, a natural BR-carrier employed as bioligand in this study, was coupled to differently functionalized EVAL membranes. Functionalization has been carried out either by direct covalent coupling of BSA to chemically pre-activated EVAL matrices or by incorporation of 65 wt.% Lewatit type cation exchange particles with a high BSA adsorption capacity. Chemical pre-activation of the EVAL membranes was carried out in the wet state with GA or sTT. In the dry state the matrix was pre-activated using a CO_2-plasma treatment. BSA-immobilized particle loaded EVAL membranes were prepared by GA cross-linking of physical adsorbed BSA.

The BSA-immobilized membranes showed in the static mode a BR retention, which was by a factor of 2.5–12.5 higher than the 2 mg/g obtained for the bare EVAL membranes. The low retention value of the bare EVAL indicates a low non-specific interaction of the ligate with the EVAL matrix and make it thus attractive as support material for affinity separation. The BR retention scaled with the amount of immobilized BSA, demonstrating that the biological activity of BSA stays intact during the different immobilization procedures. The BSA immobilized particle loaded membrane (E/MMA) displayed with 25 mg BR/g membrane the highest static bilirubin adsorption capacity. The dynamic binding capacity was somewhat lower, which could be attributed to mal distribution in the flow pattern and low adsorption kinetics. The obtained BR capacities and the high specificity and biocompatibility of both the ligand and the polymer matrix made clear that this system should be more intensely investigated as a viable alternative for the bilirubin retention from human plasma.

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