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Surface modification of microporous polyamide membranes with hydroxyethyl cellulose and their application as affinity membranes

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Abstract

Activated membranes for covalent immobilization of hydroxyethyl cellulose (HEC) were obtained by reaction of microfiltration nylon membranes (N66) with bisoxirane or formaldehyde. Covalent linkage of HEC was essential for the reduction of non-specific interactions with proteins and yielded a HEC coating as a layer of extended coils at the porous network of the membrane after both activation methods, thus reducing the water permeability of the modified membranes. Immobilization of iminodiacetic acid (IDA) onto HEC-coated membranes via standard oxirane chemistry provided IDA affinity membranes with almost identical properties to IDA chromatographic sorbents. The extended coil structure of the coating accounts for protein capacities higher than a theoretical monolayer coverage would yield. The thermodynamics of the interaction of metal chelate affinity (MCA) membranes with the proteins lysozyme, ovalbumin (OVA) and concanavalin A (Con A) demonstrated that dissociation constants $K_D > 10^{-5}$ M are unsuitable for the retention of target proteins on a single membrane disc. This was demonstrated by the separation of a protein mixture of lysozyme ($K_D \approx 10^{-5}$ M) and Con A ($K_D \approx 10^{-7}$ M) on an MCA membrane.

1. Introduction

Over the last few years affinity membranes have been introduced in downstream processing of proteins as protein adsorbers [1–4]. In order to overcome the main drawbacks of classical soft gels, such as compressibility and slow process rates, microporous membranes with various covalently bound affinity ligands were employed [5]. In such membranes the transport of the biomolecules to the affinity ligands occurs mainly by convection. The low diffusional resistance leads to faster adsorption kinetics [6,7]. These benefits were investigated in membrane chromatographic systems employing stacked membranes [8,9] or hollow fibres [10]. The high volumetric throughput combined with a membrane-based immunoaffinity purification process allowed the purification of recombinant proteins from dilute solutions [10]. In principle, these membranes are capable to handle crude cell or microbial suspensions. An integration of separation by microfiltration and adsorption of target products or contaminants by affinity membranes

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has been demonstrated by means of cross-flow filtration [11]. Typical ligands used are ion-exchange groups [2,3,9], pseudobiospecific ligands such as reactive dyes [1,8,12] or metal chelators [13,14] and biospecific ligands such as protein A, protein G or receptor molecules [10,15,16].

Most of the polymers used in manufacturing of microfiltration membranes, such as nylon, polysulfone or polyethylene, display undesirable surface characteristics leading to difficulties in processing protein solutions. Proteins tend to adsorb strongly on these membrane surfaces. Different approaches were undertaken to remedy this problem, such as preparation of copolymers for membrane formation, use of hydrophillic alloying polymers, preparation of adsorptive coatings, introduction of hydrophillic groups into polymer backbones and covalent surface coatings by hydrophillic coating polymers, as reviewed by Klein et al. [17]. This review and a patent of Azad and Goffe [18] demonstrated that end groups of polysulfone membranes can be activated by oxirane chemistry to bind hydroxyethyl cellulose (HEC) covalently. A reduction of non-specific protein adsorption was observed and a higher density of reactive sites for immobilization of protein A was produced. End group modification of nylon-6 membranes ensuing polysaccharide immobilization was reported by Klein and Feldhoff [19]. The resulting composite membrane was applicable for covalent attachment of BSA or Cibacron Blue. Also polymer-grafted membranes with ion exchange, metal chelate, reactive dye and histidine ligands were used as affinity membranes [20–22].

The objective of this study was the development of a hydrophillic membrane combining the functional properties of microporous polyamide membranes, such as high consistency of the pore size distribution and mechanical rigidity, and the combination of chemical reactivity and low protein adsorption of HEC. Nylon membranes have only a low concentration of terminal amino groups [19,23], the direct activation of the nylon matrix leading to low ligand densities using metal chelators or triazine dyes [24–26]. Another problem of nylon membranes is the non-specific adsorption of proteins [27–29]. Immobilization of HEC onto nylon membranes was expected to exhibit low non-specific binding of proteins and proper binding capacities for affinity ligands. The initial activation of the nylon matrix by bisoxirane or formaldehyde was directed at the covalent immobilization of HEC to the membrane surface via amino end-groups or amide groups in the nylon chain. The HEC coatings were compared for their non-specific protein adsorption. After the subsequent activation of the HEC hydroxyl groups by epibromohydrin or bisoxirane, iminodiacetic acid (IDA), a metal chelator, was immobilized on the HEC-coated membrane surface. In Fig. 1 a schematic illustration of such an affinity membrane is shown. The adsorption of different model proteins on the metal chelate affinity (MCA) membrane was investigated.

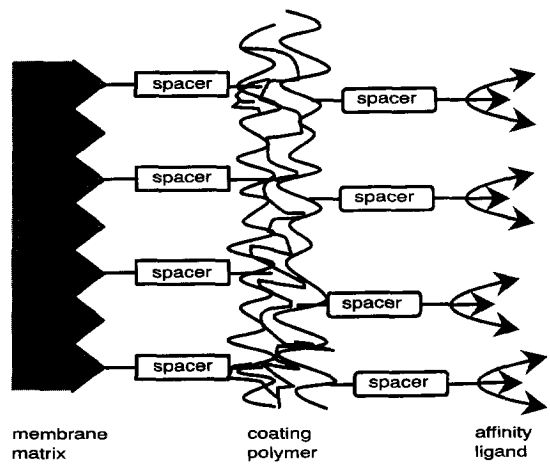

Fig. 1. Scheme of an affinity membrane as obtained by covalent immobilization of a polymer on a membrane matrix and additional linkage of affinity ligands.

2. Experimental

2.1. Materials

Microfiltration membranes (Ultipor N 66, NXG 29325, 0.45 μm) were a gift from Pall (Dreieich, Germany). Epibromohydrin, 1,4-butanediol diglycidyl ether (bisoxirane), sodium dodecyl sulphate (SDS), $CaCl_2$, Cibacron Blue

F3G-A, and hydroxyethyl cellulose (Cellosize WP 40) (HEC) were obtained from Fluka (Neu-Ulm, Germany). Acetic acid, disodium EDTA, iminodiacetic acid (IDA), $MnCl_2$, Na_2CO_3, ethanol and Triton X-100 were purchased from E. Merck (Darmstadt, Germany). HCl, NaOH, NaCl, $CuCl_2$ and formaldehyde were obtained from Riedel-de Haen (Seelze, Germany). Ovalbumin (Grade V) (OVA), concanavalin A (Con A), sodium borohydride and imidazole were obtained from Sigma (Munich, Germany). Bovine hemoglobin (2 × cryst., lyophil., research grade) and hen egg white lysozyme were purchased from Serva (Heidelberg, Germany). Porofil was purchased from Coulter Electronics (Krefeld, Germany).

2.2. Methods

Activation of nylon membranes with bisoxirane

Five membrane discs (47 mm diameter) were shaken for 15 h at 353 K in a solution of 9 ml bisoxirane–1 ml ethanol–1 ml 25 m*M* Na_2CO_3, pH 11. After activation the membranes were washed three times with water at room temperature (Fig. 2a).

Activation of nylon membranes with formaldehyde

Nylon membranes were activated with formaldehyde according to a procedure described by Cairns et al. [30] using nylon fibres, which was modified to account for membrane discs. Ten membrane discs (47 mm diameter) were incubated for 7 h at 333 K in a solution of 20 ml formaldehyde (>36.5% w/w) and 0.2 ml phosphoric acid (85% w/w). The membranes were washed several times with water at 313 K (Fig. 2b).

Coupling of HEC

Each membrane disc was shaken in 5 ml of a 2% HEC solution (w/w) for 30 min at room temperature. Then the disc was placed onto a

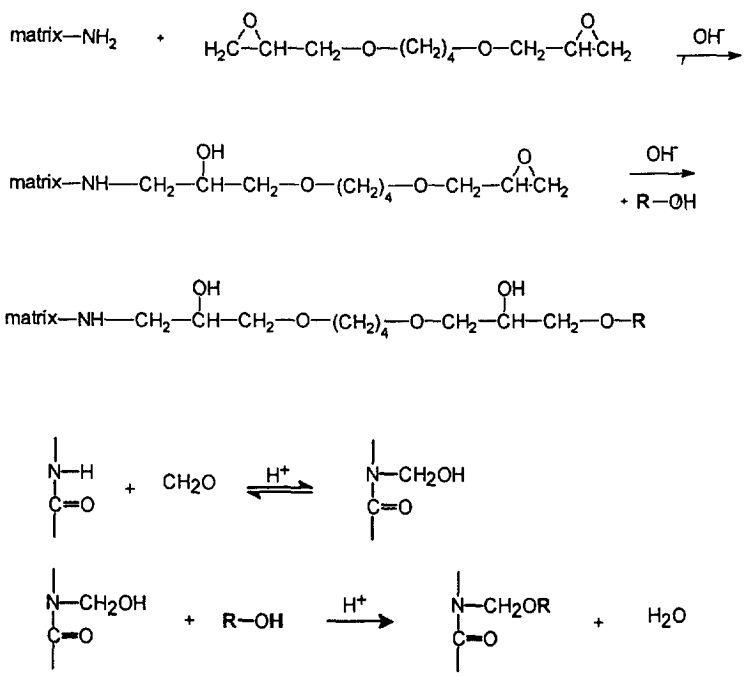

Fig. 2. Reaction scheme for covalent immobilization on nylon membranes. (a) Modification of nylon amino end groups by bisoxirane ensuing immobilization of polysaccharides (ROH), such as hydroxyethyl cellulose (HEC). (b) Activation of nylon amide groups, followed by immobilization of HEC.

sintered glass filter holder and the remaining HEC solution was sucked slowly through the membrane disc by reduced pressure until no further drop was formed at the filtrate side. Subsequently the wetted membrane disc was dried in an oven. Formaldehyde-activated membranes were coated at pH 2 and dried for 45 min at 363 K. Bisoxirane-activated membranes were dried at 393 K for 14 h after coating at pH 11. To compare the HEC coating of activated and non-activated membranes, membranes without prior activation were treated with HEC under the same conditions. The amount of bound HEC was determined after washing.

Washing procedures

In order to check the stability of the coating the membranes were treated with different washing solutions. A membrane disc was shaken three times for 1 h at room temperature in 0.1 *M* NaOH, 1% Triton X-100 or 0.1% SDS. Afterwards the membrane was shaken for 2 days with several water changes to displace the liquid applied for washing.

Determination of HEC density

Using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) the amount of bound HEC was determined. The HEC-coated membrane was incubated in 10 ml of 2 *M* HCl at 363 K for 4 h in order to decompose HEC in glucose monomers by acid hydrolysis. An amount of 2.1 ml of 1 *M* NaOH was added to 1 ml of the supernatant. Then 2 ml of BCA reagent was mixed with 0.5 ml of the glucose containing solution and incubated for 30 min at 333 K. The absorbance was monitored at 562 nm. The amount of bound HEC was calculated using a calibration plot of distinct glucose concentrations, yielded from known HEC concentrations after acid hydrolysis, versus absorbance as described above.

Activation of HEC-coated membranes

An amount of 80 mg of sodium borohydride was dissolved in 12 ml 2 *M* NaOH. Then 20 ml of water and 4 ml of epibromohydrin or 4 ml of bisoxirane were added. Three membranes were shaken in the reaction medium for 3 h either at 323 K for reaction with epibromohydrin or at room temperature for bisoxirane. Finally, the membranes were washed at least three times with water.

Immobilization of IDA and Cu(II)

A 1-g portion of IDA was dissolved in 10 ml 25 m*M* Na_2CO_3. The reaction medium was adjusted to pH 12 with 6 *M* NaOH. The immobilization occurred overnight at 323 K while the activated membrane was shaken in the solution described above. The membrane was washed several times with water and then charged with Cu(II) by shaking it for 1 h in 20 ml of 10 m*M* $CuCl_2$ dissolved in 50 m*M* acetate pH 5. Afterwards it was washed three times with water and then equilibrated with buffer as required.

Determination of non-specific binding-sites by hemoglobin adsorption

A membrane disc of 47 mm diameter was equilibrated by shaking it three times in 25 m*M* phosphate buffer–0.5 *M* NaCl, pH 7. Then the membrane was shaken for exactly 1 h at room temperature in 5 ml of 100 mg/l hemoglobin dissolved in the equilibration buffer. Control experiments without membranes were run under identical conditions. The absorbance of the supernatant was measured at 405 nm using an Ultrospec Plus 4054 UV–Vis spectrophotometer (Pharmacia, Freiburg, Germany). The protein concentration was calculated from a calibration plot. Finally, the amount of adsorbed hemoglobin was calculated from the concentration difference of the supernatants with and without membrane.

Determination of hydraulic permeability and K_L-value

K_L is defined as the pressure required to displace the wetting water from the largest pore of the membrane [31]. It was determined at the transition from diffusive air flow to capillary air flow, as the water is expelled from the largest pores of the membrane. Hydraulic permeability and K_L-value were determined to check whether

the pores of the membrane were blocked by the coating method. Therefore, pressure was applied on a water container via a Palltronic FFE 02 (Pall, Dreieich, Germany), utilizing it as a pressure controlling unit. The membrane was placed in an SM 16254 filter holder (Sartorius, Göttingen, Germany) connected to the water container. After 50 ml of pre-filtered deionized water (0.2-μm microfiltration membrane) were flushed through the membrane at 500 mbar, the time to process 100 ml water was measured and converted into the hydraulic permeability. For determination of K_L the water container was removed to reduce the dead volume.

Pore size distribution of membranes

The pore size distribution of active pores in the membranes was evaluated by means of a Coulter Porometer II (Coulter Electronics, Krefeld, Germany). The Porometer records the gas flow as a function of the applied pressure during displacement of a wetting fluid from an initially saturated membrane. This extension of the bubble-point method (ASTM F316) to the wet–dry method (ASTM 2499) is described by Klein [32]. A fluorohydrocarbon with low surface tension (Porofil) was used as wetting fluid. The dry membrane (25 mm diameter) was dipped in Porofil for 5 min at room temperature. After wetting, the pore size distribution was evaluated automatically by taking the ratios of wet to dry gas flow for a given pressure range.

Specific surface area of membranes

Specific surface areas of membranes were determined by N_2-sorption using the ASAP 2000 system (Micromeritics, Neuss, Germany) and employing the BET II Model [33]. Since N_2 is a small molecule, the specific surface area of both the large flow-through pores and the smaller pores inside the porous network of the nylon membranes are accessible by this method.

Determination of Cu(II) capacity

Cu(II)-loaded membranes were suspended for 30 min in 20 ml 20 m*M* disodium EDTA. The supernatant was fed to the furnace of an atomic absorption spectrophotometer (Perkin-Elmer, Type 2100) and the absorbance monitored at 324.7 nm. The Cu(II) concentration was calculated using a calibration plot.

Determination of protein adsorption isotherms

Single membrane discs were placed in an ultrafiltration cell (Amicon 8050, membrane area 13.4 cm^2). Constant flow-rates of 2 ml/min were maintained by means of a peristaltic pump (Pharmacia LKB P1) connected to the filtrate line. Mixing of the protein solution in the ultrafiltration cell was secured with a magnetic stirrer at approximately 300 rpm. The absorbance of the protein solution at 280 nm was monitored using a Pharmacia Uvicord S II UV-monitor with flow-through cell connected to a Pharmacia REC 101 recorder (Pharmacia, Freiburg, Germany). The solution was recirculated from the ultrafiltration cell through the UV-monitor back to the cell. A sample of protein solution of known volume (0.2–1 ml) and concentration (1–2 mg/l) was injected into 4 ml binding buffer in the ultrafiltration cell. After apparent equilibrium was achieved, i.e. no change of protein concentration in solution during circulation, another injection followed. A calibration plot was obtained in the same way without a membrane in the ultrafiltration cell. The protein concentration at each apparent equilibrium was calculated from the calibration plot. The stationary-phase protein concentration was calculated by difference and plotted against the protein concentration. Binding buffer for adsorption of lysozyme and OVA was 25 m*M* phosphate–0.5 *M* NaCl, pH 7. Con A adsorption was carried out in 25 m*M* phosphate–0.5 *M* NaCl–traces of $CaCl_2$, $MnCl_2$, pH 6, in order to bind the dimer. All experiments were carried out at room temperature.

Fractionation of a two-component model protein solution

The metal chelate membranes were charged with Cu(II) as described above. After equilibration with 25 m*M* phosphate buffer–0.5 *M* NaCl–traces of $CaCl_2$, $MnCl_2$, pH 6, a volume of 4 ml equilibration buffer was mixed in the ultrafiltration cell with 0.5 ml of 1 g/l lysozyme and 0.5 ml

of 1 g/l Con A dissolved in equilibration buffer. This solution was filtered through the membrane at a flow-rate of 2 ml/min. After washing the proteins were eluted either with 200 m*M* imidazole dissolved in buffer or 20 m*M* disodium EDTA.

Sandwich affinity membranes

The metal chelate membrane was loaded with Cu(II) as described above. Con A was adsorbed onto the membrane at a ligand density of 400 $\mu g\,cm^{-2}$ as described above. This sandwich affinity membrane, which utilizes the lectin as affinity ligand, was employed for OVA adsorption. The isotherm was determined as described for the MCA membranes.

SDS–PAGE

Protein fractions were analyzed with a Pharmacia Phast System according to the manufacturer's instructions on SDS 8–25% PAGE under reducing conditions similar to the procedure of Laemmli [34] and made visible by silver staining [35].

3. Results and discussion

According to the technical information of the manufacturer the isotropic membranes are made of nylon 66 with accessible amino and carboxylate groups in a 1:1 molar ratio. Polyester fibres are utilized as support layers. Characteristic data of Ultipor N66 (NXG 29325) are nominal pore size 0.45 μm, mass/plane surface $6.7 \cdot 10^{-3}$ $g\,cm^{-2}$, specific surface area (BET II) 7.1 m^2/g and 150 μm thickness.

3.1. Immobilization of HEC

Activation of the nylon 66 polymer with bisoxirane or formaldehyde resulted in densities of active sites of at least 80 $nmol\,cm^{-2}$ of epoxide groups or 2 $\mu mol\,cm^{-2}$ of N-methylol groups (Fig. 2), respectively [36]. The higher density of N-methylol groups is attributed to the modification of the amide groups of the nylon polymer by formaldehyde. The following immobilization of HEC provided a HEC coating which was covalently linked by reactions yielding either alkoxy- or N-alkoxymethyl derivatives (Fig. 2). As control, non-activated membranes were reacted with HEC under otherwise identical reaction conditions. The stability of these coatings was checked by different washing procedures. Afterwards the amount of immobilized HEC was determined for activated and compared with non-activated membranes. Both activation procedures resulted in a HEC density of at least 0.5 $mg\,cm^{-2}$ (Figs. 3a and b); thus no remarkable difference of the coating efficiency was found with both activation procedures.

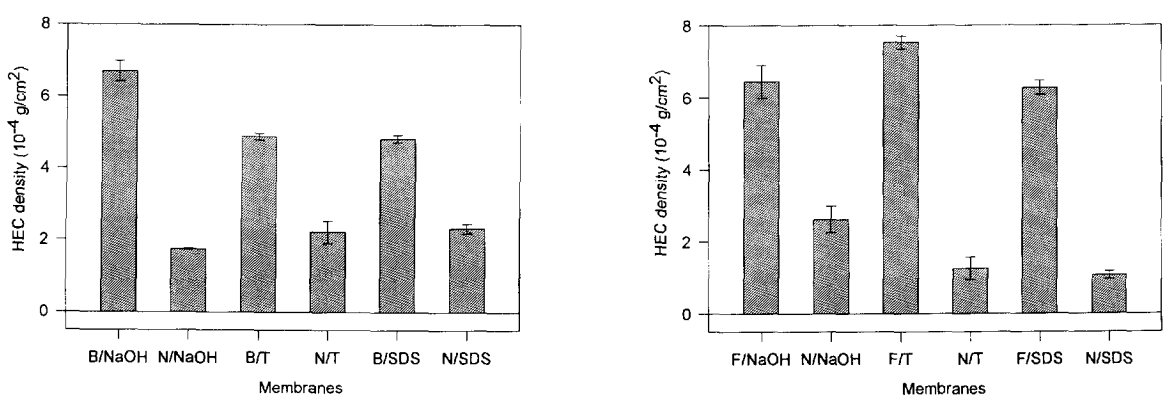

Fig. 3. Amount of immobilized HEC and influence of different washing procedures after HEC immobilization [0.1 *M* NaOH (NaOH), 1% Triton X-100 (T), 0.1% SDS (SDS)]. (a) After bisoxiran activation of the nylon matrix (B) or without nylon activation (N). (b) Amount of bound HEC after formaldehyde activation of the nylon matrix (F) or without nylon activation (N). Data were obtained as duplicates using two membranes.

It is important to note that HEC is also adsorbed to non-activated membranes. Although a reduction of HEC density was measured, especially after treatment with 0.1% SDS or Triton X-100, the adhesive forces were sufficient to hold fractions of HEC polymers in place during the different washing procedures, as displayed with non-activated membranes (Figs. 3a and b). This implies that HEC was attached in partially covalent and adsorptive manner at the inner porous surface of the membrane. However, HEC density was significantly higher for activated than for non-activated membranes.

It is well known that macromolecule chains do not adsorb on polymer surfaces in a film- or rod-like form. Instead, they build up coils on the membrane surface [37]. The coil dimension depends on the strength of interaction between matrix and the adsorbed polymer, the distance of covalent linkages and the diameter of the macromolecule in a given liquid. Therefore, it is plausible that the coated membrane surfaces consist of loops and tails of HEC chains freely moving inside the pores.

3.2. *Non-specific interactions*

The influence of HEC coating on the reduction of non-specific interactions was investigated as outlined in the Experimental section. Hemoglobin was chosen as surface active protein [38] which has been found to adsorb onto nylon membranes (Fig. 4). Significant reduction of hemoglobin adsorption on all covalently linked HEC-coated membranes was observed as compared to non-treated nylon membranes (Figs. 4a and b). Hemoglobin adsorption was also reduced on non-activated membranes which were treated with HEC under identical conditions employed for activation, however, without the activating reagent as control. The ionic detergent SDS (0.1%) and the non-ionic detergent Triton X-100 (0.1%) [39] were more efficient in the removal of physically adsorbed HEC than washing with 0.1 *M* NaOH. These results demonstrate that interactions between HEC polymers and nylon are quite strong, demanding for strong detergents if physically adsorbed HEC ought to be washed out thoroughly.

Washing exposes non-specific binding sites from both activated and non-activated membranes. Usually, HEC density and hemoglobin adsorption were inversely related with both physically adsorbed and covalently bound HEC. Hence, the presence of HEC in the pore structure of the membranes is responsible for a reduction of non-specific hemoglobin adsorption. Nylon activation prior to HEC coating was essential to yield lowest hemoglobin adsorption. If formaldehyde was chosen for activation, less non-specific interactions remained compared to bisoxirane even after washing with detergents. This could be due to a more homogeneous distribution of HEC at the membrane surface, caused by multiple covalent binding of the HEC polymer as a result of the higher density of N-methylol groups at the nylon chains.

Considerable differences in hemoglobin adsorption were also noticed after HEC coating of non-activated membranes (control experiments) employing either reaction conditions for bisoxirane or formaldehyde activation. At 393 K partial dehydration may have taken place during the 14-h reaction time, leading to some covalent linkages between nylon and HEC of undetermined origin. At the lower temperature and shorter reaction time employed with formaldehyde-activated membranes, HEC was only physically adsorbed and apparently completely displaced by the detergents. A plausible explanation of the higher hemoglobin adsorption on those membranes which demonstrated higher HEC coating after adsorption (Fig. 3a, N/NaOH, etc.) and washing with detergent compared to washing with NaOH cannot be given. One might assume that 0.1 *M* NaOH was not able to displace those polymer chains which were held closest to nylon, but the detergent did at least partially. If that is true, a more homogeneous coating would be left after washing with NaOH, despite a lower HEC density, providing less non-specific interaction with hemoglobin.

Since a slight increase of hemoglobin adsorption was noticed after washing original nylon membranes with SDS, maybe originating from

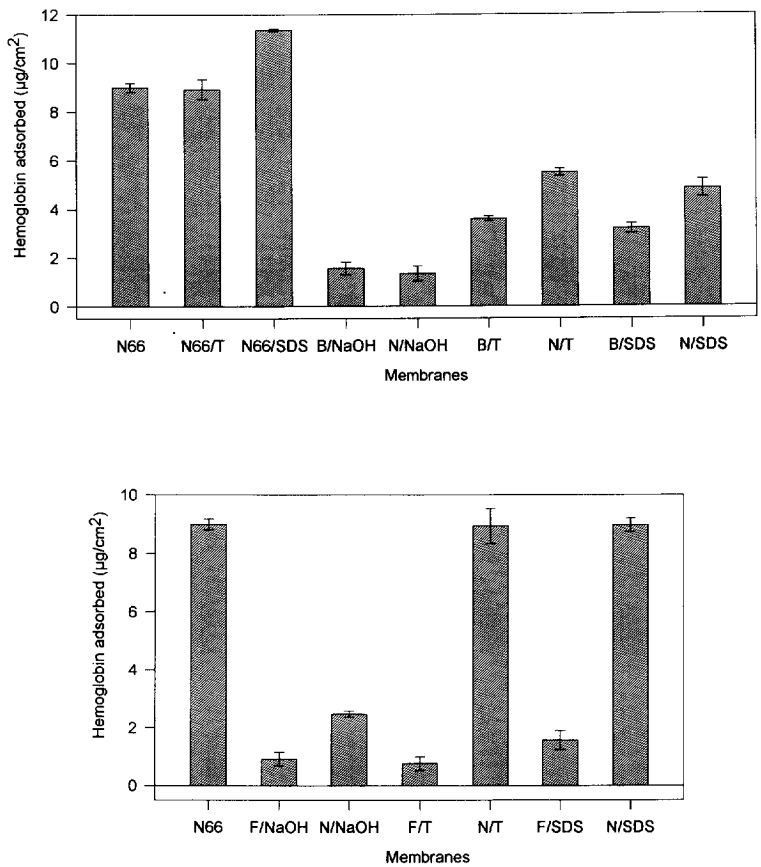

Fig. 4. Non-specific adsorption of hemoglobin on HEC-coated membranes. Influence of washing procedures. Original membrane (N66); (a) reaction conditions for HEC coating: 2% HEC at pH 11; (N) HEC coating without prior activation; (B) HEC coating on bisoxirane-activated membranes. (b) Reaction conditions for HEC coating: 2% HEC at pH 2; (N) HEC coating without prior activation; (F) HEC coating on formaldehyde-activated membranes. Washing conditions: 0.1 *M* NaOH (NaOH); 1% Triton X-100 (T), 0.1% SDS (SDS). Data were obtained as duplicates using two membranes.

adsorbed SDS, only Triton X-100 was employed in the following as washing reagent after HEC coating. No evidence was found that Triton X-100 would stick on HEC-coated nylon membranes, thus it was completely removed by washing with water.

Remaining non-specific interactions with HEC-coated nylon membranes were compared with those of non-modified nylon membranes in the filtration mode by using lysozyme as model protein (Fig. 5). Equilibrium isotherms obtained with coated membranes displayed a shallow shape, indicating very weak non-specific interactions. By contrast, approximately a three-fold amount of lysozyme was adsorbed on the original nylon membranes. No discrimination between the two activation methods was apparent which is in contrast to the hemoglobin adsorption (Fig. 4). Possibly a different mechanism accounts for the interactions of hemoglobin and lysozyme with nylon, leading to a better recognition of insufficiently coated membrane surfaces by hemoglobin.

3.3. Hydrodynamic properties

The pore sizes of the HEC-coated membranes are 40 nm smaller on average than those of the original membranes (Table 1), which represents a reduction of approximately 10%. The K_L-value

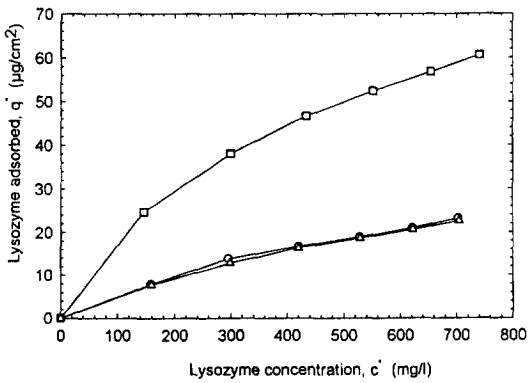

Fig. 5. Lysozyme adsorption isotherms for nylon membranes with and without HEC coating in 25 m*M* phosphate, pH 7, 0.5 *M* NaCl. (□) N66; (○) B/HEC; (△) F/HEC. Reduction of non-specific binding is observed with HEC-coated membranes independent of the activation method.

did not change significantly after modification; thus the maximum pore size of the membrane is almost not affected. By contrast, the hydraulic permeability is reduced to approximately 57% after HEC coating (Table 1). Both activation methods demonstrated comparable data as judged from these analysis.

The specific surface area of the original nylon membranes (7.1 m^2/g), as determined by N_2 sorption, is reduced after the modification process, yielding 5.9 m^2/g and 6.0 m^2/g for B/HEC and F/HEC, respectively. Taking into consideration the 8% increase in weight of the membranes after coating, the porous network of the nylon membrane, as accessible by N_2, is not affected significantly by the HEC coating. The remaining surface area still indicates the existence of a porous network after the coating process. Total blockage of the pores, as would result from a compact HEC precipitate, should have been indicated by a much lower specific surface area, accounting for the geometrical surface of the membranes only.

The decrease in hydraulic permeability indicates that the HEC polymers stay inside the pores, thus reducing the pore size. Assuming a reduction of the mean flow pore size from 0.66 to 0.63 μm, as evaluated from the porometer data, the hydraulic permeability of a capillary pore would be reduced to 91% only, employing the Hagen–Poiseuille law. One reason for the difference between the theoretical permeability and the observed value could be blockage of parts of the flow-through pores of the membrane. This would have only minor effects on the evaluation of data determined by the wet and dry method or the K_L-value, since both methods include flow-through pores only. On the other hand, the same degree of reduction of the gas flow should have been observed as for the water flow. However, this was not the case; approximately a 20% reduction of the gas flow was measured compared to approximately 40% for the water flow. Consequently, pore blockage cannot fully explain these results.

Another interpretation of the observed reduc-

Table 1
Characteristic data of nylon membranes with and without HEC coating

Membrane	Wet and dry porosity			K_L-value (mbar)	Hydraulic permeability (ml min^{-1} cm^{-2} bar^{-1})
	Minimum flow pore diameter (μm)	Mean flow pore diameter (μm)	Maximum flow pore diameter (μm)		
N 66	0.46	0.66	0.80	2550	22.4
				2550	23.0
B/HEC	0.42	0.63	0.76	2700	13.1
	0.42	0.63	0.77	2700	13.0
F/HEC	0.40	0.63	0.75	2400	12.8
	0.38	0.63	0.75	2400	12.9

Data were achieved as duplicates using two membranes, except for N66.

tion in permeability meets better with the specific properties of polymers. HEC polymers are open coils on the membrane surface in an aqueous environment. The existence of hydrogels is discussed by Klein et al. [17], although no estimation was provided regarding the space a HEC coating might require. The major problem is that the crosslinking of HEC during the coating reaction is unknown. However, a rough estimate of its structure should be possible. HEC is a flexible polymer with high extension in water as derived from polymer–solvent interactions [40]. Since data accounting for the thickness of adsorbed HEC layers in water are not available, experimental data from the adsorption of polysaccharides on silica particles may be used instead, such as described by Baudin et al. [41]. For example, pullulan, which is a neutral linear polysaccharide of β-1,6-linked maltotriose units, adsorbs in a poor affinity-type. The hydrodynamic layer thickness is 19 and 24 nm using pullulan of molecular masses 200 000 and 400 000, respectively. The radius of gyration (R_G) of these polymers in water is 19 and 28 nm; hence, it is very close to the hydrodynamic layer thickness. The environment for adsorbed polymer chains on a surface corresponds most to that of semi-dilute solutions where polymer coils overlap strongly but the volume fraction remains small [37]. If one end of the polymer chain is attached covalently to a surface, the density of chemical linkages defines the polymer's shape. Consequently, the chains are disposed like adjacent mushrooms if the distance (D) between two linkages is larger than the coil size ($D > 2R_G$). If the density of linkages is higher ($D < 2R_G$), a brush results from enlarged chains.

For HEC of molecular mass 350 000 (manufacturer information), as employed in this study, a radius of gyration of 20 nm is a conservative estimation. On the other hand, a HEC mass of $5.5 \cdot 10^{-4}$ g cm^{-2}, as determined by the BCA assay, corresponds to a HEC density of 1.6 nmol cm^{-2}. Taking into account a closed packed monolayer of HEC coils, the specific surface area of 7.1 m^2/g and the mass per plane surface of $6.7 \cdot 10^{-3}$ g cm^{-2}, a diameter of 7.6 nm for one HEC sphere is calculated. An R_G of 20 nm, as is estimated in water, would demand for strong overlapping of polymer chains at the membrane surface, which is entropically unfavourable. Therefore, HEC polymer chains should stay in an enhanced cylindrical shape inside the pores of the membrane, thereby reducing overlapping. Taking the radius of gyration as the radius of a hemisphere of adsorbed HEC at the nylon surface, a layer thickness of 20 nm would result, which is in accordance with experimental data determined for pullulan [41]. Assuming a constant volume of the HEC sphere and a radius of 10 nm at the nylon surface, which again is a conservative estimation, the sphere will deform to a cylinder with a height of 50 nm, corresponding to the layer thickness. Hence the mean flow pore size would be reduced at least from 0.66 to 0.56 μm, ensuing a reduction of the hydraulic permeability to 72%. This corresponds much better to the experimental value (57%).

Taking into consideration an enhanced cylindrical shape of the HEC coating, K_L-values and pore sizes determined by the wet and dry method should not change dramatically. Flexible polymer chains have only a minor influence on the pressure required to empty the wetted pore system, since mainly capillary forces and the surface tension need to be overcome. The gel-like structure of the polymer chains will increase mainly the viscosity of the solvent close to the membrane surface but not its general physical properties. Consequently, the pore size of the nylon network is still determining for the evaluation of these data.

3.4. Chemical properties

After having demonstrated that only minor non-specific interactions of HEC-coated membranes and proteins remain, these membranes need to be activated before immobilization of affinity ligands. In order to employ the same immobilization procedures as with hydrophillic chromatographic matrices, e.g. agarose, HEC-coated membranes were activated with epibromohydrin and bisoxirane. After that, IDA was immobilized onto the activated membranes and charged with Cu(II) ions to obtain MCA membranes. By measuring the amount of charged Cu(II) a precise method is available for

determination of the IDA density on these membranes.

In order to get high Cu(II) capacities, covalent binding of HEC is essential, such as found after bisoxirane (≈100 nmol cm^{-2} for B/HEC/B and F/HEC/B) and epibromohydrin activation (150 nmol cm^{-2} for B/HEC/E, F/HEC/E), respectively (Fig. 6). By contrast, Cu(II) capacities on non-covalently coated HEC membranes (N/HEC/E and N/HEC/B) are similar to those on membranes without HEC coating (N66/E and N66/B), which all were significantly lower than those on covalently coated membranes. Thus, the activation of hydroxyl groups of HEC is mainly responsible for these high Cu(II) capacities and not the remaining functional groups at the nylon matrix, as could be speculated.

Only minor differences among covalently coated membranes were found. Epibromohydrin activation leads to approximately 60% higher Cu(II) capacities than bisoxirane activation. On chromatographic matrices the same order is found, which is commonly attributed to a higher crosslinking of bisoxirane with the matrix due to its spacer length. Probably the same is true for HEC-coated membranes.

3.5. Adsorption isotherms of MCA membranes

Equilibrium isotherms obtained from the adsorption of lysozyme onto the different MCA membranes are displayed in Fig. 7. The data points reflect experimental results which were fitted employing the Langmuirean model for adsorption, $q^* = c^* q_m / (K_D + c^*)$. From the fitting, the apparent dissociation constant, K_D, and the maximum capacity, q_m, were evaluated. The maximum capacity for all membranes was found to be in the same range of 300 ± 30 $\mu g\ cm^{-2}$, but the shape of the isotherms differed. K_D of B/HEC/B/IDA:Cu(II) ($4 \cdot 10^{-5}$ M) was less than half the K_D of the other membranes [$(9 \pm 1) \cdot 10^{-5}$ M], indicating a stronger interaction between lysozyme and bisoxirane-linked IDA for B/HEC membranes despite a 60% lower Cu(II) capacity compared to epibromohydrin-linked IDA. Apparent K_D's of about 10^{-4} M corre-

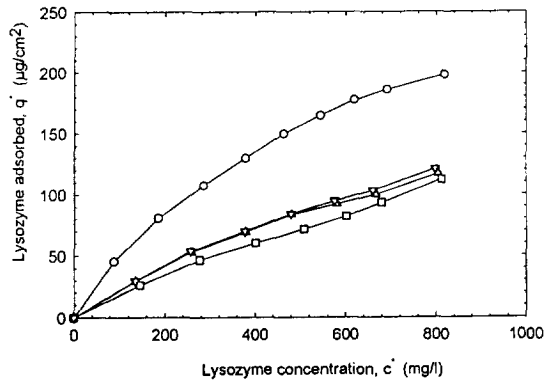

Fig. 7. Lysozyme adsorption isotherms for MCA membranes in 25 mM phosphate, pH 7, 0.5 M NaCl. (○) B/HEC/B/IDA:Cu(II); (▽) B/HEC/E/IDA:Cu(II); (△) F/HEC/B/IDA:Cu(II); (□) F/HEC/E/IDA:Cu(II).

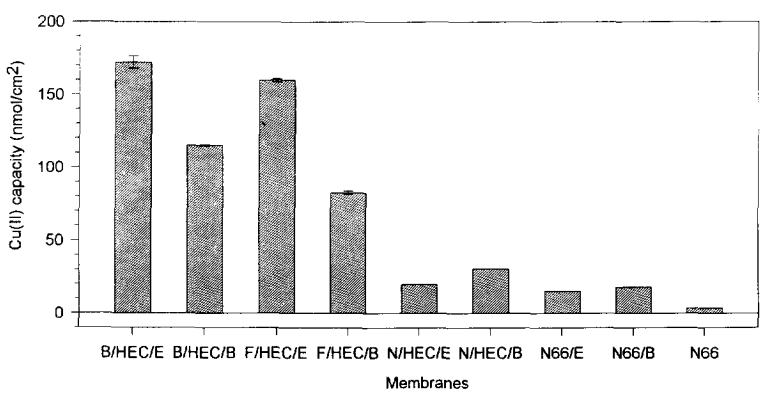

Fig. 6. Cu(II) capacity of MCA membranes obtained by IDA immobilization after different activation procedures. Covalent immobilization of HEC yields significantly higher capacities. Data were obtained as duplicates using two membranes.

spond to unusually weak interaction of IDA:Cu(II) with lysozyme, commonly being found one order of magnitude lower. Probably the accessibility of the IDA:Cu(II) chelate immobilized in the HEC coils on the membrane surface is restricted. It is known that lysozyme possesses one surface-exposed histidine responsible for binding onto metal chelate sorbents [42]. However, this histidine residue needs to come close to the coordination sphere of the membrane-bound metal chelate to be adsorbed. The longer spacer obtained after bisoxirane activation may improve this. In MCA chromatography the apparent dissociation constant is inversely related to the ligand density over a certain range [43]. Thus, for lysozyme the metal chelate density seems to be higher after bisoxirane activation than after epibromohydrin activation. Since the opposite is true, a considerable fraction of metal chelate ligands is not accessible to the protein.

The shape of the isotherms and the maximum capacity on F/HEC membranes displayed only minor differences between both activation procedures. As indicated after immobilization of IDA, less Cu(II) was bound on these membranes than on bisoxirane-activated membranes despite a slightly higher HEC density. This might reflect the more compact structure of HEC on formaldehyde-activated membranes. Consequently, spacers and chelate ligands will be less accessible on these membranes as well. Since lysozyme contains only one histidine for interaction with the metal chelate, it is more sensitive to changes in the accessibility of chelate ligands. This is found in the differences of the adsorption isotherms of lysozyme on F/HEC and B/HEC membranes, which are less marked on F/HEC membranes.

Another model protein for characterization of MCA matrices is Con A. Con A exposes several histidines at its surface; thus the discrimination of more or less accessible metal chelate ligands is not as distinct as with lysozyme. The lectin dimer binds strongly onto IDA:Cu(II) chelates at pH 6 by the interaction of several surface-located histidine residues [44,45]. The equilibrium adsorption isotherms in Fig. 8 confirm these strong

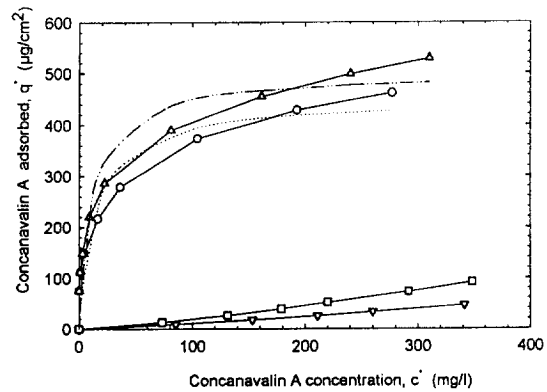

Fig. 8. Concanavalin A adsorption isotherms for different membranes, 25 m*M* phosphate, pH 6, 0.5 *M* NaCl, including trace amounts of Ca(II) and Mn(II). (△) B/HEC/E/IDA:Cu(II); (○) B/HEC/B/IDA:Cu(II); (□) N66; (▽) B/HEC. Dotted lines are obtained by iteration assuming Langmuir isotherms.

interactions also for MCA membranes. They are very steep at low protein concentrations. Fitting of the data by the Langmuirean model is not successful, as indicated by non-linearity of the double reciprocal plot (data not shown). Nevertheless, the apparent dissociation constants were estimated by iteration based on a non-linear least-squares curve-fitting algorithm from Marquardt [46], showing some deviation from the real K_D. Dissociation constants of $2 \cdot 10^{-7}$ and $3 \cdot 10^{-7}$ *M*, as obtained by iteration, confirm the strong interactions of Con A onto the metal chelates. In comparison, Con A adsorption onto the B/HEC membrane without metal chelate ligands was very low (Fig. 8). Proteins are well-known to interact weakly with many surfaces in a non-specific manner [47]. In the case of Con A this might by the reason for the non-Langmuirean adsorption of Con A on the metal chelate membranes. Another important fact to consider is the behaviour of Con A to build multimers of different extent depending on the pH and the concentration of Con A [48]. This could result in multimer or multilayer adsorption, particularly at higher equilibrium concentrations. A further explanation could be polarization on the membrane which builds up during the filtration process, leading to additional Con A adsorption at higher protein concentration.

In the case of Con A no influence of the spacer on the shape of the isotherms was observed; thus the type of interaction did not change and the metal chelates are accessible regardless whether short or long spacers were used. Therefore, the slightly higher Con A capacity obtained after epibromohydrin activation can be explained by the higher Cu(II) capacity of the membrane. This was also found by El-Rassi et al. [44].

The metal chelate membranes with formaldehyde-bound HEC coating exhibit similar Con A adsorption isotherms, with slightly lower capacities of approximately 380 $\mu g\ cm^{-2}$. Con A capacities were reproducible with all types of membranes after elution of Cu(II) and Con A by EDTA. Elution of the lectin was not observed at 200 m*M* imidazole, which again points to a very tight binding.

3.6. Separation of model proteins on MCA membranes

The distinct affinities of lysozyme and Con A for the IDA:Cu(II) chelate, as indicated by the adsorption isotherms, were utilized to fractionate a mixture of both proteins. The protein mixture was flushed once through B/HEC membranes with the chelate immobilized either by a bisoxirane or a epibromohydrin spacer. Both membranes adsorbed Con A completely; hence it was not found in a corresponding SDS–PAGE gel (Fig. 9). However, lysozyme was found in the filtrates. Rinsing with buffer caused complete elution of lysozyme from the membranes. Obviously, the relatively weak interactions between the immobilized metal chelates and lysozyme cannot retain the protein during extensive washing. Elution of large amounts of lysozyme from IDA:Cu(II) membranes was also found by Serafica et al. [13] with buffer containing 0.1 *M* NaCl. By contrast, with chromatographic sorbents elution of lysozyme is not described during washing; however, this is due to a better efficiency (larger number of theoretical plates) of the column setup.

With a 200 m*M* imidazole step gradient only small fractions of Con A elute from the IDA:Cu(II) membrane activated with bisoxirane. No Con A was found in the filtrate using membranes activated with epibromohydrin. Con A elution was achieved from both membranes by elution of Cu(II) by 20 m*M* EDTA. This is another proof that interactions between Con A and the metal chelate membrane are indeed metal chelate interactions; the proteins were not retained by non-specific interactions.

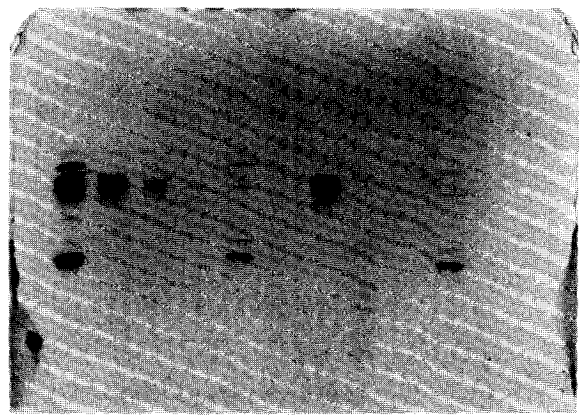

Fig. 9. SDS–PAGE gels of the fractionation of a Con A–lysozyme mixture; lane 2–5, fractionation by B/HEC/B/IDA:Cu(II); lane 6–9, fractionation by B/HEC/E/IDA:Cu(II). Lane 1: protein mixture; lane 2, 6: breakthrough fraction; lane 3, 7: washing filtrate; lane 4, 8: 200 m*M* imidazole elution; lane 5, 9: 20 m*M* EDTA elution; lane 2 and 6: lysozyme only; lane 5 and 9: Con A only.

3.7. Accessibility of large ligands

In order to investigate the accessibility of large ligands, a sandwich affinity membrane of metal chelate-immobilized Con A was chosen. Con A was immobilized onto a B/HEC/E/IDA:Cu(II) MCA membrane. Such sandwich affinity chromatographic sorbents are described by Horváth and co-workers [44,49]. The carbohydrate binding site of Con A retains completely its affinity towards carbohydrates and glycoproteins since the immobilization by metal chelate adsorption leads to orientation of the affinity ligand [45]. Affinity interactions of OVA with either the IDA:Cu(II) chelate or Con A takes place at different sites on the surface of the protein. One

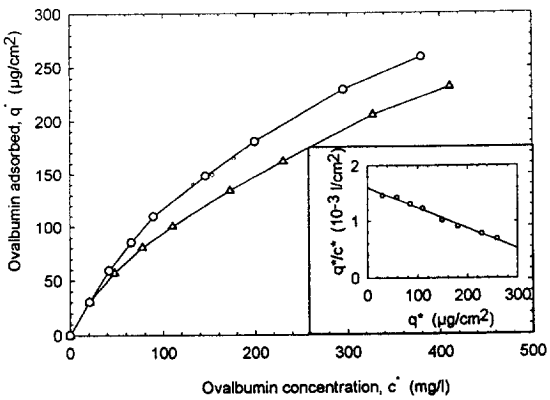

Fig. 10. Adsorption isotherms of OVA on a MCA membrane and a sandwich affinity membrane, 25 m*M* phosphate, pH 7, 0.5 *M* NaCl. (○) B/HEC/E/IDA:Cu(II):Con A; (△) B/HEC/E/IDA:Cu(II). The linearity of the Scatchard plot indicates specific interactions of the sandwich affinity membrane (inset).

histidine residue is available for interaction with the metal chelate [50], whereas the carbohydrate site is responsible for interaction with Con A. The adsorption isotherms of OVA on the metal chelate and the sandwich affinity membrane are displayed in Fig. 10. The maximum capacity of the sandwich system (453 $\mu g\,cm^{-2}$) is slightly higher than that of the metal chelate membrane (419 $\mu g\,cm^{-2}$) assuming Langmuirean adsorption. There is strong evidence that OVA binds specifically to Con A, as indicated by the linearity of the Scatchard plot. Con A capacity of the sandwich membrane was approximately 400 $\mu g\,cm^{-2}$, as estimated from the Con A isotherm on the metal chelate membrane (Fig. 8) obtained experimentally. This corresponds to 7.5 nmol cm^{-2} of the Con A dimer. The maximum capacity of OVA on the sandwich membrane corresponds to 10.1 nmol cm^{-2}. This indicates clearly the good accessibility of the large ligand Con A. The slightly higher OVA capacity of the sandwich membrane compared to the metal chelate membrane and the binding ratio OVA/Con A of 1.3 indicates that one Con A dimer can adsorb more than one OVA molecule. Possibly, both binding sites of the Con A dimer are partly accessible for OVA.

3.8. Theoretical considerations of protein adsorption on MCA membranes

Table 2 displays the thermodynamic data obtained for the adsorption of different proteins on metal chelate membranes by fitting to the Langmuirean model. The apparent dissociation constants are in a similar range as described for particulate sorbents, as used in immobilized metal chelate interaction chromatography [51,52]. This confirms the mainly specific interactions of the affinity membranes with the model proteins. The influence of the membrane matrix is only minor, almost similar to hydrophillic chromatographic matrices.

The dimensions of lysozyme, Con A dimer and OVA are $4.5 \times 3 \times 3$ nm [53], $8.4 \times 4 \times 3.9$ nm [54] and $7 \times 4.5 \times 5$ nm [55], respectively. Because the orientations of the adsorbed proteins are not known, the surface area covered by one protein molecule is calculated utilizing smallest and largest values of protein dimensions building a rectangle at the membrane surface.

Table 2
Thermodynamic constants of MCA membranes and proteins, as determined from Langmuir adsorption isotherms

MCA membrane	Protein	Maximum capacity, q_m ($\mu g\,cm^{-2}$)	Apparent dissociation constant, K_D (*M*)
B/HEC/B/IDA:Cu(II)	Lysozyme	321	$4 \cdot 10^{-5}$
B/HEC/E/IDA:Cu(II)	OVA	419	$6 \cdot 10^{-6}$
B/HEC/B/IDA:Cu(II)	Con A	451	$3 \cdot 10^{-7}$

Consequently, a range of monolayer adsorption of the proteins is calculated. Theoretically, a membrane of 1 cm^2 comprising 435 cm^2 internal surface area would adsorb 77–116 μg lysozyme (M_r 14 400), 114–245 μg Con A (M_r 53 000 for the dimer) or 93–144 μg OVA (M_r 45 000) in a monolayer. However, maximum capacities are two- to five-fold higher than theoretical monolayer capacities (Table 2). This can be attributed to the HEC coils on the membrane surface. Since nitrogen sorption is performed on a dehydrated membrane surface, the extended HEC polymer chains on the nylon membrane are collapsed and compact like a film. Thus, the specific surface area from N_2 sorption must be different from the surface "perceived" by proteins in an aqueous environment. Considering the size of the proteins employed (3 to 10 nm), adsorption is not only possible on the out-ranging positions of the HEC layer, it will take place on inner locations of the expanded HEC coils as well (≈50 nm thickness). Therefore, more proteins can be adsorbed than calculated for a monolayer. By contrast, monolayer adsorption was experienced by lysozyme adsorption onto nylon membranes (Fig. 5) [24,26]. Protein adsorption in expanded HEC coils does imply that at least partial transport by diffusion is taking place. Thus, in polymer-coated membranes protein transport does not occur by convection only.

4. Conclusions

Covalent coating of nylon microfiltration membranes by HEC is a basic requirement to minimize non-specific adsorption of proteins and to allow an effective immobilization of affinity ligands. Both activation of amino end groups by bisoxirane and amide groups by formaldehyde results in stable HEC coatings which display very little remaining non-specific interactions with proteins.

The hydroxyl groups of HEC are a suitable matrix for activation procedures known from hydrophillic chromatographic supports, such as agarose. Thus, very similar chemical reactions could be utilized to immobilize affinity ligands. Small ligands, such as metal chelates, as well as large ligands, like Con A, are accessible after immobilization onto these HEC-coated membranes.

The structure of the HEC polymer represents expanded coils on the membrane surface. These coils are responsible for the reduction of the hydraulic permeability of up to 43% but also for two- to five-fold higher protein capacities of the affinity membranes than monolayer adsorption would yield.

Formaldehyde-activated membranes produce a more compact structure of the HEC coating at close proximity to the nylon matrix compared to bisoxirane-activated membranes. This results in slightly lower protein capacities and reduced accessibilities of the immobilized ligands. However, these minor differences would not justify the preference of one of these methods.

Thermodynamic data of protein adsorption demonstrates that affinity interactions can be utilized without alteration of their specificity, which might have occurred through the modification and immobilization processes. From the current results it can be derived that dissociation constants $K_D > 10^{-5}$ M are unsuitable for the retention of target proteins on a single membrane disc. With affinity systems demonstrating stronger interactions, the potential of these affinity membranes as a tool for protein purification is indicated.

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List of abbreviations

Chemicals

Bisoxirane	1,4-butanediol diglycidyl ether,

Con A	concanavalin A,
Disodium EDTA	disodium salt of ethylenediaminetetraacetic acid,
HEC	hydroxyethyl cellulose,
Hemoglobin	bovine hemoglobin,
IDA	iminodiacetic acid,
Lysozyme	hen egg white lysozyme,
OVA	ovalbumin,
SDS	sodium dodecyl sulphate.

Washing procedures

Indicated is: membrane/washing solution.

B/NaOH	washing of HEC-coated membranes after bisoxirane activation of the nylon matrix with 0.1 *M* NaOH,
N/T	washing of HEC-coated membranes without prior activation of the nylon matrix with 1% Triton X-100,
F/SDS	washing of HEC-coated membranes after formaldehyde activation of the nylon matrix with 0.1% SDS.

Membranes

N66	original, non-treated nylon membrane,
MCA membrane	metal chelate affinity membrane,
B/HEC	HEC-coated membrane after bisoxirane activation of the nylon matrix,
F/HEC	HEC-coated membrane after formaldehyde activation of the nylon matrix,
N/HEC	HEC coating without prior activation of the nylon matrix,
F/HEC/B	bisoxirane-activated F/HEC,
B/HEC/B	bisoxirane-activated B/HEC,
F/HEC/E	epibromohydrin-activated F/HEC,
B/HEC/E	epibromohydrin-activated B/HEC,
IDA:Cu(II)	chelate formed after charging IDA with Cu(II),
IDA:Cu(II):Con A	sandwich affinity sorbent formed by adsorption of Con A on IDA:Cu(II).

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