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Membrane adsorbers for selective removal of bacterial endotoxin

D. Petsch, T.C. Beeskow, F.B. Anspach*, W.-D. Deckwer

GBF, Gesellschaft für Biotechnologische Forschung mbH, Biochemical Engineering Division, Mascheroder Weg 1, D-38124 Braunschweig, Germany

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Abstract

Surface-modified flat-sheet microfiltration membranes were functionalised with poly-L-lysine, polymyxin B, poly-(ethyleneimine), L-histidine, histamine, α-amylase and DEAE as well as deoxycholate. Their suitability to remove endotoxin from both buffers and protein solutions was examined using bovine serum albumin, murine IgG_1 and lysozyme as model proteins. In protein-free solutions reduction from 6000 EU/ml to <0.1 EU/ml was achieved with all applied ligands; only α-amylase as well as L-histidine and histamine, when immobilized via the non-ionic spacer bisoxirane, exhibited low clearance factors at neutral pH. The adsorption of endotoxin is mainly ruled by electrostatic interaction forces. Thus in multi-component systems, such as endotoxin-contaminated protein solutions, competing interactions take place: acidic proteins compete with endotoxin for binding sites at the membrane adsorbers, basic proteins compete with the ligands for endotoxin and act as endotoxin carriers. With properly chosen conditions the membrane adsorbers presented here show exceptional effectiveness also in the presence of proteins. They are generally superior to functionalised Sepharose chromatographic sorbents and allow fast processing. They may contribute to reduce the risks in the application of parenterals and diagnostics.

Keywords: Endotoxin; Proteins

1. Introduction

Endotoxins are constituents of the outer cell membrane of all Gram-negative bacteria. They are composed of a hydrophilic polysaccharide moiety which is covalently linked to a hydrophobic lipid moiety (lipid A). Lipid A anchors the molecule in the outer membrane. The sugar residues may be partially phosphorylated; thus endotoxin molecules exhibit a net negative charge. During bacterial growth endotoxin can be released. In contrast to bacteria, once released, endotoxin cannot be removed by standard methods such as autoclaving or sterile filtration [1]. Thus a sterile preparation is not necessarily endotoxin free. Especially endotoxin contamination of drugs, intended for parenteral use, is a serious problem. Intravenously administered endotoxin exhibits a broad spectrum of adverse effects [2–4], ranging from fever at concentrations as low as 1 ng per kg body weight to irreversible shock at higher doses. Therefore, all pharmacopoeias have set strict endotoxin limits for parenterals; e.g., hyaluronidase for injection may not contain more than

*Corresponding author.

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2.3 EU/unit, heparin sodium not more than 0.003 EU/unit heparin [5], corresponding to 230 and 0.3 pg/ml, respectively.

These requirements are, however, the critical point in the formulation of high molecular mass pharmaceuticals such as pharmaproteins, heparin or albumin preparations. On the one hand absence of endotoxins cannot be taken for granted since the production of these substances involves inherent sources of endotoxin (contaminated raw products as plasma or tissue, introduction of host-specific endotoxin if recombinant DNA methods are exploited, accidental microbial contamination during downstream processing or co-purification of endotoxin with the product [6]). On the other hand established methods for endotoxin removal, such as chemical or heat treatment, ultrafiltration, adsorption on activated charcoal or charge-modified depth filters, are not appropriate in the case of the mentioned substances [7]. The separation of endotoxins from these products requires more sophisticated methods.

The application of affinity techniques using L-histidine (His), histamine (Him), polymyxin B (PMB), poly-L-lysine (PLL) and poly(ethyleneimine) (PEI) immobilized on beads [8–10] seemed to provide a promising approach. Reported apparent dissociation constants in the range of 10^{-9} *M* [8] should ensure a tight binding of endotoxin. However, recent studies [11] revealed that these supports suffer from considerable decrease of efficiency in the presence of proteins. Hence, they are not in general applicable for the above mentioned problem.

In this study membrane adsorbers are employed. These sorbents became a promising alternative to well known particulate systems in recent years [12–14]. Especially with regard to the processing of large volumes membrane systems are more advantageous than particulate systems since they allow higher throughput. Isotropic Nylon microfiltration membranes coated with dextran, hydroxyethyl-cellulose (HEC) and poly(vinyl alcohol) (PVA), respectively, were used as matrices in order to combine the excellent mechanical properties of Nylon with low non-specific adsorptivity. Bare Nylon shows significant non-specific protein adsorption which is undesired in terms of avoiding protein loss [15,16]. These polymer-coated membranes were functionalised with a variety of ligands with the intention to establish a library from which can be chosen for a specific decontamination problem. The well known ligands His, Him, PMB and α-amylase as well the newer ligands PLL, PEI and deoxycholate (DOC), were examined with regard to their ability to remove endotoxin from protein-free solutions as well as from solutions of model proteins with different net charge, such as bovine serum albumin (BSA), IgG_1 and lysozyme. The anion-exchange ligand DEAE studied by Hou [17] was used for comparison.

2. Experimental

2.1. Materials

Nylon 66 microporous membranes (Ultipor N 66, nominal pore size 0.45μm) were a gift from Pall (Dreieich, Germany). DEAE-Sepharose, poly-L-lysine-agarose, polymyxin-B-agarose, histamine-agarose (all 4% beaded agarose), poly(ethyleneimine) (M_r 50 000), poly-L-lysine-hydrobromide (M_r 4000–15 000), polymyxin B sulphate, sodium borohydride, L-histidine, deoxycholate (sodium salt) and bovine serum albumin were purchased from Sigma (Munich, Germany); Sepharose 4B, hydroxyethyl-cellulose (molecular mass ≈350 000), bisoxirane, epibromohydrin, dextran (M_r 40 000), α-amylase from hog pancreas, lysozyme and 1,6-diaminohexane from Fluka (Neu-Ulm, Germany); poly(vinyl alcohol) (M_r 70 000), sodium periodate, sodium cyanoborohydride and histamine hydrochloride from E. Merck (Darmstadt, Germany); EDC from Pierce (Rotterdam, Netherlands); LAL test from Chromogenix (Mölndal, Sweden).

The antibody MAX16H5 (murine IgG_1) was a generous gift from Dr. J. Paulsen, Department of Bioseparation Techniques, GBF (Braunschweig, Germany).

A culture filtrate of *E. coli* 10 498 (Deutsche Sammlung Mikroorganismen) was used as endotoxin source rather than purified endotoxin in order to mimic naturally occurring contaminations in fluids.

Pyrogen-free water was obtained from a Milli-Q-UF system. All glassware was treated with 2 *M*

KOH containing 30% ethanol and pyrogen-free water followed by heat treatment at 180°C overnight.

2.2. Methods

2.2.1. Immobilization protocols

Membranes

Surface modification of Nylon 66 membranes. Dextran, PVA and HEC were covalently bound to Nylon membranes (Pall, nominal pore size 0.45 μm, 47 mm diameter) through bisoxirane, according to Beeskow et al. [18,19].

Activation of polymer coated Nylon membranes. For coupling of ligands either bisoxirane or epibromohydrin activation was employed following the activation protocols described by Beeskow et al. [18]. PLL, PMB, PEI and α-amylase were directly immobilized to periodate-oxidized dextran- and HEC-coated membranes, respectively (90 min incubation at room temperature in 0.2 *M* sodium periodate). PVA-coated membranes were first activated with bisoxirane. The free epoxy group was then hydrolysed (30 min incubation at pH 2.5 (HCl)) and subsequently activated with periodate as described above.

Immobilization of PLL, PMB and α-amylase. An amount of 60 mg sodium cyanoborohydride and 100 mg of PMB sulphate, PLL hydrobromide or α-amylase, respectively, were dissolved in 10 ml 0.1 *M* phosphate buffer and adjusted to pH 7. One periodate-activated polymer-coated membrane was given in each reaction solution and shaken overnight at room temperature. Afterwards the membranes were washed with 1 *M* NaCl and water extensively.

Immobilization of PEI. PEI was immobilized on periodate-activated polymer-coated Nylon membranes by the modified method of Champluvier and Kula [20]. Briefly, one activated membrane was reacted for 2 h with a solution of 0.5 g PEI and 60 mg sodium cyanoborohydride in 10 ml 0.1 *M* phosphate buffer adjusted to pH 8. After termination of the reaction the membrane was washed with 1 *M* NaCl and water thoroughly.

Immobilization of L-histidine and histamine. L-Histidine and histamine were immobilized either through bisoxirane or through diaminohexane (DAH) after epibromohydrin activation [8]. For the latter, one epibromohydrin-activated polymer-coated membrane was shaken for 2 h at 60°C in a solution of 0.2 g DAH in 10 ml water adjusted to pH 11. The DAH-immobilized polymer-coated Nylon membrane was then activated by 10 min incubation at 90°C in a mixture of 5 ml 4 *M* NaOH and 5 ml epibromohydrin and consecutively reacted with L-histidine at pH 12 or histamine hydrochloride at pH 9, respectively. Finally, the membranes were washed with 1 *M* NaCl and water.

For the bisoxirane method one bisoxirane-activated polymer-coated membrane was transferred into a solution of 0.5 g L-histidine or histamine hydrochloride, respectively, dissolved in 10 ml 0.5 *M* sodium carbonate adjusted to pH 11. The reaction was carried out for 2 h at 75°C. Then the membranes were washed as described.

Immobilization of DOC via DAH. Immobilized DAH was prepared as described above. DOC was coupled to DAH via its carboxylic function by the EDC method [21]. Briefly, one DAH-immobilized membrane was given into a solution of 0.2 g DOC sodium salt in 10 ml 0.1 *M* MES buffer adjusted to pH 6. An amount of 0.13 g EDC was added and the reaction mixture was shaken overnight at room temperature. Afterwards the membrane was washed as described.

Preparation of DEAE-membranes. DEAE was immobilized directly without incorporation of a spacer according to Santarelli et al. [22]. One polymer-coated Nylon membrane was shaken 1 h at 55°C in a solution of 0.2 g DEAE hydrochloride in 10 ml 1 *M* NaOH. The washing procedure was carried out as described above.

Chromatographic sorbents

Immobilization of dextran on Sepharose 4B. Sepharose 4B was bisoxirane-activated according to Sundberg and Porath [23]. However, the reaction time was reduced to 2 h in order to ensure a minimum loss of oxirane groups. The activated gel was suspended in an equal volume of dextran solution (20% dextran 40 000, and 0.2% $NaBH_4$ in 25 m*M* carbonate buffer adjusted to pH 11) and shaken for 60 min at room temperature. Afterwards

the dextran solution was sucked off and the gel was incubated for 24 h at 80°C.

Preparation of Sepharose-immobilized L-histidine and DOC. L-Histidine and DOC were immobilized through DAH on epibromohydrin-activated Sepharose prepared as described by Hochuli et al. [24]. The reaction conditions of the following steps were in correspondence to those employed for the membrane support.

Preparation of Sepharose-immobilized PEI. For immobilization of PEI the same procedure was used as for the membrane support after initial bisoxirane activation of Sepharose 4B according to Sundberg and Porath [23].

2.2.2. Adsorption experiments

Filtration experiments. Adsorption of endotoxins on membranes was studied in dead-end filtration mode using an ultrafiltration cell (Amicon 8050; membrane surface 13.4 cm^2), which was decontaminated with 2 *M* KOH containing 30% ethanol and pyrogen-free water prior to the experiment. A single membrane was placed in the cell and washed with 0.1 *M* NaOH containing 20% ethanol, 1.5 *M* NaCl and pyrogen-free water to ensure the absence of endotoxins on the membrane [25]. After equilibration with 15 ml 20 m*M* phosphate buffer an amount of 15 ml of buffer containing approximately 6000 EU/ml was filtered through the membrane at a flow-rate of 2 ml/min. This flow-rate was chosen since no alteration of the adsorption behaviour was evident in comparison to lower flow-rates. The endotoxin concentrations of the filtrates were determined by the LAL test. Samples were frozen to prevent microbial growth if not measured immediately after the experiment.

Chromatographic experiments. Adsorption experiments on chromatographic supports were carried out using a standard FPLC system (Pharmacia, Sweden). The system was rendered endotoxin-free by washing with 2 *M* KOH containing 30% ethanol and pyrogen-free water. The sorbents were packed in standard FPLC columns of 5 mm I.D. and 30 mm bed height and washed with 0.1 *M* NaOH containing 20% ethanol, 1.5 *M* NaCl and, finally, pyrogen-free water in order to remove endotoxin traces [25]. After equilibration with 20 m*M* phosphate buffer, 24 ml of endotoxin-contaminated buffer (≈6000 EU/ml) were loaded onto the sorbents at 0.3 ml/min flow-rate. The effluent was collected in 6 ml fractions and their endotoxin content was measured by the LAL test.

2.2.3. Analytical methods

Endotoxin assay. Endotoxin concentrations in feeds and filtrates or effluents were determined with the chromogenic limulus amebocyte lysate test (LAL test) using the end-point method, as described by the supplier. This technique uses an enzyme complex isolated from blood cells of the horseshoe crab *Limulus polyphemus*. The enzyme complex is activated in the presence of endotoxin traces and reacts to cleave *p*-nitroaniline from a synthetic substrate. Via calibration with a standard endotoxin (*E. coli* O111:B4) quantification in samples is possible by spectrophotometric measurement of the released amount of *p*-nitroaniline at 405 nm. The detection limit of the assay is 0.015 EU/ml, corresponding to 1.5 pg/ml.

Protein assay. Proteins were assayed using the method of Lowry et al. [26] with BSA and lysozyme as standards.

Determination of ligand densities of PMB membranes. Ligand densities on the different coating polymers were studied exemplary with PMB membranes using ninhydrin reagent (50 mg $SnCl_2$ and 400 mg ninhydrin were dissolved in a mixture of 10 ml DMSO and 2 ml of 2 *M* sodium acetate yielding a dark red solution).

First the hydrophilic coatings bearing immobilized PMB were decomposed by acidic hydrolysis: one PMB membrane was incubated for 4 h at 90°C with 2 *M* hydrochloric acid. Afterwards the supernatants were neutralized with 2 *M* NaOH. A sample of 100 μl of each supernatant was incubated for 15 min at 90°C with 0.5 ml of ninhydrin reagent and 0.9 ml water. The absorbance of the samples was measured at 570 nm against a supernatant from a polymer-coated Nylon membrane without ligands. The amount of ligand was calculated from calibration plots with free PMB treated in the same way as described above. The procedure was verified using

the commercial PMB support with known ligand density as control.

3. Results

3.1. Removal of endotoxins from protein-free solutions

The interaction of endotoxins with PLL, PMB, PEI, α-amylase, His, Him, DOC and DEAE immobilized on Sepharose 4B on the one hand and on dextran-coated Nylon membranes on the other hand was compared. The sorbents were contacted with contaminated buffer solutions at pH 7. In order to select the supports with high effectiveness an endotoxin content of approximately 6000 EU/ml was chosen although such high concentrations are unusual in practice.

With the Sepharose-based sorbents endotoxin removal at such high initial concentrations was not satisfactory (Table 1). Although the capacities of the sorbents were not yet exhausted [11,27] between 20 and 40 EU/ml were found in the effluents on average. For comparison: the USP standard for injection is set at 0.25 EU/ml [5]. Hence, at high endotoxin concentrations the chromatographic supports are not suitable to meet the requirements of international standards. This fact was also reported by Anspach and Hilbeck [11] who examined the influence of the endotoxin concentration on removal factors using endotoxin-specific Sepharose sorbents.

In contrast, reduction to picogram amounts (1 EU=100 pg) was obtained within one single step using the membrane adsorbers (Table 1). The low molecular mass ligands DAH::His, DAH::Him and DOC proved to be almost as effective as the polymeric ligands PLL, PMB and PEI. Remarkably even the anion-exchanger DEAE exhibits excellent clearance factors. Only His and Him immobilized through bisoxirane as well as α-amylase, an amylolytic enzyme with reported depyrogenation activity [7,28], exhibited poor effectiveness.

Control membranes, which were treated under the same conditions as the functionalised membranes without offering a ligand, were ineffective as well as bare or polymer-coated Nylon themselves (data not shown). Backflushing of the functionalised membranes with 15 ml pyrogen-free water yielded only between 1 and 10 EU/ml, which is negligible in comparison to the endotoxin content of the feed. Hence, non-specific adsorption of endotoxin can be

Table 1
Removal of endotoxin from 20 m*M* phosphate buffer (pH 7) containing 6300 EU/ml

Ligand	Matrix	
	Membrane: Endotoxin concentration in permeate (EU/ml)	Sepharose: Endotoxin concentration in effluent (EU/ml)
PLL	0.06	74 (9.1)
PMB	0.02	18
PEI	0.02	43 (7.3)
Bis::Propa	5.9	nd
Bis::His	1968	nd
DAH::His	0.09	28
Bis::Him	122	nd
DAH::Him	0.10	38
DAH	0.02	nd
DOC	0.12	51
α-amylase	2111	nd
DEAE	0.02	14

Comparison of membrane and Sepharose sorbents; results of dextran-coated Sepharose in parentheses.
Membrane area 13.4 cm^2 corresponding to 0.2 ml; flow-rate, 2 ml/min. Bed volume (Sepharose), 0.5 ml; flow-rate, 0.3 ml/min. Sepharose sorbents were loaded with 48 bed volumes, membrane adsorbers with 75 membrane volumes.
nd, not determined.

ruled out as well as size exclusion effects. These findings indicate that adsorption of endotoxin is based on its interaction with the ligands only.

Comparison of the clearance factors of the individual ligands gives rise to the assumption that electrostatic interaction forces are involved to a large extent in the adsorption mechanism. All ligands comprising a net positive charge (Table 2) show very good endotoxin removal, except Bis::Him. In contrast, with the α-amylase support, carrying a net negative charge at pH 7, almost no reduction of the endotoxin content was achieved. Also the zwitterionic nature of Bis::His does not support the attraction of endotoxin. The structurally closely related but positively charged ligand Bis::Him (Fig. 1) exhibits a clearance one order of magnitude better than Bis::His. Bis::Propa, which carries a methyl group instead of imidazole (Fig. 1), is even superior to Bis::Him. However, the most effective supports are obtained if the cationic spacer DAH is employed for immobilization of His and Him. This spacer is even slightly more effective without further modification.

The electrostatic concept is further supported by the finding that removal factors decrease dramatically with all ligands in the presence of 1 *M* NaCl. A simple electrostatic interpretation of the interaction mechanism is certainly inadequate since complete

Table 2
Overview of the p*K*- and p*I*-values of the ligands and proteins under investigation

Ligand/Adsorbant	p*K*	p*I*
Endotoxin	pK_1=1.3	–
	pK_2=8.2	–
PLL	10.8 (-NH_2)	9.7 (monomer)
PMB	>9 (-NH_2 of DAB)	–
	~10.5 (-NH_2 of DAB)	
PEI	>10.5 (NH)	–
	>9 (terminal NH_2)	
His	6.0 (imidazole)	7.6
	>9.0 (-NH_2)	–
	[>10.5 (NH of DAH)]	
Him	6.0 (imidazole)	–
	[>10.5 (NH of DAH)]	
DOC	[>10.5 (NH of DAH)]	–
DEAE	9–9.5	–
α-amylase	–	5.2
BSA	–	4.7
Lysozyme	–	11.2
IgG_1 (MAX16H5)	–	5.5

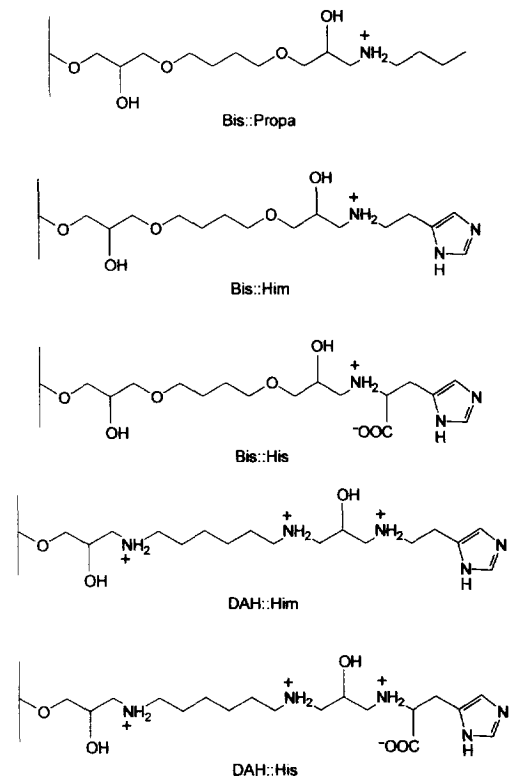

Fig. 1. Charge distributions of immobilized L-histidine, histamine and propylamine at pH 7 with different spacers employed.

elution of endotoxin cannot be achieved with salt buffers even at high molarities (1.5 *M* NaCl). Apparently, electrostatic attraction is responsible for a long-range interaction bringing endotoxin in close proximity to the ligands. This arrangement also allows short-range interactions, such as charge transfer, non-polar and polar interactions or even hydrogen bond formation to come into play, resulting in a strong multiforce binding.

3.2. Influence of the sorbent's structure on endotoxin-adsorption

The secondary structure of Sepharose consists of individual polysaccharide double helices which are aggregated to bundles of $10–10^4$ helices [29]. This macroporous gel structure is stabilized by hydrogen bonding. Crosslinking of single helices in the bundles results in an increased thermal and chemical stability but does not change the gel's pore structure. In contrast, dextran forms an open-cage-like struc-

ture by point-crosslinking of single dextran coils. One can envisage those differences of the two polymers accounting for the superiority of dextran-coated membranes over Sepharose. In order to check this aspect bisoxirane-activated Sepharose 4B beads were coated with dextran. In the second step PEI and PLL were immobilized on the coated Sepharose using the same protocols as described for the membrane adsorbers. Applying these materials for endotoxin removal from buffer solutions (pH 7) with approximately 6000 EU/ml, significantly improved clearance factors were obtained in comparison to the uncoated Sepharose (Table 1).

To clarify the influence of ligand densities, HEC and PVA were used for coating of Nylon membranes. These hydrophilic polymers exhibit microporous gel structures similar to that of dextran but exhibit lower ligand densities as is illustrated in Table 3 with PMB as representative. PVA-coated membranes and Sepharose 4B exhibit almost the same ligand densities while dextran binds between five and seven times more PMB. HEC shows intermediate PMB density. Despite the considerable differences in the ligand densities no differences in clearance factors were observed as far as the sorbent's capacity is not exhausted (Fig. 2), thus, the ligand density is not linearly correlated with clearance factors with the membrane adsorbers used. This was also indicated with PEI and PLL (data not shown). It seems that the open-cage structure of the hydrophilic polymers enhances the flexibility of the immobilized ligands thereby supporting the formation of an optimal arrangement of opposite charges decisive for effective adsorption of endotoxin. This is also supported by the results obtained with PEI and DAH::His immobilized to Nylon in the absence of a coating. These supports achieved significantly worse results

Table 3
Ligand densities as a function of the polymer applied for coating

Polymer	Immobilized PMB (mg/ml)
Sepharose	2.3
Dextran	17.5
HEC	7.2
PVA	3.3

PMB was used as model ligand. The amounts of immobilized PMB are given in mg/ml assuming a volume of 260 μl for one membrane of 47 mm diameter in order to enable a direct comparison with the chromatographic sorbents.

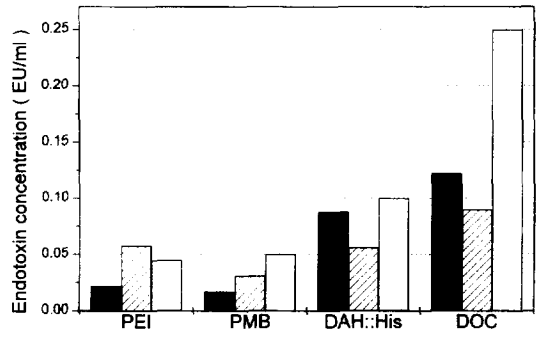

Fig. 2. Removal of endotoxins from contaminated phosphate buffer using membrane adsorbers based on different coating polymers. Both polymeric and low molecular mass ligands were applied on each coating. Black, dextran; hatching, HEC; white, PVA. 15 ml of contaminated feeds (6114 EU/ml) were loaded on each membrane; membrane area 13.4 cm^2, flow-rate 2 ml/min.

reducing 6000 EU/ml to 6.7 EU/ml (PEI) and 77 EU/ml (DAH::His) only (data not shown).

3.3. Influence of the pH on endotoxin-adsorption

In order to determine the useful pH-range of the membrane adsorbers the effectiveness of endotoxin adsorption was studied at acidic (pH 4.7) and alkaline (pH 9.4) conditions. The results are summarized in Table 4.

Varying the pH will result in changes of the charge distribution of both ligands and endotoxin molecules.

Table 4
Comparison of the effectiveness of membrane adsorbers in removing endotoxins from 20 m*M* phosphate buffer at different pH

Ligand	Permeate endotoxin concentration (EU/ml)		
	pH 4.7	pH 7	pH 9.4
PLL	0.06	0.06	0.95
PMB	0.03	0.02	>15
PEI	0.02	0.02	0.78
Bis::His	124	1967	4421
DAH::His	0.09	0.09	>15
Bis::Him	80	122	278
DAH::Him	0.44	0.10	>15
DAH	0.02	0.02	>15
DOC	0.21	0.12	11
α-amylase	65	2111	4514
DEAE	0.05	0.02	>15

Feed endotoxin concentration, 6211 EU/ml; membrane area, 13.4 cm^2; flow-rate, 2 ml/min.

At acidic conditions the density of negative charges at the endotoxin molecules decreases slightly in comparison to neutral pH while the density of positive charges increases at all ligands as can be judged from Table 2. In some cases changes of the sign of the net charge are involved: α-amylase becomes positively charged at pH 4.7. As a consequence endotoxin molecules are attracted by the immobilized enzyme allowing it to develop its depyrogenating potential which was not possible at neutral pH due to electrostatic repulsion.

A corresponding effect is observed with Bis::His. At pH 4.7 imidazole is protonated yielding a net positively charged structure (Fig. 1). Due to this circumstance Bis::His exhibits at acidic pH a clearance factor similar to that of Bis::Him at pH 7. At Bis::Him the beneficial effect of an additional positive charge is not as pronounced.

With all other ligands under investigation the contradictory development of the charge distributions at ligands and endotoxin molecules has only minor effects.

At alkaline conditions the situation is the opposite to that in acidic milieu: with increasing pH the charge density at endotoxin molecules increases but the charge density at the ligands decreases (Table 2). This leads to a considerable decrease in endotoxin clearance in comparison to pH 7. Only the polymeric ligands PLL and PEI displayed endotoxin concentrations below 1 EU/ml in the permeate.

These results indicate that decreasing the charge density at the endotoxin molecules themselves has less impact on endotoxin clearance than decreasing the charge density at the ligands. With the polymeric ligands PLL and PEI this effect is less pronounced. Generally spoken PLL and PEI can be applied over a wider pH range. Bis::His and α-amylase should be applied at pH-values below their p*I* and p*K*, respectively. All other ligands can be well applied at pH-values between pH 4.7 and 7. These results point out again the decisive role of electrostatic interaction forces for the adsorption of endotoxin.

Although it became obvious that the effectiveness of α-amylase and Bis::His can be improved by applying acidic conditions, these ligands were no longer employed in the following. Possibly the clearance with α-amylase can be further enhanced by application of prolonged contact times. Chawla et al. [7] incubated for 12 h in their batch experiments. However, this did not fit within the scope of this study.

3.4. Removal of endotoxin from protein solutions

If the feed consists of several components, e.g., an endotoxin-contaminated protein, multilateral interactions based on electrostatic attraction or repulsion must be taken into account. Proteins comprising a net negative charge, such as BSA, will also interact with the ligands, thus competing with endotoxin for binding sites. On the other hand, proteins comprising a net positive charge, such as lysozyme, will be repelled from the supports but may interact with endotoxin molecules and possibly carry them through the sorbents.

3.5. Endotoxin removal from endotoxin-contaminated BSA solutions

A solution of 1 mg/ml BSA was prepared in 20 m*M* phosphate buffer (pH 7), spiked with approximately 6000 EU/ml and pumped through the membrane adsorbers. As can be seen in Fig. 3 the endotoxin clearance decreased dramatically in the presence of the protein with the exception of DOC. Kinetic effects can be ruled out since pumping the contaminated BSA solution for 30 min in a closed

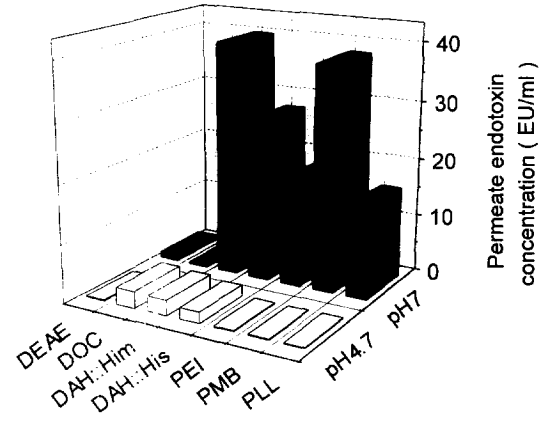

Fig. 3. Endotoxin removal from contaminated BSA solutions (1 mg/ml) at neutral and acidic pH. Endotoxin content of the feed 6610 EU/ml; a volume of 15 ml feed was applied in each case; membrane area 13.4 cm^2, flow-rate 2 ml/min.

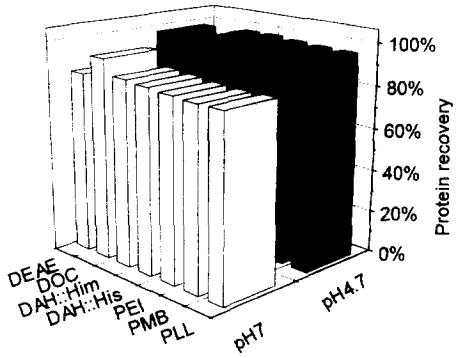

Fig. 4. Effect of pH on protein recovery during the decontamination of BSA solutions (1 mg/ml).

loop circuit provided no further reduction of the endotoxin content in the permeate. The protein recoveries (Fig. 4) indicate a competing interaction of BSA with binding sites which apparently affects the adsorption of endotoxin considerably.

As demonstrated above, application of pH values below 7 shows hardly impact on endotoxin removal. However, the interaction of BSA with the ligands should become weaker with decreasing pH due to increasing protonation of the protein (p*I*=4.7). Indeed, decontamination at pH 4.7 yielded significantly better clearance factors (Fig. 3). The polymeric ligands PLL, PMB and PEI as well as DEAE displayed endotoxin concentrations <0.15 EU/ml in the permeates. Employing DOC, DAH::Him and DAH::His, endotoxin concentrations between 1 and 3 EU/ml were found in the permeates. At the same time protein recoveries of at least 96% were obtained. This fact is of particular interest if the target protein is of high value.

Interestingly DOC was found to exhibit better removal factors and protein recoveries at pH 7 than at pH 4.7 which is most likely attributed to the apolar character of DOC. Since BSA is less polar at its isoelectric point competition with endotoxin for binding sites is more pronounced at pH 4.7 than at pH 7.

3.6. Endotoxin removal from endotoxin-contaminated lysozyme solutions

As with BSA, 6000 EU/ml were added to a solution of 1 mg/ml lysozyme in 20 m*M* phosphate buffer. A reduction of the endotoxin level to concentrations between 1 and 2 EU/ml was achieved with all ligands (Table 5). However, these data do not represent absolute removal factors. Examination of the feed streams revealed that only about 300 EU/ml of the originally added 6000 EU/ml are detectable using the LAL test. Furthermore, a proportionality between added and free amount of endotoxin was not evident. This phenomenon is also reported by Rantze [27] for a series of other basic proteins such as human IgG (p*I*=9.0), basic fibroblast growth factor (p*I*=9.6) and RNase A (p*I*= 9.4). In contrast, endotoxin was completely recovered in solutions of acidic proteins, such as BSA and ovalbumin. It was concluded that the poor endotoxin recoveries in the presence of basic proteins are rather due to a masking of endotoxin than to an inhibition of the LAL test. In fact, methods to overcome the inhibiting or stimulating effects of plasma on the LAL test, such as heat, chloroform or perchloro acid treatment [30,31], were ineffective in the case of lysozyme solutions as well as the common practice of sample dilution [32].

Two fates may occur to the lysozyme-bound endotoxin during its passage through the functionalised membranes. If the interaction of endotoxin with lysozyme is much weaker than with the applied ligand, the protein–endotoxin complex dissociates followed by adsorption of most endotoxin on the membrane. If the protein–endotoxin complex is of similar stability or even stronger than the endotoxin–

Table 5
Remaining endotoxin in lysozyme solutions (1 mg/ml) at pH 7 after the first and the second decontamination cycle using membrane adsorbers

Ligand	Cycle I Permeate endotoxin concentration [EU/ml]	Cycle II Permeate endotoxin concentration [EU/ml]
PLL	1.99	0.95
PMB	2.02	1.22
PEI	1.40	1.00
DAH::His	1.77	1.00
DOC	1.77	0.91
DEAE	1.38	1.32

6000 EU/ml from culture filtrate were added to the lysozyme solution (only 297 EU/ml were detected by the LAL test). The feeds of the second cycle were the permeates of the first; membrane area, 13.4 cm^2; flow-rate, 2 ml/min.

ligand complex, lysozyme will act as a carrier and most of the endotoxin will pass the membrane incognito. However, there is no way to prove which of the two possibilities is real since the amount of endotoxin masked by lysozyme is not detectable. Even if high salt concentrations (1.5 *M* NaCl) were employed in order to dissociate the protein–endotoxin complex, added and detected amounts of endotoxin differed by 60%. Detergents such as DOC, SDS or Triton X-100 are not recommendable since they disturb the LAL test in concentrations as low as 0.1%; phenol extraction as described by Westphal et al. [33] was not suitable either (data not shown).

In further experiments the permeates of the first decontamination cycle were pumped a second time through the membrane adsorbers after completion of a regeneration cycle. If the interactions between endotoxin and lysozyme were weak, reduction to very low endotoxin concentrations should be possible. However, the permeates of the second decontamination cycle still exhibited about 1 EU/ml on average (Table 5). This result demonstrates clearly that lysozyme acts as an endotoxin carrier: probably, free endotoxin is removed completely during pumping the solution through the membranes. However, the equilibrium condition between bound and free endotoxin will thereupon cause liberation of endotoxin from lysozyme. Consequently, a relatively high endotoxin concentration was found in the permeates. The total amount of remaining endotoxin depends upon the dissociation rate constant of the protein–endotoxin-complex. If the rate is fast a considerable amount of endotoxin will dissociate from the protein during its passage through the membrane due to a steady removal of free endotoxin by adsorption on the ligands. However, this scenario remains speculative as long as nothing can be said about the total amount of endotoxin left in the permeates. One must consider that despite the low contents of free endotoxin a considerable amount of endotoxin is still present.

3.7. Applications

In this part selected ligands were employed for practically related situations. Their suitability to remove endotoxin contaminations from commercial preparations of BSA (Sigma A-3059) and lysozyme (Fluka 62 971) was checked. These proteins exhibited 65 (BSA) and 134 (lysozyme) EU/mg, respectively. The endotoxin population of these protein preparations is not defined, as it was in the model study above, since contributions of several sources have to be considered. This circumstance makes the task challenging. In addition, a solution of a monoclonal antibody of the IgG_1 type (MAX16H5, p*I*=5.5) containing 3 mg/ml protein and 62.5 EU/ml was treated. This antibody became of growing interest due to its beneficial effects in the therapy of rheumatoid arthritis [34].

The decontamination was carried out at pH 7 for lysozyme; with BSA and IgG the pH of the protein solutions was adjusted to the proteins' p*I*-values in accordance to the previous findings. Fig. 5 and Fig. 6 illustrate that the selected ligands show very good endotoxin clearance from BSA and IgG solutions. The residual endotoxin content is far below 0.25 EU/ml except of DAH::His which left about 1 EU/ml. Also the less effective ligands Bis::His and α-amylase achieved reduction of the endotoxin-level to concentrations of 0.6 and 1.2 EU/ml at this low initial endotoxin concentration. Recently the decontamination of various IgGs was reported by Legallais et al. [35] using Bis::His immobilized to a poly(ethylene-vinyl alcohol) hollow fibre membrane. Just as in this study, satisfactory results were only obtained at initial endotoxin concentrations less than

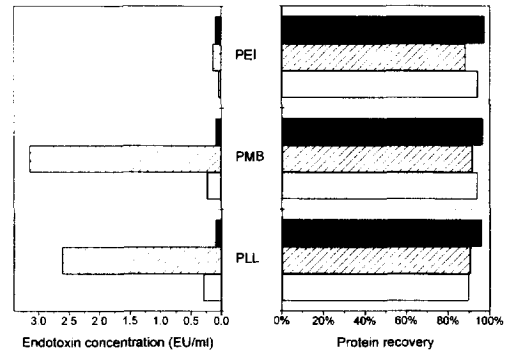

Fig. 5. Decontamination of proteins dissolved in 20 m*M* phosphate buffer using membrane adsorbers with polymeric ligands: permeate endotoxin contents and protein recoveries; membrane area, 13.4 cm^2; flow-rate, 2 ml/min. Black, commercial BSA 1 mg/ml containing 65 EU/ml (pH 4.7); hatching, commercial lysozyme 1 mg/ml containing 134 EU/ml (pH 7); white, IgG_1 (MAX16H5) 3 mg/ml containing 62.5 EU/ml (pH 5.5).

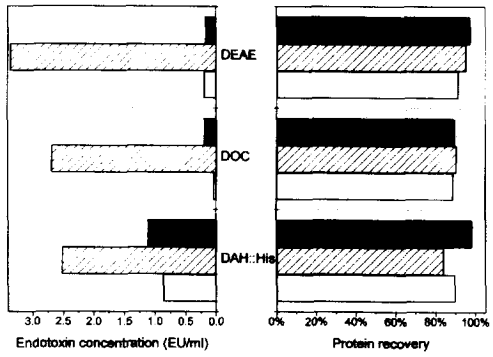

Fig. 6. Decontamination of solutions of proteins using membrane adsorbers with low molecular mass ligands: permeate endotoxin contents and protein recoveries; membrane area, 13.4 cm^2; flow-rate, 2 ml/min. Black, commercial BSA 1mg/ml containing 65 EU/ml (pH 4.7); hatching, commercial lysozyme 1 mg/ml containing 134 EU/ml (pH 7); white, IgG_1 (MAX16H5) 3 mg/ml containing 62.5 EU/ml (pH 5.5).

100 EU/ml. In all cases the clearance was accompanied by protein recoveries above 90% indicating high selectivity of the supports for endotoxin under the conditions employed.

The protein recoveries of lysozyme were found to be similar to those of BSA and IgG. However, the clearance was one order of magnitude less effective as a consequence of the endotoxin carrier function of lysozyme. Only with PEI the residual endotoxin concentration was reduced to 0.2 EU/ml in the permeate, as detected by the LAL test.

4. Discussion

Polymer-coated Nylon microfiltration membranes functionalised with PLL, PMB, PEI, DAH::His, DAH::Him, DEAE and DOC proved to be characterized by their exceptional high clearance factors for endotoxin. In protein-free solutions, permeate endotoxin levels far below 0.1 EU/ml were routinely obtained even at feed concentrations of several thousand EU/ml; in these situations Sepharose 4B sorbents carrying the same ligands failed (Table 1). Apparently both the three-dimensional structure of the coating polymers and the fluid dynamics in the membrane matrix contribute to the superior adsorption behaviour of membrane adsorbers in comparison to chromatographic beads. From the latter point, it can be expected that more efficient chromatographic matrices would perform better than Sepharose 4B. At the moment, however, nothing can be said about the extent.

The chemistry leading to a network of hydrophilic polymers with immobilized ligands is not restricted to Nylon. Membrane materials based, for example, on regenerated cellulose or poly(sulphone) can be modified by the same or slightly altered chemical procedures. It is most probable that the resulting membrane adsorbers would demonstrate similar selectivities than those investigated. However, their stability in alkaline solution may differ.

Although the harsh elution conditions (20% ethanol in 0.1 *M* NaOH) point to a multi-force attachment of endotoxin to the ligands it became obvious that electrostatic forces play a governing role in the primary interaction mechanism. Generally, very little adsorption is observed in the absence of positive net charges at the spacer–ligand system. Biospecific interactions as described for His and Him [8,25] were not evident. Indeed the results obtained with Bis::His and Bis::Him in comparison to Bis::Propa give rise to the assumption that neither His nor Him are endotoxin-specific per se. Certainly, immobilization via DAH results in very effective supports which is in accordance with data published by Minobe [8,25]. Minobe assumes a synergism of positive charges of the spacer and the imidazole ring; however, he does not provide the results with DAH alone. In our experience, DAH itself is at least as effective as DAH::Him and DAH::His, pointing out that the contribution of the imidazole ring is rather poor. The same suspect may be addressed to DOC since also immobilized via DAH. However, DOC shows a remarkable effect in the presence of acidic proteins as outlined below.

The dominating role of electrostatic forces for the adsorption of endotoxin has important consequences for the handling of multi-component systems, such as contaminated protein solutions. Since the selectivity of this type of interaction is not very pronounced, competition of negatively charged proteins for binding sites results in protein loss which is closely related to considerably decreased endotoxin clearance. Due to the different charge characters of proteins and endotoxin the selectivity of the mem-

brane adsorbers towards endotoxin can be controlled by the pH. A protein's interaction with the positively charged adsorbers can be reduced by adjusting the pH of the buffer to the protein's isoelectric point. The isoelectric points of most proteins are fairly above the pK_1 of phosphate groups of endotoxin molecules ($pK_1=1.3$) [17]. Hence, the interaction of endotoxin and adsorbent would then still be guaranteed. Indeed, BSA ($pI=4.7$) was successfully decontaminated and quantitatively recovered at pH 4.7 (Figs. 3 and 4). In contrast, at pH 7 a protein loss of 20% occurred accompanied by at least two orders of magnitude worse clearance with all investigated ligands, except DOC. It is very characteristic that DOC shows an opposite behaviour to the other ligands. This can be useful considering that some proteins precipitate at their isoelectric point. In such a case immobilized DOC could be used at neutral environmental conditions without the disadvantage of worse clearance. Here a synergism of spacer and ligand is apparent: the spacer introduces the charges prerequisite for endotoxin adsorption and the ligand acts as a shield against unwanted adsorption.

From these experiences one may conclude that pH-values below a protein's isoelectric point should ensure the best clearance and highest recovery. All the same such a proceeding is not advisable as became obvious with lysozyme ($pI=11.2$). While its interactions with the adsorbents are in fact only minor, it shows strong electrostatic interactions with endotoxin and acts as a carrier. This behaviour is not only restricted to lysozyme but probably characteristic for all basic proteins [27]. The protein-bound endotoxin is not LAL-active. However, it is doubtful that protein-bound endotoxin is inactivated in all biological effects. Although Caselli and Callerio [36] found an inhibiting effect of lysozyme concerning pyrogenicity in the in vivo rabbit test, it was reported by Ohno and Morrison [37] that lysozyme-bound endotoxin shows similar immunostimulatory effects on peritoneal macrophages, B-lymphocytes and spleenic B-cells than unbound endotoxin. In addition, in the present study it was shown that masked endotoxin is released in endotoxin-free environments, and this material is still LAL-active (Table 5). In the same way endotoxin can be set free in vivo after application of a solution containing a basic pharmaprotein. Complications may arise if proteins undergo structural changes, or after their catabolism. Hence, the potential of basic proteins to act as endotoxin carriers in a solution has to be considered critical. Further investigations should be carried out in order to elucidate the consequences of this aspect and to find ways for separation of proteins and endotoxin.

In conclusion it can be said that the pH is the most important tool for optimization of clearance factors. It has to be chosen in such a way that the target protein interacts neither with the sorbent nor with endotoxin. At the same time care has to be taken that the interaction of endotoxin and sorbent is not affected by these conditions. In this context the choice of the ligand becomes important. For example, removal of endotoxin traces from human IgG ($pI=9.0$) would not be possible using Bis::His ($pK_{imidazole}=6.0$). For decontamination of acidic proteins five ligands are available at the moment: the polymers PLL, PMB and PEI as well as DEAE and DOC (pH 7). His and Him, even when immobilized through DAH, show significantly worse clearance factors than the other ligands. Decontamination of basic proteins is connected with uncertainties arising from the endotoxin-masking properties of these proteins. It must be stressed again that endotoxin masking is different from the problem of test interferences. However, the international pharmacopoeia prescribe a gel-clot assay [32] for endotoxin detection and do by now not regard the phenomenon of endotoxin-masking by basic proteins. From this point of view PEI meets the requirements of the international pharmacopoeia at low initial endotoxin concentrations (≈100 EU/ml), thus, being the most widely applicable ligand.

Despite the intrinsic problems with basic proteins the new membrane adsorbers exhibit potential for various disciplines. Besides applications in biotechnology (treatment of process water, buffers, raw materials as well as end products) and pharmacy (treatment of parenteralia, diagnostics or dialysis buffers), application of the new membrane adsorbers for therapeutic purposes, such as hemoperfusion in acute sepsis, is conceivable. Studies examining the suitability of the membrane adsorbers for plasma decontamination are in progress. However, the guidelines set down for the development of so-called biologicals require validation in every single case.

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