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Endotoxin removal with poly(ethyleneimine)-immobilized adsorbers: Sepharose 4B versus flat sheet and hollow fibre membranes

Dagmar Petsch[a], Wolf-Dieter Deckwer[a], Friedrich Birger Anspach[a,*], Cécile Legallais[b], Mookambeswaran Vijayalakshmi[b]

[a]*GBF, Gesellschaft für Biotechnologische Forschung mbH, Biochemical Engineering Division, Mascheroder Weg 1, 38124 Braunschweig, Germany*

[b]*UTC, Université de Technologie de Compiègne, Centre de Recherches de Royallieu, Génie Biologique, BP 529, 60205 Compiegne Cedex, France*

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Abstract

Poly(ethyleneimine) was immobilized on poly(vinyl alcohol)-coated nylon flat sheet membranes, poly(vinyl alcohol) and poly(ethylenevinyl alcohol) hollow fibre membranes as well as Sepharose 4B. The resulting poly(ethyleneimine)-immobilized adsorbers were used for removal of *E. coli* derived endotoxin from buffers and bovine serum albumin solutions. The efficiency of poly(ethyleneimine) proved to be constant over a wide pH range, including phosphate buffered saline. The performance depended upon the matrix type employed: endotoxin clearance factors varied from 100 to 120 000 in protein-free solutions and 40 to 33 000 in solutions of bovine serum albumin using 6000 EU/ml as feed concentration. The best adsorber was the flat sheet membrane-immobilized poly(ethyleneimine), followed by the hollow fibre-immobilized poly(ethyleneimine) and poly(ethyleneimine)-Sepharose. The factors influencing endotoxin clearance were the mass transport (convective systems were superior to the diffusive system), the chemical composition and the surface structure of the underlying matrix. © 1998 Elsevier Science B.V.

Keywords: Endotoxin; Poly(ethyleneimine)

1. Introduction

Endotoxin is a common term for lipopolysaccharides located in the outer cell membrane of Gram-negative bacteria. These components, being essential for the viability of those bacteria, act as strong immunostimulants when entering the blood circulation of many species with possibly fatal outcome [1].

In humans endotoxin contact may occur during a bacterial infection [2,3] or via an endotoxin contaminated medicament that is given intravenously. In order to minimise risks – biological effects may arise even at concentrations of 1 ng/kg body mass – such medicaments have to comply with the endotoxin threshold limits regulated by the various pharmacopoeias; e.g., tetracycline hydrochloride may not contain more than 0.5 EU/mg, insulin for injection not more than 0.8 EU/unit insulin [4]. The term endotoxin unit (EU) describes the biological activity of an endotoxin in the limulus amoebocyte lysate

*Corresponding author.

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(LAL) test. For example, 100 pg of the standard endotoxin EC-5, 200 pg of EC-2 and 120 pg of endotoxin from *E. coli* O111:B4 have an activity of 1 EU [5]. Usually, it is taken as a rule of thumb that 1 EU corresponds to 100 pg endotoxin.

Despite sterile process conditions in the production of pharmaceuticals, endotoxin may be introduced accidentally (contaminated raw products or media) or intrinsically (recombinant DNA techniques). This fact is a critical point in the production of sensitive high-molecular-mass substances, especially pharmaproteins, since by now no routine method is available for removing residual endotoxin [6,7]. Thus, a contaminated product has to be discarded; also reprocessing is not ruled out but a costly alternative.

Recently, adsorbers based on surface-modified nylon flat sheet microfiltration membranes were described which provided exceptional endotoxin removal properties from protein solutions [8]. The adsorption characteristics were principally determined by the ligands immobilized to the membranes. The high effectiveness in comparison with commercially available affinity gels carrying the same ligands was explained by faster adsorption kinetics in membranes due to the absence of pore diffusion, which is the main transport resistance in particulate systems [9].

The present study intended the transfer of this concept to hollow fibre membranes (HFMs). Microporous poly(vinyl alcohol) (PVA) and poly(ethylenevinyl alcohol) (PEVA) HFMs were employed as basic materials since they can be easily modified. Their usefulness for immobilization of different ligands was already demonstrated [10,11]. These HFMs were compared with nylon flat sheet membranes (FSMs), which were surface-modified with PVA in order to create surface properties comparable to the HFM, as well as with Sepharose 4B as representative of a particulate adsorber.

The different matrices were functionalised with poly(ethyleneimine) (PEI), a polycationic amine (Fig. 1) which was introduced in recent years as a ligand for endotoxin removal [12,13]. This ligand was found to have the widest applicability in terms of pH and salt concentration among several other described ligands, such as polymyxin B, histidine or diethylaminoethane (DEAE) [8]. The endotoxin removal capabilities of the obtained adsorbers were compared in buffers of low and physiological ionic strengths as well as in bovine serum albumin (BSA) solutions.

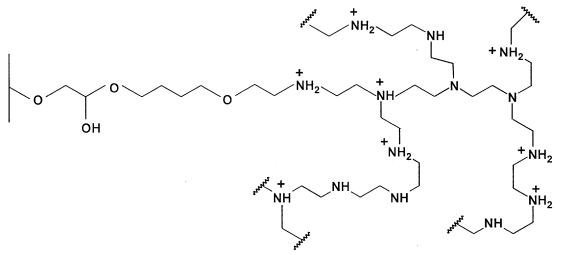

Fig. 1. Proposed structure of PEI immobilized through periodate-oxidized bisoxirane; the degree of ionization depends upon the pH.

2. Experimental

2.1. Materials

Nylon 66 FSMs (Ultipor N66, 0.45 μm nominal pore diameter) were a gift of Pall (Dreieich, Germany), PEVA (EVAL 4A, cut-off 600 kDa) and PVA (0.2 μm nominal pore diameter) HFMs were a gift of Kuraray (Osaka, Japan).

Bisoxirane (Bis), 1,6-diaminohexane (DAH), epibromohydrin and Sepharose 4B were purchased from Fluka (Neu-Ulm, Germany), PVA (M_r=70 000) from Merck (Darmstadt, Germany), BSA, glycine and PEI (M_r=50 000) from Sigma (Munich, Germany) and the LAL-test kit from Chromogenix (Mölndal, Sweden). All other reagents were of analytical grade.

A culture filtrate of *E. coli* 10498 (DSMZ, Deutsche Sammlung Mikroorganismen und Zellkulturen) was used as endotoxin source in order to mimic naturally occurring contaminations.

Pyrogen-free water was obtained from a Milli-Q UF system (Millipore, Bedford, MA, USA). All glassware were treated with 2 *M* KOH containing 30% ethanol and pyrogen-free water followed by heat treatment at 180°C overnight.

2.2. Hollow fibre modules

In order to keep the membrane volume of the different hollow fibres constant, 20 PVA and 50

PEVA fibres were fixed with epoxy glue in glass housings. These were prepared by joining two glass cylinders with screw threads and adding two fittings as permeate outlets (Fig. 2). The connectors for the feed and retentate tubings were fixed with a screw cap containing a silicone-PTFE sealing ring. In this way a flexible link was developed which prevented leaking reliably. The length of the fibres in the module was 8 cm.

2.3. Surface modification of nylon 66 FSMs

PVA was covalently bound to nylon 66 FSM as described by Weissenborn et al. [14]. Briefly, the nylon membranes were activated by incubating for 16 h at 80°C in a mixture of 9 ml Bis, 1 ml ethanol and 1 ml 25 m*M* sodium carbonate (pH 11.0). The activated membranes were washed three times for 10 min and immediately transferred into a 2% aqueous PVA solution (pH 11.0). After shaking for 15 min at room temperature, the PVA solution was sucked slowly through the membrane to ensure also wetting of the internal surface. The formation of the covalent bond was performed by 14 h incubation at 120°C. Finally, the PVA-coated membranes were washed with 0.1 *M* NaOH and water to remove excess PVA.

2.4. Surface modification of PVA HFMs with dextran

The coating with dextran was done to investigate pore size effects by mercury intrusion (see Section 2.9). At least 20 PVA fibres of ≈100 mm length were activated with Bis as described in Section 2.3. After washing, they were incubated in a dextran–$NaBH_4$–water solution (20:0.2:100, w/w/w) at pH 11.0 overnight, then removed from the solution and further treated as described above.

2.5. Ligand immobilization

PEI was immobilized to the matrices via periodate-activated bisoxirane or dextran, DAH via epibromohydrin. The immobilization protocols were recently described in detail [8]. PEVA was treated in the same way as PVA. For modification of the HFMs, the reaction fluids containing the desired activation reagent or ligand were pumped through the fibres fixed in the module.

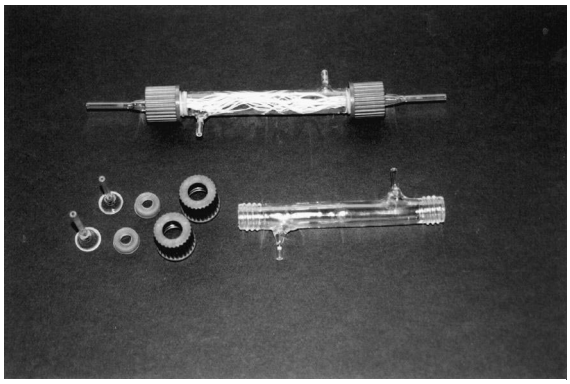

Fig. 2. Laboratory-scale hollow fibre housing. The upper part shows the ready-to-use module with fixed fibres, at the lower part the module is disassembled.

2.6. Decontamination experiments

Endotoxin adsorption on the FSMs was studied in dead-end mode using an ultrafiltration cell (Amicon 8050, 13.4 cm^2 surface). Also the hollow fibre modules were operated in dead-end mode. The experiments on the Sepharose-based adsorbers were carried out with a standard FPLC-system (Pharmacia, Sweden) in HR5/2 columns (5 mm I.D., 30 mm bed height).

All adsorbers were rendered endotoxin-free by washing consecutively with 0.1 *M* NaOH–20% ethanol, 1.5 *M* NaCl and pyrogen-free water; these conditions were also employed for regeneration. After equilibration with operating buffer the endotoxin-containing solutions were applied. The filtrates or effluents were examined for endotoxin and for proteins, if present. Samples were frozen to prevent microbial growth if not measured immediately after the experiment.

2.7. Analytical methods

Endotoxin was measured using a quantitative, chromogenic LAL-test (end-point method), following the instructions of the supplier. All samples were measured in duplicate. According to the supplier a deviation of 25% in the test results is tolerable under these conditions. This deviation should be taken into account for all endotoxin concentrations presented in this investigation.

BSA concentrations in the feeds and filtrates were assayed according to Lowry et al. [15].

The amount of immobilized PEI was determined by the ninhydrin method described recently [8]. For determination of the ligand distribution on the HFMs, the fibres were taken out of the housing, cut into four equally long pieces and assayed separately.

2.8. Determination of the specific surface areas of the membranes

The specific surface areas of original membranes were determined from N_2 sorption using an ASAP 2000 (Micromeritics, Norcross, GA, USA). The BET II model [16] was employed for the calculations. 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.

2.9. Determination of pore diameters by mercury intrusion

Pore sizes of original and functionalised PVA and PEVA HFMs were measured by mercury porosimetry on a poresizer 9320 (Micromeritics). This technique was already described for the characterization of HF ceramic membranes [17]. The pore size distribution was assessed from the mercury intrusion volume under a pressure gradient ($7.1 \cdot 10^3$–$2 \cdot 10^8$ Pa) based on the device calibration and assuming cylindrical pore shape.

3. Results

Characteristic data of original and PEI-immobilized membranes are summarized in Table 1. While the N66-FSM and the PVA HFM are typical microfiltration membranes, thickness and exclusion limit of the PEVA HFM are indicative of an ultrafiltration membrane with much smaller flow-through pores. Further evidence for the existence of small pores is the relatively high specific surface area of PEVA compared to the other membranes, as measured by nitrogen sorption. It must be pointed out that the surface area is measured in the dry state of the membranes. Hence the absolute value will change due to swelling, depending on the polymer employed. It also does not represent a surface area which is completely accessible to proteins. Yet, it provides a measure of the surface area which is accessible for small reagents used in the modification reaction.

Data from mercury porosimetry (Fig. 3A) indicated a total blockage of the small pores of PEVA membranes after immobilization of PEI (M_r = 50 000). This type of membrane was found to allow only application of low-molecular-mass ligands. In contrast, the diameter of flow-through pores of the PVA HFM did not change significantly after immobilization of PEI or dextran and PEI (Fig. 3B). Therefore these pores were not blocked by the polymers. Since analysis by mercury porosimetry requires membranes in the dry state as well, PEI or dextran molecules do not assemble coils on the membrane surface like in the wet state [18]. Rather,

Table 1
Characteristic data of the membranes employed in comparison to Sepharose 4B

	N66:PVA-FSM	PVA-HFM	PEVA-HFM	Sepharose 4B
Dimensions[a]	150 μm thickness	100 μm thickness 300 μm I.D.	20 μm thickness 200 μm I.D.	100 μm bead diameter
Nominal pore diameter[a]	0.45 μm	0.2 μm	–	–
Exclusion limit[a]	–	–	600 kDa	2000 kDa
Symmetry[a]	isotropic	isotropic	isotropic	–
Specific surface area	7.1 m^2/g	18.4 m^2/g	49.5 m^2/g	40 m^2/g[a]
PEI-density	6.4 mg/g	4.1 mg/g	–	7.0 mg/g
PEI-distribution	homogenous	homogenous	–	homogenous
Maximum endotoxin binding capacity[b]	>0.5 mg/ml	–	–	≈1.1 mg/ml

[a] Data provided by the suppliers.
[b] From high-affinity binding sites [20].

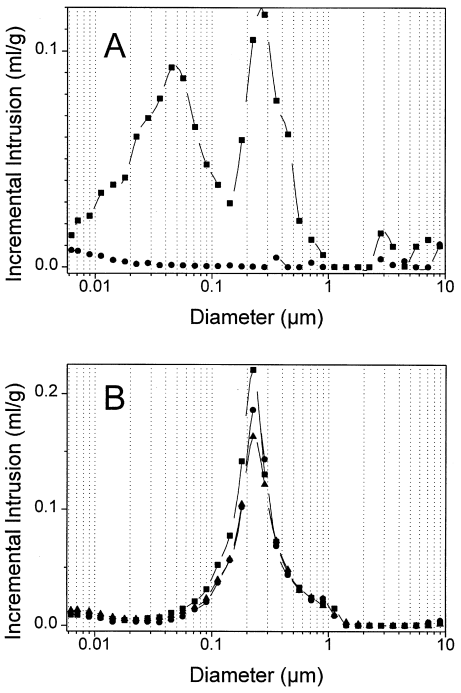

Fig. 3. Pore size distribution and incremental pore volume of membranes as measured by mercury porosimetry. PEVA (A) and PVA (B) membranes with (■) representing original membranes, (●) PEI-immobilized membranes, and (▲) a dextran-coated membrane with immobilized PEI (PVA membrane only).

they are collapsed in a more or less dense layer within the pores which is not resolved by the method. Instead, the total intrusion volume was reduced from 1.02 for PVA over 0.91 for PVA:PEI to 0.86 for PVA:Dex:PEI. This is a reliable indication that the polymers are present in the membrane pores.

The relation of intrusion volume to pore sizes of the different membranes, as represented in Fig. 3, should be interpreted with care. Pore sizes are calculated from the applied pressure and assuming a constant contact angle of mercury with solids of 130°. However, it is known that the contact angle of mercury varies slightly on surfaces with different chemical composition [19], which has a significant effect on the calculated pore size.

3.1. Endotoxin clearance from protein-free solutions

The interaction of immobilized PEI with endotoxin was studied by pumping phosphate buffer (pH 7.0) containing 6000 EU/ml through the adsorbers. In order to detect non-specific interactions, both the non-modified original materials and a negative control (Bis:Glycine) were also investigated for endotoxin adsorption. In addition, DAH was included in the studies as a positive control since immobilization of PEI on PEVA membranes caused total blockage of the pores.

As can be seen in Table 2, neither the PVA-based membranes nor Sepharose tended to non-specific interactions with endotoxin. Thus, the properties of adsorbers prepared from these materials reside in interactions of endotoxin with the immobilized ligands. In contrast, large amounts of endotoxin were lost by filtration through non-modified PEVA fibres. Backflushing of the membrane, after filtration of endotoxin solution, recovered only a negligible amount of endotoxin. Thus, polarisation or steric

Table 2
Removal of endotoxin from 20 m*M* phosphate buffer (pH 7.0)

Ligand	Matrix			
	N66:PVA-FSM 200 μl bed	PVA-HFM 190 μl bed	PEVA-HFM 126 μl bed	Sepharose 4B 500 μl bed
–	5965 (–)	4726 (–)	25 (240)	5700 (–)
–[a]	5870 (–)	4812 (–)	75 (80)	5700 (–)
Bis:Glycine	3379 (–)	4597 (–)	22 (273)	3150 (–)
DAH	0.02 (300 000)	2.0 (3000)	14 (429)	25 (240)
PEI	0.02 (300 000)	1.66 (3614)	–	43 (140)

[a] Without NaOH–ethanol treatment.

Data represent EU/ml in permeates and effluents from triplicate experiments, respectively; removal factors are in parentheses, experimental errors are ≈25% (deviation of LAL test). A 20-ml amount of feed containing 6000 EU/ml endotoxin was applied in each case; flow-rates were 2.0 ml/min for the membranes and 0.3 ml/min for Sepharose.

exclusion – conceivable since the PEVA membrane exhibits a relatively small pore size – played a minor role. Apparently, non-specific adsorption of endotoxin on PEVA took place. This was even enhanced after a pretreatment with 0.1 *M* NaOH–20% ethanol solution. Furthermore, only a marginal improvement of endotoxin clearance was achieved by introduction of DAH as positive control. Within the tolerance of the LAL-test no differences were seen between the NaOH–ethanol rinsed PEVA, PEVA functionalised with the negative control glycine and DAH- functionalised PEVA. Hence, endotoxin adsorption is not governed by the immobilized ligands but by non-specific matrix effects. The interaction forces are relatively weak: low ionic strength buffers, such as PBS, were sufficient for elution of several hundred EU/ml from both PEVA and functionalised PEVA HFMs. This was not the case with any of the other adsorbers under investigation (data not shown). Consequently, PEVA HFMs were no longer employed in the following and attention was focused on the properties of the PVA-based membranes and Sepharose.

These matrices showed, despite comparable ligand densities (Table 1), significant differences in endotoxin clearance. Under static conditions, maximum binding capacities and apparent equilibrium constants for endotoxin on FSM- and Sepharose-based adsorbers are comparable [20]. Though under dynamic conditions, the concentration of non-adsorbed endotoxin was much higher on Sepharose- than on membrane-based adsorbers (Table 2). Also, between the two membrane types a striking difference was apparent. Typically, the clearance factors achieved with the FSM-based adsorbers were two orders of magnitude higher than those based on HFMs.

Comparing ligand densities related to the membranes' specific surface area instead of volume, data from Table 1 reveals a four-times lower PEI density for the HFM than for the FSM (200 $\mu g/m^2$ vs. 900 $\mu g/m^2$). However, this was not the reason for the lower clearance efficiency of HFMs: by changing the activation conditions, a FSM with the same PEI-density per surface area as the HFM was prepared. This membrane adsorber exhibited almost identical endotoxin clearance factors as the FSM with the higher PEI-density.

Since ligand distributions are homogenous along the fibre axis, the worse results with the HFM could also not be attributed to a breakthrough on non or poorly functionalised parts of the fibres.

3.2. Influence of pH and ionic strength on clearance of endotoxin in protein-free solutions

Endotoxin clearance was best on all adsorbers at neutral or acidic pH and low or moderate ionic strength (Table 3). High ionic strength completely inhibits adsorption. This observation is in accordance with the charge interaction model [8,21]. Due to the content of phosphate and carboxylic groups, endotoxin molecules are negatively charged at pH values higher than 1.3. The primary forces of interaction with cationic species, such as PEI, were shown to be of electrostatic nature.

This concept also explains the decrease of clearance factors at elevated pH. Under these conditions PEI is increasingly deprotonated (pK>9 of terminal

Table 3
Effects of pH and ionic strength on the clearance of endotoxin by FSM- and HFM-immobilized PEI

pH of buffer (20 m*M* phosphate)	Endotoxin adsorbers		
	N66:PVA:PEI-FSM 200 μl bed	PVA:PEI:HFM 190 μl bed	PEI-Sepharose 4B 500 μl bed
4.7	0.08 (75 000)	2.13 (2817)	26.2 (229)
7.0	0.05 (120 000)	1.66 (3614)	43.1 (139)
9.4	1.5 (4000)	2.59 (2317)	>500 (–)
PBS, pH 7.2	0.05 (120 000)	4.53 (1325)	97 (62)
7+1 *M* NaCl	>1000 (–)	>1000 (–)	>1000 (–)

Data represent EU/ml in permeates and effluents from triplicate experiments, respectively; removal factors are in parenthesis, experimental conditions and data deviation as in Table 2.

NH_2– and >10.5 of NH-groups). Thus, the density of positive charges and consequently the attraction of endotoxin decreases. The effect was less pronounced with HFM-immobilized PEI while FSM-immobilized PEI and PEI-Sepharose showed a marked decrease in clearance factors. Despite this, FSM-immobilized PEI still exhibited the best clearance factor of all adsorbers under investigation with a residual endotoxin concentration of only 1.5 EU/ml even under alkaline conditions.

Comparing the clearance factors at pH 4.7 and pH 7.0, significant differences were not evident. This can be attributed to the very low pK_1 of endotoxin phosphate groups (pK_1=1.3) [21]. Probably, adverse effects will only become obvious below pH 3.

Table 3 also demonstrates that, despite the important role of electrostatic attraction, moderate ionic strengths, as in PBS (150 m*M* NaCl), had only a small impact on the interaction of endotoxin with immobilized PEI. Hence, its application is not restricted to buffers of low ionic strength.

3.3. Influence of operating modes on endotoxin clearance with PEI-functionalised PVA HFMs

In this part it was examined whether optimisation of endotoxin clearance with HFMs is possible by choosing other operation modes than dead-end (Fig. 4A). Alternatively, backflush and cross-flow modes with different permeate/retentate ratios (Fig. 4B,C) were employed. As Table 4 shows, slight differences

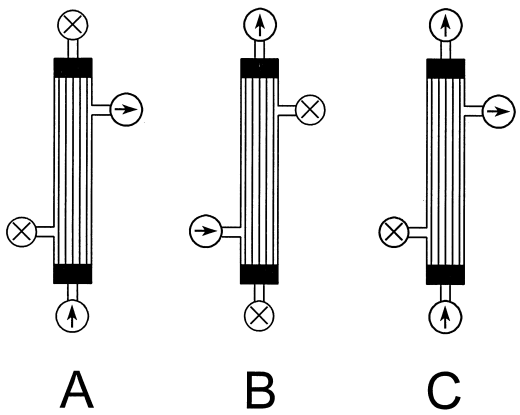

Fig. 4. Scheme of the different operating modes for hollow fibres. A=Dead-end, B=backflush, C=cross-flow.

Table 4
Endotoxin removal from 20 m*M* phosphate buffer (pH 7.0) with HFM-immobilized PEI in dependence of the operation mode

Operating mode	EU/ml in permeates (clearance factor)
Dead-end	1.66 (3614)
Backflush	2.80 (2143)
Cross-flow, P/R 1:1	1.72 (3488)
Cross-flow, P/R 1:5	1.50 (4000)
Cross-flow, P/R 1:10	2.54 (2362)

P/R=permeate/retentate ratio with permeate flow-rate of 2.0 ml/min; data are from triplicate experiments, experimental conditions and data deviation as in Table 2.

were found between the different modes employed. However, these results should not be over-interpreted. Taking into account the tolerance of 25% of the LAL-test, significant differences cannot be manifested – even a trend is absent. Hence, an influence of the operation mode on endotoxin clearance must be denied. Also, the permeate/retentate ratio in cross-flow mode can be chosen freely without consequences for endotoxin clearance.

In hollow fibre modules non-homogenous filtration rates along the fibre axis must be taken into consideration due to pressure loss. If filtration occurred preferably on a small region at the inlet, local overloading would lead to an early breakthrough of endotoxin while other parts of the fibre would hardly contribute to clearance. Although this seems unlikely in the case of the small laboratory scale modules applied, this point was examined by taking 1-ml fractions of the permeate. In the case of filtration inhomogeneities, the first fractions would contain almost no endotoxin while the later ones in increasing amounts. In the pool – as is shown in Tables 2 and 3 – an average value would be found. Though the experiment, representatively carried out in dead-end mode, revealed constant endotoxin concentrations in all fractions (data not shown). Therefore, the variation of filtration rates along the fibre axis had no effect on the endotoxin clearance under the conditions employed here.

3.4. Decontamination of BSA solutions

The clearance of endotoxin was studied in the presence of protein using BSA as model protein. The results are shown in Table 5. In contrast to protein-

Table 5
Decontamination of BSA solutions (1 mg/ml) in different buffer systems

pH of buffer (20 m*M* phosphate)	N66:PVA:PEI-FSM (200 μl bed)		PVA:PEI-HFM (190 μl bed)		PEI-Sepharose (500 μl bed)	
	EU/ml in permeate	BSA recovery (%)	EU/ml in permeate	BSA recovery (%)	EU/ml in effluent	BSA recovery (%)
4.7	0.15 (33 333)	96	3.0 (1667)	92	27.3 (220)	93
7.0	40 (125)	80	17.7 (282)	96	75.0 (80)	65
PBS, pH 7.2	0.25 (20 000)	100	3.57 (1400)	92	148 (41)	89

Data are from triplicate experiments, experimental conditions and data deviation as in Table 2.

free solutions large differences were observed as a function of the buffer conditions employed. In general, endotoxin clearance was the worst at pH 7.0 and low ionic strengths with drastically lower clearance factors compared to protein-free solutions. This can be attributed to binding site competition between endotoxin and BSA. Due to its isoelectric point (p*I*) of 4.7, BSA is negatively charged at neutral pH. Consequently, PEI does not only interact with endotoxin but also with BSA. As a result endotoxin clearance factors decrease and up to 35% of the BSA is lost. The protein loss was highest on PEI-Sepharose; however, the corresponding relative reduction of endotoxin clearance was lowest. Much more pronounced effects were seen for the membrane adsorbers with 100- and 1000-fold reduction of clearance factors for HFMs and for FSMs, respectively. Paradoxically, the clearance factor of HFM-immobilized PEI was reduced, although almost no BSA was adsorbed.

An improvement was achieved either by adjusting the buffer pH to the p*I* of BSA or by addition of salt, e.g., PBS. Both manipulations reduce the electrostatic attraction between immobilized PEI and BSA while the interaction of endotoxin with PEI is hardly affected (see above). Applying these strategies, endotoxin clearance factors were achieved similar to those obtained in protein-free solutions with nearly quantitative BSA recovery.

PEI-Sepharose showed a deviation when decontamination in PBS and 20 m*M* phosphate buffer (pH 7.0) are compared: although BSA adsorption was significantly lower in PBS and consequently a lower competition effect is expected, endotoxin clearance did not improve. As already mentioned, also no correlation was found between endotoxin clearance and BSA recovery on HFM-immobilized PEI. Although the protein recovery was relatively less dependent on the environment pH and ionic strength, the endotoxin clearance differed significantly, following the trend above mentioned. This phenomenon is not understood at present. The low adsorption of BSA gives reason to believe that adsorption is generally impaired on the PVA HFM. This might also explain the worse endotoxin clearance.

4. Discussion

This study describes the adsorption of *E. coli*-derived endotoxin by immobilized PEI with special attention to the contributions of matrix selection on the adsorption behaviour.

In order to create defined endotoxin adsorbers, matrix surfaces showing non-specific interactions with endotoxin are unsuitable. Non-specific binding is described especially for hydrophobic materials, such as polysulfone or poly(ethylene) [22,23]. However, the results with PEVA, possessing a content of about 30% poly(ethylene) [24], demonstrate that also partially hydrophobic materials should be avoided. In order to prevent any imponderability, it is strongly recommended to carefully validate each material.

For the preparation of membrane adsorbers, it is further recommended to choose microfiltration membranes with pore sizes $\geq$0.2 μm in order to allow immobilization of polymeric ligands without the risk of pore blocking. Concerning endotoxin adsorption, polymers such as PEI or poly-L-lysine, were shown to be superior to low-molecular-mass ligands, such as DEAE or deoxycholate [25] and are therefore preferable ligands.

This study indicates that the efficiency of endotoxin adsorption is not only a function of the ligand employed. Comparison of the different adsorbers, all modified by PEI, reveals that membrane adsorbers offer, in general, much higher endotoxin clearance factors than the particulate system employed. This is usually attributed to enhanced mass transport in membranes due to convection [9]. The concept is supported by the fact that immersing a PEI-immobilized FSM in an endotoxin contaminated solution is hardly suitable for endotoxin clearance. However, from this point of view the significant difference between HFM- and FSM-based adsorbers remains unexplained.

The difference in endotoxin clearance between FSM- and HFM-immobilized PEI is even larger than the difference between HFM-immobilized PEI and PEI-Sepharose. Neither ligand densities nor ligand distributions nor fluid dynamics exhibit differences which can account for the worse properties of the HFM-based adsorbers. Considering the different pore sizes (Table 1), one should expect faster adsorption on the HFM since the effective mass transport coefficient increases with decreasing pore sizes [26]. However, this is not observed.

Also, properties of endotoxins, such as their tendency to form aggregates of various size, are out of question since it concerns both membrane types to the same extent. This suggests the existence of one or more aspects that were overlooked so far.

One such aspect may be that the PVA-coating does not cover the nylon-surface like a flat film but forms a flexible network of PVA coils [14]. Such flexible network structures, which are not present in the HFMs, are reported to enhance adsorption [8]. This is not only seen in the adsorption of endotoxin but also in the adsorption of BSA (Table 5). From these results it becomes obvious that despite the higher efficiency of convective systems (membranes) compared to diffusive systems, such as Sepharose, different membrane types may vary considerably in their overall performance. Thus, development of effective adsorbers for endotoxin removal involves both screening for appropriate ligands and for appropriate matrices.

PEI is a ligand which covers a broad pH range and is applicable at ionic strengths up to 150 m*M* NaCl. In the presence of a protein some rules have to be taken into consideration: best clearance is obtained in PBS or at low ionic strength at the protein p*I*. In this way, reduction of 6000 EU/ml to a few EU/ml was routinely achieved using HFMs as matrices. This may be sufficient for many applications. However, in critical cases, such as infusion fluids, dialysis buffers and other high dose pharmaceuticals, the application of FSM-based adsorbers is indicated. These adsorbers represent the most efficient endotoxin adsorbers under investigation. Typically, they allow reduction from 6000 EU/ml to <0.1 EU/ml in protein-free solutions and <0.25 EU/ml in the presence of BSA.

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