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Review

Endotoxin removal by affinity sorbents

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Abstract

Affinity sorbents and detoxification strategies are described to remove different amounts of endotoxin. Advantages and disadvantages of the employed ligands are discussed and it is shown that both electrostatic and hydrophobic interactions contribute to the association of ligands and endotoxins. Furthermore, the flexibility of the ligand is more important than an exact structural match between ligand and ligate. Owing to the formation of endotoxin micelles and vesicles, microfiltration membrane adsorbers are particularly effective since mass transfer restrictions are almost absent in the flow-through pores. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Lipopolysaccharides; Endotoxin removal; Interaction modes; Ligand specificity; Polycationic ligands; Membrane adsorbers; Removal strategies

1. Introduction

Endotoxins show strong biological effects at very low concentrations in human beings (threshold level: 1 ng per kg body weight and hour) and in many animals when entering the blood stream. Because of this toxicity, the removal of even minute amounts is essential for safe parenteral administration. A generally applicable method for the removal of endotoxins is not available; methods used for water purification, such as

Abbreviations: BSA, bovine serum albumin; BPI, bactericidal/permeability-increasing protein; CF, clearance factor; CIP, cleaning-in-place; DAH, diaminohexane; DEAE, diethylaminoethane; DOC, deoxycholic acid; EU, endotoxin unit, 1 EU ml^{-1} ca. 100 pg ml^{-1} endotoxin; HSA, human serum albumin; IgG, immunoglobulin G; IgM, immunoglobulin M; KDO, 2-keto-3-deoxyoctonic acid; LAL, *Limulus* amebocyte lysate; LALS, anti-LPS factor; LBP, lipopolysaccharide-binding protein; PEI, poly(ethyleneimine); PLH, poly-L-histidine; PLL, poly-L-lysine; PMB, polymyxin B; PVA, poly(vinylalcohol).

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ultrafiltration, have little effect on low, but critical endotoxin levels in protein solutions. Ion exchangers, two-phase extraction, and affinity techniques have been applied with varying success. Also, tailor-made endotoxin-selective adsorber matrices for the prevention of endotoxin contamination and endotoxin removal are used for this purpose. After giving a short overview of the properties of endotoxins, this review intends to provide a picture of the various affinity techniques employed for their removal. Avenues are pointed out for optimizing methods with regard to the specific properties of endotoxins in aqueous solution.

2. Properties of endotoxins

2.1. Origin of endotoxins and clinical aspects

Gram-negative bacteria carry a heat-stable toxin, which was termed endotoxin by R. Pfeiffer (1858–1945). This term characterizes a class of lipopolysaccharides which are an integral part of the outer cell membrane of Gram-negative bacteria and which are responsible for their organization and stability [1]. As dominant surface structures they also participate in the interaction of the bacterial cell with its environment and possible hosts.

Although endotoxins are firmly anchored within the bacterial cell wall [2], they are continuously liberated into the environment. Clearly, endotoxin release does not happen only with cell death but also during growth and division. Since bacteria can grow in nutrient-poor media, such as water, saline, and buffers, endotoxins are found almost everywhere. High concentrations are found where bacteria accumulate or are being used for industrial purposes, such as in bioprocessing.

Endotoxins do not act directly against cells or organs but through activation of the immune system, especially through monocytes and macrophages. These cells release mediators, such as tumor necrosis factor, several interleukins, etc., and free radicals [3,4], having potent biological activity and being responsible for the adverse effects seen upon endotoxin exposure. These include: affecting structure and function of organs and cells, changing metabolic functions, raising body temperature, triggering the coagulation cascade, modifying hemodynamics, and causing septic shock [5].

2.2. Chemical and supramolecular structure of endotoxins

The general structure of all endotoxins is a polar heteropolysaccharide chain, covalently linked to a non-polar lipid moiety (lipid A), as schematically shown in Fig. 1 [2]. Lipid A anchors the endotoxin in the outer bacterial membrane. The heteropolysaccharide, being composed of a core oligosaccharide (core region) and a surface antigen (O-antigen), is exposed to the environment. The O-antigen is built up of a chain of repeating oligosaccharide units (each of 3–8 monosaccharides), which are strain-specific and determinative for the serological identity of bacteria. Some deficient strains, such as *Escherichia coli* K-12, lack it completely. This genetic defect neither impairs the viability of the microorganism nor the biological potency of endotoxin. The core

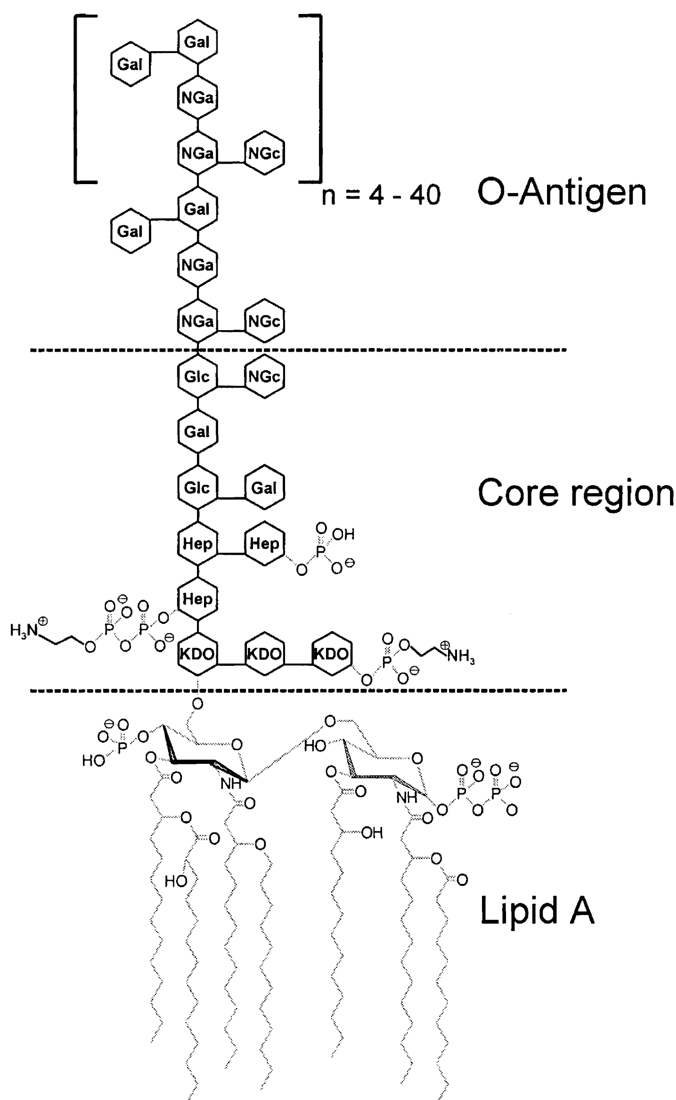


Fig. 1. Schematic view of the chemical structure of endotoxin from *E. coli* O111:B_4, according to Ohno and Morrison [16]. Hep, L-glycero-D-manno-heptose; Gal, galactose; Glc, glucose; KDO, 2-keto-3-deoxyoctonic acid; NGa, *N*-acetyl-galactosamine; NGc, *N*-acetyl-glucosamine.

oligosaccharide has a conserved structure; in *E. coli* species, five different core types are known, *Salmonella* species share only one. The most conserved part of endotoxins is Lipid A, showing very narrow structural relationship in different bacterial genera. For the removal of endotoxins it is important to point out that the core region close to Lipid A and Lipid A itself are partially phosphorylated. Therefore, at neutrality, endotoxins exhibit a net negative charge (pK_a 1.3; [6]). The molar mass of an endotoxin monomer,

Fig. 2. Supramolecular structures of endotoxin aggregates in aqueous solutions of varying composition.

as shown in Fig. 1, varies between 10 and 20 kDa, owing to the variability of the oligosaccharide chain; even extreme masses of 2.5 (O-antigen-deficient) and 70 kDa (very long O-antigen) are found.

According to molecular dynamics, the three-dimensional structure of endotoxin, especially the long surface antigen, is much more flexible than the globular structure of proteins [7]. Another typical characteristic is the formation of supramolecular structures, owing to non-polar interactions between lipid chains as well as to bridges generated among phosphate groups by divalent cations. Hence, micellar structures and vesicles are produced, having much higher molar masses and diameters up to 0.1 μm, which show high stabilities, even in dilute aqueous solutions (Fig. 2).

3. Interactions of endotoxins with other molecules

Two major differences should be mentioned in comparison to the affinity purification of other molecules. On the one hand, endotoxins are, in most cases, not a product but a contaminant. Thus, high purity and high biological activity are not the main objective. Rather, another product—in most cases a protein—must not be altered, if possible, during endotoxin clearance and should be recovered in close to 100% yield. The other difference is the extremely low endotoxin concentration in presence of substances up to 6 orders of magnitudes higher concentration, at which, owing to the high toxicity of endotoxins, still further purification may be necessary.

Several consequences derive from these circumstances. The so-called negative chromatographic mode is preferred, which allows binding of a byproduct or pollutant—here endotoxins—whereas the product passes the adsorber without considerable retention. Elution of endotoxin is not the object, and therefore irreversible adsorption is an option; it may often be welcome. This is deduced from the fact that adsorption is an equilibrium process that, due the low endotoxin threshold levels expected, requires a very low apparent dissociation constant, K_d, of endotoxin and sorbent. This condition also follows, owing to unavoidable interactions with substances (proteins) of much higher concentration, leading to competition for endotoxin. The latter leads to the unfortunate situation of the endotoxin often being carried piggyback through an adsorber, the extent being dependent on the different strengths of interactions of the many components involved.

Owing to the chemical structure of endotoxins, it is to be expected, especially at low ionic strength, that interactions occur with net-positively charged substances as well as generally with hydrophobic moieties, because of their lipophilic character, their strength

depending on the type and concentration of the salt. The distinct chemical structure is to be seen as the reason why a number of distinct processes are involved in the patent literature (adsorption, two-phase partitioning, ultrafiltration) for the removal of endotoxins [8]. None of these processes is generally applicable.

A number of proteins show strong interactions with endotoxins, such as lipopolysaccharide-binding protein (LBP), bactericidal/permeability-increasing protein (BPI), amyloid P component, cationic protein 18 [9,10], or the enzyme employed in the biological endotoxin assay (anti-LPS factor from *Limulus* amebocyte lysate (LAL) used in the LAL assay; [11]). These proteins are directly involved in the reaction of many different species upon administration of endotoxin (cf., e.g. Refs. [12,13]). Other proteins interact as well without having strong links to a biological mechanism, such as lactoferrin [14,15] or lysozyme [16,17]. Lysozyme also interferes with the rabbit test [18], giving false negative results due to masking of endotoxin, a problem occurring also in the LAL test with many—if not all—net-positively charged proteins [19]. In some cases, interaction is not linked to binding but to deaggregation of supramolecular endotoxin structures, for example with hemoglobin or transferrin [20,21]; de-aggregation usually leads to a higher toxicity of endotoxin in vivo.

In principle, it should be possible to make an affinity sorbent by immobilization of one of these proteins. Attempts to use immobilized amylase [22] or lysozyme [23] were not further explored and therefore these techniques have not succeeded. Recently, the anti-LPS factor (LALS) of *L. polyphemus* was immobilized [24]; until now, reports about its efficiency have not been published. It is questionable whether a protein-ligand-based sorbent is recommendable, as it is prone to denaturation and degradation and may therefore lead to additional contamination of the product. Peptide ligands, such as fragments of LALS [25], are generally more robust; however, again, there are still no data available on immobilized systems. The alternative of using totally synthetic endotoxin-binding ligands is, therefore, the most common route pursued so far.

4. Ligands employed for endotoxin removal

It must be strictly distinguished between the removal of endotoxin from protein-free and protein-containing solutions [26,27]. In a protein-free solution, methods, such as ultrafiltration, can be employed which take advantage of the different size of endotoxins and water as well as salt and other small molecules. However, in the presence of proteins, methods utilizing physical–chemical interaction forces must be employed among which, besides affinity chromatography, two-phase extraction has a high potential [28].

Since endotoxins are negatively charged, anion exchange ligands are also employed, e.g. diethylaminoethane DEAE (see Fig. 3) or quaternary amino groups, immobilized on chromatographic supports or in a depth filter [6,29–31]. Clearance factors of more than 5 orders of magnitude can be obtained at high endotoxin feed concentrations (> 1 μg ml^{-1}). At low feed concentrations < 10 ng ml^{-1}, ca. 3–4 orders of magnitude are feasible. A prerequisite for maximal adsorption is a low ionic strength ($\Gamma < 0.05$). If solutions with acidic proteins are to be decontaminated, protein co-adsorption is a

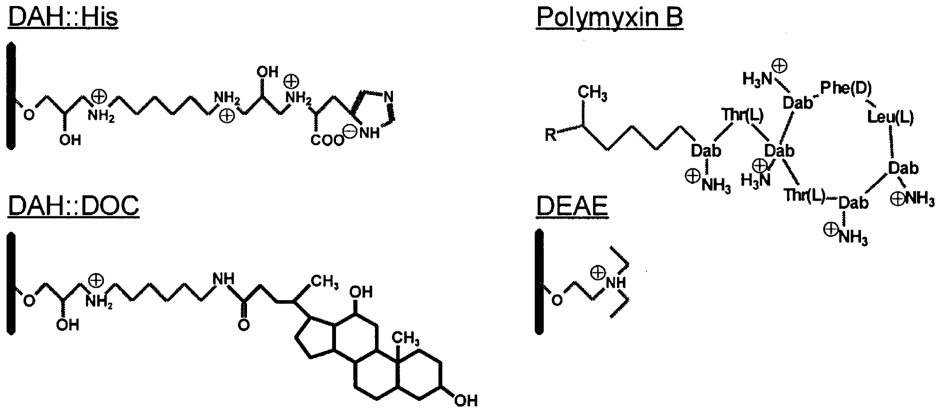


Fig. 3. Chemical structures of low-molecular-weight ligands utilized for endotoxin clearance; immobilization of polymyxin B occurs via any amino group.

problem. This has the consequence of competing interactions at binding sites. Therefore, endotoxin concentrations often cross the limits in column effluents after exhausting the binding capacity of protein adsorption. From this point of view, only proteins with net positive charge, i.e. basic proteins, ought to be treated with anion exchangers, the proteins being repelled from the anion-exchange matrix. However, competition of the ion exchanger and net-positively charged proteins for endotoxin takes place, causing proteins to drag endotoxins through the column. It will be seen that these effects are not restricted to anion exchangers, but to all affinity matrices so far used for endotoxin removal.

According to the common understanding of affinity interactions, clearance by an endotoxin-selective affinity sorbent should guarantee a recovery of other molecules of close to 100%. The many different chemical structures of endotoxins, expressed in the variability of the O-antigen and the core oligosaccharide, define the demands on the structure and function of an affinity ligand. Those with biological recognition should be targeted exclusively against the most conserved part of the endotoxin structure, which is Lipid A. Ligands interacting with other structural charac-teristics should be of diffuse recognition or should be group-selective for the small structure elements existing in various endotoxins. Because of the low concentrations to be cleared, strong interactions are mandatory.

4.1. Polymyxin B

The bactericidal activity of the antibiotic polymyxin B against Gram-negative bacteria is based on its ability to disorganize the bacterial wall after insertion [32]. The cyclic peptide (Fig. 3) is surface-active and causes dissociation of endotoxin aggregates [33]. Titration-microcalorimetric studies indicate a stoichiometry of ca. 1 with a dominating contribution of hydrophobic interactions for polymyxin B bound to the Lipid A of

endotoxin [34]. Owing to these interactions, polymyxin B has the potential to recognize endotoxins of different origin.

Its use in affinity sorbents yields clearance factors (CF) $> 10^5$ from heavily contaminated culture filtrates (1–10 $\mu g\ ml^{-1}$) of different Gram-negative bacteria [35]. Talmadge and Siebert [36] showed, at input concentrations of 6000–6700 endotoxin units (EU ml^{-1} (ca. 0.6–0.7 $\mu g\ ml^{-1}$ endotoxin), that a CF ca. 10^3 only slightly changed in the presence of up to 10 mg ml^{-1} bovine serum albumin (BSA) or human immunoglobulin G (IgG) in batch experiments, utilizing contact times of 16 h. However, other results are controversial and show distinct CFs from solutions of different monoclonal antibodies, as for an anti-horseradish peroxidase antibody (2.1 mg ml^{-1} with 20 EU ml^{-1}) and an anti-human chorionic gonadotropin antibody (0.5 mg ml^{-1} with 61 EU ml^{-1}), which were reduced only to 0.3 EU ml^{-1} (CF ca. 100) and 6 EU ml^{-1} (CF ca. 10) in 20 mM phosphate buffer, pH 6.5, respectively. A CF value of > 1000 was reported for the decontamination of bovine catalase [37].

In addition to drawbacks in view of its neuro- and nephrotoxicity [32] as well as stimulation of monocytes to release interleukin-1 [38], protein losses during passage through polymyxin B columns were reported (bovine catalase 24% loss, [37]; BSA 20% loss, [26]) going along with lower clearance rates. This is because there are positive charges located at the amino groups of this ligand, leading to electrostatic attraction of net-negatively charged proteins at low ionic strengths. This is also the reason why, in spite of 200- to 10,000-fold reduction of endotoxins from plasmid DNA preparations, DNA recovery is only about 50% [39,40].

Srimal et al. [34] concluded that recognition between polymyxin B and endotoxin is mainly due to interactions of a hydrophobic patch at one side of the peptide and Lipid A. It seems, however, that the binding kinetics of this affinity complex are relatively slow, as contact times of 16 h are described in batch adsorption experiments [36]. Slow binding kinetics may be the reason that the CFs described in batch experiments can often not be confirmed in column experiments [26] (cf. also adsorption kinetics).

In an attempt to circumvent the neuro- and nephrotoxicity of polymyxin B, Rustici et al. [41] developed peptide analogs with similar composition, which also demonstrated strong interactions with Lipid A. However, although high stabilities of some of the peptide–endotoxin complexes and also less toxicity was reported in vivo, this approach was not followed up.

4.2. Histamine and histidine

Kanoh et al. [42] discovered interaction between ribonucleic acids and endotoxins and Minobe et al. [43] later showed that besides the nucleobases adenine, cytosine, etc., histidine and histamine were equally successful for endotoxin removal from culture filtrates of various microorganisms. Although histamine was favored [44], they later switched to histidine (Fig. 3), owing to the biological activity of histamine [45]. Both ligands are about as effective as polymyxin B for culture filtrates and show decontamination potential for various proteins, including serum albumin, insulin, lysozyme, and myoglobin, with CFs ranging from 5 to 200, depending on the protein concentration and environmental conditions. As with polymyxin B, best protein recoveries and removal

efficiencies cannot be achieved independently. The presence of proteins strongly affects endotoxin removal, leading to a more than 10-fold reduction of CFs in the presence of BSA and lack of effectiveness in the presence of a murine IgG_1 of p$I = 5.5$, if the process is carried out at pH 7 [26,46].

Despite similar removal efficiencies of the various ligands employed for endotoxin removal [43], their chemical structures are quite different; this casts doubt on a molecular recognition mechanism. According to Minobe et al. [45] the mechanism of endotoxin binding is attributed to synergistic hydrophobic and electrostatic interactions, originating from the spacer diaminohexane (DAH) and imidazole (Fig. 3). However, the DAH spacer is effective without any ligand, as was shown for the decontamination of a 10% cytochrome *C* solution with 1130 EU ml^{-1} and a 20% human serum albumin (HSA) solution with 85 EU ml^{-1} (5000- and 100-fold endotoxin reduction, respectively) using a poly(vinylalcohol) (PVA)-based microfiltration membrane support [47]. Hou and Zaniewski [48] have also shown that DAH and other diaminoalkanes are effective for endotoxin adsorption, owing to a synergistic effect of ionic and hydrophobic interaction. However, they did not provide data from protein solutions. The function of the sole histidine ligand must be linked to the presence of a positive charge at the imidazole ring, as was shown by Petsch et al. [49]. Incorporating a bisoxirane-based spacer, which does not add a charge to the ligand, significant removal was achieved only at pH ≤ 5, where the imidazole ring is positively charged ($pK_{imidazole} = 6.0$).

4.3. Deoxycholic acid

As indicated by the above-mentioned examples, recognition between ligand and substrate is not restricted to a unique mechanism; but synergistic interactions are important. Therefore, the development of an endotoxin-selective ligand, utilizing deoxycholic acid (DOC) and giving rise to disorganization and rupture of micellar endotoxin structures, and a spacer with positive charge, such as DAH, should also be effective. Indeed, Anspach et al. [50,51] have demonstrated that membrane adsorbers based on the ligand DOC (Fig. 3) were as effective as others, yielding CF ca. 10^4 in the absence of proteins and endotoxin concentrations of ca. 1 $\mu g\ ml^{-1}$ when DOC was immobilized on a dextran polymer network, located in the flow-through pores (see below). Better endotoxin clearance was observed than with the ligands PLL, polymyxin B (PMB), poly(ethyleneimine) (PEI) and DEAE, which were similarly immobilized, at high concentrations of 5–10 mg ml^{-1} BSA, yielding 1.3 EU from 133 ml^{-1} and 1.8 from 303 EU ml^{-1}, respectively. Also, a better clearance efficiency was found at unfavorable environmental conditions, i.e. at pH > pI with BSA and HSA. Thus, this ligand also demonstrated best clearance factors with contaminated fetal calf serum (CF = 22) as well as human serum (CF = 3.6) and human plasma (CF = 2) at initial conditions with 20 mM phosphate buffer at pH 7 and 300 EU ml^{-1}.

Owing to the relatively low charge density per ligand, only little ionic interaction with negatively charged proteins is observed, so that a competition for binding sites is not limiting. Although a reaction limitation is found with these membrane adsorbers, the formation of the affinity complex was not significantly slower than with the other ligands under investigation [50].

4.4. Polycationic ligands

According to molecular dynamics, the three-dimensional structure of endotoxin is rather flexible compared to proteins [7]. This seems to play a decisive role during endotoxin adsorption. It is observed that only a fraction of adsorbed endotoxins can be desorbed at high salt concentrations (> 1 M NaCl), despite an effective suppression of adsorption in the presence of 0.5 M NaCl [23, 40]. Most likely, this result, which at first sight seems conflicting, is attributed to short-range interactions, evolving after an approach of ligand and endotoxin as result of long-range electrostatic interactions. Secondary binding is caused by the formation of van der Waals and hydrogen bonds after a structural adaptation to the microstructures at the surface of the sorbents. Only harsh conditions (30% ethanol with 1 M NaOH) are successful in cleaning sorbents used for endotoxin adsorption.

The possibility of structural adaption again points out that an exact structural match between affinity ligands and endotoxins is not necessary. From this point of view, an endotoxin-selective ligand should meet the characteristics of a polyanionic molecule with hydrophobic moieties. Indeed, several cationic polymers were successfully employed as ligands (Fig. 4). Mitzner et al. [52] used PEI, a hydrophilic polymer, which was immobilized on cellulose beads for the extracorporeal removal of endotoxin from plasma. They obtained efficacy similar to polymyxin B but with superior biocompatibility. Immobilization of PEI on cellulose fibers revealed greater endotoxin removal from BSA solutions than with corresponding histidine-immobilized fibers and less dependence on ionic strength [53]. Solutions of myoglobin, γ-globulin, and cytochrome *C* were almost completely cleared of endotoxins (> 98% removal efficiency with < 0.05 ng ml^{-1} remaining) at > 98% protein recovery in a batch process.

Poly-L-lysine (PLL), which is more hydrophobic due to its alkyl chains (Fig. 4), also displayed slightly better clearance of low amounts of endotoxins from BSA solution than the ligands histamine, histidine, polymyxin B, and DEAE but with a higher protein recovery [26]. In contrast to a DEAE ion exchanger, the PLL sorbent is still applicable

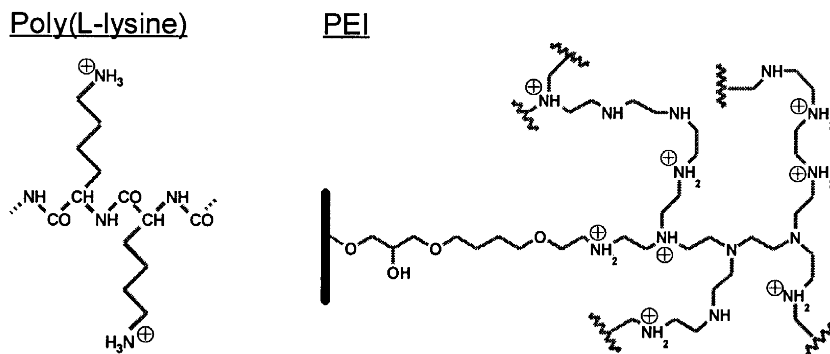


Fig. 4. Chemical structures of high-molecular-weight ligands used in endotoxin-selective sorbents; immobilization of PLL occurs either via the α- or any ε-amino group.

after exhaustion of the protein-binding capacity. Pre-coating of microplates with high-molecular-weight PLL ($M_r = 150,000–300,000$) was more effective in improving endotoxin binding to the wells than poly-L-histidine (PLH) and polymyxin B [54]. Recently, Hirayama et al. [55] have extended their concept of charged polymeric matrices (see below) by including PLL in a polymer matrix. Karl et al. [56] showed that zirconia-immobilized PLH gives better endotoxin removal from a BSA solution than the bare zirconia surface. However, PLH is quite an expensive ligand, which is moreover unstable under alkaline conditions (0.1 M NaOH, cf. Wäsche [57]).

The principle underlying these polycation–endotoxin interactions is possibly the same as in the flocculation of cells and cell debris. In flocculation, a polycation–polyanion complex is also formed initially. Then, replacement of water molecules follows after structural adaptation of both molecules, and finally flocks form—a process which is called complex coacervation [58]. If this process also took place with immobilized polycations and endotoxins, it would continuously withdraw complexes from solution. This would explain the high-affinity binding sites observed in thermodynamic investigations of these sorbents and also the selectivity of polycationic ligands in the presence of proteins [27].

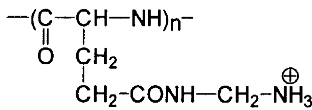
4.5. Polymeric matrices with cationic functional groups

Through amination of spherical porous poly(γ-methyl-L-glutamate) beads (Fig. 5), Hirayama et al. [59,60] obtained sorbents with better endotoxin-binding capacity than commercial endotoxin adsorbers based on histidine and chitosan. Furthermore, less dependence on the ionic strength (working range up to 0.4 M NaCl) and higher selectivity towards BSA were claimed. Additionally, penetration of proteins into the pore system is prevented by adjusting reaction conditions so as to yield beads with small pore sizes. This gives high recoveries of net-negatively charged proteins and at the same time strong endotoxin adsorption. These authors concluded that the high efficiency can be attributed to the adsorption of mainly endotoxin aggregates on the adsorber surface, while the BSA-binding capacity is rather low; binding of endotoxin monomers inside the pore system is not considered.

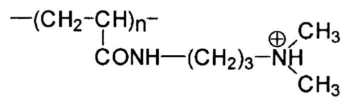
A disadvantage of these adsorbers is the low chemical stability of ester bonds, being only partially replaced by amide groups during the synthesis. Ester bonds are prone to hydrolysis under harsh environmental condition, leading to structural changes of the matrix. Therefore, cleaning-in-place (CIP) at high or low pH is ruled out. In a more recent publication [61], the same group introduced *N*,*N*-dimethylaminopropylacrylamide/*N*-allylacrylamide copolymers (Fig. 5), which are stable under CIP conditions. This concept allows also adjustment of the pore size of the beads. The charge density is manipulated by adjusting the ratio of the two monomers. Removal efficiencies were 96–99% (pH 7, $\Gamma = 0.05$) with remaining endotoxin amounts of < 1 EU ml^{-1} at 0.5 mg ml^{-1} of BSA, myoglobin, γ-globulin, or cytochrome *C*, and protein recoveries $> 99\%$.

Immobilization of ligands on microfiltration membranes may also yield polymers with cationic functional groups. The inner surface of these membranes (this is mainly the wall of the flow-through pores) is first covered by a hydrophilic polymer, such as

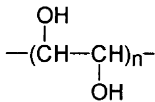
Poly(γ-aminomethyl-L-glutamine)



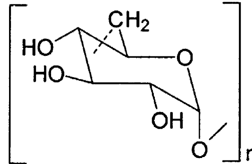
Poly(N,N-dimethylamino-propylacrylamide)



Poly(vinylalcohol) (PVA)



Dextran



OH ➤ OR with R = any endotoxin-binding ligand

Chitosan

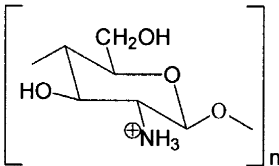


Fig. 5. Basic repeating units of polymer matrices employed for endotoxin clearance; at PVA and dextran endotoxin-binding ligands are immobilized through a spacer, which replaces one or several OH groups of the polymer chain.

dextran, hydroxyethyl cellulose, or PVA. Then, either small ligands, such as histidine, deoxycholate, polymyxin B, or DEAE, or polycationic ligands, such as PLL or PEI are immobilized inside the hydrophilic polymer network [49]. Differences between high- and low-molecular-weight ligands are not as noticeable any more; the whole polymer network acts like a cationic polymer [27]. Although these membrane adsorbers show cationic properties and therefore adsorb net-negatively charged proteins, displacement of endotoxins is not observed with the ligands PEI, PLL, and not even with DEAE after exhaustion of the protein-binding capacity. Corresponding adsorption isotherms show distinct binding sites for endotoxins and BSA [50] with high affinity binding ($K_{A(DEAE)}$ = 10,000 and $K_{A(PEI)}$ = 28,000 ml mg^{-1}) and rather normal binding (K_A = 0.5–2.0 ml mg^{-1}) for endotoxin and BSA, respectively. Under optimized environmental conditions, protein recoveries can be close to 100%, owing to the low protein-binding capacity of membranes.

Chitosan, a poly-(1,4-β-D-glucopyranosamine) (Fig. 5), and quaternized chitosan are also claimed to be endotoxin-selective ligands [62,63]. However, the complex formation of endotoxin and chitosan requires an elevated temperature and, also, complex stability seems to be a problem [64]. Furthermore, chitosan itself triggers monocytes to release

TNF-α [65], which may become a problem if some ligands are released, causing contamination of a parenteral solution.

4.6. Immunoaffinity ligands

With regard to competing interactions at the surface of sorbents and with proteins in solution, as described above, utilization of immunoaffinity ligands, recognizing endotoxins at the molecular level should be most promising at first sight. However, relevant information about the usefulness of immuno sorbents for endotoxin removal is rare (cf. Ref. [66]) and the patent literature does not provide additional data. Generally, immunosorbents are used reluctantly at the end of a purification train, as ligand leakage may lead to further contamination of an almost pure product; the problem is of similar importance as with protein ligands. However, the concept is doubtful also in another respect.

Strong efforts were undertaken in the past 10–15 years to develop therapeutic proteins, based on IgG and immunoglobulin M (IgM) antibodies, to guard against the problem of endotoxin intoxication, a common problem in hemodialysis and acute bacterial infections. In spite of promising attempts [67–69], all clinical trials have failed. A reason for these failures is the great variety of chemical endotoxin structures [70]. On the other hand, antibodies raised against the non-polar Lipid A, such as HA-1A (Centoxin), displayed mainly non-specific hydrophobic interactions, recognizing hydrophobic moieties of proteins as well [71]. However, these ligands have not been employed as ligands in immunosorbents until now and, thus, a success is not impossible. However, neither the specific recognition of only one antigen nor the non-specific interactions with proteins are favorable for endotoxin removal from protein solutions.

5. What is unique in the affinity interaction of endotoxins?

In view of the many possibilities of selective adsorption of endotoxin, it may be asked what distinct differences exist between affinity interactions with proteins and other molecules. The word affinity is used very often in the context of endotoxin clearance, but at the same time sorbents with very different functionalities are employed, raising questions about the “most suitable method”. On the basis of present examples, the discrepancy between different interpretations of affinity becomes especially clear. Often, affinity interactions are linked to a special recognition between two molecules, leading to strong binding. At the same time, it is expected that an affinity sorbent, which is characterized by the immobilization of one of these molecules, displays a high selectivity towards the purification of the partner molecule; this is often stated a specific interaction. However, each association is described by a chemical affinity between two molecules; a special recognition mechanism is not a necessary requirement. Hence, a differentiation between specific and non-specific interactions is not allowed. Correspondingly, a special selectivity of a sorbent cannot be derived, either.

It is clear that endotoxins develop specially strong binding to those kinds of adsorbers that carry positively charged functional groups. Therefore, electrostatic interactions play

an important role during endotoxin adsorption. It is common for all ligands and adsorber structures described above that they display more or less marked hydrophobic properties. It must be assumed that interactions between hydrophobic sites arise after the two-molecule approach. Owing to the adaption ability of endotoxin molecules [7], these interactions can be quite strong. If at the same time the structure of the ligands is flexible, anion cation complexes of great stability can form. Since proteins are amphoteric molecules, electrostatic interactions are not as strong as for the mainly negatively charged endotoxin. Owing to the globular structure of proteins, charged and hydrophobic groups are fixed and cannot be twisted towards functional groups or surface structures of the adsorbers. On the other hand, the flexibility of a (polymer) ligand cannot be seen as a general advantage in adjusting itself also to a protein surface; positive and negative charges alternate at the protein surface, especially at the isoelectric point, where the same number of positive and negative charges exist. Moreover, with proteins, local patches with extreme hydrophobicity, like the carbon chains of Lipid A in endotoxins are generally not found.

If a high flexibility of ligands and endotoxins coincide, an exceptionally high selectivity of a corresponding sorbent can be expected. To what extend additional characteristics of the ligand structure are of importance is difficult to state. Generalized conclusions from publications are not admissible, as a number of different endotoxin structures have been considered in combination with different proteins and affinity ligands, and this has often led to contradictory conclusions.

Owing to the supramolecular structure of endotoxins, special importance needs to be attached to adsorption kinetics. In order to allow the formation of anion cation complexes, micelles or vesicles must first get close to the binding site, where they dissociate into monomers in a second step. It is rather unlikely that micelles are adsorbed as a whole, as these could release single molecules with changing environmental conditions, but relevant information regarding such a phenomenon is missing. Clearly, mass transport of these large structures can easily be hindered if adsorbers with small pore and large particle diameters are employed. In isolated cases, breakthrough of endotoxin activity can be expected in column experiments. This might be the reason why the clearance of endotoxins is often described in batch experiments. However, this is irrelevant if the process has to be carried out under good manufacturing practice (GMP) conditions. Therefore, membrane adsorbers, such as those introduced by Nagamatsu et al. [47], Guo et al. [72] or Petsch et al. [27], cannot be just an alternative to particulate sorbents but may improve endotoxin clearance significantly. Owing to mainly convective mass transport and the short path lengths in the flow-through pores of nylon microfiltration membranes with nominal pore size of 0.45 μm, it was shown that a residence time of 6 s is enough for complete adsorption in one pass [50]. From this residence time, a mean diffusion length of 12 μm is calculated for endotoxin aggregates, which exceeds by far the diffusion length in these membrane adsorbers. At the same time, high protein recoveries are achieved as protein-binding capacities of membrane adsorbers are lower than those of particulate sorbents.

Since a selective endotoxin removal is usually at the end of a purification train (high endotoxin concentrations are usually lowered with each purification step downstream), conditions can be optimized for the separation of two components. Best clearance

conditions can be expected, if the pH of the buffer is adjusted to the pI of the protein (of course protein stability must be considered). Possibly, this pH can further be optimized, as the surface charge distribution does not necessarily have to be identical with the total charge distribution of the protein. At a very high isoelectric point, the buffer pH may need to remain lower. However, then the maximally possible pH should be chosen as well as a quaternary anion exchanger that is effective in competing with the protein for endotoxin, due to its high charge density. If, on the other hand, the pH cannot be lowered to the theoretically optimal value, cationic polymer ligands should be preferred. These may establish complexes with endotoxins, thereby shifting the competition with proteins in favor of favorable endotoxin clearance.

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