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Membrane cartridges for endotoxin removal from interferon preparations

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Abstract

The suitability of membrane cartridges for the removal of endotoxin from both distilled water and interferon preparations was examined. The endotoxin concentrations were reduced to 4.0 and 7.3 EU/ml, respectively, when about 4000 ml of distilled water with 20 and 28 EU/ml were passed through the deoxycholate and chitosan immobilized membrane cartridges. When 200 ml of interferon preparation with endotoxin concentration more than 80 EU/ml and pH 3.9 were applied to a deoxycholate immobilized membrane cartridge at a flow-rate of 9 ml/min, the endotoxin concentration was reduced to less than 10 EU/ml. However, if an interferon preparation of 450 ml, with more than 80 EU/ml of endotoxin and pH 3.9 was applied to the chitosan immobilized membrane cartridge at a flow-rate of 18 ml/min, the endotoxin concentration was reduced to less than 10 EU/ml.

Keywords: Membrane cartridges; Cellulose membrane; Interferon; Chitosan; Deoxycholate; Endotoxin

1. Introduction

Bacterial endotoxin is a term for lipopolysaccharides located in the outer cell membrane of Gram-negative bacteria. They are composed of a hydrophilic polysaccharide moiety and a hydrophobic lipid moiety (lipid A) which anchors the molecules in the outer membrane. The sugar residues may be partially phosphorylated and the endotoxin molecules exhibit a net negative charge when the pH is above 1.3. Endotoxin can be released during bacterial growth and death. It acts as a strong immunostimulant when entering the blood circulation of humans, with possibly fatal outcome [1]; hence endotoxin contamination of drugs for intravenous use is a serious problem. In order to minimize risks, such medicaments have to comply with the endotoxin threshold limits regulated by various pharmacopoeias. According to Chinese Pharmacopoeia 2000, the endotoxin content should be less than 10 EU/ml for interferon-α_{1b} preparations and 2 EU/ml for human serum albumin injections [2]. These requirements are, however, the critical point in the formulation of high-molecular mass pharmaceuticals, such as pharmaproteins, albumin preparations and recombinant

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peptides and proteins. Usually inherent sources of endotoxin involved in contamination of raw products, such as plasma or tissue, are introduction of host-specific endotoxin in recombinant DNA methods, accidental microbial contamination during downstream processing or co-purification of endotoxin with the products [3].

The established methods for endotoxin removal, such as chemical or heat treatments, ultrafiltration, adsorption on activated charcoal or charged-modified depth filters, are not completely appropriate to the substances mentioned. The separation of endotoxins from these products requires more sophisticated methods. The applications of affinity ligands using L-histidine, histamine, polymyxin B, poly-L-lysine, and poly(ethyleneimine) immobilized on beads seemed to provide a promising approach [4,5]. Poly(ethyleneimine)-Sepharose was successfully used to adsorb endotoxin for purification of recombinant basic fibroblast growth factor from a high cell density cultivation of *E. coli* [6]. Endotoxin was decontaminated from tumor necrosis factor and IL-1 by styrene–divinylbenzene copolymer having sulfonic acid groups [7]. The endotoxin in the solution of murine IgG1 was successfully adsorbed by both deoxycholate and poly(ethyleneimine) immobilized adsorbers [8]. Histidine [9], cationic charged groups 1-vinylimidazole or *N*,*N*,*N*′,*N*′-tetramethylethylenediamine [10] and chitosan [11,12] were also immobilized on cellulose membrane for endotoxin removal from water, buffer [9,10], human serum albumin, hydrocortisone, lysozyme, tetracaine and dextran 40 solutions [11].

Interferon has three types (α, β, and γ), and all have been produced by recombinant gene technology. Recombinant human interferon-α may be effective in eliminating or controlling chronic hepatitis B infection, hepatitis D infection and leucocythemia of bristle cells [13,14]. According to the production requirements for recombinant human interferon-α_{1b}, the endotoxin content must be reduced to less than 10 EU per 300 000 Interferon Units after the approved ion-exchange chromatographic separation and immobilized immunoglobulin affinity chromatographic purification. Then the interferon-α_{1b} is diluted to be semi-finished interferon preparations with human serum albumin [2]. Thus the interferon preparations were taken by membrane cartridges for endotoxin removal investigation in this study.

2. Experimental

2.1. Materials and equipments

Cellulose membranes with an average pore size of 20–25 μm (qualitative filter papers) were purchased from Hangzhou Xinhua Paper Manufactory (Hangzhou, China). Epichlorohydrin was from Tianjin Tian Tai Reagent (Tianjin, China). Chitosan was from Jinan Hai De Bei Marine Biological Products (deacetylation >90%, molecular mass ca. 350 000, Jinan, China). Sodium deoxycholate was purchased from Serva (Heidelberg, Germany). (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) was from Merck (Darmstadt, Germany). Endotoxin standard of *E. coli* O111:B4 was from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). *Tachypleus* amoebocyte lysate (TAL) for endotoxin detection was from Zhanjiang Marine Organisms Products (Zhanjiang, China). The sample water was distilled water prefiltered using a 0.45-μm membrane after distillation. Recombinant human-α_{1b} interferon preparations were supplied by the Shanghai Institute of Biological Products (Shanghai, China). All other chemicals were of analytical grade.

Bacterial endotoxin test apparatus BET-32C and the EasyBET software designed for quantifying endotoxin in medicines, biological preparations and foods were supplied by Electronic Instrument Factory of Tianjin University (Tianjin, China). DD-50B Endotoxin Removal Apparatus, consisting of a peristaltic pump, a fluid pressure controlling device and a holder for membrane cartridge, was from Dandong Saili Chemicals (Dandong, China).

Pyrogen-free water was Wahaha purified drinking water (Hangzhou, China) which passed the TAL test or available from the Shanghai Institute of Biological Products (Shanghai, China). All glassware was treated with potassium bichromate–sulfuric acid solution overnight, tap water and pyrogen-free water washings, followed by heat treatment for 1 h at 250 °C.

2.2. Membrane preparation

2.2.1. Chitosan immobilized cellulose membrane (CHI-CM)

Cellulose membranes were cut into round pieces with a diameter of 47 mm. Fifty pieces of the

membrane (8 g) were placed in a beaker containing 120 ml 1 *M* NaOH, 40 ml epichlorohydrin and 0.19 g $NaBH_4$ and activated for 18–20 h at room temperature with occasional shaking. The activated membranes were washed with plenty of distilled water until neutral and then placed in 150 ml 1% chitosan solution (dissolved in 1% acetic acid) for more than 16 h. After being washed with 1% acetic acid and plenty of water, the membranes were deoxidized by 100 ml 0.28% $NaBH_4$ solution in 0.1 *M*, pH 9.2, phosphate buffer for more than 16 h. Then the membranes were washed with plenty of water and dried using filter paper [15]. Ten pieces of the prepared membranes were constructed into the patented membrane cartridge which consisted of two round plates holding the membranes, a cylinder with an internal diameter of 47 mm holding the two plates and membranes with glue. Another cylinder connected with the former cylinder by screw thread and a ring for adjusting the distance between the two cylinders [16–18]. The cartridge had an internal area of 17.7 cm^2.

2.2.2. Deoxycholate immobilized cellulose membrane (DOC-CM)

Twenty pieces of the membrane with the same size as CHI-CM (3.2 g) were immersed in a beaker containing 50 ml 2% $NaIO_4$, and activated for 18–20 h at room temperature with occasional shaking. The activated membranes were washed with plenty of distilled water and were immersed in 50 ml 1% diaminohexane solution (dissolved in 0.1 *M* pH 9.2 phosphate buffer) for more than 16 h at room temperature. After being washed with plenty of water, the membranes were deoxidized by 50 ml 0.6% $NaBH_4$ solution in 0.1 *M* pH 9.2 phosphate buffer for more than 16 h. Then the membranes were washed with plenty of water and dried using filter paper. Deoxycholate was coupled to diaminohexane via its carboxylic function by the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide method [8,15]. Twenty sheets of the diaminohexane-immobilized membrane were immersed in 50 ml 0.1 *M* 2-[*N*-morpholino]ethanesulfonic acid buffer containing 1 g sodium deoxycholate in adjusted to pH 6. An amount of 0.5 g 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added and the reaction mixture was shaken for 18–20 h at room temperature. Then the membrane were washed with plenty of water and dried using filter paper. Ten pieces of the prepared membranes were glued with polyurethane, loaded and sealed into a patented cartridge [16–18].

2.3. Adsorption experiments

Adsorptions of endotoxin in membrane cartridges were studied in single pass filtration mode using the patented cartridge. The membrane cartridges were washed consecutively with 500 ml 0.2 *M* NaOH containing 20% ethanol, 500 ml 1.5 *M* NaCl and 500 ml pyrogen-free water to ensure the absence of endotoxins on the membrane; and these conditions were also employed for membrane cartridge regeneration [8]. The sample water was filtered through the membrane cartridge at a flow-rate of 20 ml/min. The flow-rate was chosen by pre-experiments which we do not show here. The recombinant human interferon-α_{1b} preparations at different pH values were passed through the membrane cartridge at different flow-rates. The filtrates or effluents were sampled and examined for endotoxin concentration or for interferon titer, if present. Samples were frozen to prevent microbial growth if not measured immediately after the experiment.

2.4. Analytical methods

Bacterial endotoxin test can estimate the concentration of endotoxins that may be present in the samples using LAL or TAL, obtained from the aqueous extracts of the circulating amebocytes of the horseshoe crab, *Limulus polyphemus* or *Tachypleus*, and which has been prepared and characterized for use as an LAL or TAL reagent for gel-clot formation and for turbidimetric readings [2,19].

Endotoxin concentrations of water samples were determined by the turbidimetric kinetic assays using TAL reagent in BET-32C Bacterial Endotoxin Test Apparatus [10,11]. The detection limit of the TAL reagent was 0.06 EU/ml.

The endotoxin concentrations of the interferon preparation samples were determined by the gel-clot formation [2]. The detection limit of the TAL reagent used was 0.25 EU/ml.

The titer of the interferon was determined according to the Chinese Pharmacopoeia 2000 [2].

The nitrogen content of the membrane was determined by the Kjeldahl method. The infrared

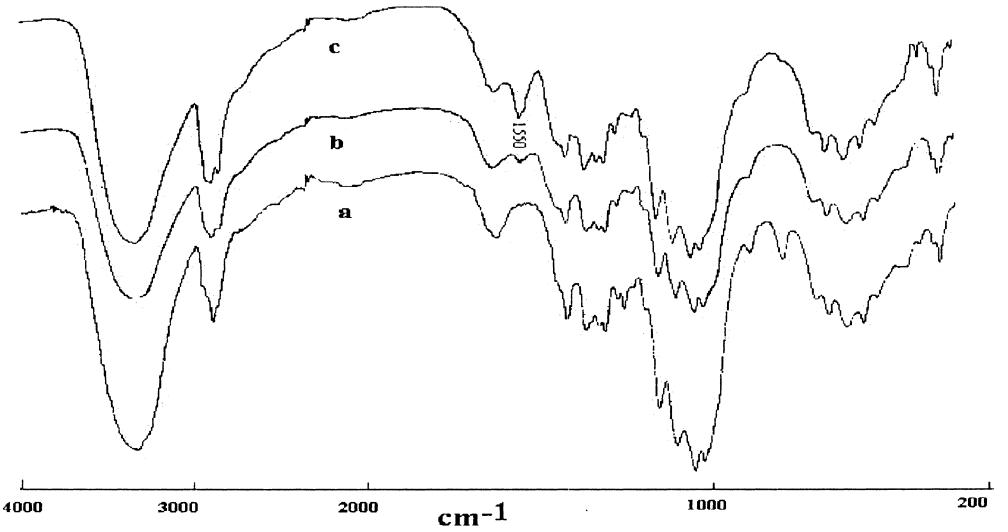

Fig. 1. FT-IR spectra of the cellulose membrane (a), diaminohexane immobilized cellulose membrane (b) and deoxycholate immobilized cellulose membrane through diaminohexane (c).

spectra were acquired on an FT-IR 170-SX infrared spectrophotometer (Nicolet, USA).

3. Results

3.1. Endotoxin removal from water

The nitrogen content of the diaminohexane-immobilized membrane was 0.63%. Fig. 1 presents the FT-IR spectra of the blank membrane, the diaminohexane-immobilized membrane and DOC-CM. The peak at 1550 cm^{-1} is the stretching vibration of –NHCO– which indicates the link of deoxycholate to the membrane through the spacer diaminohexane.

The nitrogen content of CHI-CM was 0.045%. The sample water with an endotoxin concentration of 28.0 EU/ml was passed through the CHI-CM cartridge at a flow-rate of 20 ml/min and the results are shown in Fig. 2. The total amount of endotoxin removed was 84 000 EU and the removal efficiency

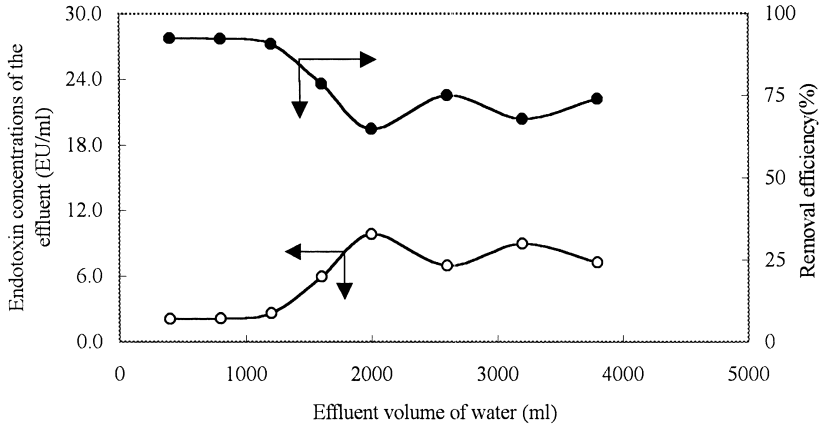

Fig. 2. Endotoxin removal graph from distilled water by the chitosan immobilized cellulose membrane cartridge.

ranged from 93 to 65% when a total of 3800 ml of the sample water was filtered.

Endotoxin removal from the sample water by DOC-CM cartridge is shown in Fig. 3 when a 4050-ml sample water with endotoxin concentration of 19.2 EU/ml was treated at a flow-rate of 25 ml/min. The total amount of endotoxin removed was 71 000 EU when the removal efficiency ranged from 98 to 80%.

3.2. Endotoxin removal from the interferon preparations

There might be several components in the interferon preparations, e.g., interferon, human serum albumin (HSA) and endotoxin-contaminated interferon or HSA. Thus multilateral interactions based on electrostatic attraction or repulsion and hydrophobicity should be taken into account. The p*I* of interferon is from 4.0 to 6.5 and that of HSA is 4.7 [16]. Both CHI-CM and DOC-CM contain ligands, the amino groups of which can interact with phosphate anion of endotoxin through electrostatic interaction. Besides, there is an additional part of hydrophobic interaction for DOC-CM. When the pH of the interferon preparation is higher than the p*I* values, proteins comprising a net negative charge will interact with the ligands, thus competing with endotoxin for binding sites. On the other hand, when the pH is below the p*I* values, proteins comprising a net positive charge will be repelled from the ligands but may interact with endotoxin molecules and possibly carry them through the membrane. So the endotoxin removal conditions of the given interferon preparations, such as pH values and flow-rates, were investigated.

3.2.1. Effects of pH

The interferon preparation, containing 91 406 IU/ml of interferon, more than 80 EU/ml of endotoxin and 2% of HSA, was pumped through CHI-CM or DOC-CM cartridges and the effluent was collected, sampled and determined for endotoxin concentrations and interferon titers. The pH of the interferon preparation was adjusted to 3.9, below the p*I* values of interferon and HSA, or to 7.0 in the physiological status, above the p*I* values of interferon and HSA. The flow-rate was 9 ml/min and the volume of the preparation was 200 ml. It is shown in Table 1 that the endotoxin contents decrease dramatically to meet the endotoxin limit of a interferon preparation (<10 EU/ml) after it was treated by the CHI-CM and DOC-CM cartridges when the sample pH was 3.9, but there were no effects at pH 7.0 for both cartridges. The interferon titer recovery was 88.3% for both cartridges at pH 3.9.

3.2.2. Effects of flow-rates

The interferon preparation, containing 93 976 IU/ml of interferon, more than 80 EU/ml of endotoxin and 2% of HSA, was pumped through CHI-CM or DOC-CM cartridges and the effluent was collected, sampled and determined for endotoxin concentrations and interferon titers. The pH of the interferon preparation was adjusted to pH 3.9. The flow-rates were 9 and 18 ml/min, while the volumes of the

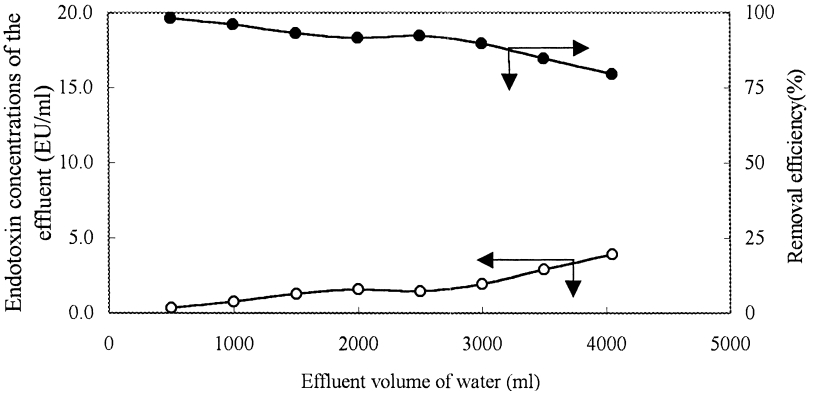

Fig. 3. Endotoxin removal graph from distilled water by the deoxycholate immobilized cellulose membrane cartridge.

Table 1
Effects of pH on endotoxin removal and interferon recovery by the membrane cartridges

Ligands immobilized on the membrane	PH of the feed	Endotoxin concentration of the effluent (EU/ml)	Interferon titer of the effluent (IU)	Interferon titer recoveries (%)
Chitosan	3.9	<10	80 684	88.3
	7	>80	Not determined	–
Deoxycholate	3.9	<10	80 684	88.3
	7	>80	Not determined	–

A solution of 200 ml interferon preparation with interferon titer 91 406 IU/ml, endotoxin concentration more than 80 EU/ml and different pH was applied to chitosan and deoxycholate immobilized membrane cartridge at flow-rate of 9 ml/min.

preparation were 200 (or 225) and 450 ml, respectively. It is shown in Table 2 that the endotoxin concentrations of the effluent decrease dramatically to less than 10 EU/ml after the interferon preparation was treated by the CHI-CM cartridge both at flow-rates of 9 ml/min (volume of 225 ml) and 18 ml/min (volume of 450 ml). The flow-rate of 18 ml/min was comparable to 20 ml/min which had been used in the endotoxin removal experiment from water. The endotoxin removal amounts were more than 31 500 EU and the overall endotoxin removal efficiency was about 87.5% which was also comparable to that from water. The interferon titer was completely recovered for CHI-CM. It may be presumed that the interferon in the preparations has less electrostatic charges at pH of 3.9; thus there is no or less competing interaction of interferon with chitosan of CHI-CM.

Contrary to CHI-CM, the endotoxin contents of the effluent from the DOC-CM cartridges were the same as that of the feed when the flow-rate was increased to 18 ml/min, although it was effective at 9 ml/min. This was also different from the results of the sample water. The flow-rate might be a limiting factor for endotoxin removal from the interferon preparations by DOC-CM.

4. Discussion

In this study membranes are employed as they are more advantageous than bead columns since they have higher throughput and endotoxin removal capacity. Cellulose membrane was used as matrix in order to combine its good biocompatibility with the high flux and low inlet pressure than that in the literature [20], which are of benefit for magnification of the cartridge and for improvement of the productivity. Chitosan was coupled to the membrane by way of epichlorohydrin activation and the coupling of sodium deoxycholate to the membrane by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide activation. The interaction mechanism of bacterial endotoxin with chitosan is rather complicated and depends on the macromolecular organization of endotoxin as well as on the degree of polymerization of the chitosan. Chitosan with a molecular mass of 20 000 reveals a higher affinity to endotoxin than that of

Table 2
Effects of flow-rate on endotoxin removal and interferon recovery by the membrane cartridges

Ligands immobilized on the membrane	Flow-rate (ml/min)	Volume of the effluent (ml)	Endotoxin concentration of the effluent (EU/ml)	Interferon titer of the effluent (IU)	Interferon titer recoveries (%)
Chitosan	9	225	<10	93 976	100
	18	450	<10	93 976	100
Deoxycholate	9	200	<10	80 684	88.3
	18	450	>80	Not determined	–

Different volume of interferon preparation with interferon titer 93 976 IU/ml, endotoxin concentration more than 80 EU/ml and pH 3.9 was applied to chitosan and deoxycholate immobilized membrane cartridge at different flow-rate.

140 000 [21]. Deoxycholate, Poly-L-lysine, polymyxin B, poly(ethyleneimine) as well as DEAE had been functionalised on nylon microporous membrane for endotoxin removal from bovine serum albumin, lysozyme solutions and phosphate buffer. Deoxycholate was found to exhibit better removal factors and protein recoveries when the sample pH was 7.0, most likely attributed to the apolar character of deoxycholate [8,20,22,23].

Cellulose membranes functionalised with chitosan and deoxycholate were proved to have the ability for endotoxin clearance from protein-free solution. The endotoxin concentration was reduced to 7.3 EU/ml with the removal efficiency of 60% for CHI-CM when the flow-rate was 20 ml/min. For DOC-CM, it was reduced to 4.0 EU/ml with a removal efficiency of 80% at a high flow-rate of 25 ml/min. The DOC-CM showed better efficiency than CHI-CM.

The endotoxin clearance from protein solutions was comparable to that from protein-free solution for CHI-CM when the flow-rate was 20 ml/min. It is apparent that the interactions between endotoxin and ligands (chitosan) are stronger than the interactions between endotoxin and proteins in the interferon preparations at pH 3.9. At that pH value, the CHI-CM can effectively remove endotoxin at a flow-rate ranging from 9 to 18 ml/min without any loss of interferon. How many volumes of the preparation can be processed by the membrane cartridge one at a time, how many endotoxin amounts can be removed by a membrane cartridge and how fast the flow-rate can be adopted is still to be tested.

4.1. Nomenclature

CHI-CM	chitosan immobilized cellulose membrane
DOC-CM	deoxycholate immobilized cellulose membrane
EU	endotoxin units
IU	interferon units
LAL	*Limulus* amoebocyte lysate
TAL	*Tachypleus* amoebocyte lysate

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