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Application Note

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Endotoxin Removal

Ion Exchange Chromatography With Sartobind® Membrane Adsorbers

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Introduction

Endotoxins (lipopolysaccharides from membranes of gram negative bacteria) make up the majority of pyrogens, which must be removed from pharmaceutical products, biologicals for injection and some media for tissue cultures. There are many factors to be considered when designing a depyrogenation scheme for media or solutions containing proteins and peptides: type of target substance, proteins or peptides, product concentration, molecular weight and isoelectric point (pI); electrolyte concentration; pH and buffer system; and interactions such as interference or aggregation. In general, these factors determine a method for an effective pyrogen removal.

Fig. 1 shows that endotoxin monomers in solution range from molecular weight of 4 to 20 kDa but they can form micelles and vesicles with diameters up to 0.1 µm. The presence of detergents, chelators and proteins promotes the formation of structures like micelles (300-1000 kDa) and monomers (10-20 kDa), while bivalent ions promote the formation of large structures like vesicles (>1000 kDa)[1]. If the target substance in the solution to be depyrogenated has a small molecular weight (e.g. buffer, salt, nucleotides, amino acids, peptides, some carbohydrates etc.) the endotoxins can be separated from the target substance by ultrafiltration with an appropriate cut off crossflow filter. However, proteins in the same molecular weight range as endotoxins cannot be separated by ultrafiltration.

Due to the negatively charged phosphoryl and carboxl groups in endotoxins, ion exchange chromatography is the most common depyrogenation method for proteins; however, it has several drawbacks which limit its usefulness as depyrogenation step. This includes handling and usage problems such as packing, channeling, low flow rates, long regeneration times, compressibility and limited chemical stability. Small flow rates and susceptibility to fouling mean that incorporating chromatography resins into process scale purification steps can be expensive and troublesome. Sartorius has introduced a high capacity, scalable and ready-to-use ion exchange membrane chromatography technology, which provides excellent performance needed for depyrogenation in the laboratory or for process scale.

Three standard strategies are available for removal of endotoxin from solutions with Sartobind® devices. Using the strong basic ion exchanger type Q and a buffer pH lower than the pI of the protein, endotoxin will be bound and protein will pass through the membrane (negative chromatography). Sartobind STIC® PA is an anion exchanger and works at the same pH conditons as Q but is also applicable at higher salt concentrations. When other contaminants such as host cell proteins have to bind at up to 20 mS/cm, Sartobind STIC® (Salt Tolerant Interaction Chromatography) may be the membrane of choice as these contaminants can be effectively removed at such conditions. Using the strongly acidic ion exchanger type S and a buffer pH lower than the pI of the protein, the endotoxin will pass through and the protein will bind and can be subsequently eluted.

Larger volumes for production scale (gram – kilogram) can be processed with Sartobind® Q or STIC disposable capsules with 4 mm bed height. Sartobind® Q capsules with 8 mm bed height may be applied as well.

The reduction of endotoxin is expressed with a log reduction value (LRV) which is the logarithmic quotient (log10) of the sample solution containing the endotoxins, divided by the concentration of endotoxins of the processed solution.

$$LRV = \log \frac{\text{EU/mL starting solution}}{\text{EU/mL filtrate}}$$

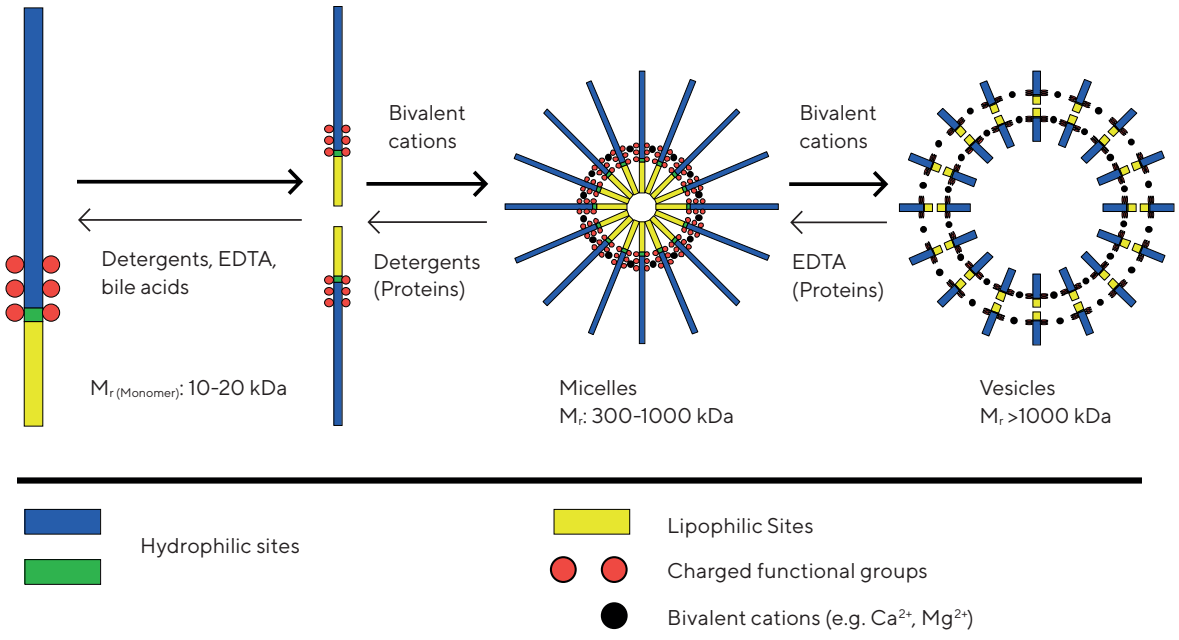

Figure 1: Structure of endotoxin aggregates in aqueous solutions of different composition. (Courtesy of Prof. F.B. Anspach, Hamburg University of Applied Sciences, Department of Natural Science Technology)

1. Purification of Clinical Vaccine Proteins[2]

Purification of five recombinant bacterial vaccine proteins was investigated. The molecular weight range was between 35 and 53 kDa. Crude E. coli lysates were purified by a combination of anionic and cationic membranes. Membrane chromatography was able to reduce the purification process development time. Salt gradient elution was compared to pH elution on 15 and 100 cm² ion exchange membranes. The pH elution produced higher yields as well as eliminated the time necessary for desalting. In order to remove very high levels of DNA and endotoxins, two consecutive anionic exchanger (Q) steps were utilized. The first Q membrane was used as a product capture step, while the second Q was a negative chromatography step. The pH condition was optimized in order to let the product run through the second Q and directly captured on a cationic exchanger membrane (C).

Membrane chromatography steps for a vaccine protein-D purification

1. Adsorption and elution from Q
- Bind at pH 8.0
- Wash with 50 mM NaCl at pH 6.5
- Elute with 100 mM NaCl at pH 4.7

2. Flow through Q and loading of C
- Load onto Q and C in series at pH 4.7
- The protein flows through Q and binds to C
- Disconnect Q from C membrane

3. Elution from C
- Elute at pH 5.5

This purification method was adapted to purify four additional recombinant protein variants. All vaccine proteins were successfully scaled to 30 mL (1100 cm²) Sartobind® disc membrane and 1.15 L (4 m²) Sartobind® modules. The 1 liter (4 m²) modules were run at a flow rate of 7 L/min/bar.

Results

Table 1: Clearance of endotoxin and DNA from protein-D

Purification step	Endotoxin [EU/µg protein]	LRV	DNA [pg/µg protein]	LRV
Sterile crude	6224	–	180	–
After first Q	32	2	30	1
After second Q/C	0.02	3	0.08	3
Sterile filtration	0.002	1	0.05	–
Total		6		4

Table 2: Scale-up results with protein-B

Scale	Purity [%]	Endotoxin [pg/µg protein]	DNA [pg/µg product]
Q 100 2.8 mL (100 cm²)	99.7	0.01	0.04
Disc 30 mL (1100 cm²)	98.2	0.002	0.10
Module 1.15 L (4 m²)	99.0	0.01	0.05

1 liter ion exchange membrane = 3.64 m²

Discussion

Process development experiments were evaluated by the ability to retain the target protein while removing endotoxins, DNA and contaminating proteins. Endotoxin removal was achieved by a 3 log removal in the first anion (Q) exchange step followed by an additional 3 log reduction in the second anion | cation (Q|C) step. DNA removal was accomplished by a 1 log reduction in the Q step followed by a 3 log reduction in the Q step. The process consistently gave purities of > 95 % and yield of 65 % of the target protein.

One of the most important advantages using Membrane Adsorbers was the decreased processing time. Small-scale purifications would yield pure product in approximately 75 minutes. When scaled-up the processing time did not increase significantly, the entire chromatographic purification could be completed in a few hours. We took advantage of this quick equilibration time and built-in pH elution steps in nearly all of the recombinant proteins. The pH elution steps allowed the processing from one ion-exchange step to the next with only a pH adjustment. This eliminated the need for time consuming buffer exchange steps. Quick binding and pH elution is not possible in bead-based columns due to the long equilibration times required.

The purification process was developed using Sartobind® Q 100 (2.8 mL, 100 cm^2) and then scaled to 30 mL (1100 cm^2) disc and 1.15 L (4 m^2) Sartobind® module (11- and 400-fold scale-up, respectively). Each of the scaled-up processes provided protein of equal purity and yields compared to the small-scale experiments. This data demonstrated that Membrane Adsorbers are easily scalable.

A cleaning procedure was developed using 1 N NaOH and 1 N HCl on the anion exchangers and 1 N NaOH on the cation exchangers. Reproducibility and regeneration was demonstrated for 40 purification cycles by overlaying chromatograms.

Conclusion

The information gathered above demonstrated that Membrane Adsorbers are a valid option compared to the classical resin technology. The Adsorbers have proven to achieve a higher throughput. They increased speed while maintaining the capacity, selectivity, and reproducibility required for industrial chromatographic separations. There was an overall reduction of labor as a result of no column packing, shorter set-up, and process times. Manufacturing capacity may quickly be expanded by simply adding more cycles.

2. Endotoxin Clearance of a Immunoglobulin Solution With Sartobind® Q 75

Sample: Cytoglobin in 10 mM potassium phosphate, pH 6.0

Device: Sartobind® Q 75

Table 3 shows that endotoxin in a protein solution was removed effectively (99.40–99.97 %) with Sartobind® Q 75. At the same time, the protein recovery was 84–86 %.

Table 3: Endotoxin clearance of an immunoglobulin solution with Sartobind® Q 75

			Control (without protein)
Endotoxin concentration [EU/mL]	1000	1000	1000
Sample volume [mL]	10	10	10
Total endotoxin in sample [EU]	10000	10000	10000
Total endotoxin in flow-through [EU]	60	60	3
LRV	2.2	2.2	3.5
Endotoxin clearance [%]	99.40	99.40	99.97
Protein in sample [mg]	5	5	-
Protein in flow-through [mg]	4.2	4.3	-
Protein in flow-through [%]	84	86	-
Bound protein [%]	16	14	-

Immunoglobulins often have isoelectric points between 7.5–9.5 and will not bind at a pH of 6.0. Due to some other protein impurities bound on the membrane, the endotoxin removal decreased compared to the protein free solution (control).

3. Scale-up of Endotoxin Clearance From Protease Solution

A. Clutterbuck[3] (Avecia Ltd., UK) successfully removed endotoxins and DNA from their GST-3C protease with Sartobind® Q. He completed the process scale-up from Sartobind® Q downscale units through pilot scale with 5" capsules for 100 L non-GMP demonstration batch to the final scale with 20" capsules for 2 × 100 L cGMP production batches.

Sample: GST-3C protease, 46 kD (genetically engineered fusion protein consisting of human rhinovirus 3C protease and GST, theoretical pI 6.8)
Buffer: 50 mM Tris, 150 mM NaCl, 3 mM reduced glutathione, pH 8 at 20 °C.

Demonstration batch

Endotoxin level of affinity column eluate: 42,900 EU/mL
Flowthrough after Sartobind® Q was taken for endotoxin every 5 L (50 L total, initial 100 L was reduced to the half before the endotoxin removal step). Endotoxin breakthrough was detected after 10 L, therefore, Sartobind® Q 20", 4 mm bed height (5 times larger membrane volume than 5") was chosen for the final cGMP runs.

Table 4: Endotoxin removal step with Sartobind® Q SingleSep* 20" at cGMP runs

	Run 1	Run 2
Process volume (Start)	50 L	47 L
Process volume (End)	60 L	59 L
Process time	~10–20 min	~10–20 min
Protein concentration (Bradford)	4.10 g/L	4.14 g/L
Conductivity	16.63 mS/cm	17.41 mS/cm
Pre use endotoxin level	26,900 EU/mg	10,100 EU/mg
Post use endotoxin level	0.13 EU/mg	0.18 EU/mg
Protein recovery over step	81.4 %	84.6 %
LRV endotoxins	5	5

Conclusion

The final product from cGMP production had great purity, quantity and activity, low endotoxin and low DNA as well. The Sartobind® Q capsule was extremely easy to use and saved up to 100 production plant hours per batch and enabled significant cost saving.

* The successor of Sartobind® Q SingleSep 20" is Sartobind® Q 400 mL.

4. Purification of Recombinant Helicobacter Pylori Urease for Immunization Against H. Pylori

Helicobacter pylori infects the gastric epithelium of approx. half of the world's population and causes a histologic gastritis. An estimated 20 % of those infected will go on to develop either peptic ulcer or gastric adenocarcinoma, which is the second-most-common cause of cancer mortality worldwide[2].

Recombinant H. pylori urease was purified, which was used in immunoassays and for immunization of mice,[4,5] or of rhesus monkeys[6]. Using Sartobind® Q, endotoxin contamination was reduced to <1.5 ng/mg of urease[4,5,6].

5. Endotoxin Removal at High Conductivity With Sartobind STIC®

In this experiment, the ability of Sartobind STIC® PA anion exchanger for endotoxin removal at high salt concentration was tested.

Materials
Endotoxin: LONZA N 185 LPS E. coli 055:B5
Buffer: 20 mM TRIS +150 mM NaCl, pH 7.5, conductivity 16 mS/cm Sartobind STIC® PA nano 1 mL (salt tolerant anion exchange membrane adsorber, order number 92STPA42DN-11--A), 3 lots, one device from each lot.

Methods
The Sartobind STIC® PA nano 1 mL was sanitised with 1 N NaOH for 30 minutes at room temperature. To avoid false negative results (NaOH in high concentration results in a detectable inhibition), the unit was then thoroughly washed with 250 mL Arium® water to reach a conductivity of <2 mS/cm (no inhibition of the test). The sample was rebuffered with 50 mL 20 mM Tris with 150 mM NaCl, pH 7.5, 16 mS/cm. Altogether 4000 mL of endotoxin sample with a concentration of 1000 EU/mL (in total 4 Mio EU) were applied to the capsule at 10 mL/min flow rate.

Results
The endotoxin concentration after the Sartobind STIC® treatment was lower than the detection limit (< 0.0125 EU/mL) using all three devices.

Table 5: Endotoxin removal with Sartobind STIC® PA nano 1 mL

	before	after	LRV
Endotoxin concentration	1000 EU/mL	< 0.05 EU/mL	> 4.3

This result shows that the Sartobind STIC® PA can reduce endotoxins from high levels down to below detection limit at higher conductivity levels compared to endotoxin removal with Q chemistry.

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