Application Note

Endotoxin Removal

The Solution With Membrane Separation Technology

Introduction to Endotoxins

Endotoxins are the single most important class of pyrogens. A pyrogen is a substance that causes a fever when injected into humans and animals. Because of their pyrogenicity, endotoxins need to be controlled and minimized in any process involving a parenteral drug.

Endotoxins are complex aggregates of LPS (lipopolysaccharides) and may contain protein material. The LPS consists of an innermost region composed of hydrophobic fatty acid groups, or lipid A, and a central and outermost region composed of hydrophilic polysaccharides. In solutions above a pH of 2, the LPS has a negative charge.

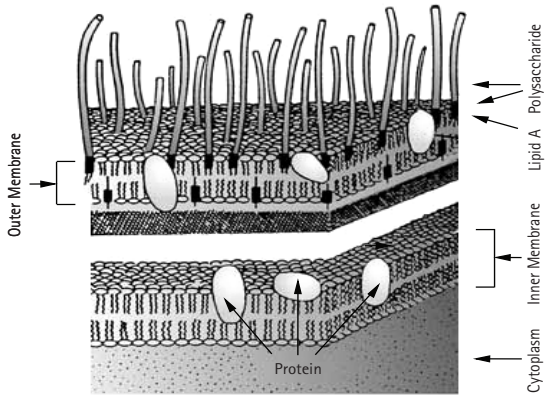


Figure 1. Gram Negative Bacterial Cell Wall

In aqueous solutions, endotoxins can exist in various states of aggregation. Whereas individual molecules have molecular weights ~10-20 kDa, aggregates can have molecular weights as high as 1 MDa. Divalent cations, such as Ca^{2+} and Mg^{2+}, are found to stabilize the aggregated structure of LPS, whereas detergents help to break down the structure into smaller subunits. These reductions in size result from disaggregation alone.

Table 1. Aggregation State of Bacterial Pyrogens as a Function of Solution Chemistry

Solution	Endotoxin Size	Aggregation Form
Water	Submicron	Vesicle
Saline	Submicron	Vesicle
$MgCL_2$	Submicron	Vesicle
EDTA	300–100 kDa	Micelle
Cholate and EDTA	10–20 kDa	Sub unit

These endotoxins will easily pass through a 0.2 µm sterilizing-grade membrane filter where size exclusion is the sole retention mechanism. Traditionally, endotoxins have been removed by reverse osmosis or ultrafiltration membranes of a 10,000 NMWL cutoff[12]. However, this approach is not practical when a component of a drug product has a molecular weight greater than 10 kDa.

Cause of Endotoxin Contamination

Endotoxins are continuously shed from the outer membrane of viable gram negative bacteria and are also released when the bacterial cell dies. Gram negative bacteria are present in the environment and are a source of concern in WFI, buffers and other fluids that go into the making of parenteral drugs.

Although bacteria are often removed by using a 0.2 µm sterilizing-grade filter, endotoxins can still be a concern in WFI distribution systems and "non-sterile" processes like chromatography steps. LPS itself is difficult to remove or inactivate because it is extremely heat and pH stable.

The amount of endotoxins required to generate a pyrogenic reaction is on the order of 0.1 ng per kg of body weight. 0.1 ng is roughly equivalent to 1 EU (endotoxin unit), the standard unit in which endotoxins data is commonly reported. Give that a typical gram negative bacterium contains 10^{-15} g of LPS, 0.1 ng would require 10^5 bacterial cells precursor.

Because endotoxins cause a pyrogenic response in humans, they must be controlled and minimized. Typical endotoxins level requirements are shown in Table 2.

Table 2. Endotoxin Limits for Various Products*

WFI	≤ 0.25 EU/mL
LVPs	≤ 0.5 EU/mL
Hlb Vaccine	≤ 50 EU/mL (≤ 25 EU/dose)
Factor IX	≤ 0.1 EU/mL (≤ 5 EU/dose)
Immune Serum Globulin	≤ 0.91 EU/mL (≤ 5 EU/dose)

* FDA Guideline on Validation of LAL as End Product.

Solutions for Endotoxin Control

Size Exclusion

Our family of ultrafiltration products (Pellicon®, Prostak™ and spirals) can be used for solutions where a 10,000 NMWL cutoff membrane does not negatively impact product yield.

Table 3. Endotoxin Removal Application Data

Solution	Membrane NMWL	Solution Permeability (LMH/bar)	Endotoxin
WFI[3]	10,000	27	>3
4% Amino Acid[3]	10,000	17	>3
10% Amino Acid[4]	10,000	25	>>1
10% Amino Acid[4]	20,000	113	>>1
Penicillin derivative[4], 30%	20,000	45	>3
Contrast Agent (35%)	10,000	3.4	>3
Contrast Agent (35%)	30,000	8.7	>2
Contrast Agent (35%)	100,000	13.0	>2

Charge Interaction

Because endotoxins are negatively charged above a pH of 2, a positively charged membrane surface can remove endotoxin. Charged Durapore® membrane has a net positive charge on its surface. Charged Durapore® has been shown to exhibit an LRV greater than 5 when challenged with 10^6 pg/mL of purified *E. coli* Type 055:B5 LPS endotoxin.

Table 4. Endotoxin Retention with Charged Durapore® Membrane

Solution	Challenge EU/mL	Capacity	LRV EU/cm^2
Water	7800	> 5x10^5	>5.0
Mannitol	10	> 400	>1.5

In the presence of competing negative ions, such as in buffer filtration, a charged membrane cannot be used because it loses its adsorptive properties.

Hydrophobic Interaction

Using the hydrophobic interaction of the lipid A component of the endotoxins and hydrophobic membrane, filter devices containing hydrophobic Durapore® can be used for removing endotoxin from buffer/ionic solutions. Phobic Durapore® comes in a variety of configurations: Opticap®, Optiseal®, Millipak®, Millidisk® and 5", 10", 20", and 30" cartridges.

Table 5. Endotoxin Retention with 0.2 µm hydrophobic Durapore® Membrane, 47 mm disks, 1 liter of solution filtered

Solution	Challenge EU/mL	LRV
15 µm NaCl Buffer	450	>3.0
150 µm NaCl Buffer	22.5	>3.35
150 µm NaCl Buffer	15	>2.67
150 µm NaCl Buffer	2.1	>2.3
Cascade 5	UBE1	UbcH4

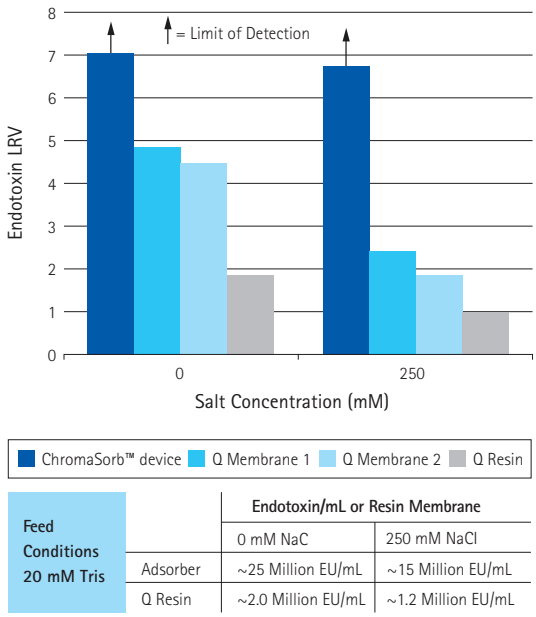
Endotoxin Removal using Anion Exchange Chromatography

Due to the charged nature of endotoxins, strong anion exchange chromatography has proven to be particularly effective at removing endotoxins from feedstreams.

ChromaSorb™ Membrane Adsorber is strong anion exchange membrane that has been optimized to remove negatively charged impurities such as endotoxins, viruses and HCP. Unlike traditional anion exchangers which use Q chemistry, ChromaSorb™ Membrane Adsorber utilizes a novel ligand, poly alyl amine (PAA). Typical Q membrane adsorbers must operate at very low salt levels (<4mS/cm). The advantage of PAA in the ChromaSorb™ Membrane Adsorber is that it retains its strength of binding at high salt concentrations. The feedstream does not have to be diluted with additional buffers, a major bottleneck in biological manufacturing.

Summary

Endotoxin was spiked in 25 mM Tris buffer at pH 8 and loaded onto two Q membrane absorbers, a Q resin and ChromaSorb™ Membrane Adsorber. Two different salt concentrations were tested: 0 and 250 mM NaCl. Flow-through fractions were collected and assayed with an Endochrome-K kit.



Feed Conditions 20 mM Tris		Endotoxin/mL or Resin Membrane	
		0 mM NaC	250 mM NaCl
	Adsorber	~25 Million EU/mL	~15 Million EU/mL
	Q Resin	~2.0 Million EU/mL	~1.2 Million EU/mL

Figure 2. Competitive bench-marking of Endotoxin Removal at low and high salt concentrations

ChromaSorb™ Membrane Adsorber achieved the highest removal of endotoxin at 0 mM (>7LRV) up to the limits of the assay sensitivity. When the high salt concentration was tested, ChromaSorb™ Membrane Adsorber maintained complete endotoxin retention up to the assay limits. The other formats with Q chemistry exhibited a significant reduction in their ability to adsorb endotoxin.

References

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