Astrea Bioseparations

Technical Note

EtoxiClear™, a Chromatography Adsorbent for Effective Removal of Endotoxins from Biopharmaceuticals

1. Summary

Endotoxins or lipopolysaccharides (LPS), also known as pyrogens, are highly toxic components of the cell wall of Gram-negative bacteria and are often present in large concentrations in bacterial cell culture expression systems such as Escherichia coli. Thus, they are ubiquitous in bioprocessing and their extreme toxicity means that there is a requirement for their complete removal from the final product.

Biomolecules can interact with LPS either by binding to specific sites or by electrostatic/hydrophobic interactions, making the separation of endotoxins from proteins difficult to achieve. These interactions can result in interference with endotoxin assays masking the presence or removal of LPS leading to inefficient removal. Many methods have been adopted for the removal of endotoxin based on adsorption, two-phase portioning and chromatography, however these methods often lead to loss of the target protein or introduction of toxic ligand components, such as polymyxin B which has been used for this purpose. Ion exchange chromatography is the most common approach to LPS removal but its effectiveness is dependent on the isoelectric point of the protein and product losses can be encountered. Additionally, many of the affinity adsorbents described for removal of endotoxins cannot be effectively depyrogenated with sodium hydroxide.

Although downstream processing can significantly reduce endotoxins level in the product, the efficient and cost-effective removal of residual endotoxins from biopharmaceutical preparations is challenging.

This paper addresses the issue of removal of endotoxin from biological preparations. Specific reference will be made to a novel synthetic ligand affinity adsorbent, EtoxiClear™.

EtoxiClear™ can be depyrogenated using sodium hydroxide and is an effective chromatography affinity adsorbent specifically designed for the removal of low residual levels of endotoxins. The bi-dentate ligand of EtoxiClear™ binds to Endotoxin in a spatially selective and optimal manner. The total binding capacity of the adsorbent for endotoxins is > 1,000,000 EU/mL adsorbent and in flow through chromatography mode, typical dynamic binding capacity is 40,000 EU/mL at a residence time of 1.2 minutes. A number of biomolecules with different isoelectric points were used to demonstrate efficient recovery and clearance of residual endotoxin across the pH range. In each case protein recovery is > 80 % with endotoxin clearance to below the acceptable threshold level of 0.1 EU/mg protein. Typical clearance of 3 log for residual endotoxin is achieved for products expressed and purified from E. coli expression systems.

2. Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria; LPS aggregate , also termed endotoxin, consists of an acidic LPS comprising of core hydrophobic fatty acid groups with central and outermost parts made up of hydrophilic polysaccharides (1, 2). Endotoxin aggregates can exist in various states of aggregation with a molecular size up to 1 MDa. Endotoxins interact with the innate immune system responsible for elimination of pathogens in mammals, resulting in the production of proinflammatory cytokines such as interleukin-1ß, interleukin-6 and tumour necrosis factor-α (3).

Bioprocessing technologies allow the production of numerous biologicals such as proteins, nucleic acids and sugars for use in medicines. Commercially available small and non-glycosylated recombinant proteins are produced in Escherichia coli (E. coli). This expression system has advantages, including rapid expression, high yields, ease of culture and low cost, but the proteins recovered after the purification may be contaminated by endotoxin. High levels of endotoxin have been reported not only in the proteins expressed in cell culture but also in human serum albumin, dextran, collagen and gelatin products (4).

The extreme toxicity of endotoxin means that their complete removal is required from the parenteral biopharmaceutical products.

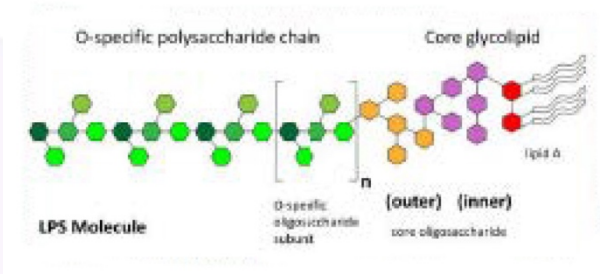
3. Endotoxin Structure

Chromatography conditions for the removal of endotoxin (low and high concentration) from a purified immunoglobulin solution using EtoxiClear™ pre-packed into 5 and 50 mL Evolve® R columns and a 385 mL Evolve® D column.

LPS molecules comprise of three domains: lipid A, a core oligosaccharide and a highly variable O-antigen. The core is covalently coupled to Lipid A and is divided into the inner and outer core (5). The Lipid A portion is the most conserved region of LPS and contains disaccharide units comprising glucosamine monomers, both of which are phosphorylated. Fatty acids are linked to the bis-phosphorylated glucosamine disaccharide by an ester and amide linkages. The oligosaccharide domain links to the disaccharide at position 6'. The oligosaccharide core typically comprises of 8 – 12 sugar residues. For the inner core, heptose may be substituted by phosphate, pyrophosphate or phosphoryl ethanolamine group (6,7). The negatively charged sugar residues and Lipid A provide the stability of LPS by interactions with cations such as Ca^{2+} and Mg^{2+}. The O-antigen is composed of repetitive subunits and can contain up to 50 identical subunits, each comprising of eight sugars units. Most frequent components of O-chain are deoxy sugars, with O- and N- acetyl phosphate and phosphoryl ethanolamine substitutions (8).

Figure 1:

Structure of bacterial lipopolysaccharides



4. Endotoxin Contamination of Biologicals

Endotoxin contamination of biologicals arises from release of the molecules from the host microbial cells or introduction by the non-sterile conditions used during the processing steps. Due to the presence of diverse negatively charged groups and hydrophobic regions, endotoxin have significant capability of interacting with both positively charged and hydrophobic compounds. Consequently, endotoxins have a significant capability of interacting with biomolecules, including proteins (9). Micelle aggregates are also formed by LPS and these can form multiple interactions with proteins, which can result in 'endotoxin masking', making it difficult to remove (10). For most parenteral (injectable) products, endotoxin limit is 5 EU/kg/hr and on the basis that the average human being weighs 70 kg, the maximum amount endotoxin that a patient can receive is 350 EU/hr.

5. Methods for Measuring Endotoxin

The regulatory authorities require that the amount of endotoxin present in final formulations of therapeutic biopharmaceuticals is determined and reported. The LAL (Limulus Amoebocyte Lysate) test method is the most common method used for testing biotechnology derived product for parenteral administration.

There are three main commercially available methods for detection of endotoxins:

a. The gel-clot method involves the reaction of horseshoe crab haemolymph with endotoxin. This method provides both quantitative and semi-quantitative results as a test limit
b. Turbidimetric method uses a dedicated spectrophotometer to measures the increase in the turbidity of the reaction
c. Chromogenic spectrophotometric method uses a synthetic chromogenic peptide substrate and blood serine protease precursors which are activated in the presence of endotoxin resulting in the cleavage of the peptide substrate releasing a chromophore.

Note: Methods based on LAL have a limitation; trace amounts (1,3)-ß-D- glucans are often present in the test samples which released from chromatography adsorbents interfere with determination of endotoxins. Modern commercially kits are supplied with an additive* that can be added to LAL assay to eliminate the interference. (* CM-curdlan, a carboxymethylated derivation of (1-3)-ß-D-glucan)

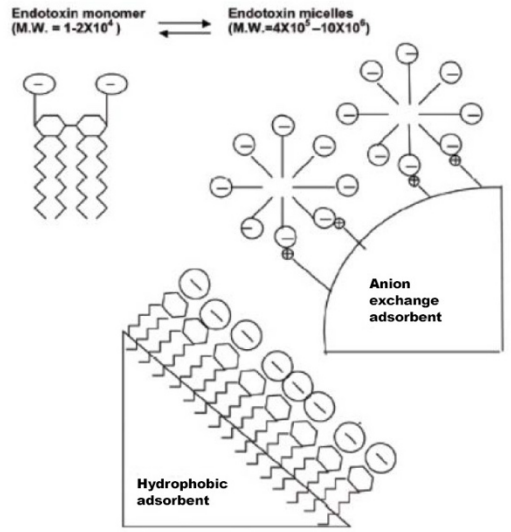
6. Endotoxin Removal using Chromatography Adsorbents

Removal of endotoxin is one of the most challenging downstream process operations during purification of biopharmaceuticals. Chromatographic methods are widely applied to exploit the size, charge, hydrophobicity and affinity (11) of endotoxin. A significant amount of effort has been directed towards the removal of endotoxins from preparations of plasmid DNA for use in gene therapy. However, most of these methods are not applicable to the removal of residual endotoxin from therapeutic proteins. This is due to stable complexes that are formed by the electrostatic and hydrophobic interactions between endotoxin and proteins. Factors to be considered for the chromatography-based methods for the removal of endotoxin include ligand safety, affinity of endotoxin, affinity of protein for endotoxin, protein loss due to non-specific adsorption, pH and temperature.

LPS has negatively charged regions and hydrophobic domains, therefore a selective ligand for endotoxin should have anion exchange /hydrophobic binding modalities (Figure 2) with low non-specific protein binding. Endotoxin removal methods mainly involve chromatography adsorbents packed in columns/utilised in batch mode and membrane absorbers.

Figure 2.

Modes of interaction of endotoxin with chromatographic adsorbents



There are several methods described for the removal of endotoxins, the most common of which involves the use of anion exchange chromatography to bind the endotoxin allowing the protein solution to flow through. However, this technique may require a considerable amount of optimisation to remove endotoxin from protein preparations (14). Positively charged proteins are repulsed by the ligand while negatively charged proteins to retained on the column competing for the ligand binding site. Consequently, anion-exchange chromatography is only suitable for the removal of endotoxin from positively charged proteins.

For specific removal of endotoxins, several affinity-based chromatography adsorbents are commercially available. Polymyxin B, an antibiotic that contains a cationic cyclopeptide with a fatty acid chain which neutralises the biological activity of endotoxins by binding to the lipid A domain of the LPS, has been immobilised on a wide variety of matrices and used for the removal of endotoxin. However, Polymixin B is also highly toxic and has slow binding kinetics making the procedure time consuming. Consequently, its use for this application is limited (15). Poly(ε-lysine) is a food preservative produced by fermentation of Streptomyces albulus and is available as a ligand immobilised onto porous cellulose beads, Cellufine™ ET clean. The performance of Cellufine™ ET clean is strongly affected by the ligand density and the pore size of the base matrix. Thus, the endotoxin binding of this matrix is primarily due to simultaneous effects of cationic properties of the ligand and hydrophobic properties of the base matrix (16).

A significant disadvantage of many of the commercially available adsorbents described is that they cannot be effectively depyrogenated using sodium hydroxide.

7. EtoxiClear™ for Endotoxin Removal

EtoxiClear™ is a novel synthetic ligand affinity adsorbent specifically developed for the removal of residual endotoxin from biological solutions e.g. proteins, buffers and neutral sugars. Astrea Bioseparations have developed a novel, non-toxic bi-dentate synthetic affinity ligand adsorbent called EtoxiClear™, designed to bind in a spatially selective and optimal manner to the LPS molecule resulting in efficient removal of endotoxin from biopharmaceutical preparations. The synthetic affinity ligand is covalently attached to a proprietary near-monodisperse cross-linked 6 % beaded agarose (PuraBead®), is resistant to high pH and can be depyrogenated using NaOH. For optimal removal of residual endotoxins from biopharmaceutical preparations.

EtoxiClear™ is best used packed in a chromatography column with a bed height of 10 cm and residence time of ~5 minutes. A range of disposable, pre-packed columns are available for this application. The scalability of EtoxiClear™ was demonstrated using disposable, pre-packed columns of 5 mL, 50 mL and 385 mL where IgG protein solutions (~5 mg/mL), containing similar starting concentrations of endotoxin, were loaded onto each of the pre-packed EtoxiClear™ columns, and both endotoxin clearance and protein recoveries were determined. The results are summarized in Table 1. Recoveries of 86 %, 98 % and 99 % IgG were achieved demonstrating low protein binding whilst achieving > 4.5 log removal of endotoxin.

Table 1.

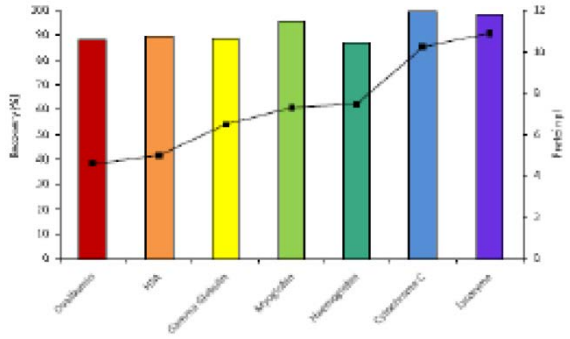
Performance and scale-up of pre-packed EtoxiClear™ columns for endotoxin removal from IgG.

Column	Initial Endotoxin Concentration (EU/mL)	Volume loaded (mL) =2cv	Total Endotoxin Loaded (EU)	Final Endotoxin Concentration (EU/mg protein)
5 mL Evolve® R	25	10	250	0.003
50 mL Evolve® R	36	100	3,600	0.03
7 x 10 cm Evolve® D	67	770	51,590	0.01

For solutions that are highly viscous such as dextran, gelatin etc., EtoxiClear™ may also be used in batch mode. EtoxiClear™ has very low non-specific protein binding and a wide range of proteins with varying iso-electric points can be processed, achieving high protein recoveries . Typical protein recoveries are > 90 % irrespective of the isoelectric point (pI) of the protein (Figure 3).

Figure 3.

Protein recoveries in the flow-through fraction of EtoxiClear™ column



Effectiveness of EtoxiClear™ for the removal of residual endotoxin from a possible therapeutic protein expressed in E. coli has been described (17). An antibody fragment, F(ab™)2 was purified from clarified cell lysate using Fabsorbent™ F1P HF, a synthetic ligand affinity adsorbent designed for the specific capture of this class of proteins. The elution pool containing the target protein with residual endotoxin was applied directly onto EtoxiClear™ column. The resulting flow through fraction had a 3 log reduction in the endotoxin concentration (Table 2).

Table 2.

Use of EtoxiClear™ for the polishing step to remove residual endotoxin from a recombinant F(ab™)2 antibody fragment expressed in E. coli.

Sample	Endotoxin (EU/mL)	Endotoxin Clearance
Cell lysate	192,000	------
Fabsorbent™ F1P HF elution fraction	46,000	<1.0 log
EtoxiClear™ flow through	19	> 3.0 log

8. Conclusions

Removal of residual endotoxin from biopharmaceutical preparations is an important consideration in the industry. EtoxiClear™, a synthetic ligand affinity adsorbent, has a high capacity for endotoxin (> 1,000,000 EU/ml) and very low non-specific binding of proteins. It is available in disposable, pre-packed, ready to use columns as well as bulk adsorbent. EtoxiClear™ provides an excellent clearance of endotoxin from final biopharmaceutical products (below 0.1 EU/mg protein) and provides > 3 log reduction in endotoxin levels for recombinant proteins.

For more information on EtoxiClear™ please visit our website:

https://www.astreabioseparations.com/applications/endotoxin-removal/

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