

Removal of Endotoxin from Protein in Pharmaceutical Processes

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Introduction

Bacterial endotoxin is the lipopolysaccharide component of the cell wall of Gram-negative bacteria, together with other cellular material that combines to form an endotoxin complex. Endotoxin is pyrogenic and it presents a risk to patients who are administered intravenous and intramuscular preparations.¹ Thus bacterial endotoxins pose a risk to many pharmaceutical processes and, where not controlled, to the finished products. There are different methods for endotoxin removal. These include depyrogenation,² such as dry-heat processes applied to glassware, and rinsing,³ as might be applied to closures. These areas receive reasonable coverage within the pharmaceutical sector. Areas that are actively discussed within biotechnology fields but which receive less attention in the wider pharmaceutical context are steps to remove endotoxin bound to protein. This article considers some of the biotechnological applications for endotoxin removal.

Pharmaceutical Processing Risks

In the pharmaceutical industry endotoxin testing of parenteral drugs is important because lipopolysaccharide is ubiquitous in waterborne bacterial species and water is the main ingredient in many parenteral products; water is also used for cleaning. As an example of the risk, a single *Escherichia coli* cell contains approximately 2 million lipopolysaccharide molecules. These molecules consist of a hydrophobic lipid A moiety, a complex array of sugar residues and negatively charged phosphate groups. Endotoxins are heat stable, making them resistant to most conventional sterilization processes and thus necessitating separate tests for viable cells (bioburden) and endotoxin.

Pharmaceutical processes and equipment are at risk from endotoxin. Hazards stem from human handling, dust, packaging, contaminated rinse water and microbial growth can all contaminate components with endotoxin.⁴ The emergence of multiple drug resistant bacteria has led to patients receiving larger doses of a number of drugs at once, each contributing to the potential endotoxin load on the patient. As medical technology improves there is also a larger number of at-risk patients such as the immunosuppressed, premature babies and the elderly, with increased sensitivity to pyrogens.



Risks not only arise as a result of contamination; they can also occur as a result of the process. With biotechnology, for example, Gram-negative bacteria, such as *E. coli*, are often used to produce recombinant DNA products like peptides and proteins. These products are always contaminated with endotoxins and removal steps are required to eliminate the endotoxin from the product.⁵

Endotoxin Removal

There are two difficulties that relate to endotoxin removal from products. The first is that the process applied must not alter the product during the step of endotoxin clearance. The second is with the relatively low endotoxin concentration in presence of the product and the difficulty in removing bound endotoxin. The binding of endotoxin can become enhanced through protein concentration processing steps.

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The basis of many techniques for endotoxin removal is the structure of endotoxin complexes themselves: an endotoxin molecule possesses hydrophobic, hydrophilic, and charged regions. These features shape a range of possible interactions with other molecules.⁶ A secondary means of endotoxin removal uses the weight of the endotoxin complex.

Two-Phase partitioning

The use of aqueous two phase systems is becoming popular with certain processes, as an alternative to organic-water solvent extraction systems, partly because the method can generate milder conditions that do not harm or denature unstable/labile biomolecules. With endotoxin removal, the hydrophobic nature of endotoxin means that two-phase partitioning serves as an effective endotoxin removal method. This involves the optimization of the conditions leading to the partitioning of the target protein into one phase while the endotoxin is separated from the product by being partitioned into another phase.⁷ Here two-phase systems

can be manipulated by altering factors like polymer mass, pH, ionic strength, and concentration of the phase component or, alternatively, through the addition of affinity ligands.

Ultrafiltration

Since endotoxin molecules tend to form micelles or vesicles in aqueous solution these can be removed from a solution by filtration. Ultrafiltration (the process works by excluding endotoxin by molecular weight using an ultra-fine filter which blocks molecules of 10,000 Daltons or greater (the molar mass of an endotoxin monomer varies from 10,000 to 20,000 Da).⁸ This process is often coupled with 0.1µm filter for bioburden control. While effective for water, methods used for decontamination of water, such as ultrafiltration, have little effect on endotoxin levels in protein solutions.⁹

Chromatography

For many applications, a negative chromatographic technique is the preferred method for endotoxin clearance.¹⁰ Affinity chromatographic methods (such as affinity ligands including DEAE sepharose, poly-L-lysine and polymyxin-B) act to bind endotoxin through binding affinity. In contrast ion-exchange chromatography uses a positive charge to attract the negatively charged endotoxin and then allowing its elution. Both processes are affected by the pH range; temperature; flow rate and amount of electrolytes in the solution.¹¹ Modifications include large bead hydrogel-based methods, including the relatively recent technique of inside-out ligand attachment.¹² In addition size-exclusion chromatography can be considered, although this depends upon the size of the proteins.¹³ Also considered are endotoxin-selective adsorber matrices designed for endotoxin removal through endotoxin-protein dissociation.¹⁴

Complications can arise, however, through the tendency of endotoxin to form micellular (cellular aggregate) structures. Thus the success of affinity and ion-exchange chromatography in separating endotoxin from proteins is affected by the properties of the target protein. With size exclusion resins, for example, the relatively large size of the micellular form of endotoxin can cause the molecule to function like a larger biological molecule. Furthermore, with anion-exchange chromatography, the negative charge of endotoxin can lead to interactions with anion exchange resins, resulting in the co-purification of endotoxins with the other biological molecules. A further downside is that negatively charged proteins pose the problem of product loss.

Electrophoresis

Although not widely used for endotoxin removal, some researchers have reported success with the application of slab-polyacrylamide or sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

These methods could be used to separate of lipopolysaccharide from protein.¹⁵

Detergents

The dissociation of endotoxin from protein solutions can through the use of non-ionic surfactants in a washing step.¹⁶ However, this can present a problem for later purification since the detergent requires removal. The additional step can lead to some loss of product yield.

Other Purification Steps

Less commonly used purification steps for endotoxin removal include ultracentrifugation (devices acceleration as high as 1 000 000 g) and enzymatic digestion of protein.¹⁷ These are not suitable for many processes.

Summary

This article has, within the space provided only surveyed some of the methods of endotoxin removal. Not every method described will be applicable and, depending upon the product of concern, alternative approaches such as bespoke endotoxin-selective adsorber matrices will be needed for the prevention of endotoxin contamination.¹⁸

Remaining with the more common methods described in this article, for approaching endotoxin removal some users elect to work through the following steps:¹⁹

1. The use endotoxin removal resin, which is especially applicable at small scale. If this is unsuccessful then,
2. Use of a cation exchanger to bind endotoxin at a low pH and wash extensively. If endotoxin remain after elution, then try:
3. Binding to anion exchanger at an alkali pH. If endotoxin remains in the eluate, then:
4. Reduce protein and dissolve in a buffer. Proceed with a cation exchanger and extensive washing. If this is unsuccessful:
5. Use a buffer containing 0.5% Triton X-100 and try repeated extractions.

Consideration of endotoxin removal steps should form part of a contamination control risk assessment. The types of removal steps selected will be process dependent. Where hazards are identified and risks cannot be completely eliminated through endotoxin removal, the critical stages of the process should be subjected to endotoxin testing.

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