Dynamic Adsorbents

Chemical Pyrogen Removal Through the Use of Activated Alumina

by Gary Witman, MD

Exciting advances in proteinomics, cellular and molecular biology over the past two decades have led to a plethora of well characterized complex pharmaceutical compounds which are amenable to scale up manufacturing using bacterial cell culture systems as "biofactories." The desired end products from such cellular manufacturing must be isolated and completely purified for clinical usage. For those drug compounds not requiring glycosylation it is most simple and cost effective to utilize bacterium as the source of the "biofactory" for cellular growth. The desired pharmaceutical may then be either released from the bacteria or isolated after bacterial cell disruption.

A cardinal problem encountered during bacterial cell culture is coping with the presence of endotoxins. There is no way to get around the fact that endotoxins are inherent when dealing with bacterium, and it is essential to assure removal of endotoxins as part of the isolation and characterization of any drug compound during industrial downstream processing.

Endotoxins are lipopolysaccharides (LPS) located on the outer layer of the cell wall (cell membranes) of gram negative bacteria and are most commonly isolated from microorganisms of the Enterobactereaceae family. These lipopolysaccharides are composed of hydrophobic fatty acid and hydrophilic carbohydrate domains. They make up the majority of pyrogens essential for removal from pharmaceutical products, biologicals for injection and media used for tissue culture. While endotoxins are associated with destruction of the cell membrane during bacterial cell death they are also continuously released during bacterial cell growth and cell division.

The presence of small amounts of endotoxin in recombinant protein preparations when injected into patients may cause systemic inflammatory reactions running the spectrum from tissue injury, to endotoxin shock and death. Highly toxic to mammalian cells, endotoxin is one of the most potent modulators of the immune system. In terms of scope, a single bacterial organism of Escherichia coli contains 2 million LPS molecules per cell. As noted above, control of LPS from Escherichia coli is important because this bacteria is the biopharmaceutical workhouse used by industry for the manufacture of many recombinant DNA products such as proteins and peptides not requiring complex glycosylation.

Pharmaceutical products produced using E. Coli and other gram negative bacterium as cellular factories are virtually always contaminated with LPS and all measurable endotoxin must be removed during the production process. Low concentrations of the LPS molecule bind to the CD 14 receptor of mammalian cells which subsequently leads to the release of a spectrum of pro-inflammatory mediators such as tumor necrosis factor (TNF), and interleukins IL-1 and IL-6. The maximum acceptable level of endotoxin for intravenous applications is set at 5 endotoxin units (EU) per kg of body weight per hour.

Endotoxins are very stable molecules, resistant to extreme temperature and pH changes and much more durable than proteins or peptides. Recognition of this molecular hardiness is essential when establishing a purification process to assure endotoxin removal while at the

same time not altering the physical, biological or chemical properties of the desired pharmaceutical compound.

Besides LPS, gram negative bacteria release peptides such as exotoxin A, peptidoglycans, muramyl peptides and still unidentified substances, all of which possess biological properties which induce the secretion of cytokines.

In aqueous solutions endotoxins can self assemble into a variety of shapes such as lamella, cubic and hexagonal inverted arrangements with diameters up to 0.1 um and 1000 kDa. Within these assemblages endotoxins achieve high stability. These arrangements are extremely heat stable and are not destroyed under regular sterilizing conditions. The chemical stability of lipopolysaccharides makes pyrogen removal so difficult. Because endotoxins are so pH and heat stable their removal is often the most difficult portion of downstream processing in protein purification.

High concentrations of acids or bases are necessary to destroy LPS within a reasonably short time. Naturally occurring LPS has a Stokes radius which is smaller than the purified endotoxin typically used to quality filters, which further adds to the uncertainty in developing effective LPS removal methods. Endotoxins when forming into lamellar and micellar forms may interact with proteins through electrostatic interactions, making their removal extremely difficult. Endotoxin can be inactivated when exposed at temperatures of 250 C for more than 30 minutes or 180 C for more than 3 hours. Acidic or alkali solutions of at least 0.1 M strength may be used to destroy endotoxin; however the removal of endotoxin from basic proteins is more difficult than removal from acidic proteins.

Levels of endotoxin are much higher in recombinant proteins derived from soluble or cytoplastic fractions than in proteins derived from insoluble or inclusion bodies. This finding is consistent with guidelines followed during conventional biopharmaceutical manufacturing processes, in which lipopolysaccharides present in cell walls are solubilized during the cell lysis procedure.

No one single purification method has been demonstrated to completely remove endotoxin. Commonly used purification techniques include LPS affinity resins, two phase extractions, ultrafiltration, hydrophobic interaction chromatography, ion exchange chromatography and membrane adsorbers. Each of these methods when used to isolate and remove endotoxin has met with varying success. Current good manufacturing processes include batch processing and column chromatography techniques to isolate peptides and proteins as part of the downstream purification process.

The superior method of endotoxin purification may be using the so called negative chromatographic method. This allows binding of the endotoxin with the peptide or protein passing the adsorber without retention. Elution of endotoxin is not the object, and therefore irreversible adsorption of the endotoxin is desired. There is a need to achieve a very low dissociation constant between the endotoxin and the adsorbent. This is critical as the amount of endotoxin to be removed may be quite low in concentration compared to the concentration of the desired final product.

Ion exchange chromatography is the most commonly used method for the removal of pyrogens when the desired end product is a recombinant protein. Limitations when using ion

exchange chromatography include handling and usage problems such as packing, channeling, low flow rates, long regeneration times, compressibility and limited chemical stability.

When hydrophobic adsorbents are used in protein solutions there is hydrophobic binding between the adsorbent and the lipophilic group of endotoxins. Important mediators include the net charge and hydrophobicity of the protein and the pH and ionic strength of the solution.

Anionic exchange chromatography is potentially useful for the decontamination of positively charged proteins such as urokinase but provides little benefit when used with negatively charged proteins due to significant loss of product through adsorption. This chromatographic technique takes advantage of the negative net charge of endotoxins.

Ultrafiltration is useful when used for small proteins such as myoglobin, but proves ineffective when used with larger proteins such as immunoglobulins (150,000 Da). Of great concern is that proteins may be sheared by physical forces during ultrafiltration, which may impact both biological and immunologic properties.

Affinity chromatography is useful as part of a two step endotoxin purification process. In one process the peptide antibiotic Polymyxin B is placed in a chromatography column and the solution containing the desired cellular product (protein or peptide) is run through the column, with the endotoxin binding to the Polymyxin. This first step is then followed by washing the column with a nonionic detergent.

Such phase separation has proven to be a useful approach towards endotoxin removal. Surfactant agents such as Triton X-114 or zwitterionic surfactants containing both negatively and positively charged moities have been used to help dissociate endotoxin from proteins in solution, with increased endotoxin removal once freely suspended in solution in the detergent phase and the upper aqueous phase contained the desired protein Of note the immunoactivity, physical integrity and biological activity of the desired protein appear to remain unchanged after this phase separation.

Specially activated alumina with surface modified chemical moieties has proven to provide a superior tool for the purification of endotoxins due to its amphoteric property. No other commercially available agent can provide such a rewarding pH response or offer a better or cost effective method for the removal of pyrogens from a protein or peptide solution. It is clear that endotoxins develop especially strong binding to adsorbants that carry positively charged functional groups. Electrostatic interactions play an important role during endotoxin adsorption. Proteins are also amphoteric. Since proteins are amphoteric molecules, electrostatic interactions are not as strong as for the mainly negatively charged endotoxin. Owing to the globular structure of proteins, charged and hydrophobic groups are fixed and cannot be twisted towards functional groups or surface structures of the adsorbants. Additional benefits provided through the use of activated alumina include low cost, limited safety issues, extremely well defined chemical characteristics and with minimal impact on the bioactivity of protein when placed into a standard manufacturing process.

Furthermore the protein binding capacity of membrane adsorbers are much lower than those of particulate sorbents. The removal of pyrogens using specially designed activated alumina can be performed using either column chromatography or batch treatment. When using column chromatography the final product is achieved by filling a column with the alumina modified to enhance pyrogen adsorbance, prewashing the packed column with a suitable

buffer and then passing the pyrogen containing solution through the column. In the method of batch treatment the final product freed of pathogens can be obtained by stirring the pryogen adsorbent in a pyrogen containing solution of the desired compound and then removing the adsorbent.

Alumina P for the removal of endotoxins and pyrogens

The isolation of therapeutic molecules from bacterial cell growth is hampered by the need to remove endotoxins and pyrogens. Many different commercial techniques are in place in an attempt to remove these endotoxins, which are lipopolysaccharide components of bacterial cell walls.

Alumina P using either column chromatography or batch processing methods has proven to be a superior tool for the isolation and removal of pyrogens. The surface of activated alumina may be chemically modified to help enhance pyrogen removal. Furthermore, the amphoteric property of activated alumina offers unique purification advantages allowing Alumina P to be used either as a single step or as part of a two step purification process for the isolation and removal of endotoxin.

DAI offers a specially designed superior Alumina which may prove to be just what you need for the removal of endotoxins and pyrogens from your bacterial cell culture system.

Talk with a member of our scientific team to discuss how we may be able to best address your production needs.

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