Development and Qualification Factors for Endotoxin Removal from Proteins Using Chromatographic Columns

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Introduction

Purification is a necessary phase in pharmaceutical drug manufacturing in order to eliminate unwanted materials that can be hazardous or which will compromise drug efficacy. This is of particular concern with protein-based drug products, where impurities include closely related non-functional proteins. To achieve the desired purification, attention needs to be paid to controlling a range of factors including temperature, time, pH, salt concentration, protein concentration, surface interaction and mechanical stresses. As part of purification process, contaminants also need to be removed; examples of contaminants include viruses and bacterial endotoxin,[1] and the risk posed by these will depend upon the source material and/or the type of end product. The presence of endotoxin, at a sufficient threshold, can cause patient harm in terms of a pyrogenic reaction[2] or endotoxic (septic) shock,[3] especially given that most biological products are administered by injection and many protein-based pharmaceutical product doses are low. Some proteinaceous products, especially those derived from starting bacterial cultures, are especially at risk from endotoxins; and proteinaceous products in general present a risk not least because endotoxins have the tendency to bind and form complexes with many types of proteins. Therefore, biomanufacturing steps must seek to both minimize endotoxin contamination through a contamination control strategy and remove ('clear') any endotoxins present at appropriate stages.[4] Even where there is no therapeutic necessity to remove endotoxin, the presence of endotoxin can cause false readings in cell-based assays, providing a further reason to remove endotoxin from the purified product.

Within biopharmaceutical manufacturing, column chromatography is a common method of protein purification[5] (the biophysical technique enables the separation, identification, and purification of proteins based on their size, shape, net charge, stationary phase used, and binding capacity).[6] The principle of chromatography is based on two phases: the mobile phase

which drives the sample and the stationary phase which allows its purification. The main separation techniques are ion exchange, surface adsorption, partition, and size exclusion.[7] Commonalities between these chromatography methods is that they each use a resin (the solid phase) with special chemical properties, which are held within a glass cylinder (the "column").

The commonly used chromatography methods to remove endotoxin are:[8,9]

1. Anion exchange chromatography using the negative net charge of endotoxin to bind endotoxin to an anion exchange resin (or the reverse, where positively charged proteins are bound to the resin).
2. The use of adsorbents to facilitate adsorption of endotoxin to matrix by electrostatic and hydrophobic interactions.

While ion exchange chromatography has traditionally been employed for endotoxin removal from protein solutions, greater success tends to be realized through the selection of affinity ligands. This article considers these methods as part of the development and qualification requirements that enable column chromatography to deliver endotoxin clearance as part of the protein purification step.[10]

Endotoxin Contamination

Many proteins will be contaminated with endotoxin, either due to the origin of the proteins (if derived from bacteria like *Escherichia coli*), variables within the manufacturing process (addition of excipients and water), or from control breakdown (the risks presented by wet equipment or uncontrolled process hold times, for example).[11] When endotoxin and protein come into contact an association is often formed primarily from electrostatic interactions although other mechanisms can occur, such as binding domains and hydrophobic interactions. These interactions can form various complexes and the bridges that form between proteins and endotoxin are very stable, making the disassociation difficult.

The weight of endotoxin capable of causing patient harm can be as low as 0.1 nanograms (depending upon the route of administration), and this quantity can be produced by around 100,000 Gram-negative bacteria,[12,13] a relatively low cell number that emphasizes the importance of manufacturing controls. Naturally occurring endotoxin is primarily lipopolysaccharide (LPS), in various aggregate forms with a typical molar mass of 10 kilo Daltons (kDa). It is a component of LPS - the hydrophobic lipid group covalently bound to a long complex polysaccharide tail (referred to as Lipid A) - that is responsible for the adverse physiological reactions that occur within the bloodstream of mammals where endotoxin induces the secretion of pro-inflammatory cytokines (such as interleukin-1 by macrophages) which triggers systemic inflammation by stimulating the hypothalamus to produce prostaglandins, which increases the body's core temperature, culminating in a fever.[10] The challenge presented by endotoxin is that it is indestructible relative to the product, in that applying methods that are capable of inactivating endotoxin – heat or chemical - will destroy proteins first. Therefore, the focus is with endotoxin removal from the product matrix by separating protein and endotoxin.[14] In the context of this article, since endotoxin is negatively charged at pH above 2 (LPS, with the Lipid-A component, is partially phosphorylated), the basis of removal is through the use of positively charged chromatography (anion exchange) or with absorption as the result of affinity chromatography, or sometimes, a synergy of the two removal processes.[15]

Ion Exchange Chromatography

Ion exchange chromatography separates ionizable molecules based on their total charge. The method technique enables the separation of similar types of molecules. The charge carried by the molecule of interest can be readily manipulated by changing buffer pH. With endotoxin, the principle is based on endotoxin, at most pH ranges saves extreme acidity, being negatively charged and the resin used in anion exchange chromatography being positively charged. Where the protein is also positively charged, the process of movement through the chromatography column will pull endotoxin away from the protein and retain it on or within the resin. Clearance factors of around 5-logs can be achieved with ion exchange chromatography, although only from high starting concentrations of endotoxin.

Depending on the process, the charge effect can also be used with certain filters where a positively charged membrane that electrostatically attracts and/or retains endotoxins,[16] although this is not practical for all applications not least due to yield losses.

Affinity Chromatography and Endotoxin Removal Resins

The most effective way to remove endotoxins from proteins to be purified is with the use of a suitable column and an endotoxin removal resin, one that also delivers the desired level of purification where the protein of interest is recovered in the flow-through. Certain chromatography resins have an affinity for endotoxin, by being able to absorb endotoxin from a solution, given sufficient contact time, through both ionic and hydrophobic interactions. Many resins come in the form of spherical particles.[17] The properties of the resin and its efficiency to achieve endotoxin absorption is a product of the resin's affinity ligands. Ligands are ions or molecules that bond to a central metal atom and function as electron pair donors, with the central atom functioning as an electron pair acceptor.[18] Hence, electrostatic interaction and hydrophobic intermolecular interaction are the principal interactions in delivering the adsorption of endotoxin.[19]

Assessing resin efficiency

Selecting the optimal resin for both achieving protein purification and removing endotoxin presents a critical choice for the development and qualification of the pharmaceutical manufacturing process. Important criteria to establish, and to verify through validation are presented below:[20-25]

- The resin ligand should be LPS-selective and possess a combination of cationic properties and hydrophobic properties. LPS binding should be non-specific.
    - The resin should function under the required physiological conditions.
- The desired and expected level of endotoxin removal (which is typically expressed as a logarithmic reduction). In terms of the desired levels, this would typically be <5 EU/mL (based on the critical quality attribute of the finished product). The Endotoxin Unit (EU) is the measure of biological activity, enabling endotoxins of different molecular weights from different bacterial species to be compared.
    - This may require an understanding of the potential challenge levels and consideration of any controls upstream of the column, since the efficiency of endotoxin removal will be part-dependent upon the challenge level (in that the higher the endotoxin challenge, the less efficient the removal process will be).
- The capacity for endotoxin removal relative to the maximum amount of pharmaceutical ingredients to be processed.
    - The ionic strength of the material passed through the column will affect process efficiency.
- The process time required, which is based on column flow rates and the contact time required between the processed material and the resin in order to remove the required level of endotoxin. There is a trade-off required between the use of a bead-based chromatography resin, which has superior absorbency, and a membrane based one that achieves better flow but is less effective at endotoxin capture.
    - The adsorption of endotoxin takes place mainly at the outer surface of adsorber particles. While adsorption can occur within the resin, the contact times required, as shown by long uptake adsorption curves from experiments, are often too long in terms of process efficiency.
- Assessing the affinity of the endotoxin for the protein and how this can be modified by factors such as temperature, pH, detergents (surfactants), solvents and denaturants.
    - Where pH can be altered, maximum endotoxin deactivation occurs at acidic pH below isoelectric point.
- The number of times the resin can be re-used, including understanding the point at which endotoxin removal efficiency decreases. This consideration will need to extend to the column regeneration process.
- To demonstrate that the resin does not introduce any impurities.
- That the resin is easy to pack into the column.

The points listed above should form part of method development and be verified as part of process validation. Some of the points will need to be developed in conjunction with the column supplier, given that the introduction of large quantities of endotoxin, although needed to demonstrate column efficiency, into the production process carries considerable risk (typically 10,000 or 100,000 EU/mL is required). Such

a study should use the actual material to be processed (or a suitable placebo), although experiments can be scaled down provided the laboratory study is appropriately representative of what will become the scaled-up process. Once this assessment is completed, the focus of the user will be with maintaining control (such as controlling the time point for resin changes and practicing column depyrogenation) and in-process monitoring (where a sample is taken from each batch processed through the column to verify absence, or sufficiently low levels, of endotoxin using an endotoxin test method, such as the *Limulus* Amebocyte Lysate (LAL) assay).[26] Batch related in process monitoring of intermediate product provides an important batch-by-batch assessment of the continuation of controls.

In addition, the ability of the resin to achieve the desired level of protein purification must also be met during pilot studies, otherwise the exercise becomes uneconomical for scale-up. Common types of resins include affinity adsorbents based on the antibiotic polymyxin-B and sodium deoxycholate; poly(ethyleneimine); more novel resins are based on lysine (poly-L-lysine) or polycationic ligands.[27,28] Generally, endotoxin will elute at pH 8 around 500 mM NaCl. The important element is the surface modification of the resin so that it has the highest possible affinity to endotoxin, meaning that lipopolysaccharide effectively binds to the resin.[29] The process can sometimes be enhanced through the presence of a surfactant, either as a pre- or post-column processing stage (a phase separation method). For example, the addition of surfactant Triton X-114 (a nonionic surfactant that has a hydrophilic polyethylene oxide chain) and subsequent incubation promotes the association with lipid A.[10,30] When followed by a refrigeration step, this can allow the surfactant to gelatinize, enabling its removal with the associated endotoxin. The use of a surfactant can be doubly useful, when seeking the simultaneous clearance of metal chelates. Additionally, Triton X-114 can be used for the washing of columns as part of the column decontamination wash.[31]

Development and Qualification Complications

It is important to demonstrate removal of endotoxin from protein since methods of endotoxin detection are limited in their ability to detect both low levels of endotoxin (at picogram levels) and some protein-endotoxin interactions can lead to masking, where endotoxin may be present but not detectable using conventional endotoxin test methods like LAL.

This places an importance on demonstrating endotoxin removal through developmental study. However, the removal of endotoxins, particularly with affinity chromatography, can be challenging when endotoxins are strongly associated with specific labile biomolecules. Complications to the endotoxin removal process also occur through the tendency of endotoxin to form micellular (cellular aggregate) or vesicular structures (as lamellar, cubic or hexagonal inverted arrangements). 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.[32]

With ion exchange, the technique is optimal for positively charged proteins; whereas, negatively charged proteins pose the problem of product loss (proteins take on different charges based on pH). Thus, the success of affinity and ion-exchange chromatography in separating endotoxin from proteins is affected by the properties of the target protein. To overcome this, instead of binding the endotoxins to positively charged surfaces and allowing protein solutions to flow through, the process is modified by using cation exchangers to bind the proteins to negatively charged surfaces and allowing endotoxins to flow through and then recovering the protein. This works most effectively at pH 4, although this is less effective in terms of yield recovery.

Column Depyrogenation

Even where resins are used that are effective at capturing endotoxin, removing endotoxin from a chromatography column is an important step since sufficient build-up of endotoxin will lead to contamination of subsequently processed materials. The process of inactivating and removing endotoxin from a column needs to be undertaken periodically (as defined by the facility contamination control strategy) and the process takes time, which needs to be built into the facility processing schedule. The most common way to achieve depyrogenation of the column is to subject the column and matrix to a wash with sodium hydroxide (NaOH). The contact time and molarity of the sodium hydroxide will be dependent upon the type of resin and the expected level of contamination (which process validation studies can provide data upon). Common protocols for depyrogenation include overnight in 0.5 M NaOH or 4 hours in 1.0 M NaOH[33] (although endotoxin inactivation may require a contact time of more than 12 hours,[34] hence this becomes an important part of the equipment and process validation. Most qualifications seek to achieve a six-log reduction in an endotoxin challenge). Following the depyrogenation, the NaOH needs to be removed from the column, which requires large quantities of Water for Injection and optimization to ensure that all valves and lines have been effectively flushed before starting the next purification process.

Conclusion

The removal of endotoxin from proteins as part of the purification step is a critical control process in biopharmaceutical manufacturing since it is the primary, and sometimes only, means to remove endotoxin. The two principal methods to achieve this are ion exchange and adsorption. These methods have their own advantages and disadvantages as well as being more suited to the purification of

certain proteins. Depending on the desired outcome, a two-phased approach is sometimes required.

While column chromatography can help with decreasing the level of endotoxin present, no single or dual method should be relied upon, and minimizing endotoxin should be considered as part of the contamination control strategy. The efficiency of any removal process is dependent upon the challenge, therefore a risk-based approach to contamination control should foremost be centered on reducing the possibility of endotoxin presence upstream of the column. An additional control requirement is with avoiding subsequent recontamination of the purified product as it continues its path downstream.

As well as control, assessing intermediate material post-purification for endotoxin serves as an important verification step to ensure that the process is working as designed (albeit the endotoxin detection method sensitivity limitations acknowledged). It is also important that controls are in place and working to prevent any recontamination of the protein by endotoxin through downstream processing, as might occur from wet equipment or from the addition of contaminated excipients. Throughout the process, the use of intermediate manufacturing endotoxin monitoring can also partly offset other concerns being grappled with by industry: the issue of finished product low endotoxin recovery.[35]

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