Current Technologies to Endotoxin Detection and Removal for Biopharmaceutical Purification

Mason Schneier[1], Sidharth Razdan[1], Allison Miller[1], Maria Briceno [1], and Sutapa Barua[1]

[1]Missouri University of Science and Technology

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Abstract

Endotoxins are considered as the major contributors to the pyrogenic response observed with contaminated pharmaceutical products. Recombinant biopharmaceutical products are manufactured using living organisms, including gram-negative bacteria. Upon the death of a gram-negative bacteria, endotoxins (also known as lipopolysaccharides; LPS) in the outer cell membrane are released into the lysate where it can interact with and form bonds with biomolecules, including target therapeutic compounds. Endotoxin contamination of biologic products may also occur through water, raw materials such as excipients, media, additives, sera, equipment, containers closure systems, and expression systems used in manufacturing. The manufacturing process is therefore in critical need to reduce and remove endotoxins by monitoring raw materials and in-process intermediates at critical steps, in addition to final drug product release testing. In this review, a discussion regarding the progression of endotoxin detection techniques, from crude to refined are presented. We provide a brief overview of the upstream processed used to manufacture therapeutic products and then discuss various downstream purification techniques widely used to purify the products off endotoxins. Finally, we investigate the effectiveness of endotoxin purification processes, both from a perspective of precision as well as cost-effectiveness.

Introduction

Pharmaceutical manufacturing deals with selection and optimization of the cell source, media composition and physio-chemical bioreactor operating conditions to maximize the culture yield and productivity (**Figure 1**) 1-3. *Escherichia coli*(*E. coli*) is a cost effective and attractive choice for producing therapeutic monoclonal antibodies, recombinant proteins and other biopharmaceuticals due to their rapid growth, minimal nutritional requirements, high product yield and transformation capability 4-11 With the millions of strains of bacteria, and gene-altering technology steadily improving, the possibilities are endless 12. One of the most recognizable products derived from genetically engineered *E. Coli* is the hormone insulin. Before being manufactured by bacteria, insulin was originally extracted from dogs and later pigs that was an extremely inefficient process, making the product rare and expensive 13. The advent of *E. coli* -produced insulin such as recombinant human insulin (Humulin) drastically increased its availability for diabetics 14. However, biopharmaceutical products manufactured using *E. coli* or other gram-negative bacteria are subject to endotoxin contamination 15-21.

Endotoxins are present in the outer cell wall of gram-negative bacteria that contribute to the organization and stability of the membrane 16-22. Endotoxin consists of three regions: a core polysaccharide, a long chain polysaccharide, and a non-polar lipid called Lipid A (**Figure 2**) 20,23. The core polysaccharide has an outer hexose region and an inner heptose region and the long chain polysaccharide is a strain-specific surface antigen (O-antigen) that consists of repeating oligosaccharide subunits 17,20. The core polysaccharide and the O-antigen are both hydrophilic while Lipid A is hydrophobic. The toxicity of endotoxin is associated with Lipid A 24-26. Lipid A triggers the production of pro inflammatory cytokines 27,28 and activation of the coagulation cascade 20,29 which can lead to sepsis and septic shock 30-34 . A pyrogenic reaction can

be caused by as little as 1 ng of endotoxin per kilogram of body weight per hour 16-21,35. The standard unit for endotoxin measurements is an endotoxin unit (EU), which is equal to the activity of 0.1 ng of *E. coli* endotoxin 36,37. For intravenous applications, a maximum of 5 EU per kilogram of body weight can be administered to a patient per hour 36,38,39, but acceptable concentrations in biopharmaceutical products vary depending on the required dose 40,41.

Endotoxin is highly stable and is resistant to destruction by heat or pH 25,39,42. Additionally, endotoxins may form stable interactions with target therapeutic compounds that further complicates separations 23,43,44. Downstream processing of recombinant protein products accounts for approximately 45-92% of the total manufacturing costs 10,45,46. In addition to the downstream processing, the detection of endotoxins is absolutely critical for the safety of patients across the globe who rely on the purity of treatments prescribed 28. The purpose of this review is to discuss these aspects of an array of endotoxin detection and removal technologies.

Endotoxin Detection

Biological Detection Techniques

Biological detection techniques include rabbit pyrogen test (RPT), limulus amoebocyte lysate (LAL) assay and bovine whole blood assay (bWBA) that use natural methods of endotoxin detection, and still in use today, although they are being phased out by newer, more accurate testing methods such as biosensors that are described after the biological detection techniques.

Rabbit Pyrogen Test (RPT)

The oldest and simplest of the endotoxin detection techniques, RPT involves injecting the biological sample in question into live rabbits and waiting for a fever to develop 23,47. This method works on the principle that rabbits and humans share similar fever patterns under influence of endotoxins. It was determined that a temperature increase of 0.5°C over a time span of 180 min after injection constituted a fever 48. It was also found that it has a detection limit of approximately 0.5 EU/ml (endotoxin unit/milliliter) or around 0.05 ng of endotoxin/ml of solution 49. As rudimentary as the technique seems, a detection rate as low as 0.1 ng was considered very accurate at the time of this methods development in 1912. This technique has been praised for its accuracy; being an *in vivo* technique, it is easy to accept the results of the test as researchers can physically see the test subject show symptoms of infection. Seeing the test subject suffering the effects of endotoxins provides a compelling argument to the presence of endotoxins in the sample. This method is often criticized 50. The scientific world is generally moving away from live test subjects where avoidable, in particular, animal testing. While this test was once considered the best in the industry, and is still being performed in parts of Japan, today it is criticized for its need for many samples, and its near-obsolete sensitivity and accuracy compared to other methods 51.

Limulus Amebocyte Lysate (LAL) Assay

Unlike RPT, LAL assay developed in the 1960s does not involve live test subjects. It does, however, rely on an extract from the blood of the*Limulus polyphemus* species of horseshoe crab 52,53. The extract is used in one of three ways. First and simplest, the gel-clot. This test involves mixing equal parts of extract and sample. If a gel has formed and the mixture remains intact in the bottom of the tube, the test shows positive 54. This means the sample has at least enough endotoxins to trigger a positive reaction, the limit of this being around the range of 0.03 EU/mL or 0.003 to 0.006 ng/ml. The other two methods are turbidimetric and chromogenic methods. Both are referred to as photometric tests as they require an optical reader for analysis. The chromogenic assay is performed by replacing a natural substrate, Coagulen, with a chromogenic, or colored one. The chromogenic substrate is cleaved by an endotoxin-activated enzyme coagulase, and the chromogenic molecule is released from the substrate into the suspension measured by spectrophotometry 55. The turbidimetric method is similar to the chromogenic method, but instead measures the turbidity of the solution 56. The rate of turbidity and absorbance (color change) are proportional to the endotoxin concentration. All three tests rely on the same protein, Factor C coagulation cascade found in horseshoe

crabs' blood (**Figure 3**). The endotoxin activates Factor C which goes onto activate Factor B following the formation of a clotting enzyme 57,58. In gel clot and turbidity assays, the clotting enzyme transforms Coagulen into Coagulin, creating the gel in the gel clot test, as well as the clouding agent in the turbidity test. The Chromogenic method follows the same pathway, but instead of using Coagulen, it uses a complex of amino acids and p-nitroaniline (pNA), as the chromogenic factor. The enzyme trims the pNA off of the complex, turning the suspension a yellow color. This color is too faint to discern by the naked eye so a spectrometer must be used 23. These tests are widely accepted as the official endotoxin test in the pharmaceutical community 59. Every drug and medical device that is tested by the US Food and Drug Administration (FDA) must undergo and pass a LAL test 60,61. As previously mentioned, this method is much more accurate than RPT, particularly the photometric methods. This method still has its drawbacks. For example, the protein cascade it relies on is disrupted in samples with free metal ions, and similarly to RPT, the method is subject to the same public outcry for its treatment of horseshoe crabs. While the phlebotomy itself is not fatal, an approximated 20% of the crabs fail to survive after being returned to sea 23. Following the discovery of Factor C as endotoxin-activated portion of the protein cascade, attempts have been made to replace the conventional LAL test, with one using recombinant Factor C 62. As technology improves, alternative techniques are being developed to ease the pressure on the horseshoe crab population.

Recombinant Factor C (rFC) Assay

rFC is an endotoxin sensitive synthetic protein that is cloned from factor C DNA to use as an alternative *in vitro* LAL test 47,63-67 . In the rFC test, the binding of endotoxin activates the synthetic rFC molecule, which then cleaves a fluorescein substrate (amino-methylcoumarin), resulting in the generation of a fluorogenic compound. The fluorescence is measured twice, first at time zero and then after the endotoxin has been introduced using excitation/emission of 380/440 nm. The difference in fluorescence is proportional to endotoxin concentrations in the sample and is used to calculate a final endotoxin result. rFC is specific to endotoxin detection eliminating the dependence on nonspecific glycan binding like that in an LAL assay avoiding false positive results.68 The enzymatic sensitivity range to endotoxin is 0.05-500 EU/ml.63

Bovine Whole Blood Assay (bWBA)

The test works by taking the whole blood from the animal and introducing it to a solution containing the pharmaceutical being tested 69. In response to endotoxin, the white blood cells in the blood produce the cytokine Prostaglandin E2 (PGE2) in an inflammatory response, similar to that of humans 70. The production of this cytokine is directly proportional to an increase in endotoxin concentration. According to several studies, the test is able to accurately detect endotoxins at concentrations of close to 25 pg/ml, whereas the concentration at which humans display symptoms of endotoxin exposure typically occurs around 30 pg/ml 70. This level of accuracy is very attractive for scientists looking to move away from LAL and RPT testing. The test also is easy to perform and takes few preparational steps 23. The test is not without its limitations. The whole blood needed for the tests can only be obtained from very young calves which makes it difficult to amass in vast quantities 71. Furthermore, due to cultural and religious practices, certain countries will not permit the collection or use of bovine blood. While its accuracy and ease of use is admirable, it still requires animal testing, and with the advancement of technology, this test may be replaced by other techniques.

Monocyte Activation Test (MAT)

The Monocyte Activation Test, or MAT, has been in development since 1995 72. The test involves using monocytes in human blood to test for a reaction to endotoxins. The human blood is exposed directly to the test surface or test therapeutic and the pro-inflammatory cytokine IL-1β is measured. The cytokine release is measured by enzyme-linked immunosorbent assay (ELISA) 73,74. The ELISA is used by attaching a protein complex, usually antibodies designed to trap the IL-1β released by the monocytes and an enzyme that will release a colored substrate when the cytokine binds to the complex, to the walls of a vessel, then adding the blood from the MAT 75. If the cytokines are present, they will bind to the protein complex, releasing the colored substrate that can either be detected by the naked eye or by spectrometers, similar to a chromogenic

LAL test 49. The test also has the added benefit of testing all pyrogens and inflammatory materials that would prove harmful to human patients 76,77. It avoids animal testing and has a detection of limit of as little as 10 pg/ml of endotoxin solution, and conveniently, this limit becomes even smaller, and the test becomes more accurate when using cryogenically-preserved human blood. This aids in storage and transportation of the human blood for testing if the blood can be cooled and preserved without sacrificing accuracy 73,78,79. The monocytes can be prepared in a variety of ways. Some experiments have used whole human blood, while others use monocytes harvested from leukocyte filters at blood donation centers 78. This method displays high precision by being able to detect non-endotoxic pyrogens and their effect on possible patients of the tested material. However, as there is often a limited supply of human blood to be used for simply testing, inconsistencies can arise when using large quantities of blood are used 72,80.

Biosensor Techniques

In attempts to modernize endotoxin detection methods, scientists have begun to develop techniques designed around more synthetic approaches. They involve more technology as opposed to pre-existing natural pathways. These techniques represent the up and coming detection methods that scientists hope will eventually replace the gold standard of RPT and LAL tests. These techniques can be split into three categories, electrochemical, optical, and mass-based.

Electrochemical

The majority of electrochemical biosensors are based on a principle called Electrochemical Impedance Spectroscopy, or EIS. Performing an EIS requires electrodes be placed within the solution desired to be tested and delivering a sinusoidal alternating current signal through the solution, usually ranging from 2-10 mV. By varying the frequency of these sinusoidal waves, an impedance spectrum can be created 81. The electrodes are coated in metal, to reduce electric resistance. Proteins that are highly selective to endotoxin components are then bound to these electrodes such that if the endotoxins come in contact with the electrode-protein complex, they bind to the proteins. These proteins are referred to as Endotoxin Neutralizing Proteins, or ENPs 82,83. When endotoxins bind to ENPs on the electrodes it increases the resistance of the electrode. This was the case in an experiment run by Yeo *et al.*84 who constructed an electrode made of gold and a complex of human recombinant toll-like receptor 4 (rhTLR4) and myeloid differentiation-2 (MD-2) proteins (**Figure 4)** . They exposed these electrodes to solutions of varying endotoxin concentrations and created impedance spectrums for each of these concentrations. The maximum current across all potential differences was lower at higher concentrations of endotoxin 74. The study also reports that this particular biosensor has high specificity towards endotoxins, in order to prevent false positive results. It then goes on to state that the sensor had a detection limit of 0.0002 EU/ml, or 2 x 10-5 ng/ml. This is drastically lower than the standard LAL test limit of 0.003 ng/ml. Metal complexes immobilized upon a gold electrode have been used and were able to detect endotoxins at concentrations as low as 0.0001 ng/ml 85. Porous silicon membranes (pSim) based electrochemical biosensors comprise of array of nano-channels which are modified using Polymyxin-B, with strong affinity to endotoxins. It shows very low limits of detection of 1.8 ng/ml. These sensors showed ability to detect endotoxins from various bacterial strains like *E. coli and S. typhimurium* and all this is done in a label free manner 86. Studies have also reported highly sensitive peptide modified gold electrode based electrochemical biosensors which are used for endotoxin detection with very low limit of detection of 0.04 EU/ml 87. These methods are faster, more accurate, and in most cases, more cost effective than biological based techniques 88. Two other electrochemical techniques are amperometric and potentiometric methods. Amperometric transducers have been described as the most common of the electrochemical sensors used for endotoxin detection 89. They work on the relying on the same principle of EIS, wherein the concentration of the analyzed sample has a linear relationship with the current measured. This method is able to use premade, disposable testing strips, for fast, cost-efficient testing 90. Potentiometric methods are worth noting because although their detection limits are relatively high, 1-5 EU/mL, they were the first biosensor to be able to detect endotoxins in real time 81,91. The methods in which the electrodes are created, as well as the ways in which they are measured and utilized, are more complicated and labor-intensive than the biological methods 92. They require more sophisticated personnel and equipment to be run effectively than RPT or

LAL tests93.

Optical Techniques

One such example is that of liquid crystal (LC) based optical sensor for highly sensitive endotoxin detection. LC based optical biosensors are developed using endotoxin specific single-stranded DNA aptamers which are the endotoxin selective probes of the biosensors. The LC based aptamer optical biosensors have linear endotoxin detection range from 0.005 to 100 ng/ml and a detection limit of 5.5 pg/ml. The biosensors have negligible cross-binding reactivity with the biomolecules thus maximizing their recovery 94. Broadly, these optical techniques can be divided into three distinct categories: luminescence, Surface Plasmon Resonance, and electrochemiluminescence that share the similar characteristic of relying on visual changes.

Fluorescence and Luminescence Techniques

For the purposes of endotoxin detection, mutant firefly luciferase shows potential in becoming a fast and reliable detection method 95,96. Experiments have been performed using a mutated version of the firefly luciferase that are able to quickly and precisely identify solutions containing endotoxins 96. The mutated form of North American luciferase used in the experiment generates a luminescence 10 times as intense as the standard, wild-type luciferase 97. The enzyme was dissolved later in a TMAT buffer solution. In short, endotoxin is detected by Factor C, previously discussed in the LAL test section. This activates the protein, which cascades to activate Factor B, which in turn activates a pro-clotting enzyme, then a clotting enzyme. The resulting protein contains Luciferin, a precursor to the light-emitting Amino-Luciferin. When this activated form of the fluorophore is created, the detectable light is emitted. The results of the experiment show a distinct increase in luminescence intensity as endotoxin concentration increases. The lowest endotoxin concentration recorded was 0.0001 EU/mL, or 1 x 10-5 ng/mL, while the researchers report a detection limit of this mutant-type luciferase bioluminescence technique was 0.0005 EU/ml, or 5 x 10-5 ng/ml 23. Another important factor to mention is that this detection limit was reported in 15 minutes. This required time is blistering in comparison to the LAL gel-clot techniques estimated required time of 138 minutes to nearly 1.5 hours 98. However, using firefly luciferase returns to the issue of animal testing that can be avoided using a recombinant version of the mutated luciferase 99. Experiments have been performed using a peptide biosensor and attached fluorescent probes, fluorescein-maleimide (F5M), and tetramethylrhodamine-5-malemide (TMR5M) 73. Recently, a fluorophore BODIPY ((4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-*s* -Indacene) with excitation and emission wavelengths of 485/20 and 528/20 nm were used to quantify presence and removal of endotoxin from biological solutions (**Figure 5**) 100-103. BODIPY dye which is a lipid biomarker, in presence of endotoxin quenches due to endotoxin binding to its surface signaling endotoxin contamination 100,101,104. Endotoxin detection studies have been conducted using Alexa Fluor-labeled fluorescent endotoxin with excitation and emission wavelengths of 490 and 525 nm 105. In this study, C-18 acyl chain modified Fe3O4/Au/Fe3O4 nanoflowers were used for simultaneous capture and detection of endotoxins from water samples 105. The lowest endotoxin detection limit using this technique was 1 ng/ml 105.

Surface Plasmon Resonance (SPR)

Zhang *et al.* have shown a smartphone biosensor platform using SPR. The disposable sensor chip utilizes the smartphone's built in flash as a light source and a compact diffraction grating and spectra dispersive unit 106, but this technology is still in development. Recent publication regarding antibiotic mediated plasmonic biosensors for endotoxin detection have shown a low limit of detection of 0.4 ng/ml 107.

Mass-based Techniques

An example of mass-based techniques is electromagnetic piezoelectric acoustic sensors, or EMPAS that has been touted as being able to measure multiple types of pathogens, not exclusively endotoxins, as well as being able to detect endotoxins in real time within human blood plasma 108. Another mass-based method is magnetoelasticity that function by placing sensors directly on to dry testing surfaces, such as medical equipment or food. The sensor filaments, whose oscillation frequencies are monitored, fluctuate within a

magnetic field. These sensors are coated in phages designed to bind with the target pathogen, like ENPs 109. Then under an applied magnetic field, the sensors will oscillate differently than before the test, due to the increase in mass from the newly-bound endotoxin. The change in mass is measured similar to EMPAS testing. This method has a reported detection limit of 0.0105 EU/mL, or close to 0.001 ng/ml, and a detection time of nearly 5 minutes.

Techniques for Downstream Removal of Endotoxins

Downstream process for pharmaceutical manufacturing comprises of three steps: (1) initial recovery by extraction or isolation, (2) purification and (3) polishing 1-3. Endotoxin removal presents a unique challenge which form stable interactions with themselves and possibly with target therapeutics.

UltrafiltrationA single endotoxin molecule in its monomeric form has a molecular weight 10-30 kDa 22 depending on the core polysaccharides and oligosaccharide chain, but endotoxins have the ability to aggregate and form micelles and vesicles with molecular weights above 1000 kDa 29 and diameters up to 0.1 μm 20. The endotoxin micelles and vesicles can be separated from water, salts, and small target therapeutic molecules through size exclusion in ultrafiltration. Factors that affect the removal of endotoxins from aqueous solutions include the size distribution of the molecules in solution, the interactions between target molecules and endotoxin, therapeutic protein concentration and the presence of detergents. The effect of protein concentration on the endotoxin removal efficiency using ultrafiltration membranes has been explored 22,110. Ultrafiltration membranes with 100 kDa molecular weight cut off (MWCO) were used to filter endotoxin contaminated protein solutions with concentrations varying between 2-30 mg/ml. The % endotoxin removal through the membrane ranged from 28.9% to 99.8%, depending on the level of protein concentration and endotoxin dilution. The more dilute the protein samples were made the higher was the rate of removal. A possible explanation for such removal efficacy is the reduction of endotoxins with each protein dilution step and the formation of endotoxin monomers compared to micelles and vesicles in dilute solutions.

Effects of detergent concentrations on the interactions between endotoxin molecules was studied and contribute towards efficient endotoxin removal. Multiple Tween 20 of 0.0%, 0.5%, 1.0%, and 2.0% were added to the protein solutions and then the respective removal efficiency was calculated 22. An increase in the Tween 20 concentration led to an increase in the passage of endotoxin into the permeate and thus removing endotoxin from protein 22. These results demonstrate that the presence of a detergents decreases the size distribution of endotoxin aggregates. As the detergent concentration increased, the equilibrium shifted from micelles and vesicles to monomers 22,110. This method is undesirable for ultrafiltration where endotoxin monomers are to be trapped within the membrane and desired protein be allowed to pass as they are less likely to be stopped by the filtration membrane compared to endotoxin aggregates.

Ultrafiltration has been used to separate endotoxin molecules from small target therapeutic drug molecules. For example, ultrafiltration was utilized to separate endotoxin aggregates from BMS-753493, a small aqueous drug molecule with a molecular weight of 1.57 kDa 111. Two membrane sizes were used to perform endotoxin decontamination of the drug molecules: 3 kDa and 10 kDa. Both ultrafiltration membranes were effective in reducing the endotoxin concentration to below 0.03 EU/mg but compared to the MWCO of 3 kDa, the 10 NMWCO had higher drug yield of around 95% unlike the 3 kDa membrane which lost around 55% of the desired product 111 . Thus, ultrafiltration membranes are an effective tool for removing endotoxins from aqueous drug molecules and other therapeutic products 111.

The main limitation associated with the ultrafiltration technique is that in most cases it can be used to remove endotoxins from molecules that are magnitudes smaller than the endotoxin aggregates. For this reason, this method is not applicable for most endotoxin separation scenarios. Ultrafiltration is best suited for removing endotoxin from water, salts, or small molecule therapeutics that do not have an affinity for endotoxin.

Extraction

Solvent extraction is used to separate endotoxins from target therapeutics based on their relative solubilities

in two immiscible liquids. Endotoxins form partition in the organic phase, while hydrophilic target molecules remain in the aqueous phase. Endotoxins have been effectively removed from the bacteriophages T4, HAP1, and F8 using 1-octanol with endotoxin removal efficiencies varying between 64 - 99.9%. 36. Additional processing is required to remove any trace quantities of 1-octanol present in the aqueous phase as the presence of 1-octanol interferes with the LAL test for endotoxin detection 36. Even though solvent extraction technique gives high endotoxin removal from various therapeutics solutions, the product yield is significantly low and varies between 30-60% impacting the profits associated with the method where it may not be a practical choice for this application 36.

Two-phase extraction using detergent Triton X-114, a non-ionic surfactant 112, has been explored to remove endotoxins from target therapeutics. Endotoxin was successfully removed from the green fluorescent protein using Triton X-114 and temperature transitions. Triton X-114 is miscible with water at a temperature of 0°C, but a phase separation occurs at temperatures above 23°C 113. Endotoxins are partitioned in the detergent phase while the target therapeutics are partitioned in the aqueous phase. Endotoxin removal efficiencies using Triton X-114 ranged between 45-99% 113. In addition to high endotoxin removal, Triton X-114 results in high product recovery of over 80% 16.Triton X-114 isothermal extraction using sodium dodecyl sulfate (SDS) has also been very effective in removing endotoxins from pDNA with residual endotoxin concentration of around 16 EU/mg. Moreover, using this extraction technique, a pDNA recovery of over 80% was reported. While isothermal extraction was proven effective for plasmid-endotoxin removal, this method is not applicable for the removal of endotoxins from protein solutions because SDS is reported to have strong interactions with proteins causing significant changes to protein conformation 114. One major disadvantage of temperature transition extraction using Triton X-114 is that the repeated heating and cooling degrades therapeutic products 114. Extraction processes provide a rapid separation that is easily scalable and can achieve high removal efficiencies, especially with high initial concentrations 36,113,115. However, final endotoxin concentrations in the aqueous phase for both solvent extraction and Triton X-114 extraction remained above desired specifications, meaning additional processing is required.

Ion exchange chromatography

Anion exchange chromatography can be used to separate negatively charged endotoxin molecules from positively charged molecules, such as basic proteins. Proteins exhibit different charges at different pHs. A protein exhibits a neutral charge if the pH is equal to its isoelectric point (pI), a negative charge if the pH is $>$ its pI, and a positive charge if the pH is $<$ its pI 116. The pI of an endotoxin molecule is ˜2 16,20,117, meaning endotoxins are negatively charged under conditions typically encountered during chromatography. At pH $<$ 2, the target protein exhibits a net positive charge and is repelled by a positive stationary phase while the negatively charged endotoxins interact with the stationary phase and leaves the column at a lower velocity 118,119. Anion exchange chromatography is not ideal for removing endotoxins from negatively charged target molecules, such as pDNA or acidic proteins 120,121.

If significant ionic interactions are present between target proteins and endotoxins or between the protein and the resin, a decrease in protein yield or an insufficient separation may be observed. If the protein and the endotoxin have a strong interaction, endotoxins leave the column bound to the target protein. If there is a strong attractive interaction between the target protein and the resin, the protein yield is low 117.

To lessen undesirable interactions, the pH of the protein solution is adjusted. The effects of resin volume and contact time in addition to pH and conductivity on the efficiencies of endotoxin removal have been explored for therapeutic products like, antigens NY-ESO-1, Melan-A, and SSX-2 117. The pIs of these antigens were 9.1, 8.7, and 6.2, respectively. NY-ESO-1 and Melan-A are both hydrophobic molecules while SSX-2 is hydrophilic 117. All tests were run using equilibrated Q XL resin. An increase in resin volume and endotoxin-resin contact time had a positive effect on endotoxin removal and the concentration of endotoxins in the permeate consistently decreased with increase in above variables. Low endotoxin concentration of ˜ 0.4 EU/µg was obtained in the permeate and a protein recovery of $>$ 80 % was obtained consistently at almost all resin volumes 117. While positively charged proteins are less likely to interact with the resin and remain in the column, they may also demonstrate an undesirable attraction to endotoxins. To minimize

protein- endotoxin interactions, the pH chosen should be high enough to avoid giving the protein a strong positive charge. Effect of different pHs on the removal of endotoxin from protein Melan-A, a hydrophobic protein with a pI of 8.7 has been studied. Melan-A exhibited a strong ionic interaction with endotoxins below its pI, causing endotoxins to leave the column with the target protein. To remedy this, the pH was increased to weaken such interactions 117. The pH tested were 7.9, 8.4, 8.9, and 9.2, which corresponded to endotoxin concentrations in the permeate of 1.4, 1.8, 0.6, and 0.5 EU/μg 117. As the pH was increased above the protein's pI, the endotoxin concentration decreased dramatically and with no significant impact on the protein yield 117.

The success of ion-exchange chromatography is highly dependent on the target molecule, but in general ion-exchange chromatography can achieve an endotoxin reduction of five orders of magnitude for concentrated solutions (>10,00 EU/ml) or three to four orders of magnitude from dilute endotoxin solutions (<100 EU/ml) 20.

Affinity Chromatography

Affinity chromatography is used to separate endotoxins from target molecules using highly specific interactions between endotoxins and a ligand bound to a stationary phase 122. Because of the specificity of the ligand, there is little to no product loss during separation 35. The target therapeutic molecule will elute with a greater velocity than endotoxin molecules due to specificity. The ligand chosen should have a strong interaction with endotoxins and a weak interaction with the target therapeutic molecule at separation conditions. Affinity chromatography is applicable to a wide range of target molecules, including proteins and pDNA 123,124.

It is important to note that the exact structure of endotoxins varies between bacteria strains based on the core polysaccharides and the long chain polysaccharide. For this reason, ligands are typically designed to interact 20 with the most conserved section 23,25,29,125 of the endotoxin molecule, Lipid A , through hydrophobic 117 and electrostatic interactions 20. Common ligands used in affinity chromatography include Polymyxin-B, histidine, dimethylamine ligands, deoxycholic acid and polycationic ligands 17,126. Hydrophobic polymers in the form of nanoparticles have been explored for removing endotoxins from water and protein solutions.100,101

One of the most commonly used ligands is Polymyxin-B (PMB), a cyclic lipopeptide with a high affinity for endotoxin (**Figure 6**). As a ligand, PMB induces the dissociation of endotoxin aggregates 127 and binds to the Lipid A section of endotoxins 128 through hydrophobic interactions 129. PMB's affinity to endotoxin can be attributed to the terminal amidine groups that are spaced such that interactions between amidine groups and the two phosphate groups on Lipid A can occur simultaneously 130. In addition to being used as a ligand, PMB is an antibiotic used to treat gram-negative bacterial infections. Despite PMB's high affinity for endotoxin, columns utilizing PMB may experience a higher than average product loss 20. This is because there are positive charges on the amino acid groups on PMB that may attract negatively charged target molecules. Additionally, PMB is both neurotoxic and nephrotoxic, which may cause a problem if the ligand is released from the column 25. Work has been going on to develop peptides with similar compositions to PMB but with a decreased toxicity. These peptide analogs displayed a strong affinity to endotoxin as well as a decreased lethality when introduced intravenously into mice 131.

The nitrogenous bases adenine, cytosine, histidine and histamine all display an affinity for endotoxin. Of these, histamine and histidine are equally as effective as polymyxin B and have been successful with separating endotoxin molecules from albumin, insulin, lysozyme, myoglobin, and others. Although histamine and histidine are considered equally effective, histamine is biologically active so histidine may be preferred 20. Deoxycholic acid (DOC) is another ligand option that may offer a higher product recovery due to a low charge density that reduces ionic interactions with negatively charged proteins 20.

Cost should be considered when choosing a ligand. If the ligand cost is too high, it will not be profitable on a manufacturing scale. Additionally, the contact time required between the solution and ligand will affect the cost. A process with a high contact time will required a larger column and therefore a greater initial

investment.

The pore size of the resin should also be considered. A small pore size will increase the retention of endotoxin in the column by size exclusion, while larger pore sizes will reduce the ionic interactions with anionic proteins 20. Studies have been conducted to study the effect of pH and ionic strength solutions on endotoxin removal efficiencies from hemoglobin samples using an Acticlean Etox affinity column. Endotoxins have been reported to form stable complexes with hemoglobin, thus complicating separation 43,132.The effect of ionic strength on endotoxin removal efficiency and hemoglobin recovery have been studied using two different salt solutions (NaCl and CaCl2). The endotoxin removal efficiency displayed a decreasing trend as the ionic strength was increased. However, the endotoxin removal efficiency for CaCl2 solutions displayed a more drastic initial decrease than that for NaCl solutions. These results indicate that not only do ionic interactions play a role in affinity chromatography, but the types of cations matter as well 43.

Unlike the endotoxin removal efficiency, the ionic strength and cation type had a limited effect on the product recovery from hemoglobin-endotoxin solutions. For all endotoxin contaminated hemoglobin solutions tested, the recovery of hemoglobin showed an increasing trend as the ionic strength was increased. Beyond, the ionic strength of 0.10 M, the hemoglobin recoveries remained relatively constant or displayed a gradual decrease with values over 95%. Though there existed interactions between endotoxins and hemoglobin that hindered separation but all the endotoxin contaminated hemoglobin solutions prepared with either NaCl or CaCl2 had hemoglobin recoveries above 99% for ionic strengths of 0.1 M, indicating that there is both an attraction between hemoglobin and the affinity resin and between hemoglobin and endotoxin which are weakened at an ionic strength of 0.1 M 43.

The effect of pH on endotoxin removal efficiency and hemoglobin recovery was tested using different buffer solutions. The endotoxin removal efficiency of resins was governed by the pI. There was a continuous and gradual decrease in endotoxin removal efficiency as the pH was increased from 4.5 to 8, and then the removal efficiency plummeted when the pH was increased from 8 to 9 because the pI of the affinity resin was 8. As the pH was increased from 4.5 to 8, the resin became less positively charged and was therefore less effective at attracting negatively charged endotoxins through electrostatic interactions but other affinity mechanisms were still present. As the pH was increased beyond 8, the resin moved from having a neutral charge to a negative charge that repelled endotoxins and overpowered some of the attractive affinity interactions. On the other hand, the pH or pI had a minimal effect on hemoglobin recovery; the recovery of hemoglobin from endotoxin solutions was above 97% for all pHs tested 43,130.

Commercial resins employing hydrophobic and/or cationic ligands to remove endotoxin from proteins and biological solutions use porous nano and/or microparticles and have shown great promise in protein purification, but the type of ligand immobilized or incorporated within the matrix still governs its intravenous application. Many of these resins have shown reasonable endotoxin binding efficiency from therapeutic proteins and biological solutions but suffer from major shortcomings like low recombinant protein recovery and difficulty in intravenous application due to nephrotoxicity and neurotoxicity associated with the endotoxin binding ligands. Toxicity related shortcoming can surely be addressed by using biocompatible endotoxin selective polymers which are non-toxic. Another major concern associated with most of the porous resins used for endotoxin removal is that they come in packed bed form which suffer from major drawbacks like high pressure drop (due to combined effect of bed consolidation and column blinding) and poor mass transfer (as intraparticle diffusion is responsible for transport of solute to the binding sites), thus making their application expensive and adding significant cost to downstream purification.

The toxicity, pressure drop and mass transfer related shortcomings were addressed by using biocompatible, rigid and non-porous particles where adsorption takes place on the surface. One such study focused on using biocompatible and environment friendly polymer, poly-ε-caprolactone (PCL) nanoparticles ~ 800 nm to remove endotoxins from water and protein solutions 100,101. The PCL nanoparticles (PolyBalls) were non-porous in nature and thus the endotoxin binding took place on the surface of the particles (**Figure 7 (a)**). PolyBalls showed high endotoxin removal efficiency of >99% from phosphate buffer saline (PBS) solution. These particles were also effective in removing endotoxin from protein solution prepared in water with more

than 90% removal efficiency 100. The removal efficiency was >99% when protein solutions were prepared in phosphate buffer saline (PBS) 100. The research also reported high endotoxin binding capacity of ˜1.5×10^6 endotoxin unit (EU) per mg of particles 100. In addition to high endotoxin removal the particles offered high protein recovery in excess of 90% thus maximizing therapeutic product recovery. High endotoxin removal in presence of PBS was attributed to the creation of shielding effect in presence of lyotropic sodium chloride salt. Considering larger-scale industry applications, combinatorial techniques were applied to construct PolyBall containing flexible and multifunctional biofilters (**Figures 7 and 8**). Contaminated samples were allowed to flow from one side of the filter to the other. The kinetics of endotoxin removal efficiency were determined as a function of concentration that also removed >99% endotoxins from water. One major advantage of the biocompatible PolyBalls and multifunctional biofilters is that they can be reused for endotoxin binding quite effectively without a major loss in binding efficiency. PolyBalls can be regenerated by breaking endotoxin-nanoparticle complexes which makes the endotoxin removal process more efficient and scalable. **Figure 8(e)** and**Table 1** showcase a comparison of different endotoxin removal products in terms of binding capacity, protein recovery and cost. Although, non-porous particles solve the mass transfer related limitation but the problem of high pressure drop during purification operation still persists. Due to the specificity of the ligand, affinity interactions offer a low product loss with a wide range of applications. Both mixed-mode chromatography and membrane adsorption use similar mechanisms and experience benefits.

Mixed-Mode Chromatography

Mixed-mode chromatography is a growing separation technique in the biopharmaceutical industry 45,133,134. While traditional chromatographic methods rely on a single dominant interaction between the ligand and the targeted molecule, mixed-mode chromatography (MMC) utilizes multiple interaction modes for an increased separation 45,134,135. When compared to traditional chromatographic methods, MMC offers an increased retention and selectivity of the targeted compound 136,137, especially for polar charged molecules 133,134. Many ligands used in affinity chromatography, such as histamine and histidine can be considered mixed-mode ligands due to their beneficial secondary interactions 121,137-139.

Membrane Adsorption

Membrane adsorption exploits the same mechanisms used in affinity and ion-exchange chromatography, but offers a reduced processing time and initial investment. Similar to affinity chromatography, a product yield near 100% can be achieved 35,140. In membrane adsorption, the same ligands used in affinity chromatography or resins used in ion-exchange chromatography are bound to a support medium. The use of a membrane greatly improves flow rates and nearly eliminates diffusion limitations. Membranes can be made of nylon, PVA, PEVA, PVDF, cellulose acetate and cellulose 25,100,140. The membrane capsules are single-use, meaning there is no need for eluting, cleaning, or regenerating. Benefits of single-use membranes include a decreased chance of product contamination as well as a decreased process time and buffer volume due to the decrease in required cleaning steps 38. Membrane adsorption requires a low initial investment when compared to traditional chromatographic methods, but membranes must be continually purchased, which will affect manufacturing costs 38.

In the past, membrane adsorbers have not been widely adopted because it had a lower binding capacity than that of traditional chromatography methods. Endotoxin removal efficiencies of histidine immobilized on a nylon membrane for different endotoxin concentrations have been carried out. The ligand density for the membrane adsorbers studied was 7.38 mg/g. As the initial endotoxin concentration was increased, the removal efficiency was greatly decreased. This demonstrates the limited binding capacity using membrane adsorbers. Even at the lowest endotoxin concentration of 387 EU/ml, the removal efficiency was only 65% 25. These results are consistent with those from previous studies that saw endotoxin removal efficiencies of approximately 70% with an initial endotoxin concentration of 6,000 EU/mL 141. Recently, membrane adsorbers with high efficiency endotoxin removal and binding have been synthesized. One such example of membrane adsorbers is that of amphiphilic carbonaceous particles (ACPs) incorporated in the polyvinylidene fluoride (PVDF) matrix. The absorbers have been successful at removing endotoxins from BSA protein solutions at >99.8% efficiency with >90 % protein recovery.140 Another study with PCL nanoparticle incor-

porated in the cellulose acetate membranes have been able to effectively remove endotoxins from water. The endotoxin binding capacity offered by the membrane adsorber was ~ 2.7×10^6 EU per mg particle compared to endotoxin binding capacity of ~ 1.4×10^6 EU per mg particle offered by PCL nanoparticles in suspension 100.

Discussion

The biopharmaceuticals industry has experienced a rapid and consistent growth over the past few years 142-145. It is predicted that half of all drugs under development will be biopharmaceuticals within the next 5-10 years 3. Developing endotoxin removal methods that are both effective and cost efficient is an ongoing challenge 45 due to the high purity required and the potential interactions present between endotoxin and target molecules. Affinity and mixed-mode chromatography are the most promising methods for a widely applicable removal method due to the highly selective interactions between endotoxins and the chosen ligand. Additional research is still required to further develop additional methods for removal and ligands that demonstrate a high affinity to endotoxins with a low toxicity and cost. There is also ongoing research to develop endotoxin-free *E. coli* strains that would eliminate the need for endotoxin removal and decrease downstream processing costs 10,146,147.

Biological techniques led the way, starting with the RPT, a crude, yet effective method of testing medicines before injecting them into humans. This was a good start, but with a detection limit of 0.5 EU/ml, and taking over two hours to perform, as well as requiring live rabbits for test subjects, it was quickly outclassed by other methods. Following close behind RPT, was LAL assay testing. This method became the industry standard in medicine and equipment testing. It still falls short of being fast enough to keep up with the modern world of testing needs, not to mention the need to move away from using horseshoe crab blood in order to protect their dwindling population. bWBA and MAT are similar to LAL in that they fall short on keeping up with the needs of the modern world. While they present attractive qualities, MAT being able to use recycled monocytes from blood banks and bWBA requiring very little preparation, they still require collection and storage of blood from living beings. This would create difficulties in collecting proper amounts of blood stores to handle testing requirements. Electrochemical techniques offer nearly endless combinations of sensor and protein-complexes, able to be designed specifically for a testing solution that could prove difficult for other tests. Optical detection methods offer incredibly high precision testing, with results ready in a matter of seconds, provided the equipment can be afforded and operated correctly. Finally, with the rise of mass-based resonance detection, the future of detection methods rely on more accurate, real-time detection, with increasingly cheap and easy to use.

There is no single purification method that fits all separation scenarios 148. The method chosen will depend greatly on the properties of the target molecule 126. Ultrafiltration is well suited for removing endotoxins from water, salts, or small molecule therapeutics, but it is not applicable to most separation scenarios. Extraction provides a high endotoxin removal efficiency for highly contaminated samples, but can possibly lead to an undesirable level of product loss. Ion Exchange chromatography provides adequate separation with acceptable product loss for molecules with a weak positive charge. Due to the specificity of the ligands, affinity chromatography and mixed-mode chromatography offer an adequate separation with high product recovery for a wide range of target molecules. Membrane adsorption offers a reduced processing time and initial cost with a high product recovery, but has a low binding capacity that limits removal efficiencies. While there is no single method that is applicable to all scenarios, ion-exchange, affinity, and mixed-mode chromatography all offer consistently high removal efficiencies and product recoveries under appropriate operating conditions. Even so, additional research is needed to develop more widely applicable and cost-effective methods that reduce product loss while meeting all governing regulations for endotoxin concentrations in biopharmaceutical products.

Conclusion

There is an increased demand for techniques capable of producing quality products at a decreased cost. This is especially true for biopharmaceuticals produced using gram-negative bacteria, where endotoxin contami-

nation is a concern. Animal-based endotoxin etection techniques will become obsolete in favor of electronic biosensors or fluorescence-based techniques. Developing endotoxin removal methods that are both effective and cost efficient is an ongoing challenge due to the high purity required and the potential interactions present between endotoxins and target molecules. Affinity and mixed-mode chromatography are the most promising methods for a widely applicable removal methods due to the highly selective interactions between endotoxins and the chosen ligand. Additional research is still required to further develop additional methods for removal and ligands that demonstrate a high affinity to endotoxins with a low toxicity and cost. These innovations will allow for an increase in product quality and yield with a decrease in manufacturing cost.

Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1: A simplified scheme of biopharmaceutical production, separation and purification steps. Biopharmaceutical manufacturing is divided into two areas: upstream fermentation or cell culture and downstream purification processes. Each area contains multiple unit operations. The primary downstream unit operation is chromatography that includes variations in modes such as affinity, cation-exchange, anion-exchange, ceramic hydroxyapatite, and hydrophobic-interaction chromatography. The process performance is mainly determined by the rate of molecule transport to the binding sites. In large chromatographic columns, small adsorbent particles provide high surface area for binding but generate a large pressure drop at high fluid velocity. On the other hand, large adsorbent particles minimize active binding site per volume as well as reduce mass transport. (Figure reproduced with permission from Jozala *et. al.* , Ref. 3)

Figure 2: (a) Schematic view of the chemical structure of endotoxin from *E. coli* . Endotoxins are lipopolysaccharides that consist of a heteropolysaccharide (O-antigen), the core oligosaccharide, and a non-polar lipid A tail. **(b)** Endotoxins form aggregates in micelle, cube, lamellar or vesicle forms exhibiting a net negative charge in pharmaceutical solutions. The negatively charged "micellar" endotoxins can be adsorbed on polycationic ligands, or the individual endotoxin monomers can be removed by hydrophobic lipid tail interactions with hydrophobic surface.

Figure 3 (a): Endotoxin induced defense mechanisms in circulating hemolymphs of horseshoe crabs. The LAL assay is designed based on the immunogenic reactions developed in the blood of horseshoe crabs. Upon

exposure to endotoxins, the electron dense large granules (L-granule) and less electron dense small granular (S-granule) amebocytes become activated by zymogen factor C.

Figure 3 (b): Coagulation cascade in horseshoe crab blood. Endotoxin activates plasma membrane-bound factor C. Factor C is a single chain glycoprotein (M.W. = 123 kDa) comprising of a heavy chain (M.W. = 80 kDa) and light chain (M.W. = 43 kDa) that plays a major key role as an activator to immune system. Upon binding with endotoxins, an autocatalytic activity triggers with the cleavage of Phe–Ile bond resulting in an activated factor C that interacts with factor B converting it into a clotting enzyme. Clotting enzyme cleaves coagulogen at two terminal of peptide C at the Arg–Lys and Arg–Gly forming insoluble coagulin gel.

Figure 3 (c): The proteolytic activity feature of the activated clotting enzyme in horseshoe crab's blood is used on synthetic chromogenic *i.e.* Gly–Arg–p-nitroaniline substrates instead of coagulogen to detect endotoxin as it separates p -Nitroaniline (p -NA). Upon addition of a chromogenic substrate, Ac-Ile-Glu-Ala-Arg-pNA, the activated protease, clotting enzyme catalyzes the release of p-nitroaniline (pNA), resulting in a yellow color that can be quantitated by measuring the absorbance at 405 nm (or absorbance at 340 nm) and extrapolating to a standard curve for correlating endotoxin concentrations.

Figure 4: (A) and (B) The design and fabrication of a new electrochemical endotoxin sensor based on a human recombinant toll-like receptor 4 (rhTLR4) and myeloid differentiation-2 (MD-2) complex. The rhTLR4/MD-2 complex, which specifically binds to endotoxin, was immobilized on gold electrodes through a self-assembled monolayer (SAM) technique involving the use of dithiobis(succinimidyl undecanoate) (DSU). **(C) – (F)** The electrochemical signals generated from interactions between the rhTLR4/MD-2 complex and the endotoxin were characterized by cyclic voltammetry (CV) and differential pulse voltammetry (DPV). **(G)** A linear relationship between the peak current and endotoxin concentration was obtained in the range of 0.0005 to 5 EU/mL with a correlation coefficient (R 2) of 0.978. The estimated limit of detection (LOD) was fairly low, 0.0002 EU/ml. The rhTLR4/MD-2 based sensors exhibited no current responses to dipalmitoylphosphatidylcholine (DPPC) bearing two lipid chains, which is structurally similar to endotoxin, indicating the high specificity of the sensors to endotoxin. Reproduced with permission from Ref. 78.

Figure 5: Assay protocol for endotoxin detection. We have developed a fluorescence-based method that measures the changes in fluorescence intensity and the corresponding endotoxin concentration. The whole process is instantaneous and can detect endotoxin as low as 0.0001 ng/ml in solutions.

Figure 6: Distinct chemical structures are seen for the removal of endotoxins. Since endotoxins are negatively charged, anion exchange ligands are employed, e.g., diethylaminoethane (DEAE), polymyxin B, histamine, histadine, poly-l-lysine, polyethylimine (pEI) and chitosan.

Figure 7: (a) PolyBall nanoparticles are synthesized using the solvent diffusion method. **(b)** PolyBalls can be lyophilized in white powder form and stored at room temperature (˜22). **(c)** PolyBalls are effective in removing >99% endotoxins (>2×10^6 EU/ml) from water (dotted line) and PBS (pH 7.4) (solid line). **(c)** Change in LPS concentrations does not compromise PolyBall's endotoxin removal efficiency.**(d)** PolyBalls efficiently remove endotoxins from a variety of protein solutions at different concentrations. **(e)** Removal of endotoxins does not affect protein recovery (>95% recovery) indicating minimal product loss and PolyBall's specificity towards endotoxins even in endotoxin mixed protein solutions.**(e)** PolyBalls can be regenerated to remove endotoxins further. Figures reproduced with permission from Ref. 97 (Razdan *et. al.*).

Figure 8: (a) PolyBall nanoparticles are embedded in a cellulose acetate (CA) biofilter. **(b)** Cross-sectional view of a CA filter without any nanoparticles (negative control) using SEM.**(c)** SEM image of a biofilter with PCL nanoparticles impregnated in it. **(d)** Our biofilter removes >99% endotoxins (solid line) while filter without PCL nanoparticles (negative control) is not as effective as the biofilter in removing endotoxins indicating the role of PCL nanoparticles in binding and removing endotoxins from solutions. **(e)** Comparison of the endotoxin removal efficiency (solid line) and protein recovery (dotted line) between our filter and other commercial endotoxin removal filters. Our filter outperforms others while removing >99% endotoxins and maintaining >95% protein recovery. Figures reproduced with permission from Ref. 97 (Razdan *et. al.*).

Table Legend

Table 1: Comparison of endotoxin removal products, their adsorption capacity, costs and regenerability

Product Name	**Maximum Endotoxin Binding capacity (EU/ml)**
Pall Acrodisc Unit with Mustang E membrane	5.0 × 105
Millipore charged Durapore cartridge membrane filters	>5.0 × 105
BioRad Proteus Endotoxin Removal Kits (Membrane based)	5.0 × 105 - 106
Sartobind Q100 membrane adsorbers (Sartorius)	1.0 × 106
Thermo scientific Pierce High capacity endotoxin removal resins	2 × 106
PCL nanoparticles	1.45 × 106
PCL nanoparticles incorporated membrane	2.8 × 106

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