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Sutapa Barua ORCID iD: 0000-0002-2385-0222

Current Technologies to Endotoxin Detection and Removal for Biopharmaceutical Purification

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

Department of Chemical and Biochemical Engineering

Missouri University of Science and Technology, Rolla, MO 65409

Abstract

Endotoxins are the major contributors to the pyrogenic response caused by contaminated pharmaceutical products, formulation ingredients, and medical devices. Recombinant biopharmaceutical products are manufactured using living organisms, including gram-negative bacteria. Upon the death of a gram-negative bacterium, endotoxins (also known as lipopolysaccharides; LPS) in the outer cell membrane are released into the lysate where they 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

[1] To whom correspondence should be addressed. Email: baruas@mst.edu. Department of Chemical & Biochemical Engineering, 110 Bertelsmeyer Hall, 1101 N. State Street, Rolla, MO 65409-1230

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systems used in manufacturing. The manufacturing process is, therefore, in critical need of methods to reduce and remove endotoxins by monitoring raw materials and in-process intermediates at critical steps, in addition to final drug product release testing. This review paper highlights a discussion on three major topics about endotoxin detection techniques, upstream processes for the production of therapeutic molecules, and downstream processes to eliminate endotoxins during product purification. Finally, we have evaluated the effectiveness of endotoxin removal processes from a perspective of high purity and low cost.

Keywords: Biosensors, Biopharmaceutical, Chromatography, Downstream purification, Endotoxin, Endotoxin detection, Endotoxin removal, Gram-negative bacteria, Lipopolysaccharides (LPS), Protein purification

Introduction

Pharmaceutical manufacturing deals with the selection and optimization of the cell source, media composition, and physicochemical bioreactor operating conditions to maximize the culture yield and productivity (**Fig. 1**) (Gronemeyer, Ditz, & Strube, 2014; Hanke & Ottens, 2014; Jozala et al., 2016). *Escherichia coli* is a cost-effective and attractive choice for producing recombinant proteins due to their rapid growth, minimal nutritional requirements, high product yield, and transformation capability (Baeshen et al., 2015; Berlec & Štrukelj, 2013; W. Chen & Qin, 2011; Gonçalves, Bower, Prazeres, Monteiro, & Prather, 2012; Mack, Brill, Delis, & Groner, 2014; McKay et al., 2017; Miyagi et al., 2002; Wilding et al., 2019). With the millions of strains of bacteria, and gene-altering technology steadily improving the possibilities are endless (X. Liu, Ding, & Jiang, 2017). One of the most recognizable products derived from genetically engineered *E. coli* is the hormone insulin. Before being produced using bacteria, insulin was originally extracted from dogs and later pigs that was an inefficient process, making the product rare and expensive (Rosenfeld, 2002). The advent of *E. coli* -produced insulin such as recombinant human insulin (Humulin) drastically increased its availability for diabetics (Hu et al., 2014). The production of large, complex molecules (*e.g.*, therapeutic monoclonal antibodies) that require specific posttranslational modifications are produced in mammalian cell lines. Although a wide variety of platforms including *E. coli*, mammalian cell lines or other expression systems have been used

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extensively, endotoxin removal from finished products is a major challenge for biopharmaceutical manufacturers (Aida & Pabst, 1990; Anspach, 2001; Gorbet & Sefton, 2005; Magalhães et al., 2007; Ongkudon, Chew, Liu, & Danquah, 2012; Dagmar Petsch & Anspach, 2000; Schwechheimer & Kuehn, 2015). A frequent source of endotoxin contamination is the water system that grows biofilms associated with viable bacteria on the walls of water distribution pipelines (Collentro, 2012).

Endotoxins are present in the outer cell wall of gram-negative bacteria that contribute to the organization and stability of the membrane and are released into the circulation upon disruption of the membrane by cell death (Aida & Pabst, 1990; Anspach, 2001; Gorbet & Sefton, 2005; Jang et al., 2009; Magalhães et al., 2007; Ongkudon et al., 2012; Dagmar Petsch & Anspach, 2000). It consists of three regions: a core polysaccharide, a long chain polysaccharide, and a non-polar lipid called Lipid A (**Fig. 2**) (Anspach, 2001; Dullah & Ongkudon, 2017). 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 (Anspach, 2001; Ongkudon et al., 2012). The core polysaccharide and the O-antigen are both hydrophilic while Lipid A is hydrophobic. The toxicity of endotoxin is associated with Lipid A (Almeida, Almeida, Fingola, & Ferraz, 2016; Amini Tapouk et al., 2019; Raetz & Whitefield, 2001). Lipid A triggers the production of pro-inflammatory cytokines (Brent, 2017; Daneshian, Guenther, Wendel, Hartung, & von Aulock, 2006) and activation of the coagulation cascade (Abbass, 2011; Anspach, 2001) which can lead to sepsis and septic shock (Angus & van der Poll, 2013; Billiau & Vandekerckhove, 1991; Das, Kumar, & Swain, 2014; Johnston & Knight, 2012; Ramachandran, 2014). A pyrogenic reaction can be caused by as little as 1 ng of endotoxin per kilogram of body weight per hour (Aida & Pabst, 1990; Anspach, 2001; Anspach & Petsch, 2000; Gorbet & Sefton, 2005; Magalhães et al., 2007; Ongkudon et al., 2012; Dagmar Petsch & Anspach, 2000). 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 (Schaumberger, Ladinig, Reisinger, Ritzmann, & Schatzmayr, 2014; Szermer-Olearnik & Boratyński, 2015). For intravenous applications, a maximum of 5 EU per kilogram of body weight can be administered to a patient per hour (de Vries et al., 2018; Hirayama & Sakata, 2002; Szermer-Olearnik &

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Boratyński, 2015), but acceptable concentrations vary depending on the required dose (S. Liu et al., 1997; Rosano & Ceccarelli, 2014).

Endotoxin is highly stable and is resistant to destruction by heat or pH (Almeida et al., 2016; Hirayama & Sakata, 2002; Miyamoto, Okano, & Kasai, 2009). Additionally, endotoxins may form stable interactions with target therapeutic compounds that further complicates separations (Branston, Wright, & Keshavarz-Moore, 2015; Dullah & Ongkudon, 2017; Kang & Luo, 2000). Downstream processing of recombinant protein products accounts for ~45-92% of the total manufacturing costs (Saraswat et al., 2013; Straathof, 2011; Wilding et al., 2019). In addition to the downstream processing, the detection of endotoxins is critical for the safety of patients across the globe who rely on the purity of treatments prescribed (Brent, 2017). 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 are still in use today, although they are being phased out by newer, more accurate testing methods such as biosensors as described after 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 (Dullah & Ongkudon, 2017; Maloney, Phelan, & Simmons, 2018). This method works on the principle that rabbits and humans share similar fever patterns under the influence of endotoxins. It is determined that a temperature increase of 0.5°C over 180 min after injection constituted a fever (Gimenes, Caldeira, Presgrave, de Moura, & Boas, 2015). As rudimentary as the technique seems, the limit of detection (LOD) is 0.5 EU/milliliter (EU/ml) that was considered accurate at the time of this method development in 1912 (Hoffmann et al., 2005). This

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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 see the test subject showing symptoms of infection. However, 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 (Studholme et al., 2019). The scientific world is generally moving away from living test subjects as 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 (Vipond, Findlay, Feavers, & Care, 2016).

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 (Jin et al., 2018; Ong et al., 2006). The extract is used in one of three ways: the gel-clot method, the chromogenic method, and the turbidimetric method. The gel-clot method involves mixing equal parts of extract and sample. If a gel has formed and the mixture remains intact at the bottom of the tube, the test shows positive (Sakai et al., 2004). This means the sample has at least enough endotoxins to trigger a positive reaction (**Fig. 3**) with a detection limit of 0.03 - 0.06 EU/ml. The chromogenic and turbidimetric methods 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 (Dolejš & Vaňousová, 2015). The turbidimetric method is similar to the chromogenic method but instead measures the turbidity of the solution (Joiner, Kraus, & Kupiec, 2002). The rates of turbidity and absorbance change are proportional to the endotoxin concentration. All three methods rely on the same protein, Factor C coagulation cascade found in horseshoe crabs' blood (**Fig. 3c**). The endotoxin activates Factor C which goes onto activate Factor B following the formation of a clotting enzyme (Iwanaga, 2007; Muta et al., 1991). In the gel clot and turbidity assays, the clotting enzyme transforms coagulen into coagulin creating the gel and the clouding agent, respectively. The chromogenic method follows the same pathway, but

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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 that is directly proportional to the endotoxin concentration in solutions (Dullah & Ongkudon, 2017). There are three types of chromogenic assays: two endpoint assays and a kinetic assay (Dawson, 1995; Lindsay, Roslansky, & Novitsky, 1989). In endpoint chromogenic assays, the reaction of the pNA release is stopped by adding acid. The difference between the two endpoint chromogenic assays is the conjugation of a di-azo compound with pNA that reads absorbance at 540 nm compared to 405 nm for pNA using the second method. The change in absorbance avoids interferences for the samples absorbing light at 405 nm. The kinetic chromogenic assay measures the absorbance of pNA at regular time intervals throughout the test. Like the chromogenic method, the turbidimetric method has two variations: an endpoint method and a kinetic method. In the endpoint turbidimetric method, samples are measured once within an incubation time. In contrast, in the kinetic turbidimetric method, optical density is measured throughout the assay. All these tests are widely accepted as the official endotoxin test in the pharmaceutical community (L. Chen & Mozier, 2013) and have different limits of detection (**Table 1**). Every drug and medical device that is tested by the US Food and Drug Administration (FDA) must undergo and pass a LAL test (Hurley, 1995; Mohanan, Banerjee, & Geetha, 2011). As previously mentioned, the LAL method is more accurate than RPT, however, it still has several drawbacks. LAL assay gives both false-negative and false-positive results. False-negative results are observed when endotoxins are masked by product formulation matrices such as buffer constituents (*e.g.*, citrate and phosphate), cell culture medium compositions and surfactants (*e.g.*, polysorbate 20 and polysorbate 80), or by aggregation with products (Nakamura, Tokunaga, Morita, & Iwanaga, 1988; Schneider, 2016; Schwarz et al., 2017). The interfering agents create a masking layer surrounding endotoxin molecules making them inaccessible to react with LAL reagents, a phenomenon known as low endotoxin recovery (LER) resulting in false-negative results (FDA, 2012; Nakamura et al., 1988; Schneider, 2016; Schwarz et al., 2017). LAL assay also causes a false-positive reaction by triggering the protease enzyme Factor G pathway by (1→3)-ß-D-glucans, a major cell membrane component of pyrogens and form the same coagulin protein end product as found in LAL reactions (Elin & Wolff, 1973; Morita, Tanaka, Nakamura, & Iwanaga, 1981). The protein cascades the LAL assay relies on is disrupted in samples with free metal ions, proteins, peptides and polymers that bind with the active site of endotoxins

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neutralizing its biological activity (Bhunia, Ramamoorthy, & Bhattacharjya, 2009; Kushibiki et al., 2014; Mares, Kumaran, Gobbo, & Zerbe, 2009; Ohno & Morrison, 1989). Following the discovery of Factor C as an endotoxin-activated portion of the protein cascade, attempts have been made to replace the conventional LAL test, with one using recombinant Factor C. As technology improves, alternative techniques are being developed to ease the pressure on the horseshoe crab.

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 (J. Ding, Navas, & Ho, 1995; Iwanaga, 1993; Levin & Bang, 1968; Maloney et al., 2018; Nakamura, Morita, & Iwanaga, 1986; Tokunaga, Nakajima, & Iwanaga, 1991). In this assay, the binding of endotoxin activates synthetic rFC molecules that cleave a fluorescein substrate, amino-methylcoumarin resulting in the generation of a fluorogenic compound. The fluorescence is measured using 380/440 nm excitation/emission in the absence and presence of endotoxins. The difference in fluorescence is proportional to endotoxin concentrations in the sample. rFC is specific to endotoxin detection eliminating the dependence on nonspecific glycan-binding like that in a LAL assay avoiding false-positive results (J. L. Ding & Ho, 2001). The enzymatic sensitivity range to endotoxin is 0.05-500 EU/ml (J. Ding et al., 1995). Despite its lower limit of detection under laboratory conditions, the rFC assay is prone to contamination in field environments that severely compromises its analytical utility (Barnett, Wadham, Jackson, & Cullen, 2012) and the assay depends on the stability of rFC proteins. A comparison of rFC with various LAL assays is summarized in **Table 1**. While the assays are comparable in terms of efficiency, the selection of a suitable assay depends on sample types, detection limits (sensitivity), detection time, the convenience of experiments, and product costs. The costs of LAL and rFC assays are comparable being ~$490 and $435, respectively to perform ~100 tests. Although rFC assay is a fluorescence-based assay that requires a fluorometer and may not be as cost-effective as quick absorbance measurements using LAL assay (Reynolds et al., 2002; Thorne et al., 2010), the availability of a fluorescence microplate reader accommodates both detection modes.

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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 (Gall, Nielsen, Yu, & Smith, 2006). In response to endotoxin, white blood cells in the bovine whole blood produce the cytokine Prostaglandin E_2 (PGE_2) in an inflammatory response, similar to that of humans (Wunderlich, Schumacher, & Kietzmann, 2015). The production of PGE_2 is directly proportional to an increase in endotoxin concentration. According to several studies, the test can accurately detect endotoxins at concentrations of close to 0.25 EU/ml, whereas the concentration at which humans display symptoms of endotoxin exposure typically occurs around 0.30 EU/ml (Schindler et al., 2006; Wunderlich et al., 2015). This level of accuracy is attractive looking to move away from LAL and RPT testing. The test also is easy to perform and takes few preparational steps (Dullah & Ongkudon, 2017). The bWBA test is not without its limitations. The major limitation associated with bWBA is the LER over time because of the intermolecular interactions of interfering assay reagents, non-specific binding of endotoxins with other blood cell receptors and masking of endotoxin monomers by assay reagents. The whole blood needed for the tests can only be obtained from very young calves which makes it difficult to amass in vast quantities. Furthermore, due to cultural and religious practices, certain countries will not permit the collection or use of bovine blood.

Monocyte Activation Test (MAT)

The Monocyte Activation Test (MAT) has been in development since 1995 (Vipond et al., 2019). The MAT is the human *in vitro* alternative to the RPT and allows the detection of the full range of pyrogens, including endotoxins and non-endotoxin pyrogens. By putting the sample to be tested in contact with human monocytic cells, it mimics the pyrogenic reactions in the human body. In the presence of pyrogens, monocytes are activated and produce several types of cytokines including interleukin-1β (IL-1β) and interleukin-6 (IL-6). The commercially available MAT kit involves using cryopreserved monocytes in human blood to test for a reaction to endotoxins. The response to endotoxin is determined by the measurement of the induced pro-inflammatory cytokines using an enzyme-linked immunosorbent assay (ELISA) (Liebers, Stubel, Düser, Brüning, & Raulf-Heimsoth, 2009; Nik Mansor et al., 2018). The

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cytokine released by monocytes in the presence of endotoxins is attached with a primary antibody and linked to a secondary antibody with avidin-conjugated horseradish peroxidase (Avidin-HRP) enzyme that metabolizes tetramethylbenzidine (TMB) substrate and develops a blue-green to yellow color product (Lequin, 2005). The absorbance of color density is measured at 450 nm by a spectrophotometer, similar to a chromogenic LAL test (Hoffmann et al., 2005). The MAT assay has the added benefit of testing all pyrogens and inflammatory materials that would prove harmful to human patients (Hasiwa et al., 2013; Stang et al., 2014). It avoids animal testing, has a LOD of 10 EU/ml and is used in cryogenically-preserved human blood without sacrificing accuracy (Koryakina, Frey, & Bruegger, 2014; Liebers et al., 2009; Spreitzer, Löschner, Schneider, Hanschmann, & Montag, 2008). 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 (Koryakina et al., 2014). 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 testing, inconsistencies can arise when using large quantities of blood (Utescher, Buosi, Botosso, & Quintilio, 2018; Vipond et al., 2019). The most important limitation for the MAT is the short half-life (< 2 h) of viable monocytes in human blood *in vitro*. An alternative endotoxin ELISA kit such as the competitive ELISA (cELISA) is available that uses a microtiter well plate pre-coated with an anti-endotoxin primary antibody (Kohl & Ascoli, 2017). Endotoxin containing samples or standards are added to the wells along with a fixed quantity of biotinylated detection antibody that competes for limited binding sites on the immobilized anti-endotoxin antibody. Avidin-HRP conjugate and TMB are used like that in ELISA to generate and measure color changes from blue to yellow at 450 nm. Both bWBA and MAT assays may not work when exposed to pyrogen like products such as cytokines (*e.g.*, interleukin IL-1β, IL-6, and Tumor Necrosis Factor-α, TNF-α). Because the product interferes with the endpoint of both assays that also measure the release of cytokines in supernatants. The LAL assay may be used as an alternative test to evaluate endotoxin activity in pyrogen like products.

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Biosensor Technique

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 Technique

The majority of electrochemical biosensors are based on a principle called Electrochemical Impedance Spectroscopy, or EIS. Performing an EIS requires electrodes to 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 (Honeychurch, 2012). The electrodes are coated in metal, to reduce electric resistance. Proteins that are highly selective to endotoxin components are bound to these electrodes such that if 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 (Priano, Pallarola, & Battaglini, 2007; Syaifudin et al., 2011). 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.* (Yeo et al., 2011) 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 (**Fig. 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 (Nik Mansor et al., 2018). The study also reported that this particular biosensor had high specificity towards endotoxins, to prevent false-positive results. The sensor had a LOD of 0.0002 EU/ml that was lower than the standard LAL test limit of 0.03 EU/ml. A major limitation of this system is a single-use electrode once endotoxins are bound to TLR4-MD-2 complexes. Metal complexes immobilized upon a gold electrode had been used and were able to detect endotoxins at concentrations as low as 0.001 EU/ml (Cho et al., 2012). Porous silicon membranes (pSim)

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based electrochemical biosensors comprise of an array of nano-channels that were modified using Polymyxin-B (PMB), with a strong affinity to endotoxins. It showed the LOD of 18 EU/ml. These sensors showed an ability to detect endotoxins from various bacterial strains like *E. coli and S. typhimurium* and all this was done in a label-free manner (Reta, Michelmore, Saint, Prieto-Simon, & Voelcker, 2019). Studies also reported highly sensitive peptide modified gold electrode-based electrochemical biosensors which were used for endotoxin detection with a LOD of 0.04 EU/ml (T. Liu et al., 2017). This method was faster, more accurate, and in most cases, more cost-effective than biological-based techniques (Heras, Pallarola, & Battaglini, 2010). 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 (Richter, 2004). They work based on the same principle of EIS, wherein the concentration of the analyzed sample has a linear relationship with the current measured. This method can use pre-made, disposable testing strips for fast, cost-efficient testing (Alahi & Mukhopadhyay, 2017). Potentiometric methods are worth noting because although their LODs are relatively high, 1-5 EU/mL, they were the first biosensor to be able to detect endotoxins in real-time (Honeychurch, 2012; Inoue, Ino, Shiku, & Matsue, 2010). The methods in which the electrodes are created, and how they are measured and utilized, are more complicated and labor-intensive than the biological methods (Yamamoto et al., 2000). They require more sophisticated personnel and equipment to be run effectively than RPT or LAL tests (Shen, Zhuo, Chai, & Yuan, 2015).

Optical Techniques

One such example is that of a 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 endotoxin selective probes of biosensors. The LC-based aptamer optical biosensors have linear endotoxin detection ranging from 0.05 to 1000 EU/ml and a LOD of 5.5 EU/ml. The biosensors have negligible cross-binding reactivity with the biomolecules, thus maximizing their recovery (An & Jang, 2019). Broadly, these optical techniques can be divided into three distinct categories: luminescence, surface plasmon resonance (SPR), and electrochemiluminescence that share a similar characteristic of relying on visual changes.

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Fluorescence and Luminescence Techniques

The bioluminescence method is based on the same principle of the LAL assay except for the endpoint material (pNA) of LAL tests is used as the starting material for the mutant firefly luciferase (Noda, Goto, Murakami, Ahmed, & Kuroda, 2010; Paul, Raichur, Chandrasekaran, & Mukherjee, 2016). Luciferin-modified pNA had been designed as the substrate for a mutated version of the North American luciferase (*Photinus pyralis*) that quickly and precisely identified solutions containing endotoxins by a bioluminescence reaction (Noda et al., 2010). The reaction generated high luminescence intensity showing a luminescence 20 times as intense as the standard, wild-type luciferase (Fujii et al., 2007). The lowest endotoxin concentration recorded using wild-type luciferase is 0.01 EU/mL, while the LOD of this mutant-type luciferase bioluminescence technique is 0.0005 EU/ml (Dullah & Ongkudon, 2017). The detection time is 15 min that is rapid in comparison to the LAL gel-clot techniques estimated required time of 138 min to nearly 1.5 h (Ostronoff & Lourenço, 2015). Experiments were performed using a peptide biosensor and attached fluorescent probes, fluorescein-maleimide (F5M), and tetramethylrhodamine-5-maleimide (TMR5M) (Liebers et al., 2009). 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, respectively was used to quantify the presence and removal of endotoxins from biological solutions (**Fig. 5**) (Barua, 2018; Bromberg et al., 2010; Donnell, Lyon, Mormile, & Barua, 2016; Razdan, Wang, & Barua, 2019). BODIPY is a highly lipophilic and intensely fluorescent boron dipyrromethene fluorophore that is quenched in the presence of endotoxins after being exposed to the blue light (~405 nm) and returns to the ground state without emission of a photon (Donnell et al., 2016; Razdan et al., 2019; Wood, Miller, & David, 2004). The difference in the fluorescence of BODIPY which indicates the degree of quenching of the dye was plotted against the amount of the corresponding endotoxin to generate standard curves and measure endotoxin concentrations precisely (R^2>0.99). Endotoxin detection had also been investigated using Alexa Fluor-labeled fluorescent endotoxin with excitation and emission wavelengths of 490 and 525 nm, respectively (Prasad, Sachan, Suman, Swayambhu, & Gupta, 2018). In this study, C-18 acyl chain modified Fe_3O_4/Au/Fe_3O_4 nanoflowers were used for simultaneous capture and detection of endotoxins in water with a LOD of 10 EU/ml (Prasad et al., 2018).

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Surface Plasmon Resonance (SPR) and Mass-based Techniques

Zhang *et al.* showed a smartphone biosensor platform using SPR. The disposable sensor chip utilized the smartphone's built-in flash as a light source and a compact diffraction grating and spectra dispersive unit (J. Zhang et al., 2018), but this technology was still in development. Recent publication regarding antibiotic mediated plasmonic biosensors for endotoxin detection has shown a LOD of 40 EU/ml (Manoharan, Kalita, Gupta, & Sai, 2019). The plasmonic biosensor was based on a facile U-bent fiber optic probe (UFOP) technology that utilized octadecyl trichlorosilane (OTS) on the surface of optical fiber probes to hydrophobically entrap endotoxin from aqueous solutions. The binding of endotoxins had been monitored in real-time by measuring the change in the refractive index (RI) in the evanescent layer (Manoharan et al., 2019). To add specificity and signal amplification, the bound endotoxins had been tagged with antimicrobial PMB conjugated gold nanoparticles (PMB-AuNPs) in a sandwich format that worked as an absorbance-based fiber optic biosensor with a LOD of 40 EU/ml and an assay time of 1 h.

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 pyrogens, not exclusively endotoxins, as well as being able to detect endotoxins in real-time within human blood plasma (Sheikh, Blaszykowski, Romaschin, & Thompson, 2016). EMPAS uses ultra-high frequency acoustic wave sensing based on an ultrathin, oligoethylene glycol-based mixed surface platform coated on piezoelectric quartz discs. The glycol end on the surface of quartz is functionalized with PMB, a cyclic peptide antibiotic that shows a high affinity for endotoxins and hence, is used as a biosensor assay for endotoxin detection. Incubation of endotoxin-spiked whole blood with PMB-bead chemistry results in the EMPAS resonant frequency shift (Δf) as a function of endotoxin concentration from 30-60 EU/ml (Sheikh et al., 2016). Another mass-based method is magnetoelasticity that functions by placing sensors directly on 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 pyrogen, like ENPs (Li et al., 2010). When bacteria such as *Salmonella typhimurium* binds with the surface of sensors, the mass of the sensor increases, resulting in a decrease in the sensor's resonant frequency. The resonant frequency of the sensors has been measured wirelessly and compared with the

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initial resonant frequency. A control sensor without phage does not show any shifts in the resonance frequency and is used to compensate for environmental effects and nonspecific binding (Li et al., 2010). The resonant frequency change of sample measurement sensors is reported to be statistically different from that of control sensors down to 500 colony forming units/ml, the LOD for the work. The number of cells bound on the sensor surface has been imaged using scanning electron microscopy (SEM) that has verified the measured resonant frequency changes due to cell binding on the sensor surface. The total assay time of the presented methodology has been reported ~30 min. While the disadvantages of phage coated magnetoelastic sensors are non-regeneration of the surface, non-specificity, and interferences from analytes, it may be advantageous as a disposable sensor due to low cost.

Techniques for Downstream Removal of Endotoxins

The downstream process for pharmaceutical manufacturing comprises three steps: (1) initial recovery by extraction or isolation, (2) purification, and (3) polishing (Gronemeyer et al., 2014; Hanke & Ottens, 2014; Jozala et al., 2016). Endotoxin removal presents a unique challenge that forms stable interactions with themselves and possibly with target therapeutics.

Ultrafiltration

A single endotoxin molecule in its monomeric form has a molecular weight of 10-30 kDa (Jang et al., 2009) depending on the core polysaccharides and oligosaccharide chains that aggregate and form micelles and vesicles with molecular weights above 1000 kDa (Abbass, 2011) and diameters up to 0.1 μm (Anspach, 2001). 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 molecules in solution, interactions with target molecules, target protein concentrations, and the presence of detergents. The effect of protein concentration on the endotoxin removal efficiency using ultrafiltration membranes has been explored (El-Moghazy, 2011; Jang et al., 2009). Ultrafiltration membrane of 100 kDa molecular weight cut off (MWCO) was used to filter endotoxin contaminated protein solutions of >100 kDa that was present in the retentate leaving relatively smaller sized endotoxin monomers in the permeate. The % endotoxin removal in the filtration

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permeate 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 solutions were made, the higher was the rate of endotoxin removal due to the shift in equilibrium from endotoxin aggregates in micelles and vesicles into monomers in dilute solutions and passing the endotoxin monomers through membranes into the permeate.

The effect of detergent concentrations on the interaction between endotoxin molecules has been studied contributing to efficient endotoxin removal. Multiple Tween 20 concentrations of 0.0%, 0.5%, 1.0%, and 2.0% have been added to protein solutions to calculate the respective removal efficiency (Jang et al., 2009). An increase in the Tween 20 concentration led to an increase in the passage of endotoxin into the permeate and thus removing endotoxin from proteins (Jang et al., 2009). These results demonstrate that the presence of detergents decreases the size distribution of endotoxin aggregates. As the detergent concentration is increased, the equilibrium shifts from micelles and vesicles to monomers (El-Moghazy, 2011; Jang et al., 2009). The addition of detergent disrupts any bonding between endotoxin and the protein of interest that allows endotoxin to pass through filter but retain the proteins for recovery from the filter retentate.

Ultrafiltration is used to separate endotoxins from small target therapeutic drug molecules. For example, ultrafiltration was utilized to separate endotoxin aggregates from BMS-753493, a small drug molecule with a molecular weight of 1.57 kDa (de Mas, Kientzler, & Kleindienst, 2015). Two membrane sizes of 3 and 10 kDa were used to remove endotoxins from BMS-753493 which was smaller in size than the membrane pore size, and therefore, permeated through the membrane retaining endotoxins in the filtrate. Both membrane sizes were effective in reducing the endotoxin concentration to <0.03 EU/ml, but compared to the 3 kDa, the 10 kDa membrane had higher drug yield of ~95%, while the 3 kDa membrane showed ~55% loss of BMS-753493 (de Mas et al., 2015). The main limitation associated with ultrafiltration is its limited endotoxin removal efficiency for molecules that are magnitudes smaller than endotoxin aggregates. This method is best suited for removing endotoxins from water, salts, and small molecule therapeutics that do not have an affinity for endotoxins.

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Extraction

Solvent extraction is used to separate endotoxins from target therapeutics based on their relative solubilities in two immiscible liquids. Endotoxin forms partition in the organic phase, while hydrophilic target molecules remain in the aqueous phase. Endotoxin has been effectively removed from T4, HAP1, and F8 bacteriophages using 1-octanol with endotoxin removal efficiencies varying between 64 - 99.9% (Szermer-Olearnik & Boratyński, 2015). Additional processing is required to remove any trace quantities of 1-octanol present in the aqueous phase because the presence of 1-octanol causes high background noises reacting with LAL reagents (Szermer-Olearnik & Boratyński, 2015). Even though the solvent extraction technique gives high endotoxin removal from various therapeutics solutions, the product yield is significantly low varying between 30-60% (Szermer-Olearnik & Boratyński, 2015).

Two-phase extraction using detergent Triton X-114, a non-ionic surfactant (Szymczyk & Taraba, 2017), has been explored to remove endotoxins from target therapeutics. Endotoxin has been 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 (Lopes et al., 2010). Endotoxins are partitioned in the detergent phase, while target therapeutics are partitioned in the aqueous phase. Endotoxin removal efficiencies using Triton X-114 range between 45-99% (Lopes et al., 2010). In addition to high endotoxin removal, Triton X-114 results in high product recovery of >80% (Dagmar Petsch & Anspach, 2000). Triton X-114 isothermal extraction using sodium dodecyl sulfate (SDS) has been very effective in removing endotoxins from pDNA with residual endotoxin concentration of ~16 EU/ml. The pDNA recovery is reported >80%. While isothermal extraction has been proven effective for plasmid purification, this method is not applicable for the removal of endotoxins from protein solutions because SDS completely denatures proteins causing significant changes to protein conformation (Ma, Zhao, Du, Tian, & Li, 2012). One major disadvantage of temperature transition extraction using Triton X-114 is that the repeated heating and cooling degrades therapeutic products (Ma et al., 2012). Extraction processes provide a rapid separation that is easily scalable and can achieve high removal efficiencies, especially with high initial concentrations (Cunha & Aires-Barros, 2002; Lopes et al., 2010; Szermer-Olearnik & Boratyński, 2015). However, the final endotoxin concentrations in the aqueous

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phase for both solvent extraction and Triton X-114 extraction have remained above desired specifications, meaning additional processing is required.

Ion Exchange Chromatography

Anion exchange chromatography can be used to separate negatively charged endotoxins 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 (Pihlasalo, Auranen, Hänninen, & Härmä, 2012). The pI of endotoxin is ~2 (Anspach, 2001; R. H. Chen et al., 2009; Dagmar Petsch & Anspach, 2000), indicating negative charges under conditions (pH>2) typically encountered during chromatography. At pH > 2, the target protein exhibits a net positive charge, is repelled by a positive stationary phase and leaves the column at a lower velocity than negatively charged endotoxins interacting with the stationary phase (Fekete, Beck, Veuthey, & Guillarme, 2015; Janson & Janson, 2011). 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 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 resin, the protein yield is low (R. H. Chen et al., 2009). Anion exchange chromatography is not ideal for removing endotoxins from negatively charged target molecules, such as pDNA and acidic proteins (Černigoj et al., 2013; Diogo, Queiroz, & Prazeres, 2005).

To lessen undesirable interactions, the effects of resin volume, contact time, and the pH of protein solutions on the endotoxin removal efficiency have been explored for therapeutic antigens such as NY-ESO-1, Melan-A, and SSX-2 (R. H. Chen et al., 2009). An increase in resin volume and endotoxin-resin contact time decreased endotoxin concentrations in the flow-through. An endotoxin concentration of ~400 EU/ml was obtained in the flow-through, and a protein recovery of >80 % was obtained consistently at almost all resin volumes (R. H. Chen et al., 2009). 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 endotoxin-protein interactions, the solution pH should be higher than the protein's pI giving the protein a strong positive charge. Melan-A exhibited a strong ionic interaction with endotoxins below its pI 8.7 causing endotoxins to leave the column with the target protein. To remedy this, pHs of 7.9,

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8.4, 8.9, and 9.2 were tested which exhibited endotoxin concentrations of 1400, 1800, 600 and 500 EU/ml, respectively in the flow-through (R. H. Chen et al., 2009). As the pH was increased above the protein's pI, the endotoxin concentration decreased dramatically with no significant impact on the protein yield. In general, ion-exchange chromatography can achieve an endotoxin reduction of five orders of magnitude for concentrated solutions (>1,000 EU/ml), or three to four orders of magnitude from dilute endotoxin solutions (<100 EU/ml) (Anspach, 2001). The resin involved with an ion-exchanger can be regenerated by washing with detergents to separate endotoxins from the resin surface and by additional washing steps (Ritzén, Rotticci-Mulder, Strömberg, & Schmidt, 2007).

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 (Hage, 1999). Because of the specificity of the ligand, there is little to no product loss during separation (Anspach & Petsch, 2000). 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 applies to a wide range of target molecules, including pDNA and proteins (Guo, Shang, Yu, & Zhou, 1997; Stadler, Lemmens, & Nyhammar, 2004).

It is important to note that the exact structure of endotoxins varies between bacteria based on the core polysaccharides and the long-chain polysaccharide. For this reason, ligands are typically designed to interact (Anspach, 2001) with the most conserved section (Abbass, 2011; Almeida et al., 2016; Dullah & Ongkudon, 2017; Wei et al., 2007) of the endotoxin molecule, Lipid A, through hydrophobic (R. H. Chen et al., 2009) and electrostatic interactions (Anspach, 2001). Common ligands used in affinity chromatography include deoxycholic acid, dimethylamine ligands, histidine, PMB, and polycationic ligands (Ongkudon et al., 2012; Serdakowski London, Kerins, Tschantz, & Mackay, 2012). Hydrophobic polymer nanoparticles are explored for removing endotoxins from water and protein solutions (Donnell et al., 2016; Razdan et al., 2019).

One of the most commonly used ligands is PMB, a cyclic lipopeptide with a high affinity for endotoxins (**Fig. 6**). As a ligand, PMB induces the dissociation of endotoxin aggregates (Ryder, Wu,

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McKelvey, McGuire, & Schilke, 2014) and binds to the Lipid A section of endotoxins (Issekutz, 1983) through hydrophobic interactions (Srimal, Surolia, Balasubramanian, & Surolia, 1996). 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 (McAllister, Hixon, Schwartz, Kubitz, & Janda, 2007). 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 (Anspach, 2001). 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 (Almeida et al., 2016). 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 (Rustici et al., 1993).

The nitrogenous bases adenine, cytosine, histidine and histamine all display an affinity for endotoxins. Of these, histamine and histidine are equally as effective as PMB and have been successful with separating endotoxins from albumin, insulin, lysozyme, myoglobin, and others. However, histamine is biologically active and may create an immunogenic response in the body (Anspach, 2001). Histidine-tag purification of a recombinant protein uses immobilized metal affinity chromatography (IMAC) in which transition metal ions are immobilized on a resin matrix using a chelating agent (*e.g.*, iminodiacetic acid). The commonly used metal ions are Ni^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} that bind histidine-tag proteins with a high affinity. The proteins are eluted usually using imidazole to remove bound proteins from the IMAC column. However, the purification of histidine-tagged proteins may be challenging due to the competition between endotoxins and proteins for the same binding sites to histidine causing product contamination (Bornhorst & Falke, 2000; Dagmar Petsch & Anspach, 2000; Saraswat et al., 2013). Two 10 consecutive histidine residues were incorporated at two terminals of amine and carboxyl residues in a peptide aptamer sequence for the purification of aptamers from endotoxins (Mack et al., 2014). It was observed that the aptamer without histidine tags removed 10-fold more endotoxins than that of histidine-tagged aptamers, indicating endotoxins retained in the Ni^{2+} chromatography column. To cleave histidine tags from aptamers, an

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enzymatic reaction was used using thrombin followed by the second round of Ni^{2+} chromatography that retained histidine tags in the column and eluted aptamers with the flow-through. This leads to additional costs for product validation and more chromatographic steps to purify target molecules. Histidine may work well for small sample volumes with a limited reduction of endotoxins, however, at the expense of large sample volumes, product losses cause low yield (Anspach, 2001; Wei et al., 2007). Alternatives to overcome the limitation of using histidine tag is the fusion of a specific binding domain to the target protein as an affinity tag. Glutathione *S*-Transferase (GST) and maltose-binding protein are the tags with specific affinities to glutathione, Ni(II)-nitrilotriacetic acid (NTA) and amylose, respectively (Arnau, Lauritzen, Petersen, & Pedersen, 2006; Cheung, Wong, & Ng, 2012; Pina, Lowe, & Roque, 2014).

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 (Anspach, 2001). While recombinant protein purification is primarily based on the use of tags, tag-free alternatives have emerged as a convenient and popular approach because of less processing time. Such an example is the purification of PspA4Pro protein with one step by washing contaminant proteins using a cationic detergent, cetyltrimethylammonium bromide (CTAB), centrifugation or cryo-precipitation of endotoxins in the precipitate, and recovery of PspA4Pro protein in the supernatant (Figueiredo et al., 2017). Comparison of CTAB wash or cryo-precipitation with ion-exchange chromatography shows higher protein recovery (~92% and 47.8% in CTAB and cryo-precipitation, respectively) than 35-62% using chromatography. The % endotoxin removal were 96.5%, 99.9% and 99.5-99.9% for CTAB, cryo-precipitation and chromatography, respectively. This new strategy does not require the use of affinity tags or large buffer volumes, thus reducing the overall costs of protein purification.

Cost-effective ligand and its binding capacity are key factors for endotoxin removal in a purification process. Poly-ε-lysine and PMB are two commonly used ligands in protein purification. **Table 2** provides a reference list of these ligands with regards to endotoxin binding capacity, protein recovery, regenerability and cost that are commercially available for use in several different product names. Additionally, the contact time required between the solution and ligand affects the investment cost. A process with a high contact time requires a larger column and therefore a greater initial investment.

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The pore size of the resin should also be considered. A small pore size increases the retention of endotoxin in the column by size exclusion, while larger pore sizes reduce the ionic interactions with anionic proteins (Anspach, 2001). 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. Endotoxin has been reported to form stable complexes with hemoglobin, thus complicating separation (Kaca, Roth, & Levin, 1994; Kang & Luo, 2000). The effect of ionic strength on endotoxin removal efficiency and hemoglobin recovery has been studied using two different salt solutions (NaCl and $CaCl_2$). The endotoxin removal efficiency displays a decreasing trend as the ionic strength is increased. However, the endotoxin removal efficiency for $CaCl_2$ solutions displays a more drastic initial decrease than that for NaCl solutions. These results indicate that not only ionic interaction plays a role in affinity chromatography, but the types of cations matter as well (Kang & Luo, 2000).

Unlike the endotoxin removal efficiency, the ionic strength and cation types have a limited effect on product recovery. The endotoxin contaminated hemoglobin solutions prepared using either 0.1 M NaCl or $CaCl_2$ had hemoglobin recoveries >99% (Kang & Luo, 2000). The effect of pH on endotoxin removal efficiency and hemoglobin recovery was tested using different buffer solutions. 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. As the pH was increased from 4.5 to 8 (the pI of the resin), the resin became less positively charged and was, therefore, less effective at attracting negatively charged endotoxins. As the pH was increased beyond 8, the resin moved from having a neutral charge to a negative charge that repelled endotoxins. The pH had a minimal effect on hemoglobin recovery that was >97% for all pHs tested (Kang & Luo, 2000; McAllister et al., 2007).

Commercial resins employing hydrophobic and cationic ligands to remove endotoxin from proteins and biological solutions use porous nano- and micro-particles 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

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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 suffers from major drawbacks like a high-pressure drop (due to combined effect of bed consolidation and column blinding) and poor mass transfer (as intraparticle diffusion is responsible for the 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 are 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 of ~800 nm to remove endotoxins from phosphate-buffered saline (PBS), protein solutions, and water (Donnell et al., 2016; Razdan et al., 2019). The PCL nanoparticle (PolyBall) is non-porous in nature, and thus, the endotoxin binding takes place on the surface of the particles (**Fig. 7 (a)**). PolyBall shows high endotoxin removal efficiency of >99% from PBS, which is effective in removing endotoxin from protein solutions prepared both in PBS and water (Razdan et al., 2019). In addition to high endotoxin removal, PolyBall offers high protein recovery of >95%, thus maximizing therapeutic product recovery. Considering larger-scale industry applications, combinatorial techniques are applied to construct PolyBall containing flexible biofilters (**Figs. 7 and 8**). Contaminated samples are allowed to flow from one side of the filter to the other. The % endotoxin removal efficiency is determined as >99% from water. One major advantage of the biocompatible PolyBall and biofilter is that they can be reused for endotoxin binding quite effectively without a major loss in binding efficiency. PolyBall can be regenerated by breaking endotoxin-nanoparticle complexes making the endotoxin removal process more efficient and scalable. **Fig. 8(e)** and **Table 3** showcase a comparison of different endotoxin removal products in terms of binding capacity, protein recovery and cost.

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 (Anspach & Petsch, 2000; Wu et al., 2017). In

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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 cellulose, cellulose acetate, nylon, PEVA, PVA and PVDF (Almeida et al., 2016; Razdan et al., 2019; Wu et al., 2017). The membrane capsules are single-use, meaning there is no need for eluting, cleaning, or regenerating. The benefits of single-use membranes include a decreased chance of product contamination, reduced process time and decreased buffer volume due to the decrease in required cleaning steps (de Vries et al., 2018). Membrane adsorption requires a low initial investment compared to traditional chromatographic methods, but frequent membrane replacement and purchase may affect manufacturing costs (de Vries et al., 2018). 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 His-tag immobilized on a nylon membrane for different endotoxin concentrations have been reported (Almeida et al., 2016). As the initial endotoxin concentration was increased, the removal efficiency was decreased demonstrating the limited binding capacity using membrane adsorbers. Even at the lowest endotoxin concentration of 387 EU/ml, the removal efficiency was only 65%. These results are consistent with those from previous studies that reported endotoxin removal efficiencies of ~70% with an initial endotoxin concentration of 6,000 EU/mL (D. Petsch, Beeskow, Anspach, & Deckwer, 1997). Recently, membrane adsorbers with high-efficiency endotoxin removal and binding have been synthesized. Amphiphilic carbonaceous particles (ACPs) incorporated in polyvinylidene fluoride (PVDF) matrix absorbers have been successful at removing endotoxins from BSA protein solutions at >99.8% efficiency with >90% protein recovery (Wu et al., 2017). PolyBall incorporated in cellulose acetate membranes showed the endotoxin binding capacity of ~ 2.7×10^6 EU per mg particle compared to the endotoxin binding capacity of ~ 1.5×10^6 EU per mg particle offered by PolyBall nanoparticles in suspension (Razdan et al., 2019).

Discussion

The biopharmaceutical industry has experienced rapid and consistent growth over the past few years (Abdullah, Rahmah, Sinskey, & Rha, 2008; Goodman, 2009; Langer & Rader, 2017; Waegeman & Soetaert, 2011). It is predicted that half of all drugs under development will be biopharmaceuticals within

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the next 5-10 years (Jozala et al., 2016). Developing endotoxin removal methods that are both effective and cost-efficient is an ongoing challenge (Saraswat et al., 2013) 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 novel methods for removal and ligands that demonstrate a high affinity to endotoxins with 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 (Mamat et al., 2015; Sanchez-Garcia et al., 2016; Wilding et al., 2019). Another development is the use of alternative expression systems other than *E. coli* such as mammalian cell lines (*e.g.*, Chinese hamster ovary or CHO and human embryonic kidney 293 or HEK293) or engineered yeasts (Gerngross, 2004). However, endotoxin contamination may originate from other sources such as additives, buffers, cell culture medium, reagents, serum, supplements, and water (Akyar, 2012; Collentro, 2012). Therefore, biomanufacturing processes focus on developing innovative and effective technologies for in-line endotoxin detection sensors and removal of endotoxins and other pyrogenic contaminants from process solutions.

Biological endotoxin detection 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 LOD 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. The LAL assay is the industry standard in medicine and equipment testing (Franco et al., 2018). Several parenteral pharmaceutical products such as Ampicillin, Cytarabine, Diclofenac, Dexamethasone, Heparin, Insulin, Gentamicin, Glucose, Saline solution, Vaccine, Vitamin, plasmid DNA, proteins, *etc.* are routinely screened for endotoxin detection using LAL tests (Di Paolo et al., 2018; Silveira, Andrade, Schmidt, Casali, & Dalmora, 2004). 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 to protect their dwindling population. The bWBA and MAT assays 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 the bWBA requiring very little preparation, they still require collection

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and storage of blood from living beings. This would create difficulties in collecting proper amounts of blood stores to handle testing requirements. These traditional endotoxin detection assays also suffer from masking of endotoxins by the constituents present in drug formulations when spiked with endotoxins (Nakamura et al., 1988; Schneider, 2016; Schwarz et al., 2017). It poses potential risks of underestimated endotoxin contamination in pharmaceutical products. Electrochemical techniques offer nearly endless combinations of sensors and protein-complexes, able to be designed specifically for a testing solution that could prove difficult for other tests. Optical detection methods offer 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 relies on more accurate, real-time detection, with increasingly cheap and easy to use. A reliable analytical method for endotoxin detection and analysis will also serve as a useful tool in the monitoring of water purification processes and water reclamation plants.

There is no single purification method that fits all separation scenarios (Magalhaes et al., 2007). The method chosen will depend greatly on the properties of the target molecule (Serdakowski London et al., 2012). Ultrafiltration is well suited for removing endotoxins from salts, small molecule therapeutics, or water but does not apply to most separation scenarios. Extraction provides a high endotoxin removal efficiency for highly contaminated samples, but can 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. Endotoxin has been removed from pDNA influenza vaccine solutions using ion-exchange chromatography with 97% purity and 47-88% yield (Bicho et al., 2015; Franco-Medrano, Guerrero-Germán, Montesinos-Cisneros, Ortega-López, & Tejeda-Mansir, 2017). Due to the specificity of ligands, affinity chromatography and mixed-mode chromatography offer an adequate separation with high product recovery for a wide range of therapeutic monoclonal antibodies. Membrane adsorption offers a reduced processing time and initial cost with high product recovery but has a low binding capacity that limits removal efficiencies. While there is no single method that applies to all scenarios, affinity, ion-exchange, 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

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widely applicable and cost-effective methods that reduce product loss while meeting all governing regulations for endotoxin concentrations in pharmaceutical 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 contamination is a concern. Animal-based endotoxin detection techniques will become obsolete in favor of electronic biosensors and fluorescence-based techniques. There is a need for real-time in-line monitoring of endotoxin concentration in process streams to take feedback actions against product contamination and maintain safety standards of the manufactured biologics and drugs. Developing simultaneous endotoxin detection and removal methods that are both effective and cost-efficient are an ongoing challenge due to the high purity required and the potential interactions between endotoxins and target molecules. Affinity and mixed-mode chromatography are the most promising and widely applicable endotoxin removal methods due to highly selective interactions between endotoxins and the chosen ligand. Additional research is still required to further develop efficient endotoxin removal methods and targeting ligands that demonstrate a high affinity to endotoxins with low cost and toxicity. 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 sites per volume as well as reduce mass transport. The figure is reproduced with permission from Jozala et al., 2016.

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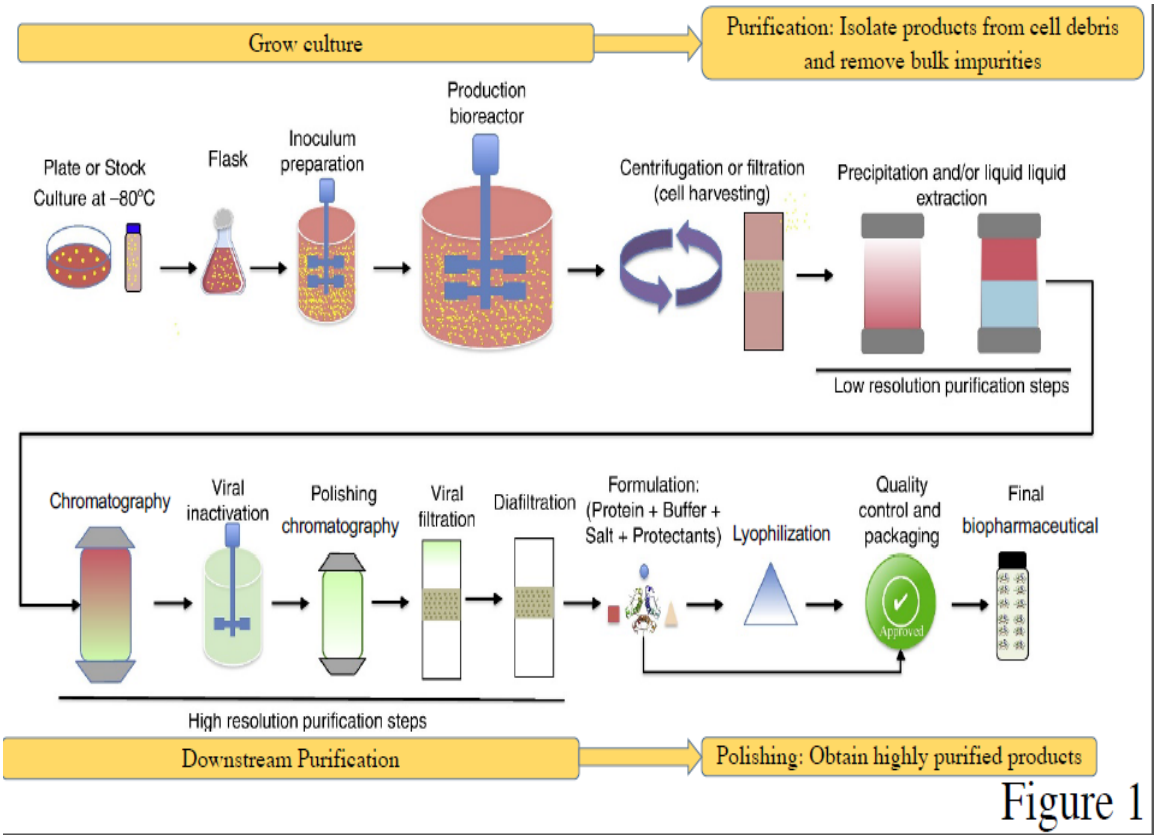

Figure 1

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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)** Endotoxin forms aggregate 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 a hydrophobic surface.

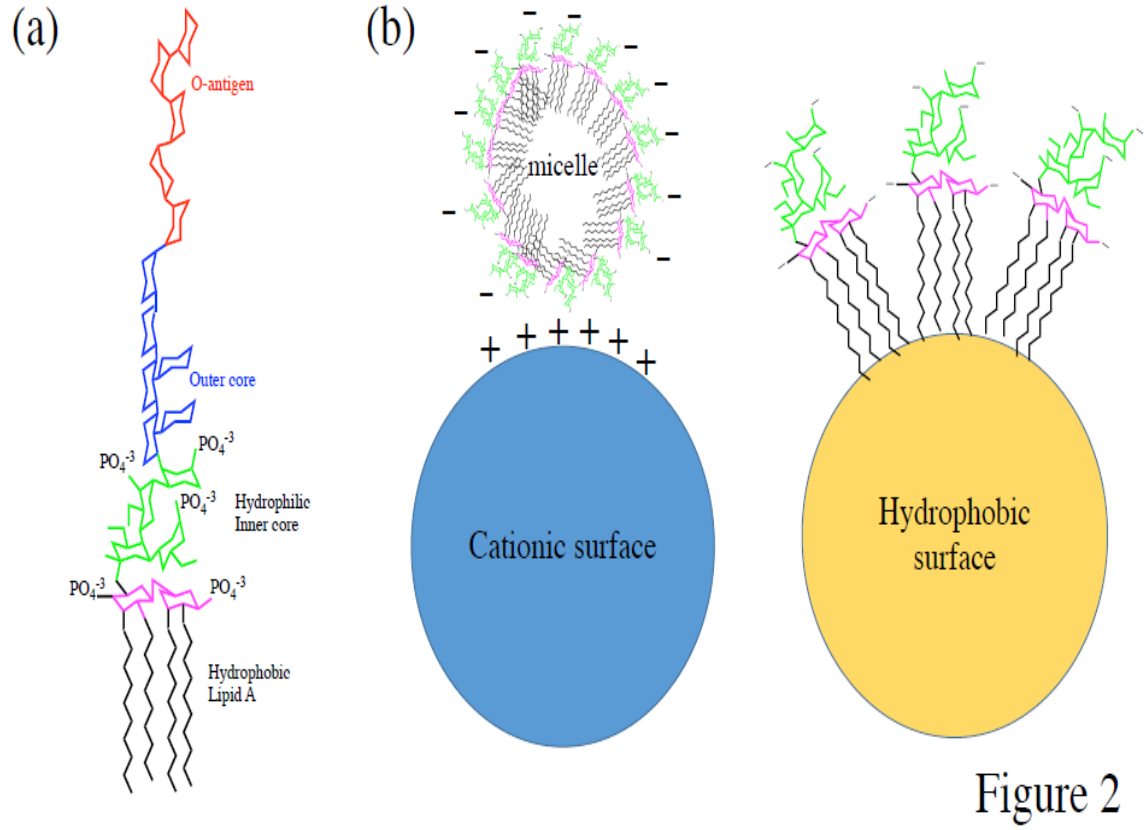

Figure 2

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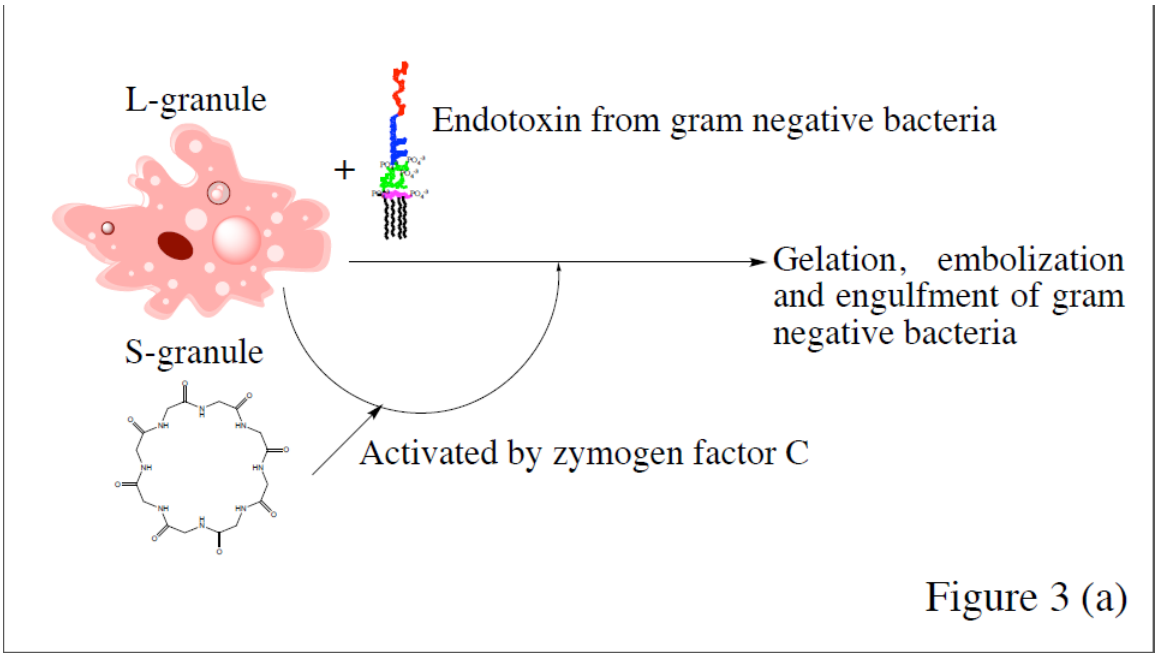
Figure 3: (a) Endotoxin-induced defense mechanisms in circulating hemolymphs of horseshoe crabs. The LAL assay is designed based on the immunogenic reactions developed in the blood of horseshoe crabs. Upon exposure to endotoxins, the electron-dense large granules (L-granule) and less electron-dense small granular (S-granule) amebocytes become activated by zymogen factor C. **(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 the immune system. Upon binding with endotoxins, an autocatalytic activity triggers 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. **(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.



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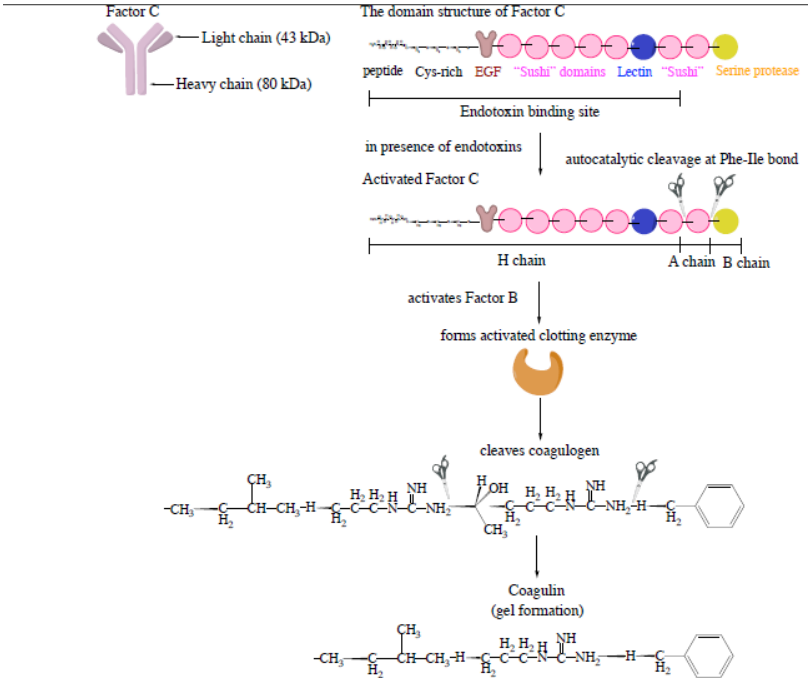

Figure 3 (b)

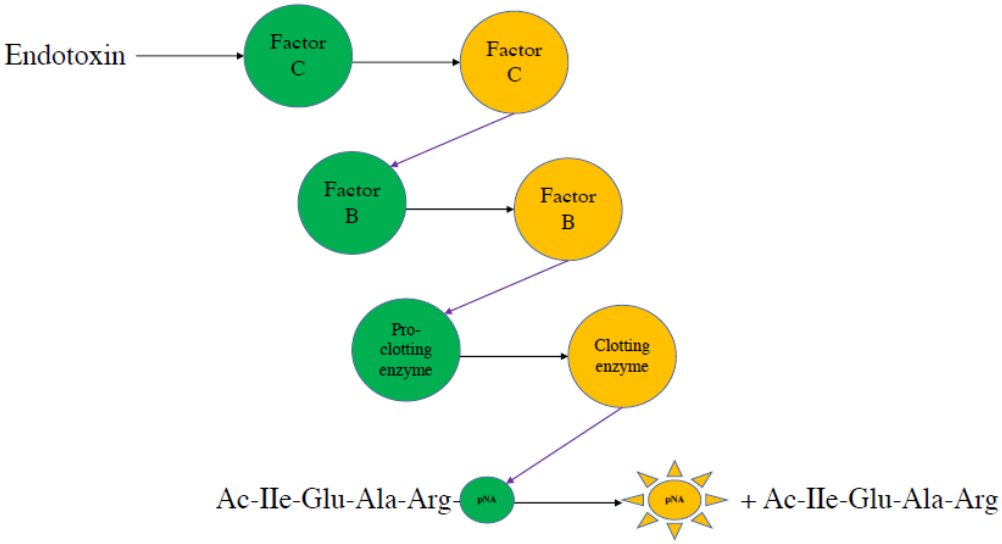

Figure 3 (c)

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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 an endotoxin, indicating the high specificity of the sensors to endotoxin. Reproduced with permission from Koryakina, Frey, & Bruegger, 2014.

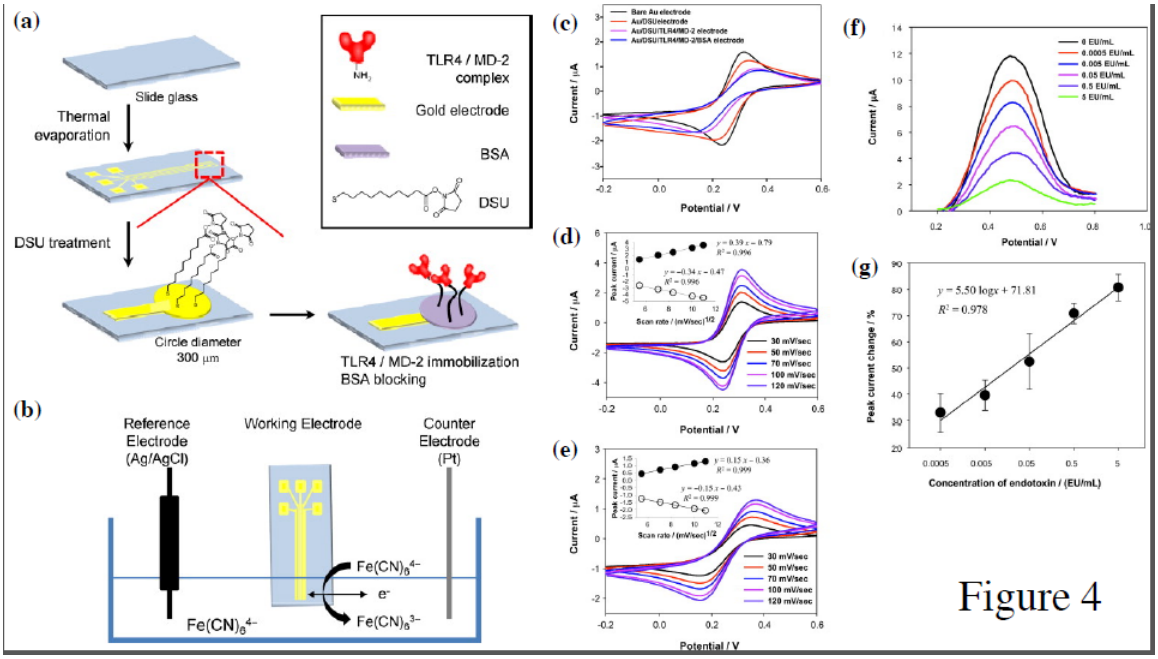

Figure 4

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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 0.001 EU/ml in solutions.

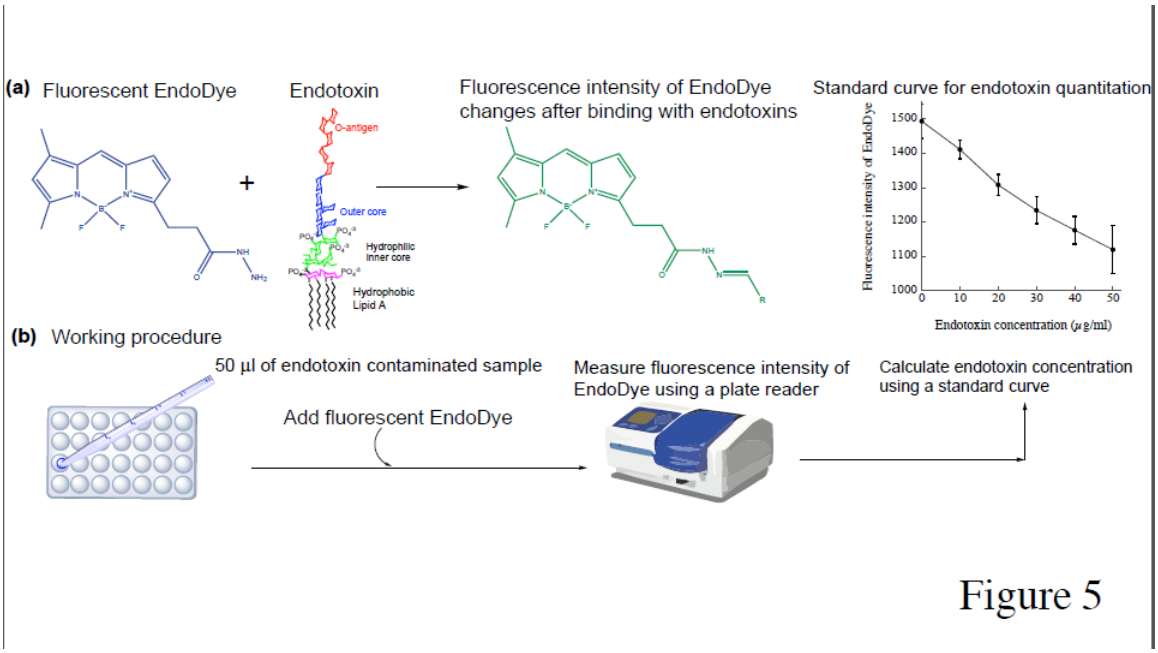

Figure 5

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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, histidine, poly-l-lysine, polyethylimine (pEI) and chitosan.

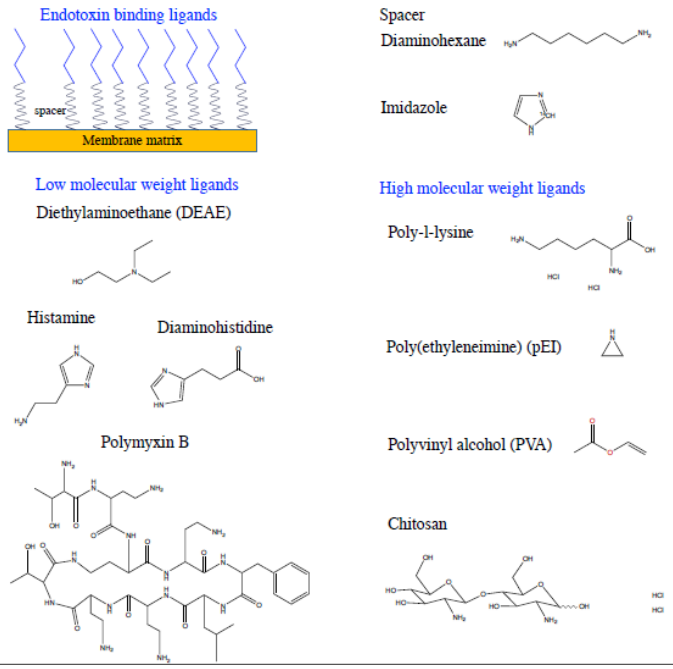

Figure 6

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Figure 7: (a) PolyBall nanoparticles are synthesized using the solvent diffusion method. PolyBalls can be lyophilized in white powder form and stored at room temperature (~22℃). **(b)** 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. **(f)** PolyBalls can be regenerated to remove endotoxins further. Figures reproduced with permission from Razdan, Wang, & Barua, 2019.

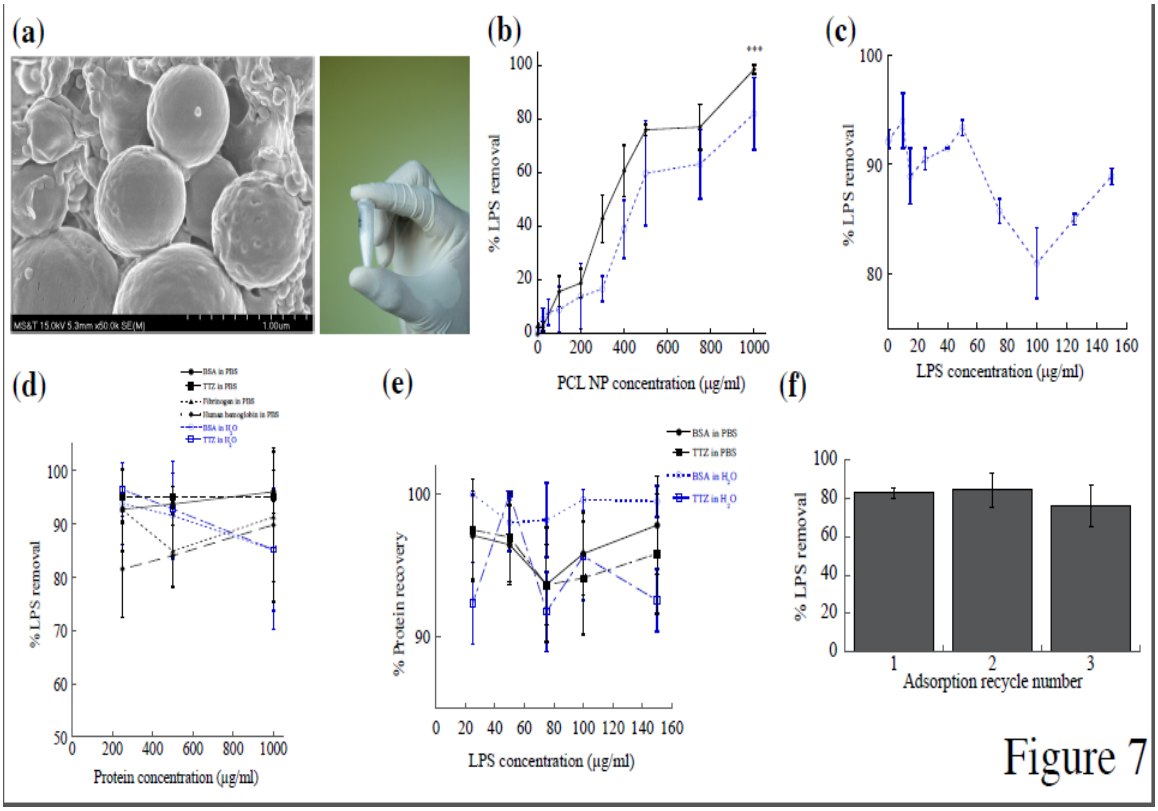

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Figure 8: (a) PolyBall nanoparticles are embedded in cellulose acetate (CA) biofilters. **(b)** The cross-sectional view of a CA filter without any nanoparticles (negative control) using SEM. **(c)** SEM image of a biofilter with PCL nanoparticles 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 Razdan, Wang, & Barua, 2019.

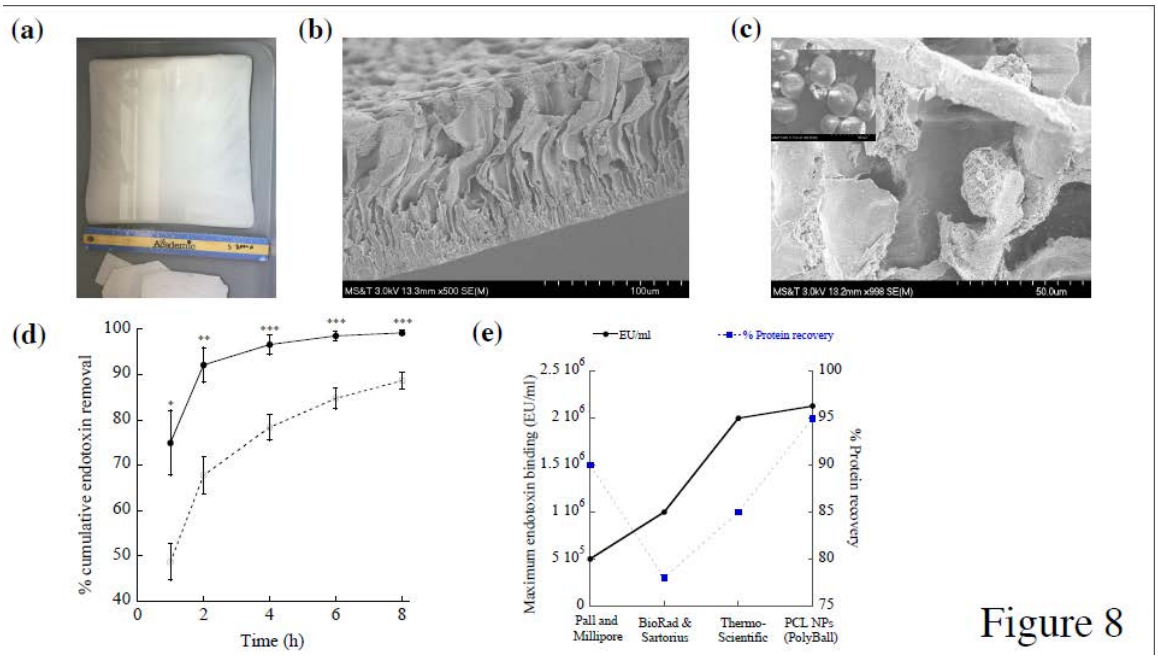

Figure 8

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Table Legend

Table 1: A comparison chart eliciting three LAL test methods: the gel clot, turbidimetric and chromogenic method and rFC assay as available in commercial endotoxin detection kits.

Kit	Model (Catalog No.)	Principle	Detection time (min)	Limit of Detection (EU/ml)	Cost (total tests)	Weblink
Lonza BioScience	QCL-1000 (50-647U)	Endpoint chromogenic assay	16	0.1	$614 (120)	LonzaBioScience
	Pyrogent 5000 (N383)	Kinetic turbidimetric assay	60	0.01	$291 (100)	LonzaBioScience
	Kinetic-QCL (50-650U)	Kinetic chromogenic assay	60	0.005	$842 (192)	LonzaBioScience
	PyroGene (rFC assay) (50-658U)	Endpoint fluorogenic assay	60	0.01	$842 (192)	LonzaBioScience
Thermo Scientific	Pierce LAL assay (88282)	Endpoint chromogenic assay	30	0.1	$338 (50)	ThermoScientific
GenScript	LAL assay (L00350)	Endpoint chromogenic assay	14	0.01	$170 (32)	GenScript

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BioVendor	ENDONEXT™ EndoZyme® II (rFC assay) (890030)	Endpoint fluorogenic assay	60	0.005	$825 (192)	BioVendor
Associates of Cape Cod	Pyrosate kit (PSD030, PSD125, PSD250 and PPT50)	Gel clot assay	30	0.25	$208 (10)	AssociatesofCapeCod

Table 2: Comparison of ligands for chromatography-based removal of endotoxins

Ligand used	**Product Name (Supplier)**	**Maximum Endotoxin Binding capacity (EU/ml)**	**% Protein recovery**	**Regenerable**	**Cost (sample size)**	**Web Link**
Poly-ε-lysine immobilized on agarose beads	ToxOut endotoxin removal resins	1.5×10^9 (eliminates >99% of endotoxins)	> 97 %	5 times	$ 195 (5 ml)	Biovision
Polymyxin B immobilized on agarose beads	PurKin endotoxin removal resins	2.0×10^6 (eliminates >99% of endotoxins)	> 85 %	5 times	$ 208 (10 ml)	Abbkine
Poly-ε-lysine immobilized on porous cellulose beads	Pierce High capacity endotoxin removal resins	2.0×10^6 (eliminates >99% of endotoxins)	> 85 %	10 times	$ 341 (10 ml slurry)	ThermoFisher

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Polymyxin B immobilized resins	Toxin Eraser endotoxin removal resins	2.0×10^6 (eliminates >99% of endotoxins)	> 90 %	5 times	$ 60 (1 ml)	GenScript
(information not available)	Proteus endotoxin removal resin	1.0×10^6 (eliminates >95% of endotoxins)	> 90 %	2 times	$ 500	BioRad
(information not available)	Endo Trap Red	$> 2.0 \times 10^6$ (eliminates >99.9% of endotoxins)	> 95 %	3 times	$ 170 (1ml column)	Lionex
(information not available)	Endo Trap HD	$> 5.0 \times 10^6$ (eliminates >99.9% of endotoxins)	> 95 %	10 times	$ 345 (1ml column)	Lionex
No ligand (PCL nanoparticles alone)	PolyBall (not commercially available)	1.5×10^6 (eliminates >99% of endotoxins)	> 95 %	3 times	$ 2.4 (1 g)	(Barua, 2018; Bromberg et al., 2010; Donnell et al., 2016; Razdan et al., 2019)

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Table 3: Comparison of membrane adsorbers for endotoxin removal, their adsorption capacity, costs, and regenerability

Product Name	**Maximum Endotoxin Binding capacity (EU/ml)**	**Cost ($)**	**Reusability**
Pall Acrodisc Unit with Mustang E membrane	5.0×10^5	$9.2 per 1 cm^2 membrane area	Yes
Millipore charged Durapore cartridge membrane filters	$>5.0 \times 10^5$	$2.7 per 1 cm^2 membrane area	Yes
BioRad Proteus Endotoxin Removal Kits (Membrane-based)	$5.0 \times 10^5 - 1.0 \times 10^6$	$12.4 per 1 cm^2 membrane area	Yes
Sartobind Q100 membrane adsorbers (Sartorius)	1.0×10^6	NA	Yes
PCL nanoparticles incorporated membrane	2.8×10^6	$0.05 per 1 cm^2 membrane area	Not tested yet

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