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REVIEW ARTICLE

Current trends in endotoxin detection and analysis of endotoxin–protein interactions

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Abstract

Endotoxin is a type of pyrogen that can be found in Gram-negative bacteria. Endotoxin can form a stable interaction with other biomolecules thus making its removal difficult especially during the production of biopharmaceutical drugs. The prevention of endotoxins from contaminating biopharmaceutical products is paramount as endotoxin contamination, even in small quantities, can result in fever, inflammation, sepsis, tissue damage and even lead to death. Highly sensitive and accurate detection of endotoxins are keys in the development of biopharmaceutical products derived from Gram-negative bacteria. It will facilitate the study of the intermolecular interaction of an endotoxin with other biomolecules, hence the selection of appropriate endotoxin removal strategies. Currently, most researchers rely on the conventional LAL-based endotoxin detection method. However, new methods have been and are being developed to overcome the problems associated with the LAL-based method. This review paper highlights the current research trends in endotoxin detection from conventional methods to newly developed biosensors. Additionally, it also provides an overview of the use of electron microscopy, dynamic light scattering (DLS), fluorescence resonance energy transfer (FRET) and docking programs in the endotoxin–protein analysis.

Keywords

Biosensors, endotoxin detection, endotoxin–protein interaction, endotoxin aggregation, lipopolysaccharide

History

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Introduction

Endotoxin mainly consists of O-antigen, core polysaccharide, and lipid A (Figure 1) components. The O-antigen is located at the outermost and hydrophilic part of endotoxin. It is made up of 1 to 40 repeating units that are unique among species resulting in the serological specificity of bacteria.[1] The core polysaccharide is divided into two parts, the inner core that connects to lipid A and the outer core that connects to O-antigen. The inner core mainly contains residues of Kdo and L-glycerol-D-manno-heptose (HEP) while the outer core is more diverse than those in the inner core.[2] Lipid A is highly conserved, and it is also responsible for the secretion of pro-inflammatory cytokines by a tool-like receptor (TLR4) on immune cells.[3]

Endotoxins exist in Gram-negative bacteria and are released upon cell death or during cell division and cell growth.[4–6] Various types of biomolecules can bind and interact with endotoxins (LPS) causing several effects especially the activation of cell-specific response and masking of biomolecules thus changing their overall physicochemical properties.[7] The change in the physicochemical properties of endotoxins has been associated with the difficulties in removing them during bioproduct purification.

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Some of the biomolecules which have reportedly been shown to interact with endotoxins are: lipopolysaccharide-binding protein (LBP), bactericidal/permeability-increasing protein (BPI), an amyloid P component, a cationic protein, enzymes utilized in the biological endotoxin assay (anti-LPS), a lysozyme and a lactoferrin.[8] Hypothetically, the interactions between endotoxins and biomolecules are influenced by either affinity interaction, hydrophobic interaction or ionic binding. The interaction of biomolecules with endotoxins is usually followed either by aggregation or disaggregation of both molecules. The physicochemical change of endotoxin aggregate is presumed to trigger toxic activities *in vivo*.[9] These interactions may also lead to the masking of endotoxin hence the difficulty of its removal in the downstream processes. The 'masking of endotoxin' effect could also tweak the Limulus coagulation cascade in LAL-based endotoxin assay thus causing false-positive results.[10] According to Chen and Vinther,[11] due to the endotoxin masking effect, endotoxin contents in some biopharmaceutical products are often underrated when LAL are used during the quantitative analysis.

Besides endotoxins, other non-endotoxin pyrogens also contribute to the challenges in bioproduction. Although non-endotoxin pyrogens could pose endotoxin-like negative effects to human, higher concentrations of the molecules are usually needed in order to trigger pyrogenic responses similar to that of endotoxins.[12] Non-endotoxin pyrogens such as lipoteichoic acid (LTA), peptidoglycan, exotoxins, and

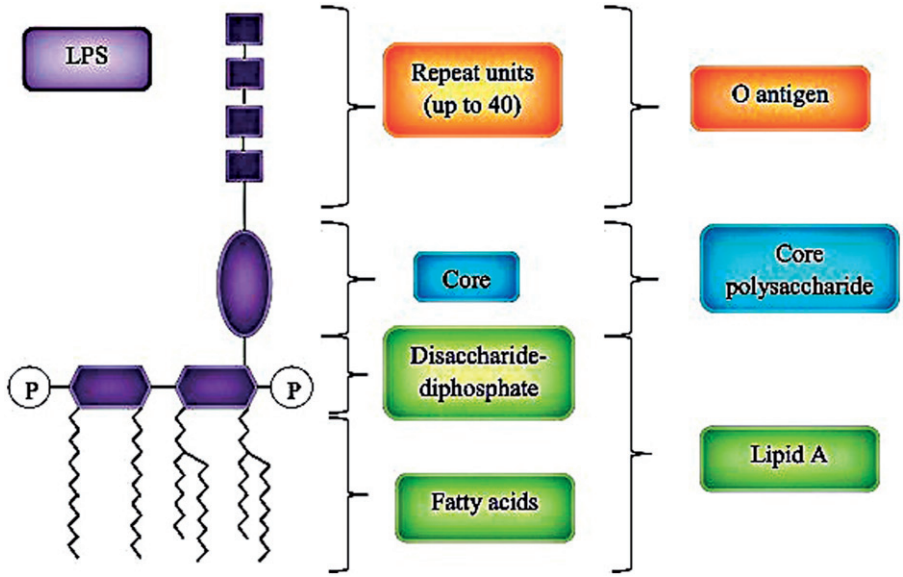

Figure 1. The major parts of endotoxin (lipopolysaccharides) structure.

enterotoxins [13] can also be detected by using the same methods as the endotoxin detection except for the LAL-based assay.[14] For the sake of this review, emphasis is on endotoxins, the major contaminants in Gram-negative bacterial products.

An overview of the current research trends is presented here in the field of endotoxin detection and analysis of endotoxin–protein interaction. It highlights the conventional and novel methods used in endotoxin detection as well as the analytical methods employed in the study of endotoxin–protein interaction. This kind of study is scarce, and the number of published report is also less. The study of endotoxin detection and endotoxin–protein interaction is crucial in order to understand the unique behavior of endotoxin in the presence of other biomolecules in different *in vitro* and *ex vitro* conditions. This information is vital especially during the developmental stage of Gram-negative bacterial vaccine purification and can save millions of dollars from initial trial and error.

Endotoxin detection

Conventional method of endotoxin detection

Rabbit pyrogen test

Rabbit pyrogen test (RPT) was first discovered by Hort and Penfold in 1912 and was further presented by Florence B. Seibert in 1920s.[15,16] Its principle of detection is based on injecting the pharmaceutical parenteral drugs into rabbits and observing the animals for a temperature rise or a fever.[16] This method can detect as low as 0.5 EU/ml of endotoxin concentration.[17] Rabbits were chosen as the test model because it showed similar sensitivity and specific immune response as in humans.[18] The *in vivo* technique plays a significant role in controlling endotoxin contamination, and it is still being executed on most blood products especially in Japan.[19] The use of animal models has restricted the application of this method especially when a large number of samples need to be analyzed. In addition, Ochiai et al. [19] reported that the RPT had limited sensitivity and accuracy compared to other conventional methods of endotoxin detection.

Limulus amebocyte lysate test

The Limulus Amebocyte Lysate (LAL) test was discovered in the 1960s by Levin and Bang.[20] It uses the blood extract of horseshoe crabs, *Limulus polyphemus* and is mostly known as the indirect animal test. This method can be divided into three basic techniques, the gel-clot, turbidimetric and chromogenic techniques (Figure 2). Turbidimetric and chromogenic LAL tests are more accurate and sensitive than the gel-clot method.[20] LAL test works by the clot formation produced by the blood extract of horseshoe crab after being exposed to endotoxins.[16] Akbar John et al. [16] stated that LAL test was 3 to 300 times more sensitive than the RPT method. It can detect as little as 0.03 EU/ml compared to RPT test that can detect only 0.5 EU/ml.[21] LAL test is also known as the Bacterial Endotoxin Test (BET) as it is based on the endotoxin-induced coagulation response activated by the binding of endotoxin to factor C in LAL.[22]

Although the LAL test has been accepted as a reliable method for measuring pyrogenicity, it is only specific for the cell wall component of Gram-negative bacteria. Thus, it is not applicable for samples containing a large amount of protein.[23] In the case of endotoxin contamination in veterinary vaccines, the LAL test is not capable for quantifying *in vivo* endotoxin activities satisfactorily.[24]

LAL test is not applicable on samples containing free metal ions as these metal ions can alter the measurement sensitivity, thus reducing the accuracy of the test result. Consequently, the development of ion-based endotoxin removal techniques has been hampered. In 2011, a modification of the LAL method was performed to minimize the variability in measurement caused by the presence of metal ions in the samples being analyzed.[25] Briefly, the modification involved reconstructing the sample so that the composition of all metal ions in each sample was analogous. A minimum sample dilution (MSD) of 1000-fold was introduced to reduce further the inhibition/enhancement

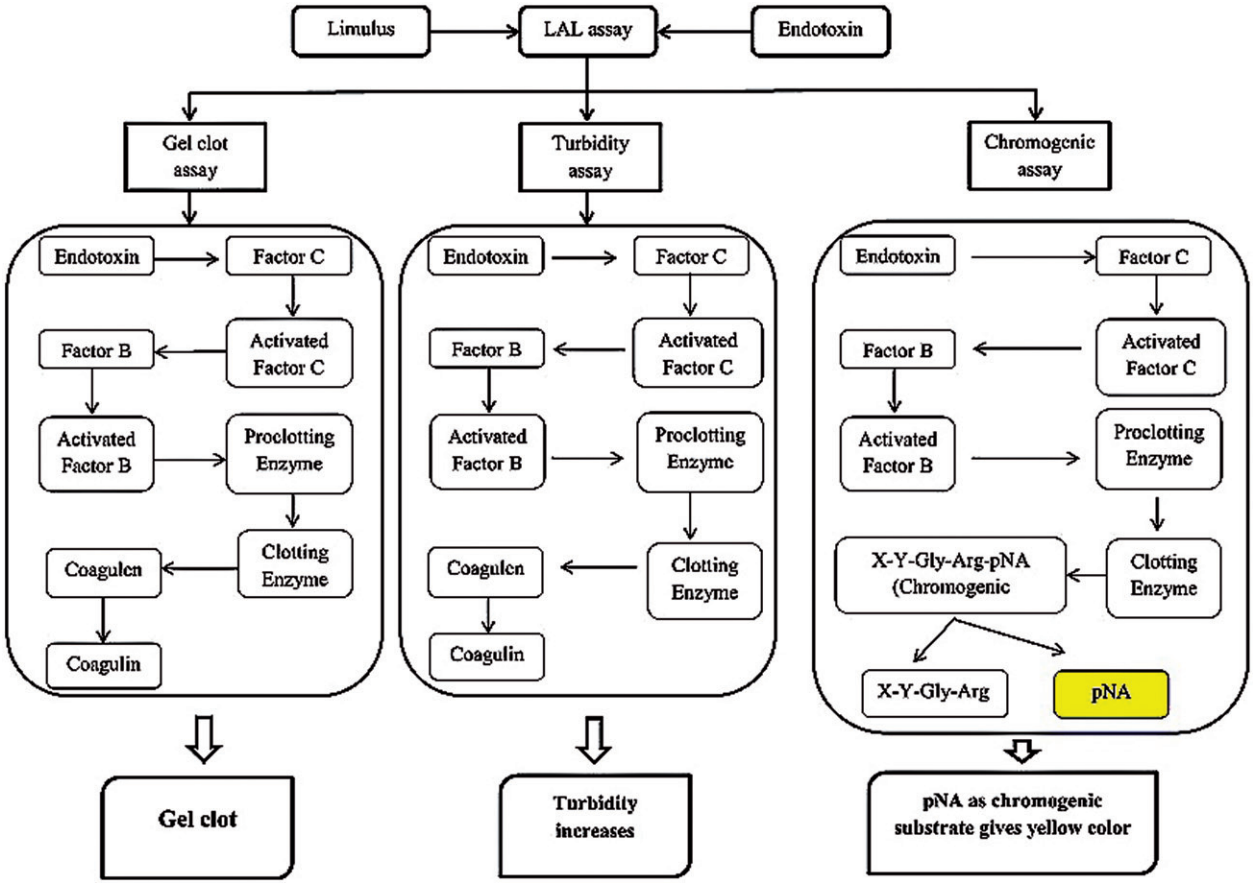

Figure 2. Types of LAL-based endotoxin detection methods.

effect of metal ions on the LAL activity without compromising endotoxin sensitivity. 5 mM EDTA was added into each sample to reduce the endotoxin–metal ion interaction.

Besides the issues on LAL sensitivity and specificity, the horseshoe crab population is also under threat due to over-fishing for its blood.[26] Although the animals are returned to the sea after the blood is collected, apparently about 20% of the horseshoe crabs fail to survive.[27] The discovery of factor C, the major endotoxin-mediated cascade component, has been transformed into recombinant LAL. According to Ding and Ho,[28] recombinant factor C acts as an endotoxin biosensor as it can detect the presence of endotoxin after full deliberation of enzymatic activity. In 2014, the European Directorate for the Quality of Medicines and HealthCare (EDQM) has revised the Guidelines for Using the Test for Bacterial Endotoxin which includes the use of recombinant factor C as a possible alternative method to the LAL test.[29]

Monocyte activation test

The Monocyte Activation Test (MAT) has been in existence since 1995. This human blood-based method can quantify all pyrogens in human patients, and it is a promising alternative to both the RPT and LAL tests.[27] Briefly, human blood is directly exposed to the surface of the test material, and the amount of pro-inflammatory cytokine IL-1b is then quantified. This method can detect as little as 10 pg/ml of endotoxin concentration and even lower using cryo-preserved blood.[30]

As MAT utilizes human blood, it is possibly the most advantageous *in vitro* endotoxin detection method since it does not involve any animal products. Although there are various sources of monocytes, the commonly used monocytes are usually derived from human whole blood, peripheral blood mononuclear cells and monocytic cell lines.[31] This method is highly sensitive, cost-efficient and most importantly regulates the reaction of the selected species.[14] The utilization of MAT can be enhanced by substantial optimization and modification. A recent study conducted by Stang et al. [32] concluded that the combination of modified MAT protocol and dynamic incubation system had great potential for directly detecting endotoxins on medical devices in the smallest amount. The modified MAT protocols comprised the rotation during the incubation period, the usage of pyrogen-free water as well as the additional concentrated saline solution used in order to observe the isotonic conditions [32] The modified MAT enhanced the pyrogen recovery rates up to 90% as indicated by the release of IL-1b from whole blood cells. The modified MAT significantly improved the safety of medical devices used on patients as observed by the decreased rate of complication resulting from the use of pyrogen-contaminated medical devices. Surprisingly, MAT is not currently widely used, and this may be due to the

limited availability and inconsistency of human blood when a large volume of samples need to be analyzed.

Bovine whole blood assay

Wunderlich et al. [33] reported that using bovine blood in endotoxin detection was preferable as it contained the Toll-like receptor equipment of bovine leukocyte similar to humans. In their study, Prostaglandin E_2 (PGE_2) was used as an indicator that enabled detection limits of 0.04 EU/ml and 0.25 EU/ml for lipopolysaccharide 0127:B8 and 0113:H10 respectively. A study by Imamura et al. [34] showed that a simple bovine whole blood culture was able to detect endotoxin activities via pro-inflammatory cytokine responses in the presence of aluminum hydroxide gel. Imamura et al. [34] also suggested that this technique was highly useful for the detection of endotoxins in Gram-negative bacterial vaccine. Additionally, this technique offers several advantages such as ease of performance, few preparation artifacts, and a mimicked physiological cell environment for studying bovine immune response.

However, the use of whole blood culture during endotoxin detection is often limited by the fresh blood availability. Nakagawa et al. [35] wrote that the donated whole blood was slightly inconsistent causing unstable monocytic cytokine responses. In addition, bovine blood is quite difficult to be acquired compared to human whole blood. Bovine blood can only be taken from very young calves as the blood from older animals is not sufficiently effective.[36] Apart from that, the collection of bovine blood for endotoxin detection can only be carried out in authorized countries.[36]

New biosensors for endotoxin detection

Electrochemical biosensors

Electrochemical impedance spectroscopy (EIS) is a detection method based on the principal component analysis (PCA), cluster analysis (CA), and multivariate discriminant analysis (MDA). In a study by Heras et al.,[37] the biosensing platform comprised a 32-amino acids fragment of an 18 kDa cationic antimicrobial protein (CAP18F) which had lipopolysaccharide (LPS) binding affinity. The electrode surfaces were also modified with a highly selective endotoxin neutralizing protein (ENP). The resulting biosensor had the ability to distinguish LPS from a mixture of proteins, nucleic acids and phospholipids, as well as to detect LPS below the threshold limit established by the pharmaceutical industry.

Cho et al. [38] fabricated a modified metal complex electrode from a gold electrode altered with copper (Cu) and nitrilotriacetic acid (NTA) complex. Basically, the principle of detection was based on the interaction of LPS with the NTA-Cu complex. This modification was considered as an upgrade of the previous EIS where the latter could detect LPS in real time thus giving more advantages during endotoxin detection.

In 2010, a zymogen-based sensor was developed based on a recombinant factor C (rFC) in which the protease zymogen was activated by endotoxin binding.[39] The working principle of this biosensor is illustrated in Figure 3. The Zymogen-based sensor can detect 5 EU/mL and 1 EU/mL of endotoxin concentrations in Tris-Ac buffer (pH 7.5, 37 °C) for reaction times of 1 and 3 h respectively. In the following year, the fabrication and design of a new electrochemical endotoxin biosensor based on human recombinant toll-like receptor 4 (rhTLR4) and myeloid differentiation-2 (MD-2) complex were established.[40] The biosensor was fabricated by immobilizing rhTLR4/MD-2 on gold electrodes via a self-assembled monolayer (SAM) technique. Additionally, this technique incorporated dithiobics succinimidyl undecanoate (DSU) in the biosensor design thus rendering the endotoxin biosensor highly specific with a very low detection limit of 0.0002 EU/ml.

Planar interdigital biosensor has been successfully fabricated with different coating materials having either a carboxylic or amine functional group that have been strictly optimized.[41] Some of the coating materials used were carboxyl-functional polymers, APTES (3-Aminopropyltriethoxysilane) and Thionine. All the coated biosensors were immobilized with endotoxin-binding Polymyxin B (PmB).[42] The APTES-coated biosensors exhibited improved selectivity for LPS. Abdul Rahman et al. [42] furthered their study on the planar interdigital biosensor by analyzing the effects of different coating thicknesses on the biosensor performance. They found that three layers of coating had higher sensitivity and selectivity towards the endotoxin compared to sensors that had five layers of coating.[43]

A portable, low-cost and easy to use biosensor for on-site endotoxin monitoring based on the LAL assay has been reported.[44] It was based on a cascade reaction of zymogen contained in LAL to generate p-nitroaniline (Figure 4).[44] Basically, the cascade reaction was triggered by endotoxins, and the induced p-nitroaniline was then electrochemically observed by differential pulse voltammetry (DPV). This type of endotoxin biosensor had an endotoxin sensitivity of 0.010 EU/mL.

An electrochemical biosensor with high selectivity for LPS despite the presence of pDNA, RNA and BSA has also been developed.[45] The biosensor was designed using LPS-specific single-stranded DNA (ssDNA) aptamer as a probe where every step of the alteration process was characterized by two methods which were the cyclic voltammetry (CV) and electrochemical impendence spectroscopy (EIS). An interesting feature of the biosensor was its ability to reform at a low pH condition hence could be used in LPS detection in a complex environment. The biosensor has been modified by conjugating the aptamers to gold nanoparticles (AuNPs) and electrochemically depositing them on the gold electrode (Figure 5).[46] The amended biosensor showed a broad linear dynamic endotoxin detection range of 0.01–10.24 ng/ml as well as a very low endotoxin detection limit of 0.005 ng/ml within 10 min post-detection.

Another aptamer-based electrochemical biosensor has been developed using non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM)-based and non-SELEX (systematic evolution of ligands by exponential enrichment) method. This technique enabled the identification of 10 different ssDNA aptamers (Table 1) that showed a specific affinity for LPS.[47] Among the 10 different ssDNAs, B2 was selected for the construction of the

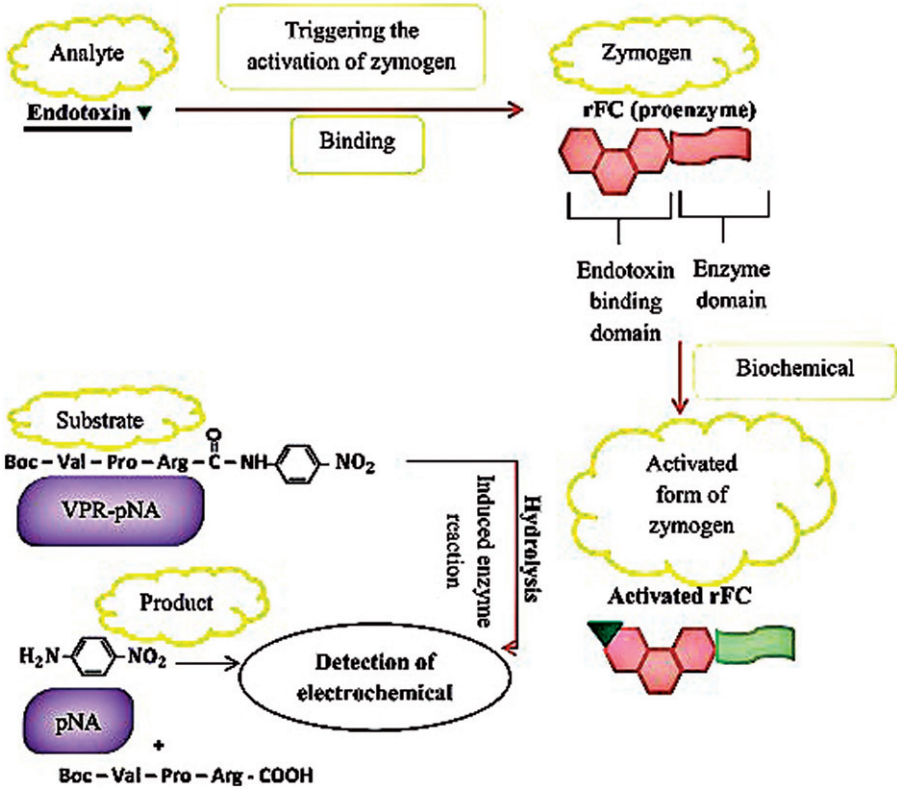

Figure 3. The principle of the rFC-based electrochemical endotoxin assay.

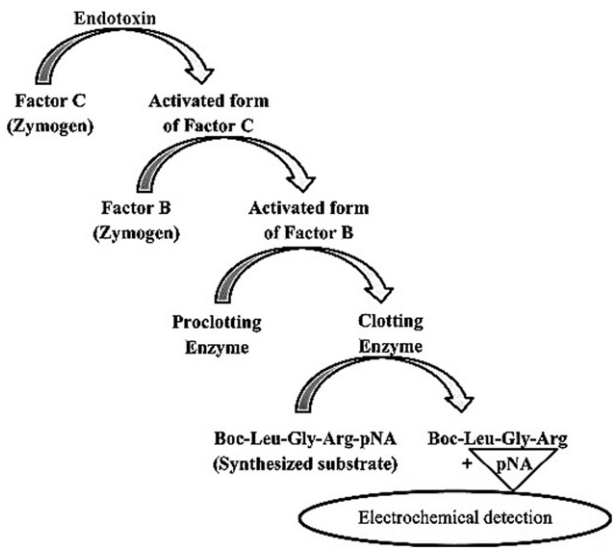

Figure 4. The principle of Limulus amebocyte lysate (LAL)-based electrochemical endotoxin assay.

impedance biosensor on a gold surface since it exhibited the highest affinity for LPS. This biosensor displayed outstanding sensitivity and specificity within the linear endotoxin detection range of 0.01–1 ng/mL as well as reduced detection time although the results were ambiguous.

The electrochemical mechanism of the LAL gel-clot process has been successfully characterized for the first time by using a highly-stable low-cost screen-printed electrode.[48] The proposed screen-printed endotoxin biosensor used electrochemical signals from the LAL gel-clot process in order to measure endotoxin concentration. The device was time-efficient, highly sensitive, durable and relatively cheaper when compared to other techniques.

Another amperometric endotoxin analysis utilizing a screen-printed electrode chip has been developed whereby the principle of detection was based on protease activity induced by the gel-cloth LAL cascade reaction.[49] This biosensor used a novel p-amoniphenol conjugated substrate (Boc-Leu-Gly-Arg-pAP) for the LAL-protease reaction where it could detect 10–1000 EU/L endotoxin within an hour. They also reported that this novel amperometric endotoxin quantification with a screen-printed electrode had dual ability since *in vitro* protease assays could also be performed along with endotoxin analysis.

Optical biosensors

One of the optical biosensors that have successfully been developed is the endotoxin assay by bioluminescence using mutant firefly luciferase. The biosensing principle is a combination of the LAL reaction and bioluminescence using mutant luciferase (Figure 6).[50] This method could detect as little as 0.0005 EU/ml endotoxins within 15 min.

Fluorescence-based biosensors also have a huge potential to be incorporated in endotoxin detection strategy. Zeng et al. [51] showed that the use of N,N-dimethyl-N-(pyrenyl-1-methyl)dodecan-1-ammonium as a fluorescent probe for sensing bacterial endotoxin allowed an extremely low endotoxin detection limit of 100 nM. Moreover, the optical biosensor possessed higher selectivity for bacterial endotoxin compared to that for other biological species.[51] Wu et al. [52] reported that by employing polydiacetylene liposomes functionalized with fluorescent pentalysine peptide derivative and histidine, a very low

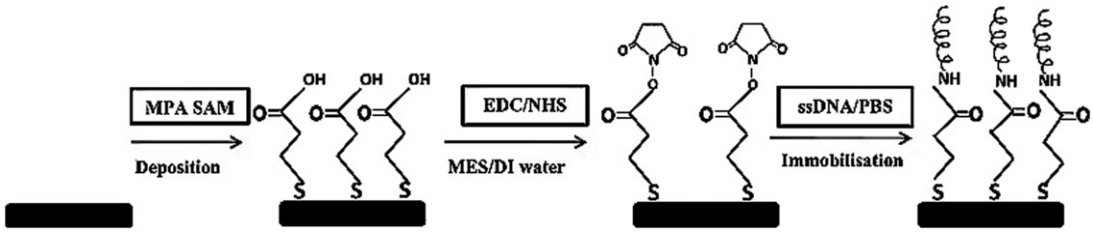

Figure 5. The preparation of the aptasensor.

Table 1. the homology of designed ssDNA aptamers.[47].

ID	Sequence (Homology)
B1	H[a]-TAGCCGGATCGCGCTGGCCAGATGATATAAAGGGTCAACCCCCCA-T[a]
B2	H-TAGCCGGATCGCGCTGGCCAGATGATATAAAGGGTCAGCCCCCCA-T
B3	H-TAGCCGGATCGCGTTGGCCAGATGATATAAAGGGTCAACCCCCCA-T
B4	H-TAGCCGGATCGCGCTGGCCAGATGATATAAAGGGTCAACCCCCCG-T
B5	H-CTAAGCACAGGGAAACCAGCTAATGAGTTAGGCCTGTCCCCCACG-T
B6	H-CAATGGACCTATTCGAGTACTGAATAGAACAGTCGGCGCTCTGGG-T
B7	H-TTCAAGACGATGCCTGGCGCGAGTTACACACTTGCATGGAGCTGG-T
B8	H-ATCCAATACCTCAGAACTCAGTTCGAGTCGTAAAGGGGAATCGCA-T
B9	H-ACCGATCCATCGAGTTTCTGAGAAAGGCCCGGAGAAACCGCGAGA-T
B10	H-TCAATCTAACCATGCATGCAGTTTAGGCAGGATTCGTTATCGCAA-T

Constant Head (H) and Tail (T) sequence regions flanking the randomized 45-mer ssDNA. H and T represent CTTCTGCCCGCCTCCTTCC and GGAGACGAGAT AGGCGGACACT, respectively.

endotoxin detection limit within a micromolar range could be achieved. The detection limit could be lowered further by performing the assay in a ratiometric fluorescence mode.[53] The ratiometric fluorescence sensing was based on the combination of two cationic dyes and one anionic dye where the complex would undergo dissociation along with significant conversion in fluorescence profile in the presence of bacterial lipopolysaccharide. Among the developed fluorescent optical biosensors, the most sensitive LPS sensor was a synthetic LPS-binding peptide, fluorescence-labeled and assembled with graphene oxides in PBS buffer solution.[54] This device was ultrasensitive with the lowest endotoxin detection limit of 130 picomolar. Another picomolar-sensitive endotoxin sensor for use in the colorimetric and fluorometric techniques was developed using the derivative of 3-phenylthiophene-based water-soluble copolythiophenes (CPTI).[55] The biosensor had high selectivity and sensitivity towards LPS in the presence of other negatively charged bioanalytes.

Mass-based biosensors

Although the mass-based endotoxin biosensor is currently less explored, a device which used an electromagnetic piezoelectric acoustic sensing (EMPAS) platform has been described for endotoxin detection in blood samples.[56] Thompson et al. [56] reported that this method could detect endotoxins in real time in human blood plasma. The LPS detection was performed using biosensing platforms that have been grafted with polymyxin B, a cyclic peptide antibiotic with high affinity for LPS in combination with ultrathin piezoelectric quartz discs that had oligoethylene glycol-based surface chemistry.[56]

Endotoxin–protein interaction

Analysis of endotoxin–protein interactions

Electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used to identify the structure and chemistry of biomolecules from micron to sub-nano resolution.[57] SEM was used by Pulido et al. [58] to analyze the surface morphology of bacterial cells after incubation with eosinophil cationic proteins [ECP] and peptides. They examined the level of damage on the cell surface and suggested that the damage was caused by the interaction between endotoxin and ECP. They also used TEM to analyze the agglutination level of the samples which tallied with the SEM analysis.

Oztug et al. [59] successfully viewed under TEM, the disaggregation of endotoxins after incubation with apolipophorin III (apoLp-III). Similarly, TEM has been used to prove that lipopolysaccharide transport protein (Lpt) could disrupt endotoxin aggregates even at low concentrations.[60]

Dynamic light scattering (DLS) analysis

DLS, also known as Photon Correlation Spectroscopy [61] has been used to determine the size distribution profile of nanoparticles in suspension.[7,62] In the study of endotoxin-apoLp-III interaction, DLS showed decreasing mean diameter of endotoxin after being incubated with apoLp-III thus further supporting the role of apoLp-III in endotoxin disaggregation.[59]

In contrast to apoLp-III, Polymyxin B has been found to drive endotoxin aggregation.[63] Results from DLS analysis confirmed that polymyxin B-endotoxin interaction was

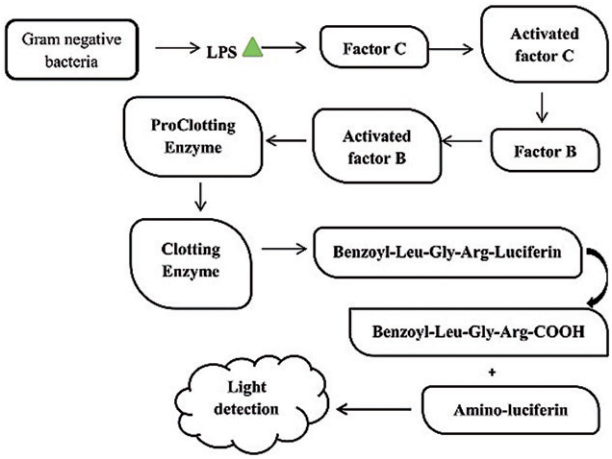

Figure 6. The principle of endotoxin detection by bioluminescence.

concentration-dependent. At an endotoxin concentration of 0.3 mg/ml, polymyxin B was unable to increase the size of endotoxin aggregates despite the high concentrations of peptides. However, at 3 mg/ml of endotoxin concentration, higher concentrations of polymyxin B could alter the endotoxin aggregates into a larger form.

DLS analysis has been used in the study of the aggregative interaction of mobile divalent metal cations with endotoxins and plasmid DNA.[7] This analysis was conducted to measure the hydrodynamic size of endotoxin aggregates in the presence of selected divalent cations. It was concluded that Zn^{2+} was able to selectively bind endotoxins compared to plasmid DNA thus resulting in >90% removal of endotoxins from plasmid DNA. Green fluorescent protein (GFPuv) has also been found to interact with endotoxins. Lopes et al. [64] performed DLS measurements on both pure GFPuv and endotoxin-GFPuv. They concluded that the aggregation of GFPuv was induced by endotoxins based on the significant correlation between GFPuv aggregate size and endotoxin concentration.

Fluorescence resonance energy transfer (FRET)

FRET works based on the energy transfer between two molecules within several nanometers in distance when applied to optical microscopy, and it is widely used today in biomedical and drug discovery research.[65] The fundamental concept of FRET consists of: (1) light absorption by a donor molecule, (2) energy emission-free transfer to an acceptor molecule by dipole–dipole coupling and (3) light emission by the acceptor.[66]

FRET has been used in the study of peptides' (Pep 19–2.5, Pep 19–2.5KO and Pep 19–8) ability to intercalate into phospholipid liposomes or endotoxin aggregates from strain R60 of *Salmonella minnesota*.[67] It was found that all three peptides intercalated nearly at the same amplitude into endotoxin aggregates. The FRET analysis also indicated that the peptides could intercalate into normal phosphatidylcholine and phosphatidylserine liposomal membranes.

Brauser [66] used FRET to study the interaction of endotoxin with antibiotic enrofloxacin. In that study, 7-nitrobenzo-2-oxa-1,3-diazole (NBD) was used as the donor while, 9-(2-carboxyphenyl)-3,6-bis(diethylamino)-xanthyliumchlorid (Rhodamine) was used as the acceptor that bound to a phosphatidylethanolamine. The endotoxin vesicles were prepared and incubated with enrofloxacin before the FRET analysis was carried out. The results showed that the signals from the donor and acceptor molecules did not change after the incubation with enrofloxacin, and thus Brauser [66] concluded that there was no interaction with the endotoxin vesicles.

Kozuma et al. [68] designed a high-throughput screening technique to obtain new compounds that blocked endotoxin binding to CD14.[31] The cell-free screening system was based on time-resolved intermolecular FRET (TR-FRET), where it successfully led to the discovery of novel inhibitors of endotoxin-CD14 interaction from the library of micro-organisms secondary metabolites.

Docking program

Docking program is a computational methodology that ''docks'' small molecules into the structures of large target molecules and 'scores' their potential complementarity to binding sites.[69] There are two major aims of using docking program in the study of endotoxin behavior: (1) to obtain an accurate structural model and (2) to obtain a correct prediction of activity.

Docking program has been used in the study of the interaction between endotoxin and antimicrobial peptides (AMPs). Kushibiki et al. [70] used docking program to understand the interaction between endotoxin and tachyplesin I (TP I), an antimicrobial peptide derived from a horseshoe crab. Based on the calculated structural model, they strongly suggested that the cationic residues of TP I interacted with the phosphate groups and saccharides of endotoxin while the hydrophobic residues interacted with the acyl chains of endotoxin. These findings provided in-depth structural knowledge about the binding mechanism between TP I and endotoxin.

Pulido et al. [58] employed the same method to study the interaction of endotoxin with eosinophil cationic protein (ECP). Based on the docking simulations of ECP and endotoxin, a high affinity interaction was observed whereby endotoxin was docked on the N-terminal cationic patch of ECP, with the protein targeting the interface between lipid A and polysaccharide moiety of the endotoxin. The results might explain the reduced antimicrobial and agglutinating activity of the protein.

Sivakamavalli et al. [71] used the docking program to understand the stimulating activity of β-1,3 glucan (β-glucan) and its interaction with lipopolysaccharide and β-1,3 glucan-binding protein (LGBP). They concluded that Arg71 was the origin of the binding selectivity of β-glucan complex with LGBP, where it formed hydrogen bonds on the binding sites of tripeptide arginylglycylaspartic acid (RGD). They also suggested that four amino acid residues in LGBP; Arg34, Lys68, Val135, and Ala146 were crucial in the binding mechanism as these amino acids had hydrogen bonds interaction on the active site of LGBP.

Characteristics of endotoxin–protein interactions

Generally, endotoxin behavior towards different types of protein under fixed physicochemical conditions (e.g. pH, temperature and viscosity) are varied. The interaction between endotoxin and lysozyme, an important component of the innate immune system, is primarily driven by the hydrophobic interaction.[72] The interaction results in the loss of lysozyme enzymatic activity through a noncompetitive inhibition and alters the biological activity of endotoxin. Additionally, endotoxins can become highly aggregated thus impeding the lysozyme activity.[73] When a lysozyme-endotoxin mixture is subjected to ultrafiltration and LAL test, a negative result will be obtained for the filtrate indicating an absence of monomeric LPS molecules.[73] Monomeric endotoxins are often associated with the inactivity of endotoxins.

The interaction of endotoxin with endotoxin-neutralizing protein (ENP) is dose-dependent.[9] A nearly complete neutralization can be achieved at an ENP/Endotoxin molar ratio of 20:1 as can be seen from the LAL assay and the TNFα production in human mononuclear cells (MNC). ENP can change endotoxin from an active into an inactive form by changing the three-dimensional structure of endotoxin, specifically the lipid A structure from a cubic to a multilamellar phase. The stability of the endotoxin-ENP binding is dependent on the endotoxin aggregate structure although the trend of dependency is still ambiguous.

Another type of protein which shows a high affinity towards endotoxin is eosinophil cationic protein (ECP), a human-secreted protein commonly used as a readout for the assessment of active inflammatory diseases. Pulido et al. [58] studied the endotoxin-agglutinating activity of ECP by determining the minimal agglutination concentration (MAC) or the minimal peptide concentration that could induce bacterial agglutination. Five *E. coli* strains (D21, D21e7, D21e19, D21f1, D21f2) with different lengths of lipopolysaccharide were used, and the agglutination was tested with ECP and peptides: [1–45] and [6–17]-Ahx-[23–36]. Based on the results, the D21f2 strain with the shortest endotoxin showed no agglutination although it had been incubated for 12 h with 5 μM ECP. Similar results were also obtained for the incubation with peptides indicating a low affinity towards endotoxins. It was also noted that the degree of endotoxin agglutination decreased with decreasing length of endotoxin strain as observed under the transmission electron microscope (TEM).

Apolipoproteins can associate with endotoxins thus giving extra protection against septic shock.[59] Apolipophorin III (apoLp-III) from *Galleria mellonell*, a moth species can be used as a model to study the apolipoproteins-endotoxin interaction and to understand its role in septic shock protection. The interaction of endotoxin with apoLp-III is essentially driven by the hydrophobic interaction, and it is dependent on the LPS phase transition temperature (30–37 °C).[74]

The interaction between endotoxin and apoLp-III has been characterized by Oztug et al.,[59] using a fast protein liquid chromatography (FPLC) technique. FPLC was used to isolate the complexes by size exclusion followed by analysis of size and analysis of apoLp-III/LPS contents. The results showed that the large LPS aggregates were transformed into smaller apoLp_III/LPS complexes, an indication of LPS disaggregation. The results were further confirmed by transmission electron microscopy where the LPS molecules were visibly transformed from a typical long-rod shape into small spheres. The binding mechanism of apoLp-III–endotoxin interaction is as shown in Figure 7. Briefly, apoLp-III first penetrates into the interior part of the endotoxin micelles thus giving access to the hydrophobic Lipid A region, hence leading to endotoxin disaggregation. Thus, an apparent change in the protein conformation enables direct interaction of the hydrophobic protein region with the lipid A region leading to the formation of a stable apoLp-III/endotoxin complex.

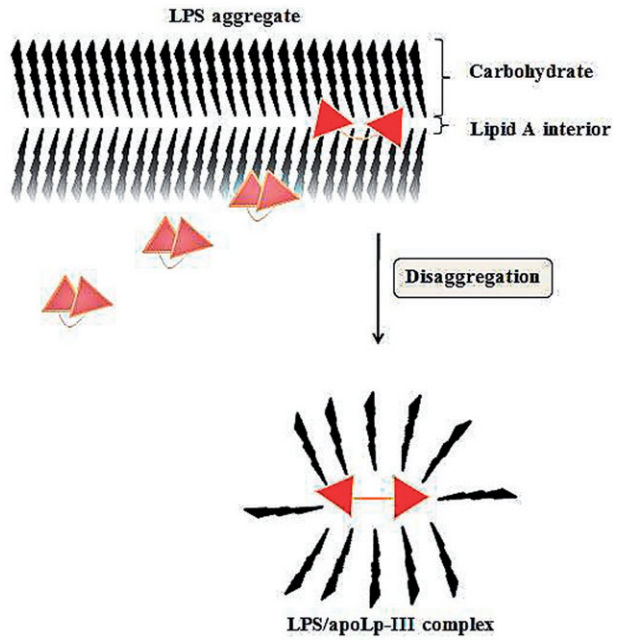

Figure 7. The proposed model of the binding of apoLp-III to endotoxi.

Previous studies have shown that some proteins which interact with endotoxins also act as mediators of endotoxin-induced cell activation. For instance, lipopolysaccharide-binding protein (LBP), a protein involved in the innate immunity has been shown to mediate the transfer of endotoxins to CD14 on macrophages or monocytes.[75–77] The LBP-mediated interaction between endotoxins and CD14 can lead to the activation of macrophages and monocytes.[78] Subsequently, the endotoxin-CD14 complex will catalyze the transfer of endotoxins to MD-2/TLR4 which will then cause cell inflammation.[79] Additionally, CD14 is reportedly a potent modifier of inflammation severity in animal models.[80]

In contrast to LBP, bactericidal/permeability-increasing protein (BPI) has been found to restrain inflammation by preventing LBP from transferring endotoxins to CD14.[81] LBP-endotoxins and BPI-endotoxins interactions are most likely driven by a competitive electrostatic interaction, with BPI showing an optimum affinity for the endotoxin.[79] The interaction of LBP and BPI with endotoxins is primarily targeted on the surfaces comprising a large number of endotoxin molecules packed closely together.[81]

Drago-Serrano et al. [82] discovered that the interaction of lactoferrin (Lf), a multi-functional protein of the innate immune system with enterobacterial endotoxin was the major reason that made Lf acted as a permeabilizing agent. The formation of bonds between Lf and endotoxin occurred via electrostatic interactions, thus leading to the neutralization of endotoxin activity and deactivation of the inflammatory response.[83]

Conclusions

Endotoxins interact with other biomolecules in multiple fashions. Briefly, it forms a stable bond with other biomolecules by affinity, hydrophobic or ionic interactions and even changes the physicochemical properties of endotoxins. These interactions make the removal of endotoxin using a standard procedure problematic. Therefore, it is imperative to conduct detailed studies on the physicochemical behavior of endotoxins in the presence of the target biomolecules. Based on these studies, an effective endotoxin removal technique can be designed for a particular situation. The state of endotoxin aggregation plays an important role in determining the stability of endotoxin–protein interaction/binding. Molecular studies on endotoxin aggregation and endotoxin–protein interactions could be the next step forward in the development of effective endotoxin removal strategies. Techniques such as electron microscopy and live imaging microscopy are promising as they could give a real-time visual indication of the interaction between endotoxins and proteins. Protein–endotoxin interaction occurs in a very short period, sometimes in milliseconds hence the suitability of the above-stated techniques.

Declaration of interest

The authors report no declarations of interest.

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