Novel Aptasensors for Endotoxin Detection Are Advancing Drug Discovery

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Introduction

Assessing levels of endotoxin during the development of pharmaceutical products and for the assessment of patients under medical care forms an important part of the pharmaceutical and healthcare system. Depending on the product type, there are complexities involved. Bacterial endotoxin can form a stable interaction with other biomolecules thus making its removal difficult especially during the production of biopharmaceutical drugs. The detection of endotoxin (generally synonymous with lipopolysaccharide where the molecule's lipid A moiety possesses most of the biological activity) is important for patient safety due to its pyrogenic properties and ability to trigger a form of septic shock.[1] In addition, endotoxin can be difficult to detect when bound with protein in the human body. This makes endotoxin testing for certain applications significantly challenging, especially for drug development and medical research, such as screening patients with severe sepsis and septic shock.

To meet this challenge, innovations in endotoxin testing are being developed in the form of biosensor technology. The most promising of these is a form of electrochemical aptasensor[2] which detects endotoxin through voltammetric determination of lipopolysaccharide. Aptamers show great affinity toward their target analytes, such as with endotoxin. The aptamer recognizes the molecular target against which it was previously *in vitro* selected. There are several such sensors in research use and the development phases are promising. Furthermore, these technologies have the potential to meet the requirements of 'rapid microbiological methods' in that they meet the criteria of good performance, accuracy, repeatability and had a shorter time-to-results. This article reviews progress in this area of endotoxin detection.

Endotoxin Detection Sensors

Due to more established laboratory-based assays not being suitable for the detection of endotoxin bound with protein, research has been underway over the past decade to produce novel endotoxin detection methods and endotoxin sensors. Investigational methods include hydrophobic interactions, localized surface plasmon resonance, mass spectrometry, Surface Enhanced Raman Spectroscopy, optical methods, voltammetric methods, shear horizontal surface acoustic wave (SH-SAW) biosensors, and electrochemistry. The focus of this article is with those biosensor methods that are most applicable to pharmaceutical drug development. Biosensors are categorized into three groups of optical-, mass-, and electrochemical-based sensors. The electrochemical type is the most likely to be commercialized due to its relatively lower cost and for displaying acceptable performance characteristics including high reproducibility, sensitivity, and stability.

To date, trials involving biosensors have been attempted based on fluorescence, chemiluminescence, electrophoresis, and electrochemical techniques. Some methods recognize and quantify the entire endotoxin molecule, others detect part of the endotoxin molecule through a chemical reaction or signal, including binding to a specific ligand. The criteria for such methods is for the end-product to be low cost, easy to operate, to produce rapid analysis, to be capable of high sensitivity and selectivity. Succeeding with this is based on producing specific recognition mechanisms and sensitive signal transformations.[3] In particular, electrochemical biosensors have a long history as as rapid, robust, cost-effective and accurate analytical tools for the detection of various target molecules.[4] This concept is being extended to lipopolysaccharide.

Electrochemical Sensors

In order to develop suitable forms of endotoxin testing, an understanding of the intermolecular interaction of an endotoxin with other biomolecules is required. It is through this understanding that progress has been made with the development of aptasensors, which demonstrate how a modified electrode can have good selectivity for lipopolysaccharide over other biomolecules. The emphasis upon selectivity is important for a nontarget biological sample may cause a higher phase shift than the actual value, which would inevitably lead to incorrect experimental results being provided. Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule (such as single-stranded nucleic acids). They possess strong binding affinity and high specificity to various target substrates, such as ions, proteins and cells.[5]

Important criteria for these types of sensors are: Electrochemical activity, electrical conductivity, surface area, ease of functionalization and biocompatibility with the samples intended to be analyzed.

An electrochemical sensor based on dual functional copper (II) cation (Cu2+)-modified metal–organic framework nanoparticles for sensitive detection of bacterial lipopolysaccharide has been developed. In terms of how this type of sensor works, lipopolysaccharide is immobilized in gold nanoparticles and reduced graphene oxide by C18 alkane thiol chains. Graphene, which can be produced through chemical vapor deposition, is a 2-dimensional material with special physicochemical properties (including excellent conductivity and high mechanical strength). Graphene has good electrocatalytic activity toward small biomolecules, and it is biocompatible when used as a sensitive layer for the immobilization of biomolecules. This step is necessary given that lipopolysaccharide can interact with the C18 alkyl chains by strong intermolecular interactions.[6] Gold nanoparticles act as the signal amplification component, together with signal output components and molecular recognition components. Gold nanoparticles have a number of useful properties, including rapid electron transfer, high surface area, excellent biocompatibility and facile synthesis.[7] The main developmental point tends to center on the successful formation of each layer on the gold electrode when forming the nanocomposite.

Following the immobilization, Cu2+-modified metal–organic framework nanoparticles are captured by the anionic groups of the carbohydrate portions of liposaccharide molecules, and this functions as the recognition unit. This signal is accentuated by the Cu2+-modified metal–organic framework nanoparticles catalyzing dopamine oxidation, which generates aminochrome. This produces a strong electrochemical oxidation signal. Primary development issues include capturing dissociative lipopolysaccharide, which can be overcome with differential pulse voltammetry.

In a study conducted at East China Normal University,[8] an electrochemical sensor based on dual functional Cu2+-modified metal–organic framework nanoparticles was investigated by differential pulse voltammetry to monitor levels of lipopolysaccharide. The resultant method demonstrated a wide linear range from 0.0015 to 750 ng/mL, with an assigned limit of detection of 6.1×10^{-4} ng/mL (electrochemical impedance spectroscopy can be used to detect varying concentrations of endotoxin, before and after exposing to samples). This is demonstrated by assessing the linear relationship with the logarithmic values of the endotoxin concentrations, with a correlation coefficient of $R^2 > 0.98$ being desirable.

Assessing endotoxin in nanograms is important from the medical perspective, where leukocytes respond to lipopolysaccharide (at nanogram per milliliter concentrations with secretion of cytokines such as tumor necrosis factor-alpha (TNF-alpha), and the association where excess secretion of TNF-alpha causes endotoxic shock.[9] While the relationship between liposaccharide weight and potency is dependent upon the bacterial species, for standard control endotoxin *Escherichia coli* 055:B5, then 1 ng of endotoxin is approximately equivalent to 0.5 endotoxin units. There are also some complexities with types of endotoxin. The structures of lipid A vary, for example, between enteric and non-enteric Gram-negative pathogens and there is also sometimes heterogeneity within organisms as well as between differences between species.

Further trials used the sensor to detect LPS in mouse blood serum, in line with research into the connection between bacterial endotoxins (especially microbiome-derived lipopolysaccharide) and the inflammatory and pathological processes associated with amyloidosis and Alzheimer's disease.[10] With this evaluation, satisfactory results were achieved, including what was reported as good reproducibility, low detection limit, and specificity. Experiments have also demonstrated the recovery upon spiking lipopolysaccharide in clinical grade insulin, again demonstrating a promising application for the trace analysis of endotoxin in the field of pharmaceutical products.

In terms of advancing this form of biosensor, design obstacles that need to be addressed include overcoming the formation of insulating films that can arise through the interaction of the analytes with their probing molecules, which can become immobilized on the electrode surfaces. It is also important that the aptamer layer contains very few defects. There can also be problems with detection in relation to pH. The pH value affects the responses of biosensors to their analytes, especially under highly acidic or alkaline environments. Extremes of pH can damage aptamers or affect the interaction between aptamers and their targets. This is a problem that exists with the conventional *Limulus amebocyte lysate* (LAL) test. As with other enzymatic tests, LAL assay results are susceptible to changes in temperature and pH and to the presence of protease and/or impurities.[11] Different materials also interfere with the conventional LAL assay, such a nanoparticles (which represent an important area of medical application for drug delivery). In particular, gold nanoparticles are known to interfere with various *in vitro* assays like LAL due to their optical properties and potential for surface reactivity. The interference does not occur with the biosensor application.

Colorimetric and Fluorometric Sensors

Alternatives to the electrochemical sensors are those based on colorimetric and fluorometric technology. An example is 3-phenylthiophene-based water-soluble copolythiophenes (colored, aromatic solids) for the detection of lipopolysaccharide). Such

sensors display high selectivity to lipopolysaccharide in the presence of other negatively charged bioanalytes as well sensitivity with the detection limit at picomolar level.[12] Copolythiophene based sensors have bene shown to be capable of rapidly discriminating the Gram-negative bacteria (with lipopolysaccharide in the membrane) from Gram-positive bacteria (without lipopolysaccharide).

A new strategy from an alternative laboratory is based on the inhibition of ion transport by lipid bilayer derived from spontaneous assembly of lipopolysaccharides. With this colorimetric method, at acidic pH values, lipopolysaccharide binds with aminophenylboronic acid modified assembled magnetic nanospheres. This results in formation of lipid bilayer around the magnetic nanospheres. Under acidic condition, the lipid bilayer inhibits the release of iron ions from the magnetic nanospheres into the solution, which decreases the oxidized extent of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt mediated by hydrogen peroxide. This enables lipopolysaccharide to be detected over the wide linear detection range with the low detection limit. One application where the technology is being considered is in relation to water testing.[13]

Flow Cytometry-Based Magnetic Aptasensors

A related development is with flow cytometry based magnetic aptasensor assays for lipopolysaccharide detection. Such methods utilize two endotoxin-binding aptamers and magnetic beads to detect endotoxin. This is in the form of an endotoxin-conjugated sandwich complex fixed to magnetic beads, developed through the application of scanning confocal laser microscopy. Trials have shown that magnetic aptasensors can rapidly detect (in under one minute) endotoxin within a detection range of 10^{-8} to 100 mg/ml (including masking with bovine serum albumin, RNA, sucrose, and glucose, materials that can interfere with conventional endotoxin assays). These materials were selected because they are most likely to coexist with endotoxin in biological liquids.[14]

Lab-On-Chip Endotoxin Detection

Researchers have also succeeded in exploiting the optical features of nanoplasmonic transducers supporting Localized Surface Plasmon Resonances (LSPRs) for lipopolysaccharide detection. With this approach, ordered arrays of gold nano-prisms and nano-disks can be created through nanospheres lithography. The resultant transducers can be integrated into a simple and miniaturized lab-on-a-chip platforms and functionalized with specific antibodies as sensing elements for the detection of lipopolysaccharide. Such devices work via interactions of specific antibodies anchored on protein A-modified sensor chips. Due to the optical and physicochemical properties of plasmonic nanostructures, the test has a robust ability to concentrate light energy in nanoscale volumes, and subsequently the increased near field intensity in relation to incident light makes creates a useful transducing platform for endotoxin detection.[15] A good linear relationship between peak shifts and the lipopolysaccharide concentration has been demonstrated for the fabricated nano-structures with a detection limit down to 5 ng/mL. This means endotoxin detection is possible through integration with a proper microfluidic platform, which could be used to assess the endotoxin content of products under development. Moreover, this concept could also see microfluidic devices integrated into wearable medical devices, where such devices have suitable flexible properties.

Summary

Endotoxins are ubiquitous microbiological contaminants, and their role can pose problems for new drug development and in the clinical field, particularly when they cannot be accurately detected. Standard traditional techniques are not always suitable, and this has led to an evolving field of agile endotoxin detection systems. These are biosensor based endotoxin detection methods, several of which are moving towards commercially available detection methods. Much of the current work is centered around the stability of the methods.

In time, such developments may further influence of omics for endotoxin detection. Of the different sensors, the greatest success has been reported with endotoxin-detecting impedance aptasensors. This is provided that the main design and operability constraints can be overcome, which primarily relate to maximum aptamer probe coverage and pH.

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