Chapter 8

Detection Methods for Lipopolysaccharides: Past and Present

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Additional information is available at the end of the chapter

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Abstract

Lipopolysaccharide (LPS) is the primary component of the outer membrane of Gram-negative bacteria. LPS aids in protecting bacterial cells, and also defines the unique serogroups used to classify bacteria. Additionally, LPS is an endotoxin and the primary stimulator of innate immune cells in mammals, making it an ideal candidate for early detection of pathogens. However, the majority of methods for detection of LPS focus on detection of the endotoxic component of the molecule, lipid A. Since lipid A is largely conserved among bacterial species and serogroups, these detection approaches are highly nonspecific. Thus, the importance of identifying the O-polysaccharide antigenic portion of LPS, which confers serogroup specificity, has received a great deal of attention in recent years. However, methods that are highly selective to the O-antigens are typically less sensitive than those that target the endotoxin. Here we present a history and comparison of the sensitivity of these methods and their value for detecting bacteria in a variety of different sample types.

Keywords: lipopolysaccharide, endotoxin, O-antigen, Serogroup, biodetection

1. Introduction

The increasing occurrence of infectious disease is a global issue. Emerging pathogens with increasing levels of drug resistance are a continuing danger to both public health and agriculture. Accurate and rapid detection of pathogens is critical to implement preventative measures to mitigate this problem. Despite this urgent need, conventional methods for bacterial detection require cell culture and serology, which can take several weeks. As new pathogens emerge, it is even more important that our detection technologies evolve to keep pace with the need to

discriminate pathogen from host flora. This requires an understanding of pathogen biology, the types of samples they occur in, and their mechanism of immune interaction within the hosts [1].

The innate immune system is able to discriminate pathogens from nonpathogens, and rapidly sense pathogen biomarkers in the complex milieu of the host. Exploiting this recognition via measurement of pathogen signatures, can provide an optimal strategy for discriminatory biodetection. A primary category of such biomarkers is virulence signatures termed pathogen-associated molecular patterns (PAMPs) [2]. PAMPs are evolutionarily conserved molecules that bind pattern-recognition receptors in the host, and activate the innate immune response [2, 3], providing a means for both early and specific pathogen detection. Biochemically, PAMPs are a diverse array of proteins, lipopeptides, lipoglycans, peptidoglycans, teichoic acids, and nucleic acids [4]. However, many detection methods have largely focused on proteins and nucleic acids [1, 5], ignoring other categories of PAMPs [2, 6–8]. Also, their small size, biochemistry, and low concentration in hosts make them difficult to target in detection assays [8, 9].

Classified as a lipogylcan, lipopolysaccharides (LPS) are small amphiphilic molecules that are associated with Gram-negative bacteria [7, 10]. LPS is an indicator of active infection, is serogroup-specific [11–13], more stable than its protein counterparts, and is released early in infection, making it an ideal candidate for detection and diagnostics. LPS serves as a biomarker that aids in serological discrimination of Gram-negative bacteria; this allows for identification and characterization of pathotypes that are essential for timely mitigation and treatment of infections. Since LPS is a pathogen-specific biomarker, it is an indicator of acute infection, which is an advantage over serological assays. In addition to medical diagnostics, LPS detection provides a method for detecting *Escherichia coli* in the food-industry, which is often associated with food-borne illnesses. Finally, LPS is also a virulence factor whose structure and function determines *E. coli* serogroup, a factor which has ramifications on vaccine design and therapeutic interventions. While many methods for LPS detection exist, most of them are not optimized for amphiphilic detection in physiological samples. An ideal measurement for LPS should be sensitive enough to detect low concentrations of the amphiphile in aqueous physiological milieu (e.g., blood), and use antibodies or ligands that provide serogroup selectivity [14]. Coupling sensitive detection platforms with surfaces designed to maximize the binding of amphiphilic PAMPs is a potential solution to achieve such an ideal.

2. Sources of lipopolysaccharides

Bacteria are classified into Gram-negative and Gram-positive [15], which release amphiphilic virulence factors such as LPS, lipoarabinomannan (LAM), and lipoteichoic acid (LTA) in the host. Species of pathogenic Gram-negative bacteria of concern to human health, include *Acinetobacter* [16], *Burkholderia* [17], *Bordetella* [18], *Campylobacter* [19–21], *Chlamydia* [22, 23], *E. coli* [20, 24], *Helicobacter* [25, 26], *Hemophilius* [27], *Klebsiella* [28], *Legionella* [20, 29], *Moraxella* [30], *Neisseria* [31], *Pseudomonas* [32], *Proteus* [33], *Salmonella* [20, 34], *Shigella* [35], *Yersinia* [36], and others, grouped into the Enterobacteriaceae family. These pathogens are contaminants in food, water, and soil, used as agents of bioterrorism, and can cause nosocomial infections [5]. Detection of these organisms, particularly *E. coli*, is an important aspect for epidemiology, disease control, and treatment.

Herein, we present a comprehensive description of the structural and biochemical properties of LPS, current methods for its detection, and potential approaches to overcome the current limitations for direct detection of the molecule in physiological matrices.

3. Lipopolysaccharide structures and conformations

Lipopolysaccharides have been the subject of intense study for over half a century [37–39]. LPS is the prototypical lipoglycan with an overall net negative charge [40–42], and is the primary component of the outer membrane of nearly all Gram-negative bacteria [11]. The bacterial membrane of each *E. coli* cell is composed of approximately 10^6 lipid A moieties and 10^7 glycerophospholipid molecules, comprising approximately three-quarters of the outer membrane [43–45]. Thus, there are approximately 62 pg of LPS per cell (for *E. coli* in log phase growth) [46]. LPS has an amphipathic tripartite structure (**Figure 1**). Lipid A is the most conserved portion of the LPS molecule, and consists of six, sometimes seven, fatty acid tails (*E. coli* and *Salmonella*, respectively), which gives the molecule its hydrophobic properties [10, 43, 45]. Lipid A is also called endotoxin [43], and is responsible for the biological effects of LPS

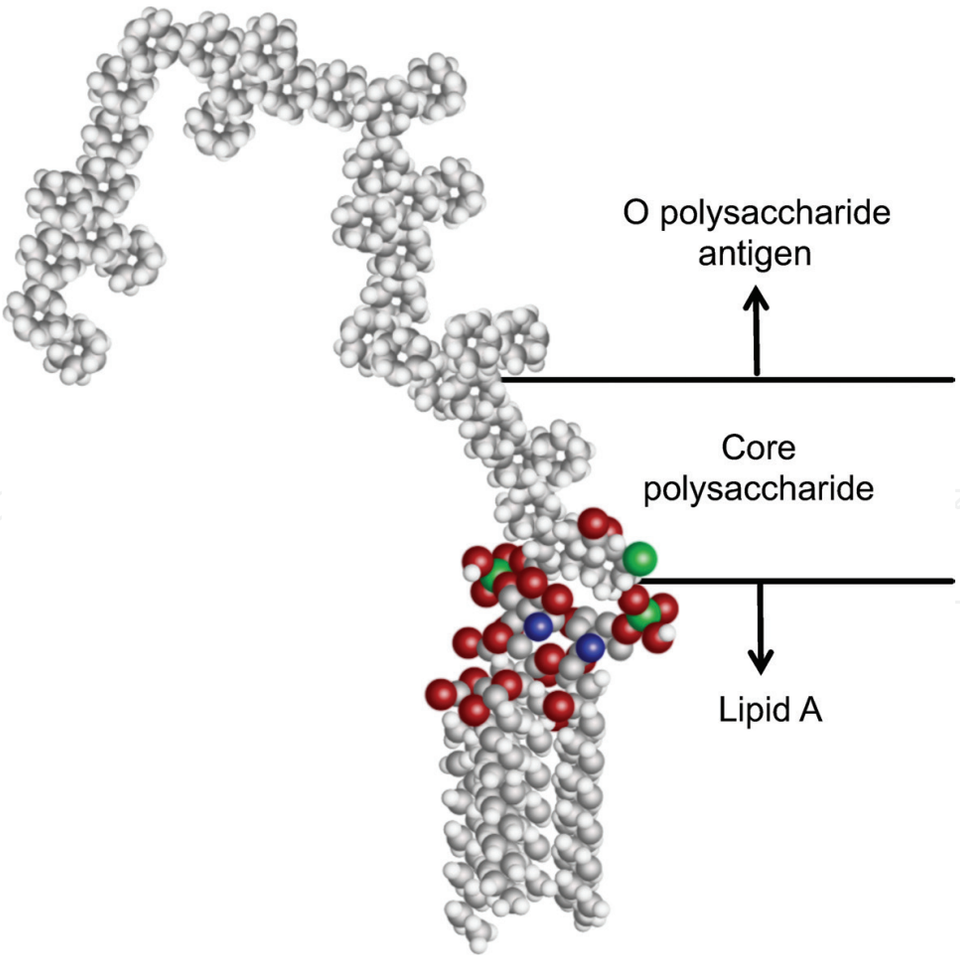

Figure 1. Representative structure of the molecular components of smooth LPS. The hypervariable O-polysaccharide antigen, core polysaccharide, and the hydrophobic lipid A group. Reprinted with permission from Ref. [74].

caused by its binding to the mammalian innate immune receptor, toll-like receptor 4 (TLR4) [11, 44, 47, 48]. Structurally, lipid A is covalently bound to the core polysaccharide, which is further divided into the inner and outer core polysaccharides, with the outer core being less conserved in both sugar moieties and location of glycosidic linkages [45, 49, 50].

There are two main forms of LPS—smooth (S-form) and rough (R-form) [42, 45, 46]. The distal end of LPS extends to a long chain O-polysaccharide antigen (O-ag(s)) in organisms possessing S-form, which is an indicator of virulence [51, 52]. R-form LPS is devoid of the O-ag [45], but can still induce an immunogenic response [53]. The O-ag is hyper-variable, and made up of repeating subunits, each composed of 1–7 glycosyl residues [45, 54]. As many as 40 size variations in subunit repeats of the O-ag have been reported just for *E. coli* O111:B4 [55], and 180 O-ag have been identified overall for *E. coli* species [47, 54]. The sugars (colitose, paratose, tyvelose, and abequose) that make the O-ag unique are seldom found elsewhere [54]. Other variations to the polysaccharide are implemented through addition of noncarbohydrate entities, such as acetyl or methyl groups [54]. These variations make discriminative detection of enteric bacteria feasible [56], but complicate antigen characterization. Therefore, LPS serves as an ideal target for early detection and identification of Gram-negative pathogens.

In aqueous solutions, amphiphiles like LPS can present in a micellar conformation [48, 55, 57–59]. This occurs at a concentration specific to the amphiphile [55], and is known as the critical micelle concentration (CMC). At or above the CMC, there is an equilibrium state between monomers, micelles or supramolecular aggregates, depending on environmental conditions [48, 55–57, 60–63]. This amphiphilic biochemistry and structural variability complicates determination of the exact molecular weight of S-form LPS. As such, LPS concentrations are reported in weight per volume, or in endotoxin units (EU), a measure of activity. As degree of endotoxicity can vary according to bacterial origin, a rough estimate of 100 pg = 1 EU is used in many cases to facilitate unit conversion [64, 65].

The large oligosaccharide region on S-form LPS makes the molecule amphipathic [54], which influences the shape of micelles in solution. Lipid A is largely responsible for shaping the LPS micelle [10, 45, 46, 56, 66–68], although other factors can also contribute. Lipid A is conserved within species in the number of fatty acid chains and the degree of saturation [44, 66] within those chains [22, 47, 69]. Shapes for LPS micelles include cubic, lamellar, and hexagonal inverted structures [56, 67, 70, 71]. Whether aggregate or monomeric forms (or both) of LPS is required for innate immune activation is debatable [56, 72, 73]. Since this process occurs in aqueous blood, it is unlikely that the molecule is presented as a monomer, unless associated with serum binding proteins.

Variation in LPS micelles [55] modifies presentation of O-ag-specific epitopes to antibodies, making detection challenging [74, 75]. This is specifically true when the heterogeneous presentation of linear [76] and conformational epitopes [49, 77] present on LPS molecules are considered. The primary structure of LPS varies in the core polysaccharide, within and between species [47, 55]. Core polysaccharides are primarily made up of common sugars such as heptose and 2-deoxy-D-*manno*octulosonic acid (a.k.a. KDO), which can be functionalized with phosphate or ethanolamine groups [45, 50, 78]. This feature contributes to varying charge distributions and differential size ratio of the hydrophobic to hydrophilic regions which influences

micelle assembly [10, 59, 79, 80]. Other factors that contribute to micelle shape [10, 79] are pH [61], ion concentration [81–86], and temperature [62]. These biochemical properties drive host-pathogen interactions and should be considered in the design of detection strategies.

4. Detection methods for lipopolysaccharides and similar amphiphiles

There have been many efforts to establish rapid and reliable detection methods for LPS in clinical samples [10, 46] and for testing pharmacological products such as infusion fluids, sterile injectables, medical device implants, and others [87]. These methods can be broadly divided into six overlapping categories: *in vivo* and *in vitro* tests, immunoassays and their derivatives; biological, chemical, and cell-based sensors. These methods span a broad range of sensitivity, but many lack the ability to differentiate between LPS serogroups.

4.1. Limulus amoebocyte lysate assay and the rabbit pyrogen test

The first method approved by the US Food and Drug Administration for LPS detection was called the rabbit pyrogen test [88–90], which simply measures the ability of an endotoxin to induce fever in an animal. Any febrile response was attributed to the presence of endotoxin [89–91]. The test, clearly, is activity-based, and nonspecific. In the case of Hepatitis B vaccine manufacturing, the rabbit pyrogen test is still the standard method for determining endotoxin contamination [91], but the test is cost prohibitive and is minimally utilized today, except in some parenteral devices [10].

In 1956, Bang discovered that amoebocytes from *Limulus polyphemus* (a.k.a. horseshoe crab) agglutinate upon addition of endotoxin [46], as a result of a protease cascade [10]. Bang and Levin [46, 92] subsequently used this concept to devise a method for endotoxin detection. Since the lysates of amoebocytes were required, it was called the limulus amoebocyte lysate (LAL) assay, and is the gold standard for the detection of lipid A. The LAL assay is prone to variability and can be inhibited through several mechanisms. The United States Pharmacopeia and the Code of Federal Regulations have consequently published guidances for the manufacturing and testing of assays for use on human products [93, 94]. Despite some challenges, the LAL assay is more rapid, cost effective, and reportedly 300 times more sensitive [46] than the rabbit pyrogen test [46].

Variants of the LAL assay use turbidimetric [95], chromogenic [46], or viscosity [10] measurements to determine results [10, 46]. A turbidimetric gel clot has more coagulen, and measures the change in turbidity over time, but does not form a solid clot [46, 95]. The viscosity assay, however, measures the degree of clotting via the change in viscosity. The chromogenic assay can be endpoint or kinetic, and utilizes a *p*-nitroaniline substrate, which is cleaved by an LAL proenzyme, providing a colorimetric readout [46]. The sensitivity of LAL assays is dependent on the sample type, processing method and time, as well as the dilution factor [46]. Additionally, the source of the LAL reagent plays a factor, as it is apparent when comparing the different limits of detection (LoD) reported for endotoxin standards. A survey of the relative sensitivities of the LAL assay is shown in **Table 1**.

Description	Sample	Detection method	Species	Sensitivity (ng/mL)*	Specific	Source
Rabbit pyrogen	Purified endotoxin	Febrile response	–	–	No	[89]
LAL	Plasma	Gelation	Multiple species	0.5–5.0	No	[96]
LAL	Blood, plasma	Gelation	*E. coli*	0.5–5.0	No	[92]
LAL	Serum plasma	Optical density	*E. coli*	0.025–0.5	No	[100]
LAL	Urine	Gelation	*E. coli*	0.5	No	[204]
LAL	Urine	Optical density	Multiple species	2.0	No	[98]
LAL	Spinal fluid/ plasma	Optical density	*E. coli, Haemophilus influenzae B*	0.1	No	[101]
LAL	Ascites	Gelation	*E. coli*	0.5	No	[104]
LAL	Cerebral/synovial	–	*E. coli*	1.0	No	[103]
LAL	Seawater	Optical density	*E. coli*	2.3	No	[41]
LAL	Purified endotoxin	Gelation	*E. coli*	1.0	No	[95]
LAL	Purified endotoxin	Gelation	*Salmonella minnesota*	10^{-11}	No	[111]
LAL	Ground beef	Gelation	*Enterobacter aerogenes*	–	No	[205]
LAL	Ground beef	Gelation	Multiple species	51.0 ng/g	No	[108]
LAL	Milk	Chromogenic	*Pseudomonas putida*	0.01	No	[107]
LAL	Purified endotoxin	Gelation	*E. coli* O114	100	No	[206]
LAL-magnetoelastic sensor	Purified LPS	resonant frequency	*E. coli* O111:B4	0.0105 EU/ mL	No	[207]
ENDOLisa® (LAL)	Purified endotoxin	Fluorescence	*E. coli* spp., *Salmonella* spp.	0.05–500 EU/ mL	No	[129]
ELISA	Milk	Abs at 405 nm	*E. coli*	100–200		[165]
LPS pull down-sandwich ELISA	Pure cultures	Abs at 450 nm	*E. coli* O157	–		[125]
LPS pull down-sandwich ELISA	Purified LPS	Abs at 450 nm	*Salmonella* spp. (31 total)	1.0	Yes	[126]
Premier EIA *E. coli* O157	Stool extract	Spectro-photometric	*E. coli* O157	–	Yes	[122]
LPS pull down	Purified endotoxin	RIA	*E. coli* O114	300	No	[206]
LPS pull down-ion (NTA-Cu)	Purified LPS	EIS	*E. coli* O55:B5	0.0001–0.1	No	[161]
Diaphorase functionalized surface	Purified LPS	Chemical	*E. coli* O127:B8	50	Maybe	[87]

Description	Sample	Detection method	Species	Sensitivity (ng/mL)*	Specific	Source
LPS pull down-SAMs with synthetic peptide	Purified LPS	Electro-chemistry	*E. coli* ATCC 35218	21.8 pg/mL	No	[189]
LPS pull down-SAMs with aptamer	Purified LPS	EIS	*E. coli* O55:B5	0.1–1.0	Maybe	[159]
LPS pull down-gold electrode w/ aptamer	Purified LPS	EIS and cyclic voltammetry	*E. coli* O55:B5	0.001–1.0	No	[160]
LPS aptamer sandwich	Purified LPS	Electro-chemistry	–	10 fg/mL	Maybe	[188]
LPS pull down-gold electrodes w/ PmB	Purified LPS	EIS	*E. coli* O111:B4	0.2	No	[162]
Polydiacetylene liposomes	Purified LPS (5 groups)	Change in Abs	*E. coli* spp, *Salmonella* spp	2.22 mg/mL	Yes	[191]
Impedance enthothelial biosensor	Purified LPS in culture medium+	Resistivity of cell monolayer	–	500	No	[169]
Macrophage microarrays on gold electrodes	Purified LPS in culture medium	FTIR	*E. coli* O111:B4	0.1 μg/mL	No	[200]
Primary culture HDME cells	Purified LPS	Fluorescence	*E. coli* O111:B4	1.0 μg/mL	No	[171]
Engineered cells secrete alkaline phosphatase	Purified LPS in culture medium+	Electro-chemistry	–	0.1	No	[170]
LPS pull down-PmB	Purified LPS	Evanescent sensing	*E. coli* O128:B12	25	No	[75]
LPS pull down-TLR4/MD2 on gold electrodes	Purified LPS	Electro-chemistry	*E. coli* O55:B5	0.0005 EU/mL	No	[180]
LPS pull down-membrane insertion	Purified LPS (3 groups)	Evanescent sensing	*E. coli*	420	Yes	[74]
LPS pull down-antibody	Pure cultures in ground beef	Evanescent sensing	*E. coli* O157	–	Yes	[166]
LPS pull down-proanthocyanidin	FITC-labeled LPS	Fluorescence	*E. coli* O55:B5	–	No	[192]
Copolythiophene interacts with LPS	Purified LPS	Fluorescence	*E. coli* O55:B5	$2.5E^{-5}$–2.0 μM	No	[164]
Polydiacetylene liposomes	–	Fluorescence	–	0.1 μM	No	[190]
Peptide-based fluorescence	Purified LPS	FRET-increase	*E. coli* O111:B4	0.15–2.0 μM	No	[194]
Pyrenyl-derived long-chain quaternary ammonium probe	Purified LPS	Fluorescence	*E. coli* O55:B5	100 nM	No	[193]

Description	Sample	Detection method	Species	Sensitivity (ng/mL)*	Specific	Source
LPS pull down-peptide on Graphene Oxide	Purified LPS (4 groups)	Fluorescence	Several species	130 pM	No	[195]
LPS pull down-PmB capture	Purified LPS spiked in blood	Acoustic sensing	*E. coli* O55:B5	1.0	No	[196]
LPS pull down-CD14 capture	Biotin-LPS	Luminescence	–	10.0	No	[176]
LPS pull down Polyaniline + ConA lectin	Purified LPS and LTA	EIS	*E. coli*, *S. aureus*	50.0	No	[208]
Aptamer sandwich on beads	Purified LPS	Fluorescence	*E. coli* O55:B5	0.01	Maybe	[163]
LPS pull down endotoxin neutralizing protein	Purified LPS	Capacitance	*E. coli*	10^{-13} M	No	[181]
LPS pull down CramoLL lectin	Purified LPS (4 types)	EIS	*E. coli* *Salmonella* *Klebsiella* *Serratia*	25.0 μg/mL	No	[185]

*Unless otherwise indicated.

Table 1. Overview of sensitivities and specificities for LPS detection methods.

In 1970, Levin discovered that samples tested in whole blood would not render a positive result [92], but if plasma was extracted in chloroform and diluted 1–10%, then endotoxin activity could be detected in the 0.5–5 ng/mL range [92, 96]. Levin correctly assumed that components of whole blood were bound to endotoxin, thereby inhibiting the reaction with the LAL reagent [46, 92, 97], or changing the reaction kinetics [46]. This is evident when the amphiphilic nature of LPS and the aqueous nature of blood are considered. In addition to blood and plasma [46, 92, 96], the LAL assay has been used in urine [46, 98], cerebral spinal fluid, synovial fluid, ascites fluid, vaginal and cervical fluids, broncho-alveolar lavage samples, seawater [46], bovine milk [99], and beef tissue [100, 101]. Virtually all of these have reported ng/mL LoDs, for endotoxin, but none are serogroup-specific. Researchers have used heat [46, 102], chemical treatment with chloroform [103], acids [104, 105], alkali [106, 107], or ether [108] to improve sensitivity with some success when using heat or chemical extraction of the endotoxin [46, 109]. However, the results show poor reproducibility between researchers (**Table 1**). Yin and Galanos [106] reported a sensitivity of 10^{-11} ng/mL for *Salmonella* spp., while Cooper et al. [89] reported 1.0 ng/mL for *E. coli* endotoxin. This disparity leaves a lot of questions and draws attention to the fact that small changes in preparation, heat or chemical treatments, usage of plastics instead of silanized glass, or addition of surfactants can result in altered assay sensitivity. This variation can also be explained by the variable biosynthesis of lipid A, as shown with *Salmonella* [47, 110]. Additionally, LAL can yield false positives upon reacting with other polysaccharides or β-(1,3)-glucans [10, 46] and depends on the source of

bacteria, as LPS/endotoxin can vary in toxicity [10, 56, 79, 68], in regards to immune stimulation [41, 46, 111].

Thus, the LAL assay and rabbit pyrogen test, both based on the native immune responses of the horseshoe crab or rabbit, exhibit significant variability in outcomes. Despite these, the LAL is still very useful for quickly detecting contamination. For example, in 1981, Jay [101] used the LAL test to determine both microbial counts and endotoxin load in 153 samples of store bought ground beef with a mean sensitivity of 7.9 μg/mL (endotoxin/beef sample) in 1 h. In 1985, Nachum and Shanbrom [46] used a chromogenic LAL system to detect between 2 and 175 ng/mL of endotoxin in 324 patient urine samples, with the assay taking between 2 and 4 h. Timely detection is valuable to both patient care and product viability. Despite being an ideal test for the presence of endotoxin, determining identity of pathogens still requires culture or enrichment.

4.2. Immunoassays for LPS detection and antibody selection

Developed in 1971 [112, 113], the enzyme-linked immunosorbent assays (ELISAs) are based on the immune reaction between antigen and antibody, with each assay being tailored for the unique antigen being tested. ELISAs were evaluated for *Salmonella* O-ags very early in development [114]. However, ELISAs for lipoglycans such as LPS suffer from low sensitivity and reproducibility [115–117]. One of the primary reasons for sensitivity issues is the amphipathic biochemistry of these molecules, leading to inconsistent binding on ELISA plates [118], and variable conformations of epitope binding sites [12, 119].

There exist two primary types of LPS-ELISAs, which detect either the LPS antigen, or LPS antibody titers. With the former, the plate surface is typically coated with a primary capture antibody specific to LPS, or with the sample to be tested [118]. After antigen capture, an epitope-specific antibody is used to detect LPS. The detection antibody can be directly labeled with an enzyme [113] or secondary antibody for colorimetric detection [120, 121]. In 1998, Mackenzie et al. [122] reported on the effectiveness of a commercial assay to screen stool samples for *E. coli* O157 antigens, and found that re-testing samples provided inconsistent results. It was speculated that this was due to inefficient washing of the microwells, yet the amphiphilic antigen preparation and its presentation to antibodies could have contributed to assay inconsistencies. It was also not considered that LPS is notorious for nonspecific and inconsistent binding on microplates [10, 116, 118]. In clinical samples, the association of LPS with host carrier molecules may affect its ability to adhere to capture surfaces [123, 124], as proteins will preferentially bind to the plate. Some groups have also reported cross reactivity or false positives with LPS sandwich ELISAs [125, 126]. Choi et al. [126] developed a sensitive capture ELISA with 24 species of *Salmonella*, but cross reactivity was observed. To mitigate cross reactivity, attempts have been made to substitute antibodies with other ligands. Grallert et al. [127, 128] coated microplates with proteins isolated from bacteriophages, which are specific to core polysaccharides in order to capture LPS, followed by detection with Factor C (a component of the LAL assay). This sandwich ENDOLisa®, a microplate assay for direct detection of endotoxin, reports sensitivity between 0.05 and 500 EU/mL. This technology is sold as the Endotoxin Sample Preparation (ESP™) kit, and is one of the few kits available for direct

detection of endotoxin in blood or serum [129]. However, the assay is unable to differentiate between serogroups.

The second type of ELISA measures LPS antibody titers to screen for Gram-negative bacterial infections. Here, the surface of the plate is functionalized with the antigen to pull down antibodies (Immunglobulins A, G, and M (IgG, IgA, IgM)) from serum. Since this method is based on adaptive immunity, there is a lag between initial exposure to the pathogen, and increased antibody titers [130], making early detection difficult. This assay is not specific for active infection, but has been used to monitor population health and track epidemiology of infections. Screening has been used to detect exposure of military personnel to *Shigella* [131], obstetric patients with *Chlamydia* spp. [132], patients with *Salmonella* [133], and other pathogens [114, 134–137]. Suthienkul et al. [136] used an indirect ELISA to passively adsorb LPS onto polystyrene plates, and measure associated IgG/IgM titers in cholera patients. The results indicated discrepancies between the titers of IgG and IgM in young versus older patients, which could either be due to the inconsistency of LPS coating or associated with cross reactivity [138]. Suthienkul also acknowledged that antibody levels in infants screened could be inherited from the mother [136, 139].

Functionalizing ELISA plates with amphiphilic LPS is a technical challenge [12], since the surfaces are optimized for protein binding. In the late 1970s, it was discovered that polymyxin B (an antibiotic, PmB) interacted with LPS monomers in a 1:1 ratio [86, 140], and can be used to functionalize surfaces for Gram-negative detection [119]. However, PmB recognizes the conserved lipid A group of LPS, and does not allow for discriminative detection. Takahashi et al. [118] showed that precoating the plate with high molecular weight poly-L-lysine increases surface adsorption and allows for detection of 1 μg/mL LPS, with no cross reactivity. Others have studied the effects of ions such as calcium and magnesium [141], trichloroacetic acid [142], mixing the antigen in chloroform/ethanol, and drying on the plate surface [135], or complexing LPS with a protein such as bovine serum albumin [143] to improve performance and reproducibility. Functionalization of ELISA plates with proteins known to bind LPS, such as high- or low-density lipoproteins (HDL, LDL), chylomicrons, and LPS-binding protein (LBP) have also been evaluated [123, 124] and offers promise for the reliable detection of LPS antigen in complex samples.

Other limitations for LPS detection include the fact that many LPS antigens have not been isolated [144] and thus are not available for the development of screening assays, limiting accessibility of specific antibodies as well [145–150]. However, there is also a need to refine methods for selection of tailored antibodies. While there are variations [10], ELISA plates are typically functionalized with whole dead bacteria to screen monoclonal antibody cultures [145, 146, 148], giving rise to potentially cross reactive clones [10, 144] that are then screened against a multitude of bacterial strains [146, 149, 150]. It is noted that it is impossible to screen clones against all epitopes of LPS, even amongst the many *E. coli* serotypes. In 2000, Jauho et al. [12] addressed this issue by covalently linking purified LPS O-ags to polystyrene ELISA plates using anthraquinone and UV irradiation. This technique could prove useful in developing serogroup-specific antibodies against LPS, as conserved antigens like lipid A and core polysaccharide are absent. Alternative methods for antibody screening have utilized immunoblotting [144, 149, 151–153] and flow cytometry [154–156]. In addition, ELISAs can suffer from high background due to nonspecific interactions limiting their sensitivity [10, 122, 135, 157]. Particularly,

endogenous endotoxin present in reagents, on glassware, or plastics [158], may contribute to false positive results. Factors identified above have to be carefully considered in the development of ELISAs targeting LPS.

4.3. Biological and chemical-based LPS sensing

Many advanced methods such as electrochemical impedance spectroscopy (EIS) [159–161], antimicrobials [75, 162], aptamers [163], synthetic polymers [164], optical immunoassays [122, 125, 165], waveguide technology [75, 166, 167], lipid bilayers [9, 74, 168], and *in vitro* [169–171] assays have been applied for LPS detection. These technologies involve functionalizing biosensors with proteins or molecules to pull down LPS from a sample.

LBP [10], a relatively small protein (~60 kDa) that transports LPS in blood, shuttles the antigen to the cluster of differentiation 14 (CD14) protein in the extracellular matrix, or to the membrane of immune cells, such as macrophages [10]. After LPS binds CD14, it is passed to the hydrophobic binding pocket of myeloid differentiation factor 2 (MD-2) [7, 10], a necessary cofactor for the activation of TLR4. Also, the serum carrier lipoproteins (HDL and LDL), are carriers for LPS in blood. In addition to these, LPS has been demonstrated to bind aptamers [159, 160], various peptides [87, 109, 162, 172], and metal/cation complexes [84, 86, 161, 173–175]. Such carrier moieties are exploited in the development of novel detection methods for LPS, as outlined below.

For electrochemical (EC) sensing of LPS, a recognition ligand (similar to ELISA) and a transducer are required to measure the variation in signal [161]. For fluorescence-based sensing, a receptor captures LPS, while another molecule emits a fluorescent signal when bound to the antigen. Burkhardt et al. [176] used solubilized LBP to transfer LPS to a CD14 functionalized surface, with a LoD of 10 ng/mL using an electro-chemiluminescent assay. This method enforces the role of LBP as a lipid transfer protein, as demonstrated by Wurfel et al. [177, 178] and shows that CD14 can bind monomeric LPS in the absence of TLR4 [179]. Highly sensitive (LoD = 0.0005 EU) EC sensors have also been developed using a recombinant TLR4 + MD-2 complex for recognition of LPS [180]. Yet, these assays are unable to discriminate between LPS serogroups. Priano et al. [10] developed a competitive EC assay using recombinant endotoxin-neutralizing protein (ENP) on a dextran matrix, with a detection range of 1–100 ng/mL. ENP has also been used in a capacitive biosensor with an extremely low LoD (1.0×10^{-13} M) [181]. The sensitivity differences may be due to variations in surface functionalization. Priano et al. [10] used the dextran matrix, and Limbut et al. [181] used self-assembled monolayers, which provide low background interference [182–184]. Inoue and Takano [10] used a recombinant factor C in an EC hybrid LAL biosensor, with a sensitivity range of 5×10^{-4}–1.0 EU/mL [10]. Kato [87] and Iijima [10] labeled PmB with ferrocene-bound LPS in solution, and captured it on a nanocarbon-film electrode with a detection range of 2–50 ng/mL in 5 minutes [10]. Ding et al. [162] functionalized an electrode with PmB and performed EIS with a detection range of 0.2–0.8 ng/mL which is more sensitive, but has a smaller range. A broader detection range was demonstrated by Rahman et al. [172] who functionalized interdigitated electrodes with PmB and tested 0.1–1000 μg/mL of LPS O111:B4 in food samples, using impedance spectroscopy. Sugar binding proteins, such as lectins and polyaniline coated electrodes, have been used for detecting LPS [10], as with an EIS sensor functionalized with the lectin, cramoLL, with a detection range of 25–200 μg/mL [185].

Several assays have been developed using aptamers as the detection ligand. Su et al. [160, 186, 187] used aptamers attached to gold nanoparticles to detect LPS using EIS, with an impressive detection limit of 0.1 pg/mL [10]. Aptamers have also been used in a magnetic aptasensor to detect LPS in medias containing BSA, sucrose, glucose, or RNA [163], and provide a detection range of 0.01–1.0 × 10^6 ng/mL (LPS O55:B5) by flow cytometry within 1 minute. Bai et al. [188] developed an EC sensor where aptamers that bind LPS were hybridized with capture probes, which were hybridized to complementary DNA sequences on gold nanoparticles with a very sensitive range (10 fg/mL up to 50 ng/mL). However, multiple aptamer libraries against O-ag would be essential before this method could be implemented for serogroup discrimination. Modifications to improve sensitivity include use of SAMs to functionalize sensors with peptides [189], PmB [162], antibodies [10], and aptamers [159]. Despite optimal surface capture methods, some of these assays suffer from poor detection limits or range of performance [10, 159].

Investigators have utilized the interaction of LPS with synthetic systems such as copolythiophene copolymers [164] and polydiacetylene liposomes [190, 191]. Johnson et al. [192] demonstrated an endotoxin capture technique by functionalizing a bead matrix with proanthocyanidins and binding with fluorescein isothiocyanate-labeled LPS [192]. Pyrenyl-derived quaternary ammonium probes, developed by Zeng et al. [193] exhibited fluorescence when bound to LPS and detected nanomolar concentrations, while fluorescently labeled CD14 synthetic peptides demonstrated an increase in Förster resonance energy transfer when bound to LPS, but were only able to detect μM concentrations [194]. Lim et al. [195] used a functionalized graphene oxide to develop a fluorescence quench-recovery method for LPS, targeting the lipid A component. Thompson et al. [196] designed a tandem system to both detect (LoD = 1.0 ng/mL) and filter LPS from blood using piezoelectric quartz discs functionalized with PmB.

Other methods have taken advantage of the amphipathic nature of LPS. Harmon et al. [197] demonstrated that disrupting the hydrophobic association of LPS with liposomes increases the sensitivity of the LAL assay. Stromberg et al. [74, 198] were able to detect 4.20 μg/mL of amphiphilic LPS O157 in beef lysates on a waveguide biosensor using a technique called membrane insertion, which has previously been applied to other amphiphiles such as LAM and phenolic glycolipids [8, 9, 199]. Membrane insertion uses the natural association of amphiphiles with a lipid bilayer to facilitate detection and fluorescent detection of a labeled antibody is performed within an evanescent field [168, 199]. Many biosensors report exquisite sensitivity, even down to the picogram [164] and femtomolar [9, 168, 199] range, but very few are capable of physiological presentation of amphiphiles to facilitate discriminative detection of O-ag groups [74, 167, 198].

4.4. Cell-based LPS detection systems

Cell systems are ideal for recognizing endotoxin, although interpreting the signal response can be challenging. Bouafsoun et al. [169] functionalized the surface of an impedance biosensor with endothelial cells, and measured the decrease in impedance with LPS binding, with a sensitivity of 500 ng/mL. Veiseh et al. [200] patterned macrophage cells onto gold electrodes to detect LPS concentrations of 0.1–10 μg/mL. However, cells were concurrently stained with necrosis and apoptosis markers in parallel studies, and no staining effect could be seen in cells using concentrations less than 10 μg/mL. This is an interesting effect, as in many *in vitro* studies,

cytokine response is induced at much lower concentrations of endotoxin [97, 201, 202]. It can be deduced that Veiseh used serum supplemented media in the experiments, and the lipoproteins and LBP in serum could have a protective effect on cells [46, 202, 203], and attenuated assay sensitivity. The most sensitive cell-based assay was developed by Inoue et al. [170] with a LoD of 0.1–1.0 ng/mL. Here, cells were engineered to secrete alkaline phosphatase in the presence of LPS, and patterned on the surface of an amperometric biosensor to measure voltage change upon LPS binding. Cell-based *in vitro* assays are prone to errors and contamination, so developing a robust and fieldable assay based on this technology is not plausible. However, by studying LPS in cell-based systems, knowledge about interactions with receptors and cell membranes can be gained, which can facilitate better detection methods.

5. Conclusions

Many novel approaches have been used for the detection of amphiphilic LPS, not all of which are functional in physiological matrices or have the required sensitivity or ease of use. One major reason for this is the failure to incorporate the amphiphilic properties of the antigen into assay design. The presentation, conformation, and host-interactions of the antigens should be considered for the development of effective assays. While both LAL and EC assays are the most sensitive for testing endotoxicity, identifying O-ag with a high degree of selectivity remains elusive, and limited to methods that use specific recognition ligands, such as membrane insertion and ELISAs. By far, the greatest limitation has been the lack of sensitive and selective ligands for the serogroup-specific detection of the antigen. Thus, as repositories of these necessary recognition molecules expand to include more serogroups, so too will our ability to selectively detect LPS.

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