

# Lipopolysaccharide (LPS) and Protein-LPS complexes: Detection and Characterization by Gel Electrophoresis, Mass Spectrometry and Bioassays

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## Abstract

A prerequisite to the discovery and characterization of lipopolysaccharide (LPS) interaction with specific receptors and the resulting pathophysiological effects is the comprehensive structural analysis of LPS species. This brief review is aimed to summarize the use of gel electrophoresis linked to other biochemical technologies for detecting and characterizing LPSs. Lipopolysaccharide aggregates alone or mixtures containing LPSs and proteins/peptides can be separated by native agarose gel electrophoresis (NAGE), after which LPSs are detected with imidazole and zinc salts. A double-staining process with Coomassie brilliant blue R-250 enables the use of NAGE for detecting and studying protein-LPS interactions. For compositional analysis, the LPS aggregates are separated, with high resolution, by surfactant-polyacrylamide gel electrophoresis. After reverse staining with zinc-imidazole and elution from gel microparticles, glycoform-specific LPSs are ready for structural and biological analysis. For sequence analysis based on tandem electrospray ionization mass spectrometry (ESI-MS/MS) of oligosaccharides, LPSs are subjected to mild acid hydrolysis, dephosphorylation and permethylation. Also, O-deacylated LPS forms can be analyzed by matrix-assisted laser desorption/ionization-time of flight-MS. By comparison to spectra of unpurified LPSs, mass spectra of the micropurified LPSs show reduce heterogeneity and increased signal-to-noise ratios. Furthermore, the micropurification of LPSs prior to MS allows a higher sensitivity of detection for less abundant LPS glycoforms. The micropurified LPS fractions can be used to form self-assembled nanoaggregates which may be detected by dynamic light scattering. The effect of the O-side chain length on the Z-potential of LPS aggregates may be estimated by measurements based on laser Doppler electrophoresis. The thus obtained glycoform-specific LPSs are not only intact chemically but also biologically active as tested by e.g. *Limulus amoebocyte lysate* test, TNF- $\alpha$  assay and agonistic effect on human Toll-like receptor 4.

**Keywords:** Lipopolysaccharides; Aggregates; Agarose; Electrophoresis; Zinc stain; Mass spectrometry; ESI-MS; MALDI-TOF-MS; TLR4

## Abbreviations

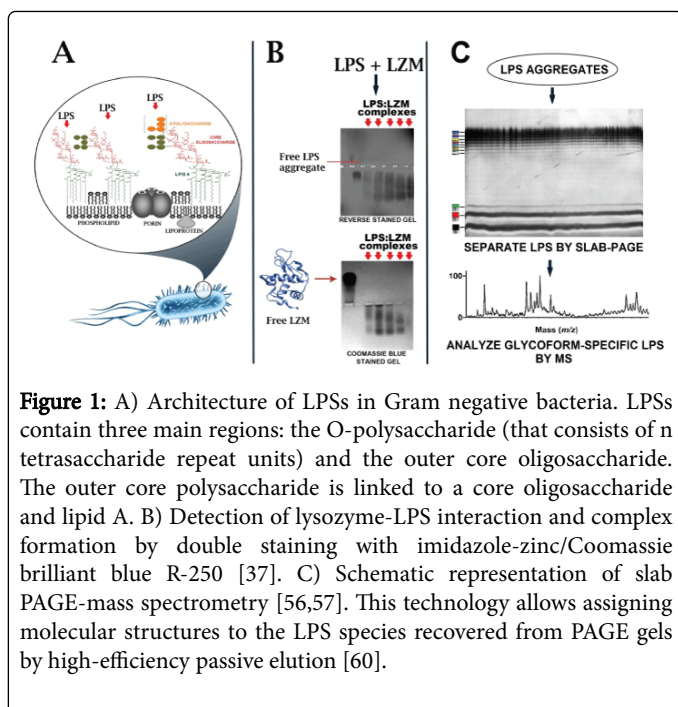
LPS: Lipopolysaccharide(s); NAGE: Native Agarose Gel Electrophoresis; SDS: Sodium Dodecyl Sulfate; PAGE: Polyacrylamide Gel Electrophoresis; S: Smooth; R: Rough; TLR-4: Toll-like Receptor-4; TNF: Tumor Necrosis Factor; DOC: Sodium Deoxycholate

## Introduction

Lipopolysaccharides (LPS) are the major biological macromolecules of the surface from Gram-negative bacteria (Figure 1A) [1]. Due to their pluripotential immunomodulatory properties (e.g., activation of B lymphocytes, macrophages and other immunocompetent host cells), these biomolecules are of huge interest in medicine [2-4]. Small amounts of LPS can be beneficial, but this situation radically changes when larger amounts are present in the bloodstream. In fact, enterobacteria- or antibiotic-released LPS arising during severe Gram-negative bacterial infections generally cause diverse pathophysiological effects (e.g., fever, leukopenia, tachycardia, hypotension, disseminated intravascular coagulation, and multiorgan failure). This may end in

septic shock, producing a high mortality rate (from 20-50%) and causing thousands of deaths annually worldwide [5,6]. Ironically, LPSs are essential for the survival of Gram-negative bacteria by playing a vital role in a variety of recognition processes and by providing a permeation barrier for harmful substances [7,8].

The general structure of LPS molecules deduced from more than one hundred enterobacterial species is described as consisting of a three part molecular architecture: the lipid A, core oligosaccharide, and outer core oligosaccharide and O-polysaccharide side chain (Figure 1A). Typically, lipid A is a dimer of  $\beta$ -(1-6)-linked glucosamine units with (R)-3-hydroxy fatty acids, attached via ester and amide linkages, of which two are regularly acylated further on their 3-hydroxyl groups. One phosphate group is usually present on each carbohydrate unit. Attached to the 6-position of one glucosamine residue is a short core oligosaccharide that is generally comprised of two unusual sugars (KDO [3-deoxy-D-manno-2-octulosonic acid] and heptose). Extending from the short core oligosaccharide may be a more heterogeneous outer core oligosaccharide that is then attached to an O-polysaccharide extension. The O-polysaccharide extension is comprised of oligosaccharide repeat-subunits of between 3 and 5 glycosyl monosaccharide residues that appear to vary substantially up to 40 repeating subunits in length [9-11].



**Figure 1:** A) Architecture of LPSs in Gram negative bacteria. LPSs contain three main regions: the O-polysaccharide (that consists of n tetrasaccharide repeat units) and the outer core oligosaccharide. The outer core polysaccharide is linked to a core oligosaccharide and lipid A. B) Detection of lysozyme-LPS interaction and complex formation by double staining with imidazole-zinc/Coomassie brilliant blue R-250 [37]. C) Schematic representation of slab PAGE-mass spectrometry [56,57]. This technology allows assigning molecular structures to the LPS species recovered from PAGE gels by high-efficiency passive elution [60].

LPS studies have shown significant progress during the past few years. Examples are: (i) the demonstration that lipid A, the glycolipid moiety from lipopolysaccharides, is the critical unit for the immunobiological activity of LPS [12], (ii) the successful chemical synthesis of lipid A [13-16], (iii) the first total synthesis of the naturally occurring Re-type LPS [17,18], and (iv) the discovery of Toll-like receptor-4 (TLR-4) as a key cellular receptor molecule for LPS [19-21].

Apart from the current biological and clinical studies concerning LPSs, great efforts are also underway to find new or improved ways to exploit LPSs for the benefit of both research and pharmaceutical uses. The reasons for this are many-fold. For instance: a) LPSs are powerful molecular tools to study a variety of cellular activation mechanisms involving the production of lymphokines and monokines (e.g., interferons, interleukins 1 and 6, tumor necrosis factor, lipid A-induced biochemical pathways) [22,23]. b) LPSs or derivatives are very potent stimulants of the immune system (i.e., these biomolecules sensitively activate such cells as monocytes, macrophages, mast cells, etc) [24]. c) LPS-derived lipid A with a reduced number of acyl chains (for example; four) can serve as an inhibitor of immune activation induced by Gram-negative bacteria, and synthetic versions of these inhibitors are in clinical trials for the prevention of harmful effects caused by Gram-negative bacterial infections [25]. d) Potent endotoxin antagonist may be obtained by slightly modifying the lipid A structure (e.g., E5564), provided the correct overall shape and intrinsic conformation of the lipid A are preserved [26,27].

In the adjuvant field, lipid A has been chemically modified to generate less toxic forms that are used as components of vaccines to improve their effect. Monophosphoryl lipid A is a key component in many of the novel adjuvant systems (e.g., from GSK) from investigational cancer prophylactic (e.g., against HPV-caused cervical cancer) to immunotherapeutic vaccine approaches (e.g., against breast, lung, melanoma, prostate cancers). Clinical experience with monophosphoryl lipid A-containing vaccine formulations is currently being extended, with tens of thousands of subjects already vaccinated

[28-30]. These adjuvant formulations containing monophosphoryl lipid A have improved both antibody responses (kinetics and titers) and cell-mediated immune responses.

Despite the many recent advances in the understanding of LPS biology and chemistry, much remains to be learnt on the mechanisms, at the molecular level, that are responsible for the LPS-associated pathogenicity and cellular activation [31-34]. Fresh discoveries are needed for: (i) the finding of new therapeutic interventions targeting components of the receptor clusters responsible for bacterial recognition or LPS/lipid A biosynthetic pathway, (ii) clinical trials aimed at evaluating LPS-related therapies against Gram-negative infections, septicemia or septic shock, (iii) the development of new LPS applications in other fields (e.g., adjuvants, drug delivery).

For fresh discoveries, improved lipopolysaccharide preparations that are well-characterized in terms of different LPS-related functionalities (e.g., the O-specific chain, core oligosaccharide, lipid A) will be needed. Having a means to separate LPS-specific glycoforms prior to a more in-depth characterization of their structural elements may be undoubtedly highly desirable. Therefore, this brief review will focus on the use of gel electrophoresis linked to mass spectrometry and bioassays as a useful approach for detecting and characterizing LPS aggregates.

## Materials and Methods

We are not attempting to provide a comprehensive summary on the analytical chemistry and biological effects of lipopolysaccharides but rather to provide our perspective on how gel electrophoresis can be connected to other biochemical techniques for a fine characterization of LPS aggregates and glycoform-specific LPSs. Thus, the goal of this report is to present a brief review of methods developed in our laboratory and lessons gathered from our experiences that may be useful to other researchers.

For this review, we used peer-reviewed, published papers that were identified by electronic searches in Medline and Google Scholar. The search strategy included different combinations of key words such as “lipopolysaccharide”, “agarose gel electrophoresis”, “polyacrylamide gel electrophoresis”, “mass spectrometry”, “biological activity”, “Limulus amoebocyte”, “tumor necrosis factor”, and “toll-like receptor 4”. No language limits were applied. Search period was not longer than 10 years. In addition, we performed manual searches in reference lists from specific papers to find additional pioneer and older studies that could have been overlooked by the electronic search.

## Separation and detection of LPS aggregates

The lipopolysaccharide molecules, which are amphiphilic, have a strong tendency to form high-molecular-mass supramolecular aggregates such as filaments, spherical-like particles, ribbon-like structures and micelles. The size and shape of LPS aggregates are governed by LPS concentration, temperature, pH, water content, concentration of mono- and divalent cations and other physico-chemical parameters [35]. The conformation and hydrophobicity of the contributing glycoform-specific LPSs also influence the aggregation of lipopolysaccharides. Indeed, whether the formed LPS aggregates are micellar, lamellar, or nonlamellar inverted in shape is determined by the ratio of the cross sections of the hydrophilic and hydrophobic moieties [36].

Very recently, we have reported the reversible negative staining with imidazole and zinc salts of LPS aggregates separated in agarose gels [37]. After incubating the electrophoresed agarose gel in 0.2 M imidazole solution, reagents (e.g., sulfate groups, Tris) that can potentially hamper the reverse staining of LPS aggregates are washed out of the gel matrix [38]. Once the imidazole-equilibrated gel is soaked in 0.2 M zinc solution, negatively charged LPS molecules will attract electrostatically zinc ions, which diffuse into the gel matrix. The strength of the charge interaction between the positively charged zinc ions and groups (e.g., negatively charged phosphates, cis-diols in the sugar moiety and carboxylate groups in sialic acids) from the LPSs will depend on the degree of ionization of these molecules. Simultaneously, phosphate and carboxylate groups will repel most of the counterions from the immediate vicinity or gel zone occupied by the LPS molecules, thus reducing the anion concentration that can locally precipitate zinc salts. Since the ionic product of free zinc and counterions does not equal or exceed their corresponding solubility product constant ( $K_{sp}$ ), regions of the gel containing zinc-LPS complexes will remain transparent and colorless. In contrast, LPS-free regions will be replete with zinc imidazolite ( $ZnIm_2$ ) [38], provided that the ionic product is greater than the corresponding  $K_{sp}$ .

This new stain requires a few  $\mu$ g of LPS aggregates for detection, and combines desired features such as simplicity (3 steps), speed (less than 30 min after electrophoresis) and the use of only inexpensive and readily available reagents and equipment. Also, the chemicals used do not necessitate specialized disposal efforts.

### Interaction of proteins with LPS aggregates

There is interest in finding proteins or peptides that protect from sepsis by strongly binding LPS, breaking its aggregates and by suppressing LPS-induced pro-inflammatory responses. In line with this, varieties of cationic proteins and peptides (e.g., polymyxin B, LPS-binding protein, anti-LPS factor, lysozyme [LZM]) which form complexes with lipopolysaccharides have been discovered. In addition, these proteins/peptides may be the target compounds of interest for understanding the biology of LPS in solution [39]. Certainly, the lipopolysaccharide disaggregation rate, whether LPS are partially or completely disaggregated, and how protein/peptide concentration influences protein/peptide-LPS interaction are still subjects for further investigation.

Using lysozyme as a model protein, we have found that imidazole-zinc may be combined with Coomassie brilliant blue R-250 into a double-staining process to enable the use of NAGE for detecting and studying protein-LPS interactions (Figure 1B) [37]. Specifically, our results validate that lysozyme can disrupt LPS aggregates and that concentration of either LPS or LZM has a significant effect on the amount of LPS disaggregated and the amount of LZM-LPS complexes formed. In a wider scope, the results of this study indicate that three different complementary criteria may be considered to infer a strong interaction and stable binding of cationic proteins/peptides to LPS. These criteria are: (i) any change in NAGE-separated LPS pattern (e.g., increased electrophoretic mobility), (ii) any shift in electrophoretic mobility of cationic proteins/peptides, and (iii) Coomassie blue-based specific detection of charged protein/peptide-LPS complexes. The benefit of using NAGE combined with the double-staining detection is that it neither alters the LPS aggregates structurally nor disrupts the native state of the proteins and LPS. Furthermore, the NAGE method can resolve LPS complexes from free cationic proteins [37].

### Compositional analysis of LPS aggregates using slab-PAGE

Among the different separation and resolution methods possible for LPS glycoforms, slab-polyacrylamide gel electrophoresis (slab-PAGE) has now been developed to offer unsurpassed resolution combined with higher reproducibility, rapidity, low cost, and simplicity. In fact, the successful application of this technique for resolving complex mixtures of LPS glycoforms in a wide molecular mass range has greatly facilitated the structural analysis of these macromolecules [40-43]. The LPS glycoform banding patterns generally consist of orderly spaced doublet bands for smooth (S)-type LPSs. Such bands represent LPS glycoforms having different numbers of repeating units in their O-side chains [44-48]. The fastest moving doublet band generally corresponds to an LPS glycoform with a complete core oligosaccharide only, the second fastest band should consist of an LPS glycoform with an additional single O-polysaccharide extension repeat unit (i.e.,  $n = 1$ ); and so on.

Separation inconsistencies such as doublet diffusion or anomalous slow-migrating glycoform bands should be the result of an incomplete LPS aggregate dissociation during electrophoresis [49,50]. This problem can be overcome either by applying a pre-electrophoresis step on the gel prior to LPS glycoform separations [51] or by increasing the concentration of sodium dodecyl sulfate (SDS) in gels [45]. Alternatively, SDS can be replaced with sodium deoxycholate (DOC) [52] to promote LPS aggregate dissociation thereby generating a well-resolved ladder of individual LPS glycoform bands ranging from a variety of S-type LPS to compact rough (R)-type LPS. Alternatively, an increased resolution of LPS can be obtained with SDS-PAGE using a Tricine buffer system [53], or by employing polyacrylamide gel concentration gradients within the gel [54,55]. Accordingly there are numerous ways of dissociating LPS glycoform aggregates into individual glycoforms and reproducibly separating the native LPS glycoforms with high resolution by slab-PAGE [56,57].

Given this, either NAGE-separated or unfractionated LPS aggregates can be electrophoresed in a second-dimension analytical SDS-polyacrylamide gel (2D-PAGE) for compositional analysis [37]. The LPS molecules fall into bands of discreet sizes, which are then stained (e.g. with silver) to make them visible to imaging techniques and reveal the composition of LPS aggregates. As described below, a wealth of information can be obtained by slab-PAGE alone or in combination with other characterization techniques (e.g., mass spectrometry, bioassays). This includes purity of LPSs, glycoform-specific composition, molecular weight of components and primary (e.g., oligosaccharide) sequences, minor structural changes due to mutations in genes responsible for LPS biosynthesis, and cell-based biological activities.

### Structural analysis of glycoform-specific LPS by mass spectrometry

Broadly speaking, LPS variants may be classified as smooth, short smooth (short S) or rough depending respectively upon whether they contain a full size O-polysaccharide extension, truncated O-chains or no O-polysaccharide extension at all [9-11]. Most "pure" LPS preparations that are extracted from Gram-negative bacteria exist as a complex mixture of all three LPS molecule classes. Even a preparation of S-type LPS may contain over 20% (w/w) of R-type LPS. Within these three types of LPS, further levels of heterogeneity also exist; for instance the number of KDO units in the core oligosaccharide may vary, so too may the degree of phosphorylation or



phosphorylethanolamine derivatization. In addition, the lipid A moiety can vary according to the degree of phosphorylation, the presence of alternative amino-glycopyranoses, and the number, chain length, and position of attached acyl groups, such that the lipid A part in certain S-type LPSs can be quite substantially chemically different from that in R-type LPSs [9-11].

Glycoform-specific LPSs are difficult to recover from silver-stained PAGE gels, because the silver staining process chemically modifies and irreversibly fixates LPSs into the gel. To overcome this obstacle, a sensitive and non-destructive reverse staining method of PAGE-separated LPSs has been established (Figure 1C) [58,59]. Reverse stained LPS fractions can diffuse out of gel matrix microparticles into solution in a fast and efficient way in 5% triethylamine solution [60]. Micropurified LPS glycoforms are in a highly purified state, essentially free of bacteria- or purification-associated pollutants (e.g., phospholipids, buffers, salts, detergents) or contamination by other interfering LPS fractions, and therefore they are amenable to structural analysis by MS either ESI or MALDI-TOF types [56,57]. Specifically, the sensitivity of ESI-MS analysis is increased by the use of dephosphorylated and permethylated oligosaccharides released by mild acid hydrolysis of the gel-separated LPS fractions [56]. MS allows determining the relative abundance and structure characteristics of LPS molecular species contained in each slab-PAGE band such as their exact molecular mass, oligosaccharidic sequence, and lipid A phosphorylation status.

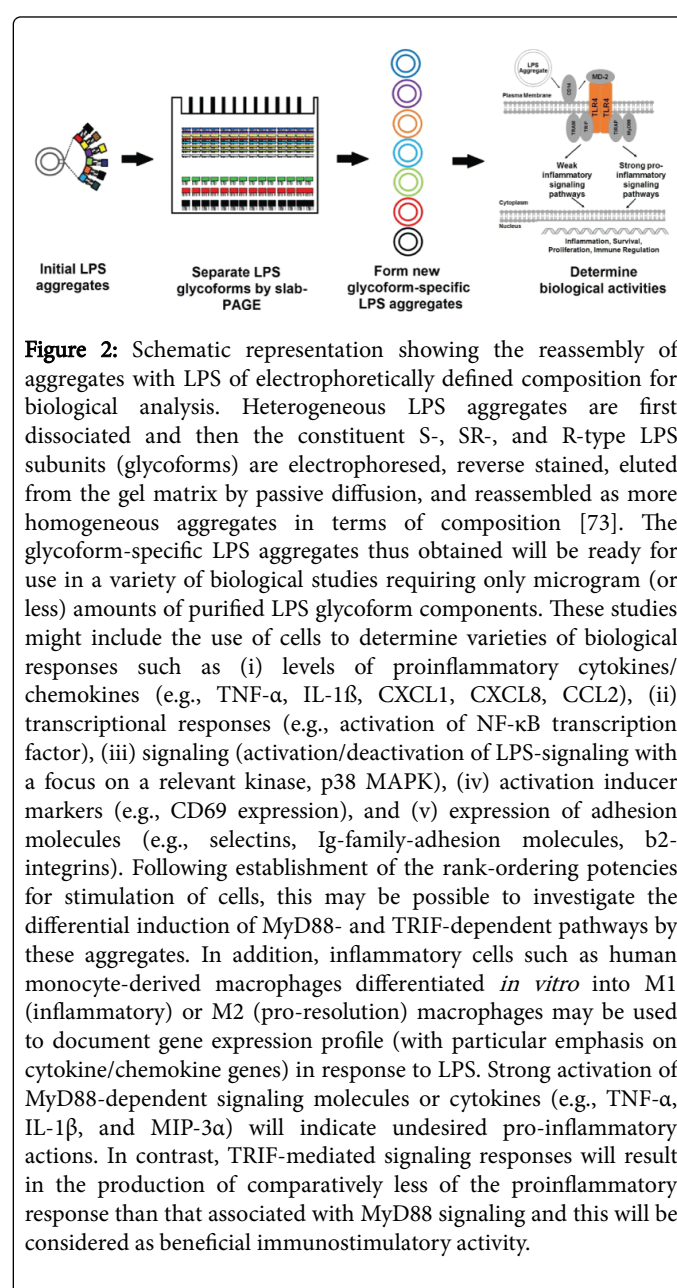
### Biological analysis of glycoform-specific LPS

Without doubt, the architecture of individual LPS variants has a strong influence on LPS activities as shown by differences between S-type and R-type LPS preparations. Examples of differential biological properties of S-type versus R-type LPS are: (i) The hepatic uptake of S-type LPSs from the blood is effected solely by sinusoidal (Kupffer) cells, while the uptake of the R-type LPSs is effected both by Kupffer cells and hepatocytes [61], (ii) There is a preferential transfer of S-type LPSs to plasma lipoproteins by acute-phase concentrations of LPS-binding protein (LBP) [62], (iii) R-type LPSs are very potent in their ability to induce chemiluminescence in granulocytes whereas S-type LPSs are virtually inactive [63], (iv) S- and R-types show distinct qualitative differences in their interactions with the complement system [64], (v) Macrophage-like cultured cells are activated by S-type LPSs in a different way to R- and/or short S-type LPSs from *S. enterica* serovar abortus equi [65], (vi) Under physiological conditions, S-type LPSs primarily activate cells that express mCD14, while in the absence of CD14 and LBP R-type LPSs stimulate cells that express TLR4/MD-2 receptors [66], (vii) S-type LPS from *Salmonella* enhances the activation of the TLR4-MyD88-independent pathway, while S-type LPS from *Escherichia coli* O55:B5 and *Vibrio cholerae* LPS dominantly induce the TLR4-MyD88-dependent pathway. *Neisseria meningitidis* lipooligosaccharide is a potent inducer of both pathways [67].

Amazingly, in spite of such obvious differences, many reports that discuss the biological activities and binding characteristics of LPS variants tend to describe data developed with LPS mixtures from wild-type Gram-negative (e.g., enteric) bacteria only and have paid surprisingly little attention to the importance of LPS heterogeneities [65,66,68-70]. Given this, many studies on biological effects of LPS molecular variants actually report on effects of heterogeneous mixtures of what are known as LPS glycoforms that can assemble into higher molecular weight LPS structures [e.g., 68]. Our research, which addresses this very limitation, is thus both novel and complementary

to previous studies. The fractionated LPS glycoforms are structurally unmodified [56,57], essentially free of the chemicals used in the purification method or contamination by other interfering bacterial components (e.g., proteins, nucleic acids, phospholipids) or LPS fractions. Therefore, the micropurified LPSs are compatible with subsequent biological tests (e.g., TNF- $\alpha$  induction [71], *Limulus* amoebocyte lysate activity [72]).

After excess surfactant (e.g., 0.4–0.5% DOC) is effectively removed (e.g., by centrifugal diafiltration), aggregates of more homogeneous composition are formed (Figure 2). These aggregates, which may show remarkable differences regarding their Z potential and are detectable by dynamic light scattering [73], show markedly different capacities to activate TLR4 and downstream pro-inflammatory NF- $\kappa$ B signaling pathways in engineered HEK293 cells.



**Figure 2:** Schematic representation showing the reassembly of aggregates with LPS of electrophoretically defined composition for biological analysis. Heterogeneous LPS aggregates are first dissociated and then the constituent S-, SR-, and R-type LPS subunits (glycoforms) are electrophoresed, reverse stained, eluted from the gel matrix by passive diffusion, and reassembled as more homogeneous aggregates in terms of composition [73]. The glycoform-specific LPS aggregates thus obtained will be ready for use in a variety of biological studies requiring only microgram (or less) amounts of purified LPS glycoform components. These studies might include the use of cells to determine varieties of biological responses such as (i) levels of proinflammatory cytokines/chemokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ , CXCL1, CXCL8, CCL2), (ii) transcriptional responses (e.g., activation of NF- $\kappa$ B transcription factor), (iii) signaling (activation/deactivation of LPS-signaling with a focus on a relevant kinase, p38 MAPK), (iv) activation inducer markers (e.g., CD69 expression), and (v) expression of adhesion molecules (e.g., selectins, Ig-family-adhesion molecules, b2-integrins). Following establishment of the rank-ordering potencies for stimulation of cells, this may be possible to investigate the differential induction of MyD88- and TRIF-dependent pathways by these aggregates. In addition, inflammatory cells such as human monocyte-derived macrophages differentiated *in vitro* into M1 (inflammatory) or M2 (pro-resolution) macrophages may be used to document gene expression profile (with particular emphasis on cytokine/chemokine genes) in response to LPS. Strong activation of MyD88-dependent signaling molecules or cytokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ , and MIP-3 $\alpha$ ) will indicate undesired pro-inflammatory actions. In contrast, TRIF-mediated signaling responses will result in the production of comparatively less of the proinflammatory response than that associated with MyD88 signaling and this will be considered as beneficial immunostimulatory activity.

Because micropurified LPS species are powerful inducers of diverse biological responses (e.g., induction of cells to produce TNF- $\alpha$  or nitric oxide *in vitro*, or lethal toxicity in mice sensitized with D-galactosamine *in vivo*), we look forward to analyzing the activity of glycoform-specific LPSs in other biological assays.

## Conclusion

NAGE in combination with the imidazole-zinc and Coomassie brilliant blue R-250 stains allows detecting both LPS aggregates and the interaction between lipopolysaccharide-binding proteins/peptides and LPS aggregates. This may become a useful tool offering the opportunity of screening a large number of proteins or host defense peptides to be further evaluated as anti-sepsis or anti-infective agents. For identification and further characterization LPS aggregates, either separated by NAGE or unfractionated, can be electrophoresed in slab-PAGE gels and visualized with silver or by reverse staining with zinc-imidazole. Gel extrusion and passive elution allows the isolation of R- or S- type LPS to electrophoretic homogeneity. The glycoform-specific LPSs thus obtained are amenable to both sensible structural analysis by mass spectrometry and biological activity evaluations.

Additionally, we believe that having reassembled glycoform-specific LPS aggregates will be useful to further study differences in physicochemical and biological properties of S- and R-type LPS preparations in the context of immunology or vaccine-related research. In fact, although LPS studies have shown significant progress during the past few years in the understanding of LPS biology and chemistry, the discovery of LPS glycoforms with real potential utility as vaccine adjuvants has lagged behind. This gap suggests that there should be critical bottlenecks hampering the process of translating basic research discoveries in the LPS field into finished immunological adjuvants. Research over the past decade has given rise to the idea that one of these bottlenecks is caused by the enormous complexity of LPS preparations. Ultimately, this research will provide a clearer picture as to how target (e.g., dendritic) cells are stimulated by glycoform-specific LPS aggregates, subsequently leading to T cell activation and antibody production.

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## References

- Putker F, Bos MP, Tommassen J (2015) Transport of lipopolysaccharide to the Gram-negative bacterial cell surface. *FEMS Microbiol Rev* 39: 985-1002.
- Tan Y, Kagan JC (2014) A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide. *Mol Cell* 54: 212-223.
- Gnauck A, Lentle RG, Kruger MC (2015) The characteristics and function of bacterial lipopolysaccharides and their endotoxic potential in humans. *Int Rev Immunol* 25:1-31.
- Bohannon JK, Hernandez A, Enkhbaatar P, Adams WL, Sherwood ER (2013) The immunobiology of toll-like receptor 4 agonists: from endotoxin tolerance to immunoadjuvants. *Shock* 40: 451-462.
- Opal SM (2010) Endotoxins and other sepsis triggers. *Contrib Nephrol* 167: 14-24.
- Opal SM (2007) The host response to endotoxin, antilipopolysaccharide strategies, and the management of severe sepsis. *Int J Med Microbiol* 297: 365-377.
- Simpson BW, May JM, Sherman DJ, Kahne D, Ruiz N (2015) Lipopolysaccharide transport to the cell surface: biosynthesis and extraction from the inner membrane. *Philos Trans R Soc Lond B Biol Sci* 370: 1-8.
- May JM, Sherman DJ, Simpson BW, Ruiz N, Kahne D (2015) Lipopolysaccharide transport to the cell surface: periplasmic transport and assembly into the outer membrane. *Philos Trans R Soc Lond B Biol Sci* 370: 1-8.
- Caroff M, Karibian D (2003) Structure of bacterial lipopolysaccharides. *Carbohydr Res* 338: 2431-2447.
- Molinaro A, Holst O, Di Lorenzo F, Callaghan M, Nurisso A, et al. (2015) Chemistry of lipid A: at the heart of innate immunity. *Chemistry* 21: 500-519.
- Kilar A, Dornyei A, Kocsis B (2013) Structural characterization of bacterial lipopolysaccharides with mass spectrometry and on- and off-line separation techniques. *Mass Spectrom Rev* 32: 90-117.
- Brandenburg K, Schromm AB, Gutschmann T (2010) Endotoxins: relationship between structure, function, and activity. *Subcell Biochem* 53: 53-67.
- Fujimoto Y, Adachi Y, Akamatsu M, Fukase Y, Kataoka M, et al. (2005) Synthesis of lipid A and its analogues for investigation of the structural basis for their bioactivity. *J Endotoxin Res* 11: 341-347.
- Akamatsu M, Fujimoto Y, Kataoka M, Suda Y, Kusumoto S, et al. (2006) Synthesis of lipid A monosaccharide analogues containing acidic amino acid: exploring the structural basis for the endotoxic and antagonistic activities. *Bioorg Med Chem* 14: 6759-6777.
- Kusumoto S, Fukase K, Shiba T (2010) Key structures of bacterial peptidoglycan and lipopolysaccharide triggering the innate immune system of higher animals: chemical synthesis and functional studies. *Proc Jpn Acad Ser B Phys Biol Sci* 86: 322-337.
- Kusumoto S, Fukase K (2006) Synthesis of endotoxic principle of bacterial lipopolysaccharide and its recognition by the innate immune systems of hosts. *Chem Rec* 6: 333-343.
- Yoshizaki H, Fukuda N, Sato K, Oikawa M, Fukase K, et al. (2001) First Total Synthesis of the Re-Type Lipopolysaccharide. *Angew Chem Int Ed Engl* 40: 1475-1480.
- Shimoyama A, Saeki A, Tanimura N, Tsutsui H, Miyake K, et al. (2011) Chemical synthesis of *Helicobacter pylori* lipopolysaccharide partial structures and their selective proinflammatory responses. *Chemistry* 17: 14464-14474.
- Park BS, Song DH, Kim HM, Choi BS, Lee H, et al. (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458: 1191-1195.
- Bryant CE, Spring DR, Gangloff M, Gay NJ (2010) The molecular basis of the host response to lipopolysaccharide. *Nat Rev Microbiol* 8: 8-14.
- Ohto U, Fukase K, Miyake K, Shimizu T (2012) Structural basis of species-specific endotoxin sensing by innate immune receptor TLR4/MD-2. *Proc Natl Acad Sci U S A* 109: 7421-7426.
- Plociennikowska A, Hromada-Judycka A, Borzecka K, Kwiatkowska K (2015) Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci* 72: 557-581.
- Brown J, Wang H, Hajishengallis GN, Martin M (2011) TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. *J Dent Res* 90: 417-427.
- McAleer JP, Vella AT (2010) Educating CD4 T cells with vaccine adjuvants: lessons from lipopolysaccharide. *Trends Immunol* 31: 429-435.
- Ianaro A, Tersigni M, D'Acquisto F (2009) New insight in LPS antagonist. *Mini Rev Med Chem* 9: 306-317.
- Barochia A, Solomon S, Cui X, Natanson C, Eichacker PQ (2011) Eritoran tetrasodium (E5564) treatment for sepsis: review of preclinical and clinical studies. *Expert Opin Drug Metab Toxicol* 7: 479-494.
- Raja SG, Dreyfus GD (2008) Eritoran: the evidence of its therapeutic potential in sepsis. *Core Evid* 2: 199-207.
- Dubensky TW Jr, Reed SG (2010) Adjuvants for cancer vaccines. *Semin Immunol* 22: 155-161.

29. Cluff CW (2010) Monophosphoryl lipid A (MPL) as an adjuvant for anti-cancer vaccines: clinical results. *Adv Exp Med Biol* 667: 111-123.
30. Garçon N, Van Mechelen M (2011) Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems. *Expert Rev Vaccines* 10: 471-486.
31. Savva A, Roger T (2013) Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. *Front Immunol* 4: 387.
32. Martin L, van Meegern A1, Doemming S1, Schuerholz T1 (2015) Antimicrobial Peptides in Human Sepsis. *Front Immunol* 6: 404.
33. Zielen S, Trischler J, Schubert R (2015) Lipopolysaccharide challenge: immunological effects and safety in humans. *Expert Rev Clin Immunol* 11: 409-418.
34. Zariri A, van der Ley P (2015) Biosynthetically engineered lipopolysaccharide as vaccine adjuvant. *Expert Rev Vaccines* 14: 861-876.
35. Richter W, Vogel V, Howe J, Steiniger F, Brauser A, et al. (2011) Morphology, size distribution, and aggregate structure of lipopolysaccharide and lipid A dispersions from enterobacterial origin. *Innate Immun* 17: 427-438.
36. Gutsmann T, Schromm AB, Brandenburg K (2007) The physicochemistry of endotoxins in relation to bioactivity. *Int J Med Microbiol* 297: 341-352.
37. Rodriguez C, Hardy E (2015) Lipopolysaccharide aggregates in native agarose gels detected by reversible negative staining with imidazole and zinc salts. *Anal Biochem* 485: 72-80.
38. Fernandez-Patron C, Castellanos-Serra L, Hardy E, Guerra M, Estevez E, et al. (1998) Understanding the mechanism of the zinc-ion stains of biomacromolecules in electrophoresis gels: generalization of the reverse staining technique. *Electrophoresis* 19: 2398-2406.
39. Yermak IM, Davydova VN (2008) Interaction of bacterial lipopolysaccharides with host soluble proteins and polycations. (*Suppl Ser A, Membr Cell Biol*), *Biochem (Mosc)* 2: 279-295.
40. Rivera M, Bryan LE, Hancock RE, McGroarty EJ (1988) Heterogeneity of lipopolysaccharides from *Pseudomonas aeruginosa*: analysis of lipopolysaccharide chain length. *J Bacteriol* 170: 512-521.
41. De Castro C, Parrilli M, Holst O, Molinaro A (2010) Microbe-associated molecular patterns in innate immunity: Extraction and chemical analysis of gram-negative bacterial lipopolysaccharides. *Methods Enzymol* 480: 89-115.
42. Apicella MA (2008) Isolation and characterization of lipopolysaccharides. *Methods Mol Biol* 431: 3-13.
43. Marolda CL, Lahiry P, Vinés E, Saldías S, Valvano MA (2006) Micromethods for the characterization of lipid A-core and O-antigen lipopolysaccharide. *Methods Mol Biol* 347: 237-252.
44. Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 119: 115-119.
45. Goldman RC, Leive L (1980) Heterogeneity of antigenic-side-chain length in lipopolysaccharide from *Escherichia coli* 0111 and *Salmonella typhimurium* LT2. *Eur J Biochem* 107: 145-153.
46. Wang X, Zhou A, Cai W, Yu D, Zhu Z, et al. (2015) Highly sensitive fluorescent stain for detecting lipopolysaccharides in sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Electrophoresis* 36: 1795-1800.
47. Zhu ZX, Cong WT, Ni MW, Wang X, Ma WD, et al. (2012) An improved silver stain for the visualization of lipopolysaccharides on polyacrylamide gels. *Electrophoresis* 33: 1220-1223.
48. Zhu ZX, Cong WT, Zhou X, Chen M, Choi JK, et al. (2012) Negative staining of lipopolysaccharides on polyacrylamide gels by using eosin B. *Anal Biochem* 426: 1-3.
49. Hitchcock PJ (1983) Aberrant migration of lipopolysaccharide in sodium dodecyl sulfate/polyacrylamide gel electrophoresis. *Eur J Biochem* 133: 685-688.
50. Peterson AA, McGroarty EJ (1985) High-molecular-weight components in lipopolysaccharides of *Salmonella typhimurium*, *Salmonella minnesota*, and *Escherichia coli*. *J Bacteriol* 162: 738-745.
51. Palva T, Makela H (1980) Lipopolysaccharide heterogeneity in *Salmonella typhimurium* analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. *Eur J Biochem* 107: 137-144.
52. Komuro T, Galanos C (1988) Analysis of *Salmonella* lipopolysaccharides by sodium deoxycholate-polyacrylamide gel electrophoresis. *J Chromatogr* 450: 381-387.
53. Lesse AJ, Campagnari AA, Bittner WE, Apicella MA (1990) Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J Immunol Methods* 126: 109-117.
54. Noda K, Kubota K, Yamasaki R (2000) Separation of lipooligosaccharides by linear gradient gel electrophoresis. *Anal Biochem* 279: 18-22.
55. Inzana TJ, Apicella MA (1999) Use of a bilayer stacking gel to improve resolution of lipopolysaccharides and lipooligosaccharides in polyacrylamide gels. *Electrophoresis* 20: 462-465.
56. Gulin S, Pupo E, Schweda KHE, Hardy E (2003) Linking mass spectrometry and slab-polyacrylamide gel electrophoresis by passive elution of lipopolysaccharides from reverse stained gels: analysis of gel-purified lipopolysaccharides from *Haemophilus influenzae* strain Rd. *Anal Chem* 75: 4918-4924.
57. Pupo E, Phillips NJ, Gibson BW, Apicella MA, Hardy E (2004) Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry of lipopolysaccharide species separated by slab-polyacrylamide gel electrophoresis: high-resolution separation and molecular weight determination of lipooligosaccharides from *Vibrio fischeri* strain HMK. *Electrophoresis* 25: 2156-2164.
58. Hardy E, Pupo E, Castellanos-Serra L, Reyes J, Fernandez-Patron C (1997) Sensitive reverse staining of bacterial lipopolysaccharides on polyacrylamide gels by using zinc and imidazole salts. *Anal Biochem* 244: 28-32.
59. Hardy E, Pupo E, Santana H, Guerra M, Castellanos-Serra LR (1998) Elution of lipopolysaccharides from polyacrylamide gels. *Anal Biochem* 259: 162-165.
60. Pupo E, Lopez CM, Alonso M, Hardy E (2000) High-efficiency passive elution of bacterial lipopolysaccharides from polyacrylamide gels. *Electrophoresis* 21: 526-530.
61. Freudenberg MA, Freudenberg N, Galanos C (1982) Time course of cellular distribution of endotoxin in liver, lungs and kidneys of rats. *Br J Exp Pathol* 63: 56-65.
62. Hamann L, Alexander C, Stamme C, Zahringer U, Schumann RR (2005) Acute-phase concentrations of lipopolysaccharide (LPS)-binding protein inhibit innate immune cell activation by different LPS chemotypes via different mechanisms. *Infect Immun* 73: 193-200.
63. Kapp A, Freudenberg M, Galanos C (1987) Induction of human granulocyte chemiluminescence by bacterial lipopolysaccharides. *Infect Immun* 55: 758-761.
64. Morrison DC, Kline LF (1977) Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS). *J Immunol* 118: 362-368.
65. Suda Y, Kirikae T, Shiyama T, Yasukochi T, Kirikae F, et al. (1995) Macrophage activation in response to S-form lipopolysaccharides (LPS) separated by centrifugal partition chromatography from wild-type LPS: effects of the O-polysaccharide portion of LPS. *Biochem Biophys Res Commun* 210: 678-685.
66. Huber M, Kalis C, Keck S, Jiang Z, Georgel P, et al. (2006) R-form LPS, the master key to the activation of TLR4/MD-2-positive cells. *Eur J Immunol* 36: 701-711.
67. Zughair SM, Zimmer SM, Datta A, Carlson RW, Stephens DS (2005) Differential induction of the toll-like receptor 4-MyD88-dependent and -independent signaling pathways by endotoxins. *Infect Immun* 73: 2940-2950.
68. Morrison DC, Silverstein R, Luchi M, Shnyra A (1999) Structure-function relationships of bacterial endotoxins. Contribution to microbial sepsis. *Infect Dis Clin North Am* 13: 313-340.
69. Trent MS, Stead CM, Tran AX, Hankins JV (2006) Diversity of endotoxin and its impact on pathogenesis. *J Endotoxin Res* 12: 205-223.

70. Liu B, Knirel YA, Feng L, Perepelov AV, Senchenkova SN, et al. (2014) Structural diversity in Salmonella O antigens and its genetic basis. *FEMS Microbiol Rev* 38: 56-89.
71. Pupo E, Lindner B, Brade H, Schromm AB (2013) Intact rough- and smooth-form lipopolysaccharides from *Escherichia coli* separated by preparative gel electrophoresis exhibit differential biologic activity in human macrophages. *FEBS J* 280: 1095-1111.
72. Pupo E, Hardy E (2007) Isolation of smooth-type lipopolysaccharides to electrophoretic homogeneity. *Electrophoresis* 28: 2351-2357.
73. Hardy E, Kamphuis T, Japaridze A, Wilschut JC, Winterhalter M (2012) Nanoaggregates of micropurified lipopolysaccharide identified using dynamic light scattering, Zeta-potential measurement and TLR4 signaling activity. *Anal Biochem* 430: 203-213.

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