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# Endotoxin detection in a competitive electrochemical assay: Synthesis of a suitable endotoxin conjugate

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**Abstract**

A biotin–lipopolysaccharide (biotin–LPS) conjugate was synthesized from LPS smooth from *Salmonella minnesota*, yielding a conjugate with a biotin/LPS ratio equal to 1:1 and endotoxic activity of 0.08 EU $ng^{-1}$. The conjugate was used in an amperometric competitive assay to determine endotoxins with endotoxin-neutralizing protein (ENP) as the recognition element. The assay is performed on a modified electrode, involving the covalent binding of carboxymethyl dextran (CMDex) to a cystamine-modified gold electrode and then the covalent binding of the recognition protein, ENP, to CMDex. The assay is carried out by incubating the modified electrode in an LPS sample to which biotin–LPS was added. Both species compete for the recognition sites on the modified surface. After the incubation stage and a careful rinsing, the electrode is immersed in a solution containing neutravidin–horseradish peroxidase conjugate (N-HRP), which binds to the sites containing biotin–LPS on the electrode. The system is rinsed and a current signal is generated by the addition of hydrogen peroxide and a redox mediator. The assay is able to detect LPS from *Salmonella minnesota* at concentrations as low as 0.1 ng $ml^{-1}$, equivalent to 0.07 EU $ml^{-1}$.


*Keywords:* Lipopolysaccharide; Competitive assay; Biotin-conjugated lipopolysaccharide

Gram-negative sepsis is a common and serious clinical problem. The primary trigger in gram-negative shock syndrome is due to the lipopolysaccharides (LPSs)[1], a major constituent of the gram-negative bacteria outer membrane, also termed endotoxins. In events of gram-negative bacterial infection, LPS is the well-known activator of the humoral and cellular components of the host defense system. Activation of the host defense is essential to fight such an infection, but uncontrolled stimulation can result in excessive release of inflammatory cytokines, leading to septic shock and death [1,2].

Several institutions have imposed regulations regarding the maximum allowable presence of LPS. For example, U.S. Pharmacopoeia establishes a limit of 0.25 EU $ml^{-1}$ for injectable drugs [3], and the Association for the Advancement of Medical Instrumentation recommends a limit of 2 EU $ml^{-1}$ for dialysis baths [4].

LPSs are complex lipid-linked carbohydrate negatively charged molecules. Usually, they are composed of three distinct regions: a fatty-acylated, highly conserved region called lipid A; a short oligosaccharide, the core region; and an O-antigen portion composed of a polymer of repeating oligosaccharide units with a composition that varies greatly among gram-negative bacteria. Lipid A is responsible for many of the pathophysiological effects associated with gram-negative bacterial infection; therefore, it is the active moiety of LPS [5]. It consists of a hydrophilic, negatively charged bisphosphorylated disaccharide of

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[1] *Abbreviations used:* LPS, lipopolysaccharide; ENP, endotoxin-neutralizing protein; LBP, lipopolysaccharide-binding protein; LPS–HRP, horseradish peroxidase-labeled lipopolysaccharide conjugate; CMDex, carboxymethyl dextran; N-HRP, neutravidin–horseradish peroxidase conjugate; LALF, limulus anti-LPS factor; EDTA, ethylenediaminetetraacetic acid; EDC, l-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; NHS, *N*-hydroxysuccinimide.

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glucosamine backbone covalently linked to a hydrophobic domain of six (*Escherichia coli*) or seven (*Salmonella*) acyl chains (12–16 carbon atoms) via amide and ester bonds [6–8]. Although LPS itself is chemically inert, the presence of LPS in blood (endotoxemia) sets off a cascade of exaggerated host responses affecting the structure and function of organs and cells, changing metabolic functions, raising body temperature, modifying hemodynamics, and causing septic shock [1].

Endotoxins are detected either by the use of amoebocytes from horseshoe crab (the LAL test) or by a live assay on rabbits. Both methods are expensive and troublesome. One of the main challenges currently posed by endotoxin detection is to develop a fast and simple method capable of, for example, performing on-line measurements in purified water lines or in aqueous saline solutions used for hemodialysis or intravenous infusion.

During the past 10 years, great improvements have been made in the study of chemical interactions between LPSs and antibacterial agents produced by many organisms. Some of the systems that have been studied include endotoxin-neutralizing protein (ENP) produced by horseshoe crabs [9], lipopolysaccharide-binding protein (LBP) from humans [10], and polymyxin produced by *Bacillus polymyxia* [11]. These findings allow the development of an assay equivalent to assays based on antigen–antibody interaction, where the selective recognition is combined with a physicochemical change that is used as a transducer element generating a signal proportional to the concentration of the analyte to be detected.

In a previous work [12], we proposed a competitive electrochemical assay using a modified gold electrode with a recombinant ENP from *Saccharomyces cerevisiae* as a recognition element. Two strategies were used to immobilize the protein onto the electrode: one based on electrostatic interactions (electrostatic configuration) and the other based on trough covalent binding (covalent configuration). The test was carried out by competition of the LPS in the sample with a horseradish peroxidase-labeled lipopolysaccharide conjugate (LPS–HRP). Sensors constructed according to the electrostatic configuration were able to detect the presence of endotoxins in concentrations of 0.2 EU $ml^{-1}$, below the American Pharmacopoeia standard for injectable drugs. Alternatively, the covalent configuration showed a broader dynamic range but a higher detection limit (1.5 EU $ml^{-1}$).

In spite of the promising results achieved, the system presents some limitations. One of them is the surface coverage, estimated at 0.08 pmol $cm^{-2}$ LPS–HRP on a covalently modified electrode Au/Cys/carboxymethyl dextran (CMDex)/ENP, whereas Limoges and coworkers reported a surface coverage 50 times higher for a monolayer of neutravidin–horseradish peroxidase conjugate (N-HRP) [13]. Our results suggest a low affinity between the LPS–HRP conjugate and the modified electrode. Several factors have an effect on the amount of LPS–HRP incorporated onto the electrode. Steric factors are detrimental due to the molecular size of this conjugate given that it is synthesized from LPS from *E. coli* 026:B6 [14]. Another factor to take into account is the low endotoxic activity of the LPS–HRP determined in the homogeneous phase through the LAL test (0.012 EU $ng^{-1}$), compared with the typical values for LPS (1 EU $ng^{-1}$), because endotoxic activity correlates with ENP affinity toward LPS [15].

To improve the characteristics of the conjugate needed for a competitive assay, it is important that the biological properties and the binding capacity of the LPS portion are not altered by means of the conjugation; on the other hand, the introduced label needs to be present in a good ratio and to be stable.

Given the complexity of the molecule, the synthesis of an LPS conjugate is not simple. Endotoxins in solution do not constitute a homogeneous sample, and at the concentrations required for the synthesis aggregates of high molecular weight are formed [16–18]. To preserve the active portion of LPS, the covalent binding of low-molecular weight probes to the O-antigenic portion of the LPS has been carried out. The objective is more ambitious than the objectives presented in previous reports for LPS conjugates [19,20]; in the current case, it is necessary that, on average, all of the LPS molecules are labeled to avoid losing sensitivity in the competitive assay.

By means of the controlled oxidation with sodium periodate, reactive aldehydes could be generated on the saccharide portion of the LPS that later was reacted with the markers through their nucleophile groups [21]. Taking into account the well-developed biotin–avidin technology, a conjugated biotin–LPS was synthesized. The interaction between biotin and the protein avidin is one of the strongest noncovalent affinity interactions in nature ($K_D = 10^{-15}$ M) [22], and it has been used for a wide range of applications, including immunoassays, protein purification, and diagnostics [23]. The strong interaction is achieved without significant perturbation of the terciary or quaternary structures of the protein and is stable over a wide range of pH values and temperatures [24]. Sometimes, neutravidin is used instead of avidin. One major difference between them is that avidin carries a positive charge at neutral pH, whereas neutravidin is nearly neutral.

In this work, a biotin–LPS conjugate was synthesized from a purified fraction of LPS from *S. minnesota*, yielding a conjugate with improved endotoxic activity and applied in our recently developed molecular recognition device based on ENP [12]. The assay is performed according to the covalent configuration reported previously, Au/Cys/CMDex/ENP, which involves the covalent binding of CMDex to a cystamine-modified gold electrode and then the covalent binding of the recognition protein (ENP) to CMDex (Scheme 1).

The steps involved in the assay are depicted in Scheme 2. A modified electrode Au/Cys/CMDex/ENP is incubated in an LPS sample to which biotin–LPS was added. Both species compete for the recognition sites on the modified surface. After the incubation stage and a careful rinsing, the