Use of an Antimicrobial Protein for Endotoxin Detection in a Competitive Electrochemical Assay

Graciela Priano and Fernando Battaglini*

INQUIMAE—Departamento de Química Inorgánica, Analítica y Química Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, C1428EHA Buenos Aires, Argentina

Endotoxins, also referred to as pyrogens, are part of the cellular walls of Gram-negative bacteria and are capable of inducing fever when entering into the blood stream. Current methods of detection involve the use of either amoebocytes from horseshoe crab that clots upon exposure to endotoxin or a live assay on rabbits. In this work, the detection of endotoxins by an electrochemical competitive assay is presented. Two configurations of modified electrodes were constructed using a recombinant endotoxin neutralizing protein (ENP) as recognition element. A modified lipopolysaccharide with horseradish peroxidase was used for the competitive assay. Modified electrodes constructed by electrostatic interaction of ENP and an electroactive polymer can detect the presence of endotoxins in concentrations as low as 0.2EU mL⁻¹, below the limit imposed for water in injectable drugs in the American Pharmacopoeia. Modified electrodes constructed by covalent linking of ENP to a carboxymethyl dextran matrix bound to the electrode show a better dynamic range but a higher detection limit.

Gram-negative sepsis is a common and serious clinical problem. It is one of the leading causes of overall mortality, and the number one cause of deaths in intensive care units,¹ accounting for some 200 000 fatalities in the United States annually.

The primary trigger in Gram-negative shock syndrome is endotoxin, a constituent of the outer membrane of all Gram-negative bacteria. Endotoxins, also referred to as pyrogens, consist of a polysaccharide portion and a lipidic portion called lipid A and are therefore also called lipopolysaccharides (LPS; Figure 1). The polysaccharide portion consists of an O-antigen-specific polymer of repeating oligosaccharide units, the composition of which is highly varied among Gram-negative bacteria. Lipid A is the active moiety of LPS² and is composed of a hydrophilic, negatively charged bisphosphorylated diglucosamine backbone and a hydrophobic domain of six (Escherichia coli) or seven (Salmonella) acyl chains in amide and ester linkages.³⁻⁵ Whereas LPS itself is chemically inert, the presence of LPS in blood (endotoxemia) sets off a cascade of exaggerated host responses, known as septic shock.⁶

Governmental regulations impose limits on endotoxin concentration allowed in injectable drugs, for either medical or veterinary applications, as well as in medical devices, dialysis baths, and foodstuff. Current methods of detection involve either a live assay on rabbits or the Limulus amebocyte lysate assay (LAL assay). The LAL assay is based on the discovery that Gram-negative bacteria cause lymph coagulation in Limulus polyphemus crabs.⁷

Figure 1. Structure of lipopolysaccharides.

¹ Gelfand, J. A.; Shapiro, L. New Horizons 1993, 1, 13–22.
The LAL assay is a semiquantitative test. It takes more than 1 h, and gel formation is not always considered a positive result, which casts doubt on the quality of the tested product. The LAL assay has other variants such as the use of turbidimetry as a detection method (with all the problems involved in this technique) and also a chromogenic assay, in which a color develops as a result of the hydrolysis of a peptide, hence liberating \( \text{p-nitroaniline} \). This compound has a low extinction coefficient; therefore, a second reaction is necessary to generate a dye that makes the assay somewhat more sensitive.

Many organisms produce antibacterial proteins/peptides that participate in their defense against Gram-negative bacterial infections. A great improvement has been made in the study of chemical interactions between lipopolysaccharides and these antibacterial agents during the last 10 years. Some of the systems that have been studied are as follows: endotoxin neutralizing protein (ENP), produced by horseshoe crab; lipopolysaccharide binding protein, produced by humans; peptides, derived from sequences of the above-mentioned proteins; polymyxin produced by \textit{Bacillus polymyxia}; cationic antibacterial proteins from humans and rabbits, among others. These findings allow the development of an assay equivalent to those based upon antigen-antibody interaction, where the selective recognition is combined with a physicochemical change, which is used as a transducer element generating a signal proportional to the concentration of the analyte to be detected.

An assay using these features has been recently described by Novitsky et al., in which the ENP is labeled with a fluorophore, and detection is carried out following changes in fluorescence polarization. This method is highly dependent on temperature and medium viscosity, and in addition, it is not as sensitive as other fluorescent techniques. Furthermore, it is not suitable for making a sensor where the protein is immobilized since, upon immobilization the diffusion rotational, which is the physicochemical change being recorded, is lost. Lorch et al. immobilized antibodies capable of recognizing the O-antigen moiety of LPS on a surface and evaluated LPS content by surface plasmon resonance; the use of antibodies is limited, since the polysaccharide portion of LPS is extremely variable and serologically distinct for each strain of the same species of Gram-negative organisms. Another alternative was presented by Rangin and Basu, where LPS is recognized by employing polydiacetylene sensors based on the principle of the electronic tongue. These authors reported the generation of a set of unique fingerprints using diacetylene liposomes for recognition of LPS from different strains, allowing detection of concentrations in the order of milligrams per milliliter.

One of the main challenges currently posed by endotoxin detection is to make available a fast and simple method capable of, for example, performing online measurements in purified water lines, in aqueous saline solutions used for hemodialysis, or for intravenous infusion.

In this work, we present a competitive electrochemical assay using a gold electrode modified with a recombinant endotoxin neutralizing protein from \textit{Saccharomyces cerevisiae}. Detection is carried out by competition of the LPS in the sample with a horseradish peroxidase-labeled lipopolysaccharide conjugate

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\[(\text{14})\] Novitsky, T. J.; Ridge, R. J.; Sloyer, J. L. U.S. Patent 6,171,807.


Two electrode configurations were tested: the first one built by electrostatic interactions of an electroactive polymer with the recognition peptide (Scheme 1) and the second one by covalent binding of carboxymethyl dextran (CMDex), bound onto the surface of the electrode, with the recognition peptide (Scheme 2). The electrostatic configuration presents a lower detection limit, but is less sensitive and more susceptible to nonspecific binding. The covalent configuration presents a better dynamic range, though a higher detection limit. In the first case, the system is able to detect LPS below the specified limit values established by the U.S. Pharmacopoeia for injectable drugs (0.25 EU mL$^{-1}$) and the recommended limit established by the Association for the Advancement of Medical Instrumentation (AAMI) for dialysis baths (2 EU mL$^{-1}$).

**EXPERIMENTAL SECTION**

**Materials.** LPS–HRP was supplied by AlerCHEK, Inc. (lot 115001, 0.22 mg mL$^{-1}$; the HRP activity of this conjugate was 255 units mg$^{-1}$). LPS was supplied by Sigma from *E. coli* serotype O26:B6 (endotoxic activity 2 EU ng$^{-1}$) and from *Salmonella minnesota* (endotoxic activity 1.5 EU ng$^{-1}$). (Note: LPS is a pyrogen. May cause fever. May be harmful by inhalation, ingestion, or skin absorption. Good laboratory technique should be employed: wear lab coat, gloves, and safety glasses. Work in a well-ventilated area. Avoid contact with open wounds.)

ENP was supplied by Associates of Cape Code (Seikagaku America), this protein is a recombinant version of the *Limulus* anti-LPS factor (LALF; pI 8.5; MW 12 200 (105 AA residues); lot 9-054). Apyrogen water was produced by bidistillation of water previously distilled, passed through a MilliQ water system, and collected in apyrogen glass material (5 h at 180 °C and 2 h at 220 °C). The soluble redox mediator [Os(bpy)$_2$(pyCOOH)Cl]$^+$ (with bpy = bipyridine and pyCOOH = nicotinic acid) (Figure 2a) and the redox polymer, poly(allylamine) containing a pyridine-based osmium complex (PAAOs; Figure 2b), were synthesized in our laboratory as previously reported. Cystamine dihydrochloride was provided by Sigma and CMDex (MW 24 200) was from Fluka. All other reagents were analytical grade.

**Characterization of LPS–HRP and Water Quality.** LPS–HRP was characterized by electrophoresis following the procedure developed by Tsai and Frasch. The conjugate does not show free LPS and shows small amounts of free HRP. To check the corresponding endotoxin units (pyrogen activity) of the LPS–HRP, the LAL test was carried out (QCL 1000, Biowhitaker). The LPS–HRP conjugate gave a value equivalent to 0.012 EU ng$^{-1}$.

The water used in our laboratory was also tested by LAL test; values below 0.01 EU mL$^{-1}$ were routinely obtained.

**Construction of Modified Electrodes.** The 1-cm$^2$ gold flags were used as electrodes. The gold surface was cleaned by immersion for 5 h in a saturated alkaline KMnO$_4$ solution (KMnO$_4$ in 1 M NaOH) at 60 °C. Then, they were immersed in 1:3 30% H$_2$O$_2$/98% H$_2$SO$_4$ solution (piranha solution) for 3 h. (Note: This solution is highly corrosive and reacts violently with organic materials; precautions must be taken at all times when handled.) Clean electrodes were rinsed with MilliQ water, and the cleanness of the surface was checked by cyclic voltammetry in 1.8M H$_2$SO$_4$ between 0 to +1.6 V versus Ag/AgCl at 0.05 V s$^{-1}$. When the cleaning step was satisfactorily verified, the electrodes were ready to be modified.

**PAAOs Modified Electrodes.** Thiol adsorption (Au/MPS): clean electrodes were immersed in a 20 mM 3-mercapto-1-propane-sulfonate (MPS) solution for 24 h. Then, they were immersed in a solution of 0.1 M ENP in 1.8M H$_2$SO$_4$ for 24 h. The electrodes were rinsed with MilliQ water, and the cleanness of the surface was checked by cyclic voltammetry in 1.8M H$_2$SO$_4$ between 0 to +1.6 V versus Ag/AgCl at 0.05 V s$^{-1}$. When the cleaning step was satisfactorily verified, the electrodes were ready to be modified.

**PAAOs Modified Electrodes.** Thiol adsorption (Au/MPS): clean electrodes were immersed in a 20 mM 3-mercaptopropane-

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sulfonate solution in 10 mM H$_2$SO$_4$ for 30 min. Fresh solutions were used in each preparation.

PAAOs adsorption (Au/MPS/PAAOs): The modified electrodes with thiol were immersed in an aqueous solution of the polymer (0.4% w/v) for 10 min.

ENP adsorption (Au/MPS/PAAOs/ENP): The protein was adsorbed onto the modified PAAOs electrode from a 50 mM Tris-HCl buffer (pH 8.5) for 15 min.

Bovine serum albumin (BSA) adsorption: The modified electrodes were immersed in solutions of BSA in 50 mM Tris buffer (pH 7.5) for 10 min or at pH 8.5 if ENP was previously adsorbed.

CMDex Modified Electrodes. Cystamine adsorption (Au/Cys): Clean gold electrodes were immersed in a 20 mM cystamine solution in absolute ethanol/water (9:1) for 2 h. Fresh solutions were used in each preparation.

Carboxymethyl-dextran Modification (Au/Cys/CMDex). CMDex was bound to cystamine through the formation of an amide bond. In a 150 mM EDC and 27 mM NHS solution in 10 mM PIPES buffer (pH 6.5) was dissolved 10 mg mL$^{-1}$ CMDex and left for activation 30 min under stirring. Then, Au/Cys electrodes were immersed and left overnight at room temperature under stirring. The CMDex-modified electrodes were immersed in a 1 M ethanolamine solution to quench the activated carboxylate groups when no subsequent modification was carried out, rinsed with a 1 M NaCl solution, and finally rinsed with pyrogen water.

ENP Modification (Au/Cys/CMDex/ENP). The binding of ENP to the CMDex modified electrode was carried out in a way similar to the previous steps. Au/Cys/CMDex electrodes were immersed in a solution containing 100 mM EDC, 10 mM NHS in PIPES buffer (pH 6.5) for 30 min to activate of carboxylate groups, and then they were rinsed with pyrogen water and immersed in 1 $\mu$g mL$^{-1}$ ENP solution in 10 mM HEPES buffer (pH 8.0), unless otherwise stated. Finally, the electrodes were immersed in a 1 M ethanolamine solution to quench the activated carboxylate groups and finally rinsed with pyrogen water.

Ferrocenecarboxyaldehyde Modified Electrodes. Modification of Au/Cys and Au/Cys/CMDex with ferrocenecarboxyaldehyde. Both types of electrodes were incubated in a saturated ferrocenecarboxyaldehyde solution (25 mM MOPS, pH 7.0) with 10 mg mL$^{-1}$ sodium cyanoborohydride and left overnight at room temperature under stirring. The presence of the ferrocene moiety was determined by cyclic voltammetry in 0.2 M KNO$_3$, 50 mM Tris buffer (pH 7.5) between 0 to +600 mV versus Ag/AgCl at 0.05 V s$^{-1}$. Blank experiments were carried out exposing the electrodes to a saturated solution of ferrocenecarboxyaldehyde without sodium cyanoborohydride; in this case, no currents were observed after rinsing the electrodes.

**Electrochemical Measurements.** A standard three-electrode system was used together with a purpose-built operational amplifier potentiostat (TEQ-02). The system consisted of a working electrode, a platinum mesh counter electrode, and an Ag/AgCl reference electrode. Working electrodes were freshly prepared for every analysis.

Current Normalization. For Au/MPS/PAAOs/ENP electrodes, currents were normalized by the following procedure: For each jth electrode, current oxidation peak of the adsorbed osmium moieties was established ($i_0$) by cyclic voltammetry at 50 mV s$^{-1}$ in 0.2 M KNO$_3$, 50 mM Tris buffer solution (pH 7.5). From these experiments, an average peak value was obtained ($\bar{i}_0$). The current obtained for each electrode in subsequent experiments was normalized by the factor $i_0$/\bar{i}_0. This criterion was taken since the reduction of the enzyme is through the immobilized mediator. The peak average is calculated from the measurement of 25 modified electrodes.

In the case of Au/Cys/CMDex/ENP electrodes, the signals were standardized by the geometric area, since for experiments taking several seconds with species in solution, the roughness factor has not effect.

**Amperometric Assays for Both Configurations of Modified Electrodes.** The time-based measurements were performed at fixed electrode potential, 50 mV versus Ag/AgCl. For all the assays described below, the electrodes were carefully rinsed and introduced into the electrochemical cell containing 0.2 M KNO$_3$, 50 mM Tris-HCl pH 7.5 buffer solution. In the case of Au/Cys/CMDex/ENP, [Os(bpy)$_2$Cl(pyCOOH)]Cl was added to a final concentration of 45 $\mu$M. The modified electrode was connected to the potentiostat, left to equilibrate with the solution, and hydrogen peroxide was added to give a final concentration of 1 mM; an immediate change in the current was observed that reached a constant value due to the catalytic current produced, which was proportional to the surface concentration of LPS$–$HRP.

(a) Adsorption of LPS$–$HRP. For both configurations of modified electrodes, adsorption of the conjugate was carried out from solutions of different LPS$–$HRP concentrations in 50 mM Tris buffer (pH 7.5) for 60 min. A blank experiment was carried out with 50 $\mu$g mL$^{-1}$ HRP (activity 230 U mg$^{-1}$).

(b) Competitive Assays. Competitive assays with both configurations of modified electrodes were carried out by adsorption of a mixture of LPS (previously sonicated) and LPS$–$HRP (at the fixed concentrations specified in the figures) for 60 min; in all cases, solutions were prepared in 150 mM NaCl, 20 mM Tris buffer (pH 7.5).

After incubation, the electrodes were rinsed with buffer and then with pyrogen water. The current informed for each concentration is the average of two independent experiments, unless otherwise stated.

**Ellipsometric Measurements.** Ellipsometry is an optical method that records the change in polarization of elliptically polarized light, when it reflects on a sample surface. If the surface is optically modified, e.g., by protein adsorption, the associated
change in polarization is detected. The difference in the polarization state between the incident and the reflected light is usually described by the measured parameters $\Delta$ and $\psi$, referred to as ellipsometric angles. These are related to the ratio ($\rho$) of the overall complex reflection coefficients of the light components polarized parallel, $r_p$, and normal, $r_n$, to the plane of incidence by

$$\rho = \tan \Psi \exp(i\Delta) = \frac{r_p}{r_n}$$

$r_p$, $\Psi$, and $r_n$ can, with the aid of Maxwell’s theory, be expressed as functions of the wavelength of the light $\lambda$, the angle of incidence $\Phi$, and the optical properties of the reflecting system. In the case of a reflection at one interface, $r_p$ and $r_n$ are the Fresnel coefficients of the interface, which depend on the angle of incidence and the refractive indices of the two phases. When a planar and isotropic film is located between bulk media, the overall reflection coefficients also depend on the thickness and refractive index of this layer. Therefore, the refractive index, $n_t$, and the optical thickness, $d_t$, of the film can be estimated by the changes in the ellipsometric angles ($\Delta$, $\Psi$).\(^{22,23}\)

In this work, different models have been employed to analyze $\Psi - \Delta$ plots. Fitting and simulation iterative programs for models with one and two isotropic layers were used in order to study the optical properties of the systems (CMDex and mixed CMDex/ENP layers) on the thiolated gold as an effective substrate.\(^{24}\) The ellipsometric data taken after each modification step or time can be simulated with these models. As the film components does not adsorb at the working wavelength, it was assumed $k = 0$.

The amount of CMDex ($\Gamma_{\text{dextran}}$) in the hydrated film (in situ measurements) were estimated from the layer thickness of the film, $d_t$, its refractive index, $n_t$, and the refractive index of the ambient buffer by assuming linearity of the refractive index increment $dn/dc$, according to the formula derived by de Feijter et al.\(^{20}\)

$$\Gamma_{\text{dextran}} = d_t \left( \frac{n_t - n_{\text{ambient}}}{dn/dc} \right)$$

For mixed CMDex/ENP layers, the refractive index was assumed to be a linear combination of the refractive index increments of the components of the concentration $c_i$:

$$n_t = n_{\text{ambient}} + \sum (dn/dc)(\Gamma_{d_i}/d_i)$$

assuming $n_{\text{ambient}} = 1.331$ and values of 0.15 and 0.19 mL/g for refractive index increment of CMDex\(^{28}\) and ENP (as proteins) in water, respectively.

A Senetch SE 400 rotating-analyzer automatic ellipsometer, equipped with a He/Ne gas laser (632.8 nm), 10 mW (maximum output power), was used. Ellipsometric parameters were collected after each modification step and time between 5 and 10 times. All modification steps were carried out bearing in mind the system alignment and avoiding any variations of the wafer position. Ex situ measurements were performed at different angles. Thiolated gold substrates were modified with CMDex at different times, 15, 30, 60, 120, 180, and 1200 min as previously described; and also a thick film of CMDex was formed by evaporation of the solvent to obtain its refractive index, which was included in the seed file for the fitting process. In situ measurements were performed at an angle of incidence of 70.00°. These were carried out after covalent modification with CMDex (20 h) and then a further modification with ENP at different times: 1, 2, 3, and 18 h. Ex situ parameters were obtained in air once the surface was dried with N$_2$ for 1.5 min, while in situ measurements were performed under water using a cell fitted with two glass optical windows.

**Droplet Depletion Method.** Onto a surface of Au/Cys/CMDex/ENP electrode (0.5 cm$^2$), 50 µL of a solution of 3300 ng mL$^{-1}$ LPS from *S. minnesota*, purified in our laboratory according to ref 27, was deposited for 1 h in a saturated water atmosphere. Then 25 µL was taken and LPS was determined by IAL test. Results are the average of two independent experiments.

**RESULTS**

**PAAO Modified Electrodes.** Modified gold electrodes were constructed by successive adsorption to the metal of 3-mercaptopropanesulfonic acid, poly(allylamine) containing PAAOs (see Figure 2b) and finally ENP at different concentrations (Au/MPS/PAAOs/ENP, Scheme 1).

The modified electrodes were immersed in LPS–HRP solutions, rinsed, and placed as working electrodes in a cell containing a buffer solution (pH 7.5) and support electrolyte. The electrode was held at 50 mV versus Ag/AgCl. Once stabilized, hydrogen peroxide was added to a final concentration of 1 mM and the following catalytic cycle took place:

$$\text{H}_2\text{O}_2 + \text{LPS–HRP (red)} \rightarrow \text{H}_2\text{O} + \text{LPS–HRP (ox)}$$

PAAOs (II) + LPS–HRP (ox) $\rightarrow$

PAAOs (III) + LPS–HRP (red)

PAAOs (III) + e $\rightarrow$ PAAOs (II)

The observed current is proportional to the concentration of LPS–HRP.

Assays, in the absence of ENP, and using different ENP concentrations (0.1, 1 µM), were carried out. The results presented in Table 1 show that, for LPS–HRP concentrations lower than 11 ng mL$^{-1}$, the conjugate does not form stable complexes with the modified electrode. However, at a concentration of 110 ng mL$^{-1}$, a sharp difference is appreciated between modified electrodes with and without ENP. At concentrations over 1100 ng mL$^{-1}$, ENP-containing electrodes exhibit higher currents; however, under the same conditions, the electrode without ENP gives rise to a substantial current (high signal at ENP, 0), suggesting the occurrence of nonspecific binding probably due to the interaction of amino groups present in the PAAOs with lipopolysaccharides, as was described for similar compounds.\(^{28}\)

Similar experiments carried out with HRP (50 µg mL⁻¹) in contact with the modified electrode, instead of the conjugate, produced a negligible current, showing that the LPS moiety, rather than the HRP group, is determining the adsorption.

To design a balanced competitive assay, the pyrogen activity of the conjugate must be assessed and compared with that of LPS. In our case, we find that the pyrogen activity of LPS–HRP is low (0.012 EU ng⁻¹) compared with typical values for LPS (1 EU ng⁻¹), indicating that higher concentrations of the former will be required in order to obtain a balanced competitive assay of LPS versus LPS–HRP. Such increment of conjugate concentration shifts the system to ranges in which the consequent increase of nonspecific binding is significant.

To reduce nonspecific adsorption at these needed high conjugate concentrations, blocking of the exposed amino sites with BSA was attempted. In the absence of ENP, exposure of the electrodes to a 10 µg mL⁻¹ BSA solution results in a notable decrease in nonspecific adsorption. Addition of ENP in the presence of the said concentration of BSA shows that the electrode remains active, while at higher concentrations of BSA the whole electrode is blocked.

Figure 3 shows the results of a competitive assay between LPS–HRP and LPS from E. coli with an endotoxin activity of 2 UE ng⁻¹. As the concentration is represented in a logarithmic scale, samples without LPS are plotted as 0.01 ng mL⁻¹, this concentration represents the appyrogen water used in this work, and it is slightly above the maximum value obtained by LAL test. An important signal change is observed between samples without and with 0.1 ng mL⁻¹ LPS; while at higher LPS values, the signal levels off. Considering that the pyrogen activity of the LPS used in the experiment is 2 UE ng⁻¹, the assay is able to differentiate solutions containing more than 0.2 EU mL⁻¹ from those pyrogen free. The limit established by U.S. Pharmacopoeia for water in injectable drug products is 0.25 EU mL⁻¹; therefore, the system is potentially useful for providing a threshold alarm for detecting endotoxin concentrations overlapping a specified level within that range. Despite the low detection limit, this assay has a severe limitation; at higher concentrations, the signal immediately levels off, suggesting an important nonspecific binding; therefore, the device can only work as an on/off alarm.

**Carboxymethylxanthin Modified Electrodes.** Several authors have shown the adsorption of LPS on matrixes of molecules bearing amino groups in an aliphatic backbone such as penta-

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**Table 1. Catalytic Currents Obtained by Adsorption of LPS–HRP on Electrodes Modified with Different Amounts of ENP**

<table>
<thead>
<tr>
<th>LPS–HRP/µg mL⁻¹</th>
<th>[ENP] = 0 µM</th>
<th>[ENP] = 0.1 µM</th>
<th>[ENP] = 1 µM</th>
</tr>
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<tbody>
<tr>
<td>11</td>
<td>8</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>110</td>
<td>11</td>
<td>65</td>
<td>50</td>
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<tr>
<td>1110</td>
<td>630</td>
<td>830</td>
<td>1110</td>
</tr>
</tbody>
</table>

* The 0, 0.1, and 1 µM are the different ENP concentrations to which each electrode was exposed before being dipped in the LPS–HRP solutions.

midine, chlorhexidine, etc. PAAOs constitutes a polymer with similar characteristics containing an aliphatic backbone from which amino groups are pending. Therefore, interactions are to be expected between the phosphate groups and lipid moieties of LPS on one hand, with the positive charged residues (amino groups) and the hydrophobic backbone, respectively, of PAAOs on the other. Exploring possible improved models for the electrode probe, we tried to avoid these interactions by using as a binding layer of CMdex, which is a carbohydrate with 16% of its OH groups forming ether bonds, OCH₂COOH, thus exposes a hydrophilic surface to which ENP can be in this case covalently bound.

Construction of the electrodes was as follows (Scheme 2): Cystamine was adsorbed onto gold by its sulfur atoms. Its amino groups were bound to some carboxylate groups in the carbohydrate with the coupling reagents EDC/NHS, leaving the rest of the carboxylates available for further reactions. Addition and reaction with ENP, and further quenching of the remnant carboxylate groups, completed the electrode.

Ellipsometric studies confirmed CMdex and ENP binding in the modified electrodes. After coupling the CMDex (to 1200 min), both the thickness and the refractive index of the CMDex layer were estimated by ex situ measurements. A refractive index of ~1.54 and a layer thickness of 2.0 ± 0.5 nm were obtained. From these parameters, a surface loading of 3.5 ± 0.7 ng mm⁻² was calculated by assuming a density of 1.56 g cm⁻³ for this refractive index. The properties of the CMdex layer under aqueous medium (in situ measurement) determined were, for the refractive index, 1.340 ± 0.002, and for the layer thickness, 71 ± 9 nm; these results indicate a strong swelling of the layer (% water ≥ 95). The CMdex content in the hydrated layer was calculated as, according to de Feijer, 6.4 ± 1.5 ng mm⁻² (26 ± 6 pmol cm⁻²).

To describe the incorporation and distribution of ENP within the CMdex hydrogel, two simplified models can be proposed and referred to as an isotropic single layer and isotropic two layers.

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In the isotropic single-layer model, ENP molecules are randomly distributed throughout the entire CMdex hydrogel given a protein–hydrogel layer with \( d_{\text{ENP}} = d_{\text{dex}} = d_{\text{layer}} \) for each time. When ENP is added to the system, both, the refractive index and the layer thickness increase in proportion to the amount of material incorporated. Under the conditions used in the competitive assay, a refractive index of 1.347 ± 0.002 and a layer thickness of 107 ± 4 nm were estimated. From these parameters, the calculated ENP content calculated was 4.0 ± 1.5 ng mm\(^{-2}\) (33 ± 12 pmol cm\(^{-2}\)).

In the isotropic two-layer model, the protein layer thickness is assumed to be different from the hydrogel thickness; the hydrogel layer is seen as divided into two isotropic and homogeneous layers: an inner layer containing the CMdex hydrogel and an outer one with ENP incorporated into the CMdex hydrogel. In this case, both refractive index and thickness of the inner layer are assumed to be unaffected by the incorporation of ENP molecules within the outer layer. According to this model, and carrying out iterative simulations, the following values were obtained: \( n_{\text{inner}} = 1.339 ± 0.001, d_{\text{inner}} = 56 ± 1 \text{ nm}, n_{\text{outer}} = 1.450 ± 0.005, \) and \( d_{\text{outer}} = 11 ± 1 \text{ nm}. \) The calculated ENP content resulting from this model is 4.2 ± 1.4 ng mm\(^{-2}\) (34 ± 11 pmol cm\(^{-2}\)).

It is noteworthy that both models give similar ENP loading results; however, further information would be needed to choose one or both.

To establish that free carboxylate groups are available for reaction with ENP, studies using ferrocene carboxylic acid as electrochemical probe were carried out.

The reaction between amino groups of cystamine adsorbed on gold electrodes with aldehydes (in this case ferrocenecarboxyaldehyde) in the presence of cyanoborohydride is well established. The difference between charges measured after linking the ferrocenealdehyde moiety to the electrode, in the presence or absence of CMDex, allows us to estimate the amount of CMDex bound to the electrode. The ferrocene moiety bound to the Au/Cys electrode yields a charge of 87 \( \mu \text{C cm}^{-2} \), which is equivalent to 901 pmol cm\(^{-2}\); a value close to the one obtained for cystamine adsorption by Wirde et al.\(^{29}\) After addition of CMDex (Au/Cys/CMdex), the charge due to the ferrocene moiety decreases to 42 \( \mu \text{C cm}^{-2} \). This indicates that 435 pmol cm\(^{-2}\) amino groups were available after modification with CMDex and, therefore, were free to react with the ferrocene derivative, which is equal to saying that 466 pmol cm\(^{-2}\) carboxylate groups have been attached to cystamine in the electrode.

To determine how many carboxylate groups remain free in the surface, the surface concentration of CMDex may be determined by ellipsometry. We obtained a value of 26 pmol cm\(^{-2}\); since CMDex contains ~66 carboxylic acid mol/mol of polymer we may conclude that 1716 pmol cm\(^{-2}\) carboxylic acid is present. Knowing that 466 have reacted, still 1250 pmol cm\(^{-2}\) carboxylate remains free and available in large excess for binding with ENP (33 pmol cm\(^{-2}\), obtained by ellipsometry).

Exposing Au/Cys/CMdex/ENP electrodes to different concentrations of LPS–HRP, the amperometric signal is seen to increase with concentration (Figure 4, diamonds); an important change in the current is observed at conjugate concentrations in the order of 10 000 ng mL\(^{-1}\). The same figure presents the values measured in absence of ENP (squares) attributable entirely to nonspecific binding of LPS–HRP directly on the CMdex matrix. The results show an optimal response at concentration of conjugate up to LPS concentrations of 10 000 ng mL\(^{-1}\).

Figure 5 shows a competitive assay between LPS and LPS–HRP, at a fixed LPS–HRP concentration of 10 000 ng mL\(^{-1}\), which is the amount closest to saturation of the electrode without formation of micelles.\(^{24}\) The response to LPS shows the typical sigmoidal shape observed in this kind of assays.

At low LPS concentrations, the observed signal corresponds to saturation with labeled LPS. As LPS concentrations increase it competes with the labeled LPS, decreasing the signal; and finally at high concentration of the analyte, the labeled LPS is completely displaced and the signal is due to nonspecific adsorption (over 100 ng mL\(^{-1}\)). At concentrations less than 1 ng mL\(^{-1}\), variations are within experimental error. Thus, this electrode performs

DISCUSSION

The different detection limits observed for the two configurations can be analyzed considering both the three-dimensional structure of the ENP macromolecule and the immobilization procedures used in each case for fixing the molecule to the electrode.

ENP is the recombinant form of Limulus anti-lipopolysaccharide factor. The crystallographic structure\(^{10}\) shows that it is a triangular-shaped protein comprising three \(\alpha\)-helices and three \(\beta\)-sheets joined by a disulfide bridge between Cys 31 and Cys 52. This domain of ENP is characterized by an alternating series of positively charged and hydrophobic residues that, by virtue of the extended protein, can be adsorbed by van der Waals forces to the polyallylamine backbone, leaving the positive charged domain exposed to the solution, initially attracting the negative charged lipid A and then stabilizing the complex by hydrophobic interactions, as described by Surolia et al. for interactions of lipid A with different proteins.\(^{11,13}\)

Instead, when the protein is covalently attached to CMDex, as in Au/Cys/CMdex/ENP, amide bonds are formed by the amino groups of the lysine residues, most of them in the above-mentioned loop. Therefore, part of the positive charge is lost, and the conformation flexibility of the protein is restricted. It is reasonable to expect that both factors will diminish to some extent the affinity of the bound conjugate for LPS.

Further studies, binding ENP through other residues to suitable chemical groups coating the electrode surface, may shed more light allowing testing and confirming this interpretation.

To improve the performance of the detection strategies presented here, an important point to focus on is the synthesis of a conjugate with higher endotoxin activity.

For better understanding and interpretation of the differences in affinities of LPS and conjugate toward the modified electrodes, estimations of the molecular coverage of the surfaces by both ENP and LPS molecules are important.

Limoges co-workers\(^{34}\) studied the adsorption of HRP conjugated with neutravidin (NHRP) on a biotin modified electrode. These authors developed a model and established kinetics parameters for HRP when it reacts with the substrate together with an osmium complex mediator, \([\text{Os}^{(bpy)}_{2}pyCl]Cl\). The authors were able to predict the registered current as a function of adsorbed HRP, hydrogen peroxide concentration, and mediator concentration. From the currents obtained, a surface concentration of NHRP of 3.8 pmol cm\(^{-2}\) was established, which corresponds to a monolayer.

CMDex and ENP coverage of the Au/Cys/CMdex/ENP electrode surface were estimated using ellipsometry, yielding 26 and 35 pmol cm\(^{-2}\), respectively, and a layer of \(\sim\)100 nm exceeding what can be considered a monolayer. Considering a 1:1 stoichiometric ratio of ENP to LPS,\(^{10}\) and taking into account that, according to the manufacturer, the conjugate bears 2 HRP/LPS molecules, a maximum of 70 pmol cm\(^{-2}\) HRP molecules could possibly be adsorbed, consistent with the findings for ENP. However, this is sterically not realistic since HRP is a bulky molecule and the conjugate presents two such moieties for each LPS.

In our case, the LPS–HRP adsorption on Au/Cys/CMdex/ENP produced a maximum current of 1.4 \(\mu\)A cm\(^{-2}\). Taking into account the activity of the HRP in the conjugate, considering the mediator and hydrogen peroxide concentration used in this work, and using the model proposed in ref 34, a surface coverage of 0.08 pmol cm\(^{-2}\) may reasonably be estimated, suggesting a low affinity between the conjugate and the modified surface.

To establish the affinity of this surface for LPS, we adopted the droplet depletion method.\(^{24}\) It consists of depositing a droplet of LPS solution on the surface of the modified electrode and determining, before and after an incubation period, the activity of the LPS contained in the droplet using the LAL test. On account of the large ratio of electrode surface/droplet volume, a sizable amount of dissolved LPS is depleted from the drop. This amount of LPS normalized to the surface area of the electrode corresponds to the amount adsorbed to the electrode surface. Using a purified LPS from \(S. minnesota\) of 20,000 Da average molecular mass,\(^{35,36}\) the amount of LPS adsorbed from a solution containing 3300 ng mL\(^{-1}\) is 12.3 pmol cm\(^{-2}\), a value that is close to the one observed by ellipsometry for ENP. This means that a native LPS is more efficiently adsorbed than the conjugate and explains why very low concentrations of LPS can completely displace LPS–HRP from the electrode.

One of the problems generated by using a high concentration of the conjugate is the tendency of LPS to form aggregates. For example, LPS from \(S. minnesota\) has a critical micellar concentration (cmc) of 11 \(\mu\)g mL\(^{-1}\) and LPS from \(E. coli\) a cmc of 14 \(\mu\)g mL\(^{-1}\).\(^{35,36}\) At high concentrations of LPS, aggregation with LPS–HRP may explain the behavior observed in both competitive assays since aggregates will not compete for the binding sites on the electrode surface.

Another aspect that should not be overlooked is that LPS can be adsorbed not only by the specific recognition element ENP but also by polymeric matrixes containing a hydrophobic backbone combined with positive charges as is the case for poly(allylamine). This raises a warning to possible interferences of protein material with the proposed LPS assay. Nevertheless, if the technique is oriented to measuring endotoxin in highly pure aqueous solutions containing mainly inorganic ions, interferences and fouling by possible biological pollutants (proteins, carbohydrates, etc.) would be negligible. It must be borne in mind that


HRP does not bind to polyallylamine even at concentrations of 50 µg mL⁻¹ and other proteins are not adsorbed by this polymer when they are present even at higher concentrations. Dextran, the other matrix used in this work, is a water-soluble hydrophilic organic macromolecules conveniently used as a coating to prevent nonspecific adsorption; therefore, in this system, the presence of other biological species should not interfere with the signal produced by LPS.

CONCLUSIONS
This study explores successfully the possibility of determining low concentrations of endotoxin using a chemical recognition strategy through a competitive assay. A recombinant version of an antimicrobial protein present in the innate immunosystem of the horseshoe crab was incorporated into an electrode. The assay was carried out between a fixed concentration of a conjugate (LPS–HRP) and the LPS present in the sample, allowing the detection of endotoxin in low levels through a more simple assay than the one currently in use. However, several issues have to be addressed, such as improving the endotoxin activity of the conjugate, avoiding side effects of the matrix, optimization of the sample incubation period, and correlating results of LPS from different sources and the current accepted method (LAL test).

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