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Surfactant-Assisted Lipopolysaccharide Conjugation Employing a Cyanopyridinium Agent and Its Application to a Competitive Assay

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The activation of a lipopolysaccharide (LPS) with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) in the presence of a surfactant allows an efficient conjugation with dansyl hydrazine or horseradish peroxidase (HRP) in an aqueous medium maintaining its biological activity. In order to promote the reaction a series of amphiphilic compounds were tested, sodium deoxycholate being the most suitable. The method presents several advantages: it is carried out in a mild environment, good conjugation ratios are obtained, it is suitable for any label bearing amino, hydrazine, or hydrazide groups, and the LPS endotoxic and HRP enzymatic activities are preserved. The HRP conjugate is applied in an amperometric competitive assay for the detection of lipopolysaccharides in an electrode array combined with a multipotentiostat able to carry out simultaneous determinations. The system is able to detect samples in concentrations as low as 100 pg mL^{-1} of LPS.

Lipopolysaccharides (LPS) are complex lipid-linked carbohydrate negatively charged molecules. Usually, they comprise 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.[1] Lipid A consists of a hydrophilic negatively charged bisphosphorylated disaccharide of glucosamine backbone, covalently linked to a hydrophobic domain of acyl chains (12−16 carbon atoms) via amide and ester bonds.[2−5] It always exhibits an amphiphilic character and the ability to form aggregates.[6] Although LPS itself is chemically inert, the presence of LPS in blood (endotoxemia) sets off a cascade of host responses affecting the structure and function of organs and cells, changing metabolic functions, raising body temperature, modifying hemodynamics, and causing septic shock.[7]

Currently, the most widely used assay to detect and quantify endotoxin in aqueous samples is the *Limulus* amebocyte lysate (LAL) assay. However, several issues affect its performance, such as changes in pH, temperature, and the presence of β-glucans derivatives.[8] Due to the high toxicity of LPS and the limitations of the different LAL test versions, there are efforts toward the development of sensors and assays able to specifically detect small amounts of LPS. In the last years, several approaches in this area have been presented.[9−14]

In a previous work,[9] a competitive assay was presented for the detection of endotoxins. Even though the possibility of determining low LPS concentrations using a protein able to recognize lipid A was shown, the assay still had some pitfalls that need to be addressed for improving its performance. Particularly, it is important to reduce the conjugate nonspecific adsorption, improve the handling of the assay platform for speeding up the throughput of results, and ameliorate the conjugate endotoxic activity.

Studies on different matrixes were carried out to effectively avoid the nonspecific adsorption of hydrophopic macromolecules on modified electrodes,[15] showing that carboxymethyl dextran (CMDex70, MW 70 kDa) is the most suitable matrix, due to its rejection properties and the ability to be further modified. Contemporarily, a gold electrode array as an electrochemical

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(1) Takahashi, I.; Kotani, S.; Takada, H.; Tsujimoto, M.; Ogawa, T.; Shiba, T.; Kusumoto, S.; Yamamoto, M.; Hasegawa, A.; Kiso, M.; Nishijima, M.; Amano, F.; Akamatsu, Y.; Harada, K.; Tanaka, S.; Okamura, H.; Tamura, T. *Infect. Immun.* **1987**, *55*, 57–68.

(2) Rietschel, E. T.; Kirikae, T.; Schade, F. U.; Ulmer, A. J.; Holst, O.; Brade, H.; Schmidt, G.; Mamat, U.; Grimmecke, H. D.; Kusumoto, S.; Zahringer, U. *Immunobiology* **1993**, *187*, 169–190.

(3) Zahringer, U.; Lindner, B.; Rietschel, E. T. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 211–276.

(4) Taylor, A. H.; Heavner, G.; Nedelman, M.; Sherris, D.; Brunt, E.; Knight, D.; Ghrayeb, J. *J. Biol. Chem.* **1995**, *270*, 17934–17938.

(5) Trent, M. S.; Stead, C. M.; Tran, A. X.; Hankins, J. V. *J. Endotoxin Res.* **2006**, *12*, 205–223.

(6) Gutsmann, T.; Schromm, A. B.; Brandenburg, K. *Int. J. Med. Microbiol.* **2007**, *297*, 341–352.

(7) Amersfoort, E. S. v.; Berkel, T. J. C. v.; Kuiper, J. *Clin. Microbiol. Rev.* **2003**, *16*, 379–414.

(8) Roslansky, P. F.; Novitsky, T. J. *J. Clin. Microbiol.* **1991**, *29*, 2477–2483.

(9) Priano, G.; Battaglini, F. *Anal. Chem.* **2005**, *77*, 4976–4984.

(10) Kilar, A.; Kocsis, B.; Kustos, I.; Kilar, F.; Hjerten, S. *Electrophoresis* **2006**, *27*, 4188–4195.

(11) Voss, S.; Fischer, R.; Jung, G.; Wiesmuller, K. H.; Brock, R. *J. Am. Chem. Soc.* **2007**, *129*, 554–561.

(12) Priano, G.; Pallarola, D.; Battaglini, F. *Anal. Biochem.* **2007**, *362*, 108–116.

(13) Kato, D.; Iijima, S.; Kurita, R.; Sato, Y.; Jia, J. B.; Yabuki, S.; Mizutani, F.; Niwa, O. *Biosens. Bioelectron.* **2007**, *22*, 1527–1531.

(14) Ding, S. J.; Chang, B. W.; Wu, C. C.; Chen, C. J.; Chang, H. C. *Electrochem. Commun.* **2007**, *9*, 1206–1211.

(15) Pallarola, D.; Domenianni, L.; Priano, G.; Battaglini, F. *Electroanalysis* **2007**, *19*, 690–697.

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platform for simultaneous assays was developed to increase the throughput of results.[16]

Regarding the conjugate quality, an efficient method for the conjugation of LPS with probes bearing primary amino and hydrazine moieties through the activation with cyanogen bromide was introduced.[17] The reaction produces conjugates with an excellent ratio, the endotoxic activity of LPS is preserved, and there is the possibility of combining LPS with a myriad of probes bearing the before mentioned functional groups. In spite of all these advantages, working with CNBr is not an amenable task. The reagent is one of the most dangerous to operate in a laboratory; the activation reaction has to be carried out at −15 °C, with problems in the reagents manipulation and the choice of a suitable solvent. Moreover, these conditions are quite unfavorable for further modification of LPS with an enzyme such as horseradish peroxidase (HRP).

Activation of hydroxyl groups in carbohydrates can also be achieved by treatment with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP).[18−22] In this case the activation and further carbohydrate modification are produced under milder conditions in an aqueous solvent, that is, a friendlier environment for an enzyme. On the other hand, in an aqueous environment LPS forms micelles; to overcome this problem different amphiphilic agents can be used to disaggregate LPS.[23−26]

In this context, the present work proposes the conjugation of LPS in an aqueous solution in the presence of amphiphilic molecules. Sodium dodecyl sulfate (SDS), Triton X-100, and sodium deoxycholate (NaDC) were tested at different concentrations using a fluorescent probe (dansyl hydrazine). From these experiments, NaDC was found to be the best suited. LPS from *S. minnesota* is conjugated with good labeling ratios with the fluorescent probe (110 nmol mg^{-1}), preserving 70% of its endotoxic activity.

For the conjugation with HRP, diamino poly(ethylene glycol) (DAPEG) was used as spacer. The spacer was bound to a periodate-oxidized HRP (oHRP) and then reacted with the CDAP-activated LPS. The conjugate LPS−DAPEG−oHRP presented a good conjugation ratio (29 nmol HRP/mg LPS) and preserved its endotoxic and enzymatic activities. Finally, this conjugate was used in combination with a gold electrode array modified by the immobilization of endotoxin neutralizing protein (ENP) on the new matrix. The assay was able to detect LPS concentrations in the order of 100 pg mL^{-1} (equivalent to 0.12 EU mL^{-1}) with better reproducibility, sensitivity, and less nonspecific adsorption than the previous version.[9]

MATERIALS AND METHODS

Materials. Smooth-form lipopolysaccharide from *Salmonella enterica* sv. Minnesota was supplied by Sigma. ***Warning***: *LPS is a pyrogen. It may cause fever. It may be harmful by inhalation, ingestion, or skin absorption. Good laboratory technique should be employed: wear a laboratory coat, gloves, and safety glasses. Work in a well-ventilated area. Avoid contact with open wounds.* Chromogenic LAL test was provided by Biowhitaker. Milli-Q water was obtained from a Millipore ultrapure water filtration unit. Apyrogen water was produced as previously reported.[9] Dansyl hydrazine and sinapinic acid were provided by Fluka; HRP was provided by Biozyme. CDAP, bovine serum albumin (BSA), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), triethylamine (TEA), ethanolamine, pyrogallol, SDS, NaDC, Triton X-100, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and cystamine dihydrochoride (Cys) were provided by Sigma. Protein assay dye reagent concentrate was provided by Bio-Rad. Carboxymethyl dextran (MW 70 kDa, COOH/monomer ratio 1:10) synthesis is described elsewhere.[15] 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). DAPEG, MW 1000 Da, was provided by NOF (White Plains, NY). RC Spectra/Por dialysis membranes (3500 and 12 000 Da molecular weight cutoff (MWCO)) were obtained from Spectrum Laboratories. Filtration membranes (30 kDa MWCO) were provided by Millipore.

LPS Purification. Smooth-form LPS from *S. minnesota* was chromatographically purified using a column of Sephacryl HiPrep 16/60 (S-200 HR, GE Healthcare) in an ÄKTA Explorer FPLC system, as previously reported.[17] The LPS collected fractions were dialyzed, lyophilized, and characterized by their content of proteins (determined by Bio-Rad protein assay), adenosine ribonucleic acid (an A_{260nm} value of 0.1 corresponds to 4 μg mL^{-1} of RNA),[27] Kdo,[28] and endotoxic activity (determined by means of the LAL test). The fractions were stored at −20 °C until use. The synthesis of the conjugates was carried out using the fraction eluted between 47 and 53 mL (47−53 fraction). This fraction presented an endotoxic activity of 0.65 EU ng^{-1}, 0.2% w/v RNA and 0.3% w/v proteins; before purification, the LPS contained 46% w/v RNA and 0.8% w/v proteins.

LPS−Dansyl Conjugate. Fraction 47−53 of purified LPS was used to perform the conjugation. An amount of 0.5 mL of 2 mg mL^{-1} of an aqueous LPS solution was vortexed for 3 min and further sonicated for 15 min at 25 °C. Then, 20 μL of 100 mg mL^{-1} CDAP in acetonitrile was added; after 30 s 20 μL of 0.2 M TEA in water was added. The mixture was left to react for 150 s in the dark at 25 °C under stirring. Thereafter, 500 μL of 2.0 mg mL^{-1} dansyl hydrazine in 0.1 M sodium borate buffer pH 9.3 was added. The mixture was left to react for 2 h in the dark at 25 °C under stirring. The reaction was quenched with 50 μL of ethanolamine. The product of the reaction was passed through two Hitrap desalting columns in series (GE Healthcare, 5 mL each) using 50 mM HEPES buffer, pH 7.5 as eluent, at an elution rate of 2 mL min^{-1}. The conjugate was dialyzed against Milli-Q water using a 3500 MWCO membrane at 4 °C in the dark. Then, the sample was characterized by fluores-

(16) Priano, G.; Gonzalez, G.; Günther, M.; Battaglini, F. *Electroanalysis* **2008**, *20*, 91–97.
(17) Pallarola, D.; Battaglini, F. *Anal. Biochem.* **2008**, *381*, 53–58.
(18) Lees, A.; Nelson, B. L.; Mond, J. J. *Vaccine* **1996**, *14*, 190–198.
(19) Shafer, D. E.; Toll, B.; Schuman, R. F.; Nelson, B. L.; Mond, J. J.; Lees, A. *Vaccine* **2000**, *18*, 1273–1281.
(20) Bystricky, S.; Machova, E.; Bartek, P.; Kolarova, N.; Kogan, G. *Glycoconjugate J.* **2000**, *17*, 677–680.
(21) Bystricky, S.; Paulovicova, E.; Machova, E. *Immunol. Lett.* **2003**, *85*, 251–255.
(22) Kossaczka, Z.; Szu, S. C. *Glycoconjugate J.* **2000**, *17*, 425–433.
(23) Olins, A. L.; Warner, R. C. *J. Biol. Chem.* **1967**, *242*, 4994.
(24) McIntire, F. C.; Sievert, H. W.; Barlow, G. H.; Finley, R. A.; Lee, A. Y. *Biochemistry* **1967**, *6*, 2363.
(25) Shands, J. W.; Chun, P. W. *J. Biol. Chem.* **1980**, *255*, 1221–1226.
(26) Panda, A. K.; Chakraborty, A. K. *J. Colloid Interface Sci.* **1998**, *203*, 260–264.
(27) Bergethon, P. *The Physical Basis of Biochemistry*; Springer-Verlag: New York, 1998; pp 254−256.
(28) Lee, C. H.; Tsai, C. M. *Anal. Biochem.* **1999**, *267*, 161–168.

cence spectroscopy,[17] Kdo content,[28] and with the LAL test for endotoxicity.

LPS−Dansyl Conjugate in Presence of a Surfactant. This synthesis was similar to the one presented before with the exception that a surfactant (deoxycholate, SDS, or Triton X-100) was added. Different concentrations were used. The synthesis consisted in the following: 0.5 mL of 2 mg mL^{-1} aqueous LPS solution was vortexed for 3 min and sonicated for 15 min at 25 °C, then 0.5 mL of one of the amphiphilic compounds at given concentration plus 50 μL of 2.5 mM EDTA was added. The solution was stirred for 30 min at 37 °C, sonicated for 15 min, and again stirred for 30 min at 37 °C. Then 20 μL of 100 mg mL^{-1} CDAP in acetonitrile was added, and the same procedure described before was followed thereafter.

Periodate-Oxidized Horseradish Peroxidase. An amount of 10 mg mL^{-1} HRP was oxidized in 25 mM aqueous sodium periodate solution in the presence of 10 mM Ca^{2+} in the dark, for 30 min at 25 °C. After the oxidation, the reaction was quenched with 50 μL of ethylene glycol. The oHRP was separated from the low molecular mass saccharides using two Hitrap desalting columns in series (GE Healthcare, 5 mL each) using 100 mM sodium bicarbonate pH 9.5 as eluent at an elution rate of 2 mL min^{-1}. A photometric procedure[29] was used to determine the enzymatic activity of the purified oHRP given a value of 236 U mg^{-1}. The molecular mass was 42 160 Da, determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) analysis using a procedure previously reported.[17]

DAPEG−oHRP Conjugation. oHRP is added dropwise for 3 min in a 34 mM DAPEG solution in 100 mM sodium bicarbonate pH 9.5 containing sodium borocyanohydride in excess. The mixture was left to react in the dark for 2 h at 4 °C under stirring. The reaction was quenched with 50 μL of ethanolamine. The enzyme was separated from the low molecular mass compounds as before. The purified DAPEG−oHRP peroxidase activity[29] was 188 U mg^{-1}, and the molecular mass was 46 724 Da, determined by MALDI-TOF analysis.[17]

LPS−DAPEG−oHRP Conjugation. Three milligrams of fraction 47−53 of LPS from *S. minnesota* was dissolved in 1.5 mL of Milli-Q water. The LPS solution was vortexed for 3 min and further sonicated for 15 min at 25 °C. Then, 1.5 mL of 4.5 mM NaDC plus 150 μL of 2.5 mM EDTA were added. The solution was stirred for 30 min at 37 °C, sonicated for 15 min, and again stirred for 30 min at 37 °C. Then, 60 μL of 100 mg mL^{-1} CDAP in acetonitrile was added; after 30 s, 60 μL of 0.2 M TEA in water was added. The mixture was left to react for 150 s in the dark, at room temperature under stirring. Thereafter, 2 mL of 12 mg mL^{-1} DAPEG−oHRP in 0.1 M sodium bicarbonate pH 9.5 was added. The mixture was left to react for 2 h in the dark at 25 °C under stirring. The reaction was quenched with 100 μL of ethanolamine. The product of the reaction was concentrated to 3 mL using a 30 kDa MWCO membrane and purified using a series of two Hitrap desalting columns (GE Healthcare, 5 mL each) with 50 mM Tris buffer pH 6.5 at an elution rate of 2 mL min^{-1}. Unreacted peroxidase was separated from the conjugate by anion-exchange chromatography as previously reported[17] (ANX FF, high sub, 20 mL, GE Healthcare) using 50 mM Tris buffer, pH 6.5 as binding buffer and 50 mM Tris buffer, pH 6.5, 1 M NaCl as elution buffer.

Conjugation Ratio. Throughout the text the conjugation ratio is presented as nanomoles of probe per milligram of LPS. This is due to the fact that LPS does not have a well-defined molecular mass. In the case of LPS−dansyl, dansyl concentration was determined by fluorescence at concentrations where LPS was not aggregated. LPS was determined by Kdo analysis with a calibration curve constructed with the same LPS fraction used in the conjugation. For LPS−DAPEG−oHRP, oHRP was determined by visible spectroscopy of its heme group at 403 nm and from its protein content obtained by Bio-Rad protein assay using HRP as standard. LPS was determined by Kdo analysis.[28]

Construction of Modified Electrodes. The electrode array was constructed as previously reported.[16] A purpose multipotentiostat system consisting of eight working electrodes,[16] a gold counter electrode, and a Ag/AgCl reference electrode was used in the amperometric experiments. The soluble redox mediator was $[Os(bpy)_2pyCl]PF_6$ (with bpy = bipyridine and py = pyridine).

The platform of eight gold electrodes of 0.6 cm^2 area were cleaned by cyclic voltammetry in 1.0 M $HClO_4$ between −0.2 to 1.6 V versus Ag/AgCl at 0.2 V s^{-1}. When the cleaning step was satisfactorily verified, the electrodes were rinsed with Milli-Q water and modified with cystamine and CMDex70, according to the procedures described previously[9] with some minor adjustments. A concentration of 20 mg mL^{-1} CMDex70 was used in the modification step. Then, CMDex70-modified electrodes were first rinsed with 100 mM PIPES buffer, pH 7.5, 150 mM NaCl and finally with 50 mM MES buffer pH, 5.5. The incorporation of ENP to the electrode was carried out in a solution containing 100 mM EDC, 125 mM NHS in MES buffer, pH 5.5 for 30 min to activate the carboxylate groups. Unless otherwise stated, after the activation step, the electrodes were rinsed with MES buffer, pH 5.5 and immersed in 1 μM ENP solution in 50 mM HEPES buffer, pH 8.0 for 2 h at 22 °C under stirring. Then, the electrodes were immersed in a 1 M ethanolamine solution to quench the activated carboxylate groups and finally rinsed with 50 mM HEPES buffer, pH 8.0 and apyrogen water.

Adsorption of LPS−DAPEG−oHRP. The modified electrodes were immersed in a solution containing 5 μg mL^{-1} LPS−DAPEG−oHRP in apyrogen 50 mM Tris buffer, pH 7.5, 100 mM NaCl for 60 min at 22 °C under gentle stirring. After the adsorption step, the electrodes were rinsed with apyrogen buffer and with apyrogen water. A blank experiment was carried out with 10 μg mL^{-1} HRP (enzymatic activity 280 U mg^{-1}).

Competitive Assays. Competitive assays of modified electrodes were carried out by adsorption of a mixture of different LPS concentrations (endotoxic activity 1.2 EU ng^{-1}) and LPS−DAPEG−oHRP at a fixed concentrations of 5 μg mL^{-1} in apyrogen 20 mM Tris buffer, pH 7.5, 100 mM NaCl for 60 min at 22 °C under gentle stirring. After incubation, the electrodes were rinsed with apyrogen buffer and then with apyrogen water. The reported current for each concentration is the average of three independent experiments.

(29) Chance, B.; Maehly, A. C. *Methods in Enzymology: Preparation and Assays of Enzymes*; Academic Press: London and New York, 1955; pp 773−775.

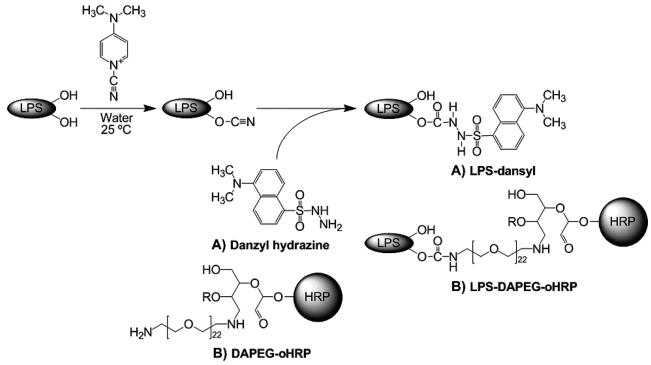

Figure 1. Steps involved in the conjugation of LPS with (A) dansyl hydrazine and (B) DAPEG−oHRP.

Amperometric Measurements. The time-based measurements were performed at fixed electrode potential, 50 mV versus Ag/AgCl. For all the assays described bellow, the electrodes were carefully rinsed and introduced into the electrochemical cell containing 0.2 M KNO_3, 50 mM Tris buffer, pH 7.5. $[Os(bpy)_2(py)Cl]PF_6$ was added to a final concentration of 20 μM. The modified electrodes were connected to the multipotentiostat, left to equilibrate with the solution, and hydrogen peroxide was added to give a final concentration of 0.9 mM; an immediate change in the current was observed that reached a constant value due to the cathodic catalytic current produced, which was proportional to the surface concentration of LPS−DAPEG−oHRP.

RESULTS AND DISCUSSION

LPS Activation and Fluorophore Conjugation. The activation of hydroxyl groups in carbohydrate moieties with CDAP has competed with CNBr in the last years. In comparison to CNBr, CDAP is less hazardous and easier to use, can be employed at a milder pH, and has fewer side reactions, finding application in the synthesis of conjugate vaccines and immunological reagents.[18−22]

The first attempt for the activation of LPS with CDAP was to dissolve a purified fraction of LPS in an aqueous solution and then sonicate for 15 min in order to obtain a homogeneous solution. Afterward, LPS was treated with CDAP, prior to the addition of dansyl hydrazine (Figure 1). Working under these conditions, the conjugation ratio obtained was very poor, 1.4 nmol dansyl/mg LPS. The same reaction carried out with a soluble carbohydrate polymer (dextran, MW 70 kDa) produced a conjugation ratio of 70 nmol dansyl/mg dextran. This value indicates that the aggregation of LPS in solution plays an important role in the possible activation of the hydroxyl groups.

To increase the exposition of these groups to the solution, different amphiphilic molecules were tested to improve the degree of modification. Tested molecules were NaDC, Triton X-100, and SDS. These molecules were chosen because their use for LPS disaggregation has been extensively described in the literature.[23−26] Triton X-100 is a nonionic detergent, whereas SDS and deoxycholate are negatively charged. Since these molecules are anionic or nonionic surfactants, the disaggregation effect on LPS of these amphiphilic compounds is mainly attributed to the interaction of their hydrophobic part with the fatty acids of lipid A. To optimize disaggregation, it is very important to consider the number of molecules needed to surround the lipid A moiety but, also, the surfactant concentration for which it will have a stronger interaction with lipid A than with itself. Therefore, the critical micellar concentration (cmc) of each surfactant was taken into account (Table 1).[30] The same amount of LPS (0.5 mL of 2 mg mL^{-1} LPS) was dissolved in the presence of one of the amphiphilic compounds at a given concentration, plus 50 μL of 2.5 mM EDTA, and then activated with CDAP and reacted with dansyl hydrazine to find the best conditions for the conjugate synthesis. The excess of surfactant and unreacted dansyl was removed by size exclusion chromatography, and the desired product was then dialyzed and characterized.

Table 1. Properties of the Amphiphilic Molecules Employed in This Work (Ref 30)

molecule	cmc (mM)[a]	no. aggregation	MW micelle (Da)
SDS	8.1	84	24200
Triton X-100	0.25	140	90000
NaDC	1.5	5	2000

[a] Values in water.

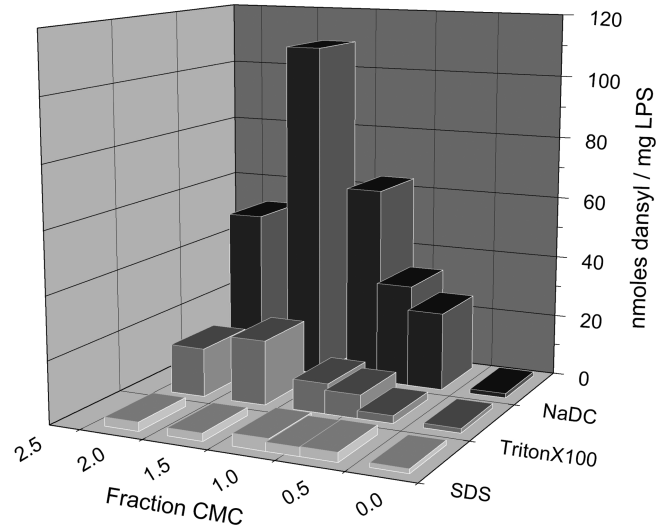

Figure 2. Modification of LPS with dansyl in the presence of amphiphilic molecules at different concentrations.

Figure 2 shows the effect of the surfactants on the conjugation yield. In the case of SDS, there is no significant improvement when increasing its concentration. The higher conjugation yield (4 nmol dansyl/mg LPS) is obtained at its cmc. This value is close to the one obtained in absence of surfactant (1.4 nmol dansyl/mg LPS). The conjugation ratio changes significantly for Triton X-100 and NaDC. The use of these compounds improves notably the conjugation yield with respect to the one obtained in their absence. For these surfactants, the best conditions correspond to 1.5 times their cmc. This behavior can be interpreted as follows: at low concentrations there is not enough surfactant to wholly disaggregate LPS, whereas at higher concentration the interaction of the surfactant with itself is predominant. This behavior coincides with the decrease of conjugation for concentrations above the cmc.

NaDC shows better behavior than Triton X-100, achieving the introduction of 110 nmol dansyl/mg LPS. The conjugate synthe-

(30) Abelson, J. N.; Simon, M. I.; Deutscher, M. P. *Methods in Enzymology: Guide to Protein Purification*; Academic Press: London and New York, 1990; pp 239−253.

sized in these conditions shows an endotoxic behavior corresponding to 70% activity (0.45 EU ng^{-1}) of the original endotoxic behavior (0.65 EU ng^{-1}).

LPS−HRP Conjugation. In a previous work[17] we have conjugated LPS with HRP through activation with CNBr. Even though we have maintained the endotoxic behavior of LPS, the use of organic solvents in the different reaction steps produced a conjugate with low enzymatic activity. To avoid the inactivation of the enzyme, in this work a different strategy for the conjugation was taken. DAPEG, MW 1000 Da, was used as spacer. First, HRP was treated with sodium periodate producing the partial oxidation of its carbohydrate shell, and then the oxidized enzyme (oHRP) was incubated with an excess of DAPEG. MALDI-TOF analysis showed that DAPEG/oHRP ratio achieved was ca. 4:1.

Finally, DAPEG−oHRP was reacted with LPS previously activated with CDAP in a synthetic pathway analogous to the dansyl conjugation (Figure 1). Once the reaction was finished, the conjugate was separated from the excess of unreacted enzyme using an anion-exchange column. The unreacted enzyme eluted first, with practically no interaction with the column, and then the conjugate eluted when the ion strength was increased. At the buffer conditions employed, DAPEG−oHRP was positively charged; on the other hand, LPS−DAPEG−oHRP bore negatively charged groups from the phosphates present in LPS. The second peak collected was desalted and characterized by its Kdo and protein contents and enzymatic and endotoxic activity. The analysis of LPS−DAPEG−oHRP showed a conjugation of 29 nmol HRP/mg LPS, similar to the one reported before,[17] but with a markedly improvement in its enzymatic activity corresponding to 120 U mg^{-1} HRP. This value is close to the one reported for HRP conjugates commercially available (e.g., neutravidin−HRP sold by Pierce). Regarding its endotoxic activity, the LPS conjugate retained an activity of 0.3 EU ng^{-1}, which was 50% of the original value.

Competitive Assay. In a previous work[15] the use of different hydrophilic polymers has been proposed to decrease the nonspecific adsorption of a hydrophobic model protein, neutravidin. Good results were obtained modifying the electrode surface with carboxymethyl dextran. In particular, CMDex70 combines a good resistance to the nonspecific adsorption with the ability to be further modified by well-known derivatization reactions. On the other hand, the redox mediators preserve their electrochemical reversible behavior on the modified surface. Figure 3 shows the modification of the electrode surface carried out in this work.

The behavior of this platform for the nonspecific adsorption of LPS−DAPEG−oHRP is shown in the inset of Figure 4. The degree of adsorption is determined by measuring the catalytic current generated by the addition of hydrogen peroxide and a redox mediator. It can be observed that the adsorption of the conjugate on bare gold is mainly due the LPS moiety present in the conjugate, since HRP is practically not adsorbed. This behavior drastically changes when the surfaces is modified with CMDex70. An important reduction in the nonspecific conjugate adsorption is observed, while some adsorption can be observed in native HRP, suggesting an electrostatic interaction between the negatively charged CMDex and the positively charged enzyme. It should be emphasize that in this experiment the concentration of the enzyme is at least twice than the one used for the conjugate, and

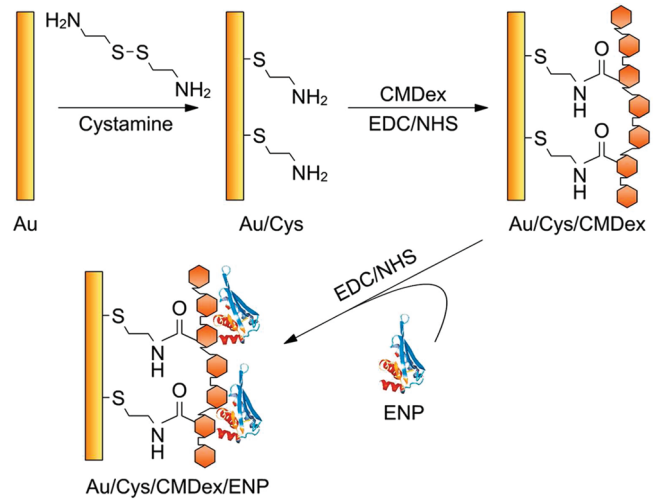

Figure 3. Construction of modified electrodes. CMDex is immobilized by means of an amide bound to the surface of the gold electrode modified with cystamine, and then ENP is incorporated by means of the same reaction.

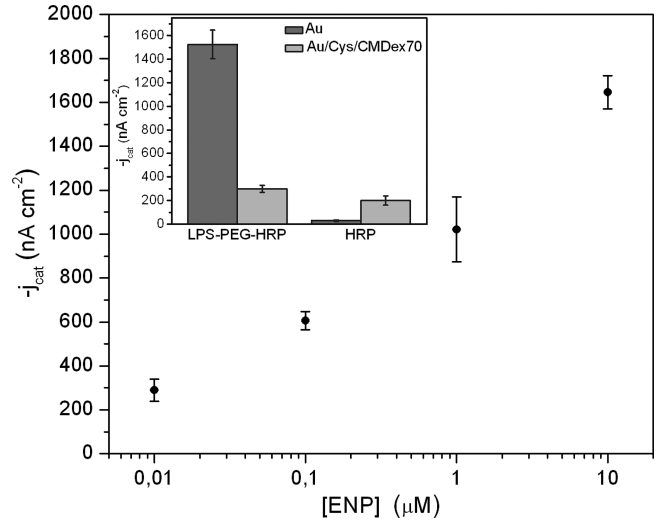

Figure 4. Catalytic current due to specific adsorption of LPS−DAPEG−oHRP 5 $\mu g\ mL^{-1}$ on Au/Cys/CMDex70/ENP electrodes constructed at different concentrations of recognition agent. Inset: catalytic current due to nonspecific adsorption of LPS−DAPEG−oHRP (5 $\mu g\ mL^{-1}$) and HRP (10 $\mu g\ mL^{-1}$) on different electrodes.

the native HRP has an enzymatic activity 3 times higher than the conjugate; therefore, in moles, the nonspecific adsorption of HRP is less than the one experienced by the conjugate.

The specific response of the system was evaluated incorporating the recognition agent (ENP). Figure 4 shows the signal obtained when the surface was modified with different ENP concentrations. The catalytic current generated by the adsorption of the conjugate on ENP-modified surfaces is plotted versus the ENP concentration used in the surface modification. For the lowest ENP concentration, the response is low and similar to the one obtained with a CMDex70-modified surface without the recognition element (300 nA cm^{-2}). The specific response increase proportional to the ENP concentration used to modify the surface. Considering that in a competitive assay a limited number of recognition sites is needed for achieving a difference

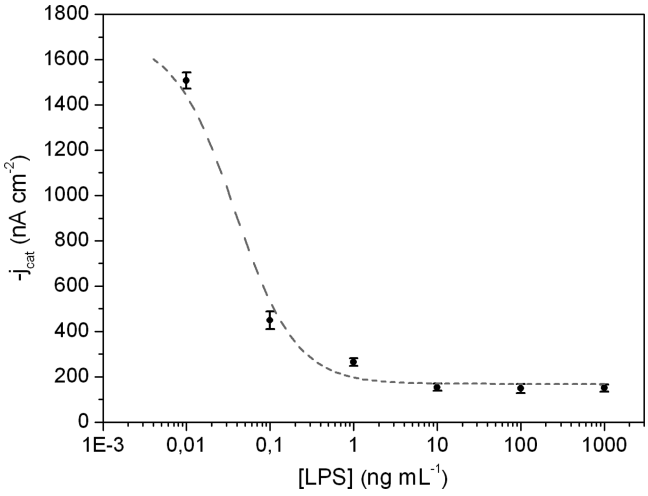

Figure 5. Competitive assay carried out between a fixed concentration of LPS−DAPEG−oHRP (5 μg mL^{-1}) and different concentrations of LPS (1.2 EU ng^{-1}).

at low concentrations, the surface modification was carried out with a 1 μM ENP solution in the competitive assay.

Once the electrodes were constructed, the samples were incubated onto them and treated with LPS−DAPEG−oHRP in a final concentration of 5 μg mL^{-1}. This concentration was chosen to achieve a good signal remaining below the cmc.[31] After the sample was rinsed, the assay was developed with a buffer solution containing 0.9 mM hydrogen peroxidase and 20 μM of the osmium complex as redox mediator.

Figure 5 shows the results obtained for samples of LPS from *S. minnesota* with an endotoxic activity of 1.2 EU ng^{-1}. At low LPS concentrations, the observed signal corresponded to saturation with LPS−DAPEG−oHRP. As the LPS concentration was increased, it competed with the labeled LPS, decreasing the signal. Finally, for high analyte concentrations, the labeled LPS was completely displaced and the signal was due to nonspecific adsorption (over 10 ng mL^{-1}). It can be observed that the method is very sensitive to low concentrations of LPS, producing an important signal change between samples containing 0.01 and 0.1 ng mL^{-1} (0.012 and 0.12 EU mL^{-1}, respectively, according to determinations carried out with the LAL test). In comparison to the previous assay,[9] three important improvements can be remarked: the high sensitivity at low endotoxic activity, the reproducibility of the results (a lower standard deviation observed for each concentration), and the lower nonspecific adsorption of the conjugate (see the signal at high LPS concentrations). Due to the low nonspecific adsorption an expanded signal range is attained. Previously, the maximum and minimum currents observed were 1400 and 900 nA cm^{-2}; in this case, they are 1500 and 150 nA cm^{-2}, respectively. In contrast, the dynamic response does not present the expected sigmoidal shape for a competitive assay; the sharp decrease between 0.01 and 0.1 ng mL^{-1}, followed by a smooth change, can be attributed to the orientation and effective number of the recognition element, a variable still not fully investigated.[32]

CONCLUSIONS

A mild and efficient method for the conjugation of LPS was developed, preserving its endotoxic activity. The conjugation is carried out by CDAP activation of LPS suspended in an aqueous solution with NaDC. The developed method provides the conjugation with fluorophores bearing primary amino, hydrazine, or hydrazide groups allowing synthesizing conjugates able to work at practically any wavelength. These conjugates can be useful not only for the research of LPS pathway mechanisms in biological studies[33] but also in surface studies regarding its rejection[34] or its removal from solutions.[35]

The mildness of this procedure allows the conjugation with labile molecules like enzymes, preserving their activity. In this study HRP was used as a model system. From these results, an improved amperometric competitive assay for the detection of endotoxins was developed, comprising a gold multielectrode platform, CMDex70 as the matrix for the immobilization of the recognition agent ENP, and an LPS−DAPEG−oHRP conjugate. The results evidence the feasibility of differentiating samples containing 0.12 EU mL^{-1} from pyrogen-free solutions; therefore, the assay can be implemented as an alarm in the purification of water in the production of injectable drugs where the limit established by the United States Pharmacopoeia is 0.25 EU mL^{-1}.

The use of a multielectrode platform allows the analysis of eight different samples simultaneously providing a low standard deviation and improving the sample/time analysis ratio. The tested LPS−DAPEG−oHRP conjugate shows an improved performance compared to a similar assays previously presented.[9,12] All these improvements lead to a lower detection limit compared to other recently proposed techniques.[10,11,13,14]

This new conjugation method will allow the introduction of more suitable probes in LPS conjugates for the study of the biological effects of endotoxins in living organism. On the other hand, the presented experimental setup for a competitive assay, embracing an improved conjugate and better reproducibility, will contribute to further study important issues in the development of an LPS sensor as, for instance, the orientation of the recognition element, the response to different LPS strains, and the effect of possible interferences present in more complex samples of clinical relevance.

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(31) Aurell, C. A.; Wistrom, A. O. *Biochem. Biophys. Res. Commun.* **1998**, *253*, 119–123.

(32) Davies, C. In *The Immunoassay Handbook*; Wild, D., Ed.; Nature Publishing Group: New York, 2001; pp 19−26.

(33) Vasselon, T.; Hailman, E.; Thieringer, R.; Detmers, P. A. *J. Exp. Med.* **1999**, *190*, 509–521.

(34) Gudipati, C. S.; Finlay, J. A.; Callow, J. A.; Callow, M. E.; Wooley, K. L. *Langmuir* **2005**, *21*, 3044–3053.

(35) Johnson, B. J.; Delehanty, J. B.; Lin, B.; Ligler, F. S. *Anal. Chem.* **2008**, *80*, 2113–2117.