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An efficient method for conjugation of a lipopolysaccharide from *Salmonella enterica* sv. Minnesota with probes bearing hydrazine or amino functional groups

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ABSTRACT

A conjugation method for coupling probes bearing hydrazine or primary amino groups to a lipopolysaccharide (LPS) is described. LPS is modified through the hydroxyl groups present in its O-antigen moiety by activation with cyanogen bromide in aqueous acetone using triethylamine to enhance the electrophilicity of CNBr. The method yields conjugates with good labeling ratios, preserving the endotoxic activity of the lipid A moiety, which in blood exerts pleiotropic effects on many tissues and organs, resulting in multiple-organ damage, circulatory collapse, and death. Conjugation of smooth-form LPS from *Salmonella enterica* sv. Minnesota to dansyl hydrazine yielded a labeling ratio of 330 nmol dansyl/mg LPS, with nearly no loss of the original endotoxic activity. In the case of horseradish peroxidase, in which a spacer was introduced, the ratio was 28 nmol HRP/mg of LPS, preserving 65% of the original endotoxic activity. This work shows that under these conditions of CNBr activation, the labeling process has practically no effect on the endotoxic behavior of LPS. The method can be used effectively for the conjugation of LPS to probes bearing primary amino, hydrazine, or hydrazide functional groups.

Lipopolysaccharides (LPSs),[1] also called endotoxins, are the major components of the outer membrane of Gram-negative bacteria, and are able to activate a variety of biochemical pathways [1]. LPSs are complex, negatively charged lipoglycans. Usually, they comprise three distinct regions: a fatty-acylated region called lipid A, a short oligosaccharide, which is the core region, and an O-antigen portion with a composition that varies highly among Gram-negative bacteria. Lipid A is responsible for many of the pathophysiological effects associated with Gram-negative bacterial infections; therefore, it is the active moiety of LPS [2]. All Gram-negative bacteria, with the exception of the few that make sphingolipids in place of LPS, synthesize lipid A through minor variations in the constitutive *Escherichia coli* pathway [3]. Lipid A consists of a hydrophilic, negatively charged bisphosphorylated disaccharide with glucosamine backbone, covalently linked to a hydrophobic domain of acyl chains via amide and ester bonds, with variations that can occur in both the hydrophilic disaccharide region and the hydrophobic acyl chain region [4], and always exhibiting an amphiphilic character and the ability to form aggregates [5]. Although LPS itself is chemically inert, its presence in the bloodstream (endotoxemia), even in small amounts, causes a violent reaction in the immune system, affecting the structure and function of organs and cells, changing metabolic functions, raising body temperature, modifying hemodynamics, and causing septic shock [6].

Because of the chemical properties of LPS, studies of its biological pathways [7–9], its interactions with recognition molecules [10,11], and its interactions with modified surfaces [12] are carried out mainly using labeled LPS. Labeling can be achieved with radiochemicals by metabolic incorporation [13–16], a laborious method with the typical concerns involved in the manipulation of radioactive material, or by chemical conjugation. Fluorescein isothiocyanate (FITC) [7,9], horseradish peroxidase (HRP) [11,17], and biotin [8,18], among other compounds, have been extensively used to label LPS; yet its conjugation poses some difficulties with respect to yield because of its chemical inertness.

Looking at the structure of the molecule, the only group susceptible to being modified without severely losing biological activity is the hydroxyl group, present in significant amounts in the O-antigen moiety. FITC has been used for such a purpose, but with limited yield [9]. Troelstra et al. envisaged a way to improve yield using Re595 LPS [7], by converting LPS molecules into the monomeric state, and maintaining them in this state during the labeling steps. Yet, the method is limited only to probes bearing an isothiocyanate moiety.

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[1] *Abbreviations used:* LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; GO, galactose oxidase; Fmoc, N^{α}-(9-fluorenylmethoxycarbonyl)-; PEG, polyethylene glycol; BSA, bovine serum albumin; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; NHS, *N*-hydroxysuccinimide; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; TEA, triethylamine; SDS, sodium dodecyl sulfate; MWCO, molecular weight cutoff; CMC, critical micelle concentration; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy.

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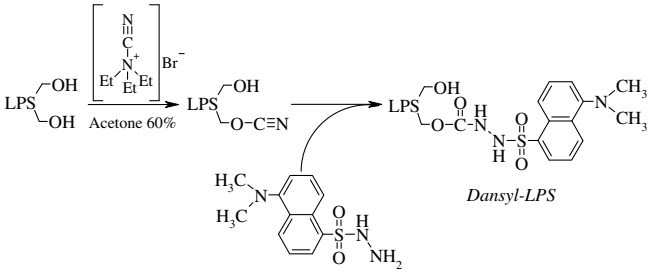
Primary amine, hydrazine, and hydrazide functional groups are present in a myriad of fluorescent probes, spacers, and proteins; therefore, the development of methods involving conjugation of LPS to molecules bearing these functional groups provides multiple labeling options, allowing fine-tuning of the properties of the selected probes. The simplest way to achieve this is through oxidation of the O-antigen moiety of LPS with sodium periodate, followed by the coupling containing an hydrazide probe, for example, biotin-LC-hydrazide. Using this method, Luk et al. [8] obtained conjugates with good endotoxic activity; however, the degree of labeling was low. Our group has synthesized biotin-labeled LPS by oxidation of the carbohydrate moiety with a good labeling ratio, yet with modest remnant endotoxic activity [18]. Further, other authors labeled Re595 LPS, previously oxidized using an enzymatic method, with Alexa 488 hydrazide [19]. The enzymatic method is based on the fact that galactose oxidase can form C-6 aldehydes on terminal D-galactose or *N*-acetyl-D-galactose residues. When galactose residues are penultimate to sialic acid, another enzyme, neuraminidase, must be used to remove the sialic acid sugars, exposing galactose as the terminal residue. The combined action of both enzymes generates C-6-aldehyde derivatives from the galactose, which can be conjugated later to amino or hydrazide groups. The authors note a very good yield for Re595 LPS [19], but it does not react with smooth-strain LPS [18], thus limiting its use to only one type of LPS.

As one of the characteristics of LPS is the formation of aggregates, we shifted our attention to reactions carried out on insoluble carbohydrates, such as the activation of agarose for the construction of affinity chromatography materials. One of the most efficient methods for this purpose is CNBr activation, producing a reactive cyanate ester (Scheme 1). It is important to control the conditions under which these reactions are carried out, as cyanate esters can be hydrolyzed to inert carbamates. For example, the use of NaOH to enhance the nucleophilicity of hydroxyl groups may lead to the formation of inert carbamates. Temperature control is another important factor in the activation process, as the activated molecule is very sensitive to this parameter. To overcome both these problems, a convenient activation method is the use of triethylamine as a "cyano-transfer" agent in an aqueous acetone solvent at temperatures bellow 0 °C, avoiding the need for a strong basic medium [20,21].

In the work described here, we used a conjugation method to couple smooth-form LPS to probes bearing hydrazine, or primary amino groups, through CNBr activation of the hydroxyl groups present in the O-antigen moiety of LPS. Similar to the modification of affinity chromatography materials, labeling of LPS with CNBr is a two-step process (Scheme 1). First, hydroxyl groups are activated by the introduction of cyanate esters; second, the selected label is covalently attached to the activated molecule.

As a model system, we used the conjugation of smooth-form LPS from *Salmonella enterica* sv. Minnesota to dansyl hydrazine and horseradish peroxidase (HRP) through primary amino groups. With this technique a labeling ratio of 330 nmol dansyl/mg LPS may be achieved, preserving practically all the original endotoxic activity. In the case of peroxidase, with previous addition of a spacer molecule, 28 nmol HRP/mg LPS is obtained, preserving 65% of the original endotoxic activity.



Scheme 1. Steps involved in the conjugation of dansyl hydrazine to LPS.

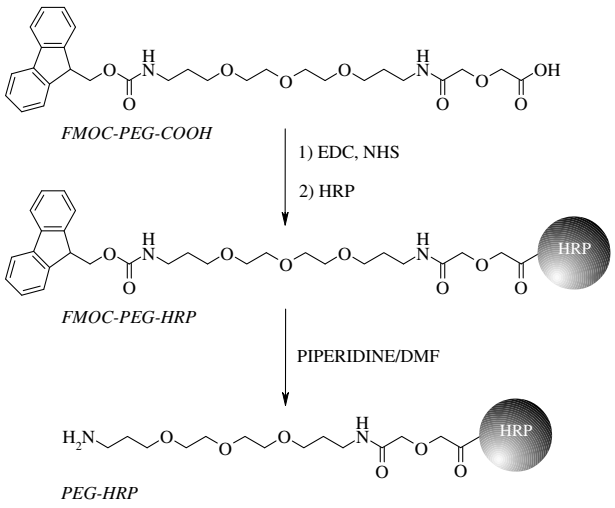
Experimental

Materials

Smooth-form LPS from *Salmonella enterica* sv. Minnesota was supplied by Sigma. LPS is a pyrogen. It may cause fever. It may be harmful if inhaled, ingested, or absorbed through the skin. Good laboratory technique should be employed: wear a lab coat, gloves, and safety glasses. Work in a well-ventilated area. Avoid contact with open wounds. The chromogenic LAL Test was provided by Biowhitaker. Deionized water was obtained from a Millipore ultrapure water filtration unit. Apyrogen water was produced by bidistillation of water previously passed through a MilliQ water system, and collected in apyrogen glass material (5 h at 180 °C and 2 h at 220 °C); values less than 0.01 EU mL^{-1} was routinely obtained (determined by means of the LAL Test). Dansyl hydrazine and sinapinic acid were provided by Fluka; HRP was provided by Biozyme. Bovine serum albumin (BSA), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), triethylamine (TEA), ethanolamine, pyrogallol, glycine, β-mercaptoethanol, sodium dodecyl sulfate (SDS), and cyanogen bromide were provided by Sigma. CNBr may be fatal if swallowed, inhaled, or absorbed through the skin. Vapors cause severe irritation to eyes and respiratory tract. CNBr causes burns at any area of contact. Contact with acids liberates poisonous gas. CNBr affects the blood, cardiovascular system, central nervous system, and thyroid. Impure material may explode. Wear a lab coat and apron, gloves, and safety glasses. Work in a well-ventilated hood. Acrylamide and bisacrylamide were obtained from J.T. Baker. Protein assay dye reagent concentrate was provided by Bio-Rad. FMOC-PEG-COOH (Scheme 2) was provided by Novabiochem. RC Spectra/Por dialysis membranes (3500 and 12,000 molecular weight cutoffs) were obtained from Spectrum Laboratories.

LPS purification

Smooth-form LPS from *S. minnesota* was chromatographically purified at 23–25 °C with a column of Sephacryl HiPrep 16/60



Scheme 2. HRP modification with the PEG spacer.

(S-200 HR, GE Healthcare) in an ÄKTA Explorer FPLC system. As elution buffer, 10 mM Tris buffer, pH 8.0, containing 0.2 M NaCl, 1 mM EDTA, 0.02 % sodium azide, and 0.25 % sodium deoxycholate was used. Fifty to sixty milligrams of LPS was dissolved in 3 mL of buffer. The sample was injected and eluted isocratically at 0.5 mL min^{-1} and collected in 1-mL fractions. The purified LPS elutes between fractions 35 and 68. Then, fractions containing LPS were passed through a series of two Hitrap desalting columns (GE Healthcare, 5 mL each) with 15 mM phosphate buffer, pH 7.5, 15 mM NaCl at 2 mL min^{-1} elution rate, for the purpose of removing excess detergent. The product was then extensively dialyzed against MilliQ water using a 3500 MWCO membrane at 4 °C. The dialyzed fractions were lyophilized and characterized by their protein content (determined with the Bio-Rad Protein Assay), adenosine ribonucleic acid (an A_{260nm} value of 0.1 corresponds to 4 μg mL^{-1} RNA [22]), Kdo [23], and endotoxic activity (determined by means of the LAL Test). The fractions were stored at −20 °C until use. For synthesis of the conjugates, the fraction that eluted between 41 and 47 mL was chosen (fraction 41–47). This fraction has an endotoxic activity of 1.7 EU ng^{-1}, 0.3% RNA, and 0.3% proteins; before purification, the LPS has 46% RNA and 0.8% proteins.

LPS-dansyl conjugate

Fraction 41–47 of purified LPS was used to perform the conjugation. Three milligrams of LPS from *S. minnesota* was dissolved in 4 mL of 60% aqueous acetone; the solution was cooled to −15 °C in an ethanol–water bath. To the LPS solution 50 μL of 1 M CNBr and 400 μL of 0.15 M triethylamine were added dropwise over 3 min; all of the reagents were dissolved in aqueous acetone (Scheme 1). Then, 200 μL of 4 mg mL^{-1} dansyl hydrazine in DMSO was added. The solution was left at 4 °C for 2 h under stirring in the dark. One milliliter of 1 M ethanolamine was added, and the solution was left to react for at least 30 min. The unreacted dansyl was removed using a series of two Hitrap desalting columns (GE Healthcare, 5 mL each) with 15 mM phosphate buffer, pH 7.5, 15 mM NaCl, at an elution rate of 2 mL min^{-1}. The conjugate was dialyzed against MilliQ water using a 3500 MWCO membrane at 4 °C in the dark. Then, the sample was lyophilized and characterized by spectrophotometry (Shimadzu UV-160A), fluorescence, Kdo, and with the LAL test for endotoxicity.

Fluorescence spectroscopy

After purification, LPS-dansyl conjugate was dissolved in MilliQ water and subjected to fluorescence spectroscopy performed in polystyrene cuvettes using a PTI Quantamaster spectrofluorometer. The excitation wavelength was set at 325 nm; emission was recorded from 350 to 750 nm, and the emission area was determined. A calibration curve was constructed using standards of dansyl hydrazine from 0.6 to 12.0 μM in MilliQ water. The LPS:dansyl ratio was calculated from samples of LPS-dansyl 5 μg mL^{-1}, below the critical micelle concentration (CMC) of lipopolysaccharide [24].

PEG-HRP modification

All reagents were dissolved in dry acetonitrile. To 6 mg of Fmoc-PEG-COOH (Scheme 2) dissolved in 5 mL, 1.2 mL of NHS (2 mg mL^{-1}) and 2 mL EDC (5 mg mL^{-1}) were added under argon atmosphere. The mixture was left to react under stirring for 1 h. Then, 5 mL of HRP (10 mg mL^{-1}) and 0.25 mL of 0.3 M TEA were added and left stirring for 90 min. Afterward, acetonitrile was removed by vacuum distillation at 25 °C. The dry product was dissolved in MilliQ water and purified using a series of two Hitrap desalting columns (GE Healthcare, 5 mL each) using 15 mM phosphate buffer, pH 7.5, 15 mM NaCl at an elution rate of 2 mL min^{-1}. The collected fraction was dialyzed against MilliQ water using a 12,000 MWCO membrane at 4 °C in the dark and lyophilized.

The modification of HRP can be checked by the appearance of a sharp band at ca. 300 nm corresponding to the Fmoc group. The modification was also confirmed by a shift in the molecular mass on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectroscopy.

To remove the Fmoc protecting group, the solid was dissolved in a 20% piperidine solution in dimethyl formamide and left to react for 30 min at 25 °C. Removal of Fmoc was followed by thin-layer chromatography and MALDI spectroscopy.

MALDI-TOF-MS analysis

Positive MALDI-TOF mass spectra were recorded using an Omniflex Bruker Daltonics mass spectrometer operated in the linear mode at an accelerating voltage of 19 keV. The photomatrix solution was prepared by dissolving sinapinic acid to 1% in an acetonitrile-0.1 % TFA (70:30 v/v) solution. One millimolar solutions of HRP, PEG-HRP, Fmoc-PEG-HRP, and BSA were prepared in MilliQ water. The samples were made by mixing the protein solutions with the photomatrix solution in a 1:10 ratio. One microliter of the sample solution was applied to the target and dried down. Desorption/ionization was accomplished with a nitrogen UV laser (337 nm). The instrument was calibrated against BSA as an external standard. [$(M+H)^+$ = 66,431 and $(M,+,2H)^{2+}$ = 33,216] [25].

LPS-PEG-HRP conjugation

Three milligrams of fraction 41–47 of LPS from *S. Minnesota* was dissolved in 4 mL of 60% aqueous acetone; the solution was cooled to −15 °C in an ethanol–water bath. To the LPS solution 50 μL of 1 M CNBr and 400 μL of 0.15 M TEA were added dropwise over 3 min; all the reagents dissolved in aqueous acetone. The mixture was left to stir for 5 min, then 1.5 mL of PEG-HRP (10 mg mL^{-1}) from the deprotection step was added. The solution was left at 4 °C under stirring for 2 h. The reaction was quenched by the addition of 1 mL of 1 M ethanolamine and left for 30 min. Then, the product was injected onto a series of two Hitrap desalting columns (GE Healthcare, 5 mL each) using 15 mM phosphate buffer, pH 7.5, 15 mM NaCl, at an elution rate of 2 mL min^{-1}. Afterward, the separation of unreacted peroxidase was carried out by ionic chromatography.

LPS-PEG-HRP purification

Chromatographic separation of unreacted peroxidase was achieved, on an analytical scale, on an ANX FF (high sub) (1 mL, GE Healthcare). One hundred microliters of unpurified LPS-PEG-HRP (ca. 300 μg) was loaded onto the column. Binding buffer and elution buffer were 50 mM Tris buffer, pH 6.5, and 50 mM Tris buffer, pH 6.5, 1 M NaCl, respectively. The chromatographic separation was performed at a flow rate of 1.0 mL min^{-1}. Binding buffer was applied to the column over 6 CV. Elution was achieved with a gradient to elution buffer over 6 CV, from 0% to 100 %, and maintained for 8 CV. The UV–Vis detector was set at 403 nm. A larger-scale purification was achieved on an ANX FF (high sub) (20 mL, GE Healthcare). The injection volume was scaled up to 3 mL. The flow rate was increased to 4 mL min^{-1}. Binding buffer was applied over 6 CV and elution buffer from 0% to 100 % over 3 CV. All experiments were performed at 23–25 °C. The conjugate was characterized by electrophoresis, Kdo, enzymatic activity [26], and (with the LAL test) endotoxicity.

Electrophoresis

Polyacrylamide gels 16 cm (length) × 14 cm (wide) × 0.2 cm (depth) were run using 25 mM Tris buffer, pH 8.8, 0.19 M glycine, o.1% SDS. Resolving gel was formed with 12.5% acrylamide, 0.375 M Tris buffer, pH 8.8, 0.1% SDS, and stacking gel with 5% acrylamide, 60 mM Tris buffer, pH 6.8, 0.1% SDS. Samples were dissolved with loading buffer containing 2% SDS. Samples detected by silver staining were dissolved in a buffer containing 5% β-mercaptoethanol and boiled for 5 min. Samples detected following peroxidase activity assay were just dissolved in sample buffer without β-mercaptoethanol and were not boiled. Electrophoresis was carried out at 30 mA at 4 °C for 6 h, until the tracking dye reached the bottom of the gel. The gel was divided into two halves and developed. Silver staining was carried out following the method described by Tsai and Frasch [27]. Detection of peroxidase activity was carried by rinsing the slab with 1.5% Triton X-100 for 1 h, then twice with water for 15 min each. Finally, the slab was immersed in a solution containing 0.6 g L^{-1} pyrogallol and 0.8 g L^{-1} hydrogen peroxide in 100 mM phosphate buffer, pH 6.0.

Conjugation ratio

Dansyl-LPS conjugate

Dansyl was determined by fluorescence, whereas the amount of LPS was established by Kdo analysis of the conjugate [23], with a known concentration of the LPS fraction used in the synthesis to build a calibration curve. For LPS-HRP, the procedure was similar for LPS, and HRP was determined from the absorbance at 403 nm.

Results

Dansyl-LPS conjugate

A purified fraction of LPS was conjugated with dansyl hydrazine, after previous activation with CNBr. LPS was dissolved in a 60% acetone aqueous solution at −15 °C and activated with CNBr; to this, dansyl hydrazine was added from a DMSO solution (Scheme 1). After completion of the reaction, the solution was eluted through a desalting column to separate the excess fluorophore from the conjugate. Finally the conjugate was dialyzed and freeze-dried, appearing pale green because of the presence of the dansyl moiety.

The new conjugate was characterized by spectrophotometry and fluorometry. The absorption spectra of both the conjugate and free dansyl are similar. With respect to the fluorescence spectra, the maximum emission wavelength of the free dansyl remained constant as concentration increased (Fig. 1A), whereas for the conjugate, the maximum emission wavelength shifted from 495 to 502 nm (Fig. 1B). Also, in this last case, the fluorescence intensity leveled off at higher concentrations (Fig. 1A, inset), probably as a result of LPS aggregation.

At concentrations below 10 μg mL^{-1}, both dansyl conjugate and free dansyl exhibited similar behavior, presenting an emission intensity that scales linearly with concentration, with no shift in the maximum wavelength emission, consistent with the CMC for LPS from *S. minnesota*, namely, 11 μg mL^{-1}. By use of the emission figures for a 5 μg mL^{-1} solution of conjugate, the mass ratio of dansyl molecules to LPS was calculated, 330 nmol dansyl/mg LPS. This result represents great improvement with respect to commercially available conjugates obtained using isothiocyanate dyes. For example, the product supplied by Sigma has a maximum conjugation of 26 nmol of FITC/mg of LPS, and for the Re595 LPS conjugated to FITC, as reported by Tobias et al., the ratio is 130 nmol/mg LPS [9], similar to the values for the product described by Troelstra [7] also using FITC and Re595 LPS. The ratio reported here is also considerably high when compared with values for conjugates in which hydrazine groups were introduced by previous oxidation of diol groups to aldehydes with periodate. The same strain modified by the latter method has a degree of conjugation of 50 nmol/mg of LPS [18].

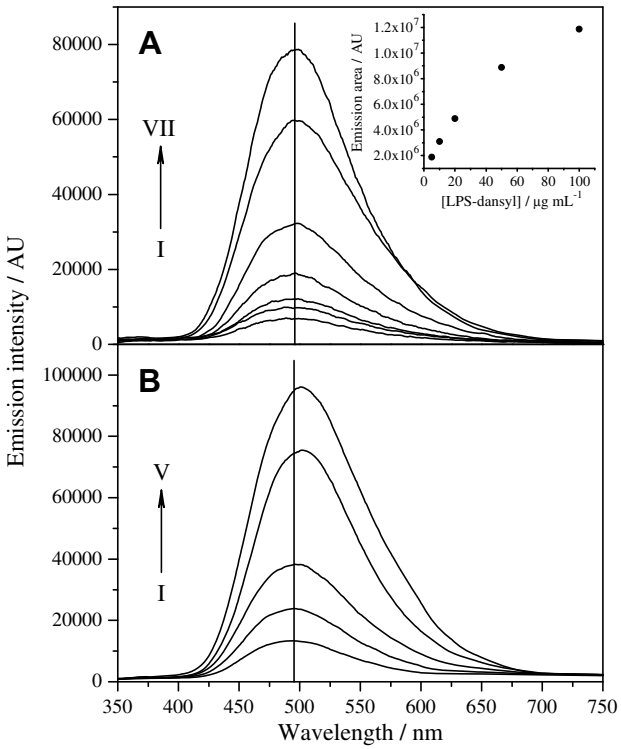


Fig. 1. Fluorescence emission spectra. (A) Free dansyl hydrazine in MilliQ water. I to VII represent 0.6, 1.0, 1.3, 2.2, 4.5, 9.0, and 12.0 μM dansyl hydrazine. (B) LPS-dansyl conjugate in MilliQ water. I to V represent 5, 10, 20, 50, and 100 μg mL^{-1} dansyl conjugate. The line perpendicular to the *x*-axis is fixed at 495 nm as reference. Inset: Fluorescence emission as a function of the concentration of conjugate in MilliQ water.

The endotoxic activity of this new conjugate is 1.5 EU ng^{-1}, which compares remarkably well with the 1.7 EU ng^{-1} of the LPS fraction used as starting material; considering that conjugates synthesized by periodate oxidation have endotoxic activities 20 times lower [18], this constitutes an important improvement.

PEG-HRP conjugate

HRP is an enzyme with four lysine residues, potentially allowing the reaction with the activated LPS through their primary amino groups. However, in our trials the direct labeling of LPS with HRP did not produce a conjugate with a good labeling ratio. This can be understood by considering that both LPS and HRP are bulky molecules with a high degree of steric interference. With this assumption in mind, HRP was previously modified with a spacer (Fmoc-PEG-NH_2, Scheme 2) to facilitate the labeling reaction. The spacer acts as a bifunctional PEG derivative which can bind to HRP with good yield. The MALDI spectra for HRP and the derivative (Fig. 2A and B) reveal a mass shift of 2560 U (Fig. 2A, inset), corresponding to the addition of ca. 4 mol of Fmoc-PEG-NH_2 per HRP. Also, the peak for the modified HRP is wider than the corresponding one for the native enzyme, probably because of the different PEG:HRP ratios obtained. Further, the removal of Fmoc was also followed by MALDI spectroscopy (Fig. 2C), showing a shift to lower mass values as a result of the loss of Fmoc.

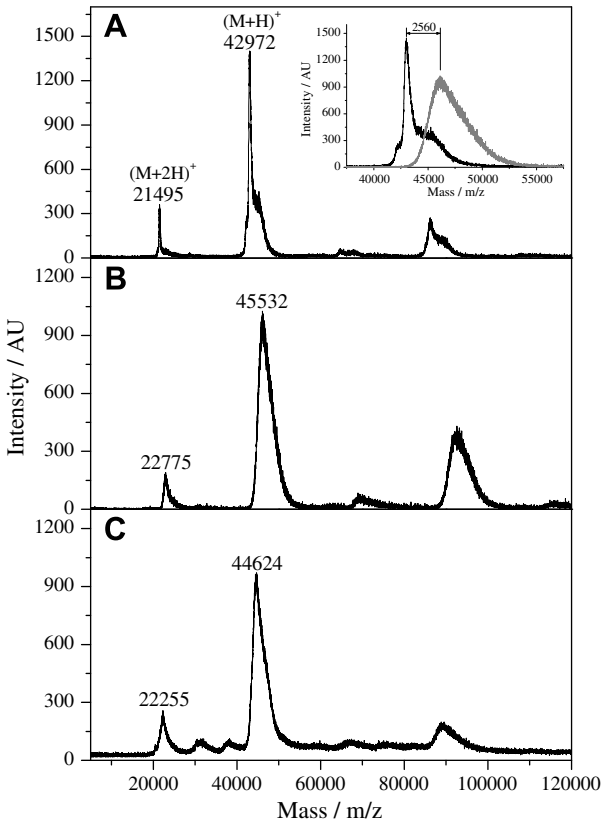


Fig. 2. Positive ion MALDI mass spectra of (A) HRP, (B) Fmoc-PEG-HRP, and (C) H_2N-PEG-HRP. Inset: Mass shift between $[M+H]^+$ ions from HRP (black) and Fmoc-PEG-HRP (gray). Sinapinic acid was used as matrix.

Further, a purified fraction of LPS from *S. minnesota*, was activated with CNBr as described above. This activated LPS easily reacted with the exposed amino groups of the modified HRP added in excess. The product of the reaction, previously desalted and dialyzed, was purified by anion-exchange chromatography. Fig. 3 is the elution profile, where the first peak corresponds to the unreacted PEG-HRP. Because of the buffer conditions, PEG-HRP is practically neutral; on the other hand, LPS-PEG-HRP bears negatively charged groups from the phosphates present in LPS. With the onset of the increase in ionic strength of the elution buffer, the LPS conjugate is removed from the column (second peak). The peak areas allow us to estimate that half of the peroxidase used reacted with LPS.

The second peak collected was dialyzed, lyophilized, and characterized by polyacrylamide gel electrophoresis (PAGE), and differed with respect to the native HRP and LPS (Fig. 4A). The conjugate shows a ladder pattern typical of those observed in LPS samples. It is noteworthy that the conjugate presents higher mobility than the LPS, even though its molecular mass is higher. This is probably due to the fact that the denaturation treatment exposes the hydrophobic sites of the protein, which become surrounded by the SDS, allowing its faster migration, whereas for LPS, this treatment is effective only in the lipid A moiety of the molecule. Also, it can be observed that in the conjugate, there are no bands in the position corresponding to the unmodified LPS, indicating that the conjugate is not contaminated with free LPS. When the gel was run under nondenaturing conditions, and then developed by activity, we observed that native HRP could hardly move from the starting point, whereas the conjugate, with a higher molecular mass but also a higher charge, ran a longer distance (Fig. 4B). This behavior suggests that the hydrophobic parts of the protein are not exposed under nondenaturing conditions; therefore, the protein cannot be effectively charged by SDS, and it is mainly the lipid A moiety of the conjugate that provides the molecule with charge. The pattern of this gel confirms that there is no free HRP in the conjugate sample. All these results are consistent with the conclusion that the method produces a conjugate with good yield and with a stoichiometric ratio of 28 nmol HRP/mg LPS. The conjugate maintains both good endotoxic activity, namely, 1.1 EU ng^{-1}, and good enzyme activity, as indicated by the color development observed in Fig. 4B. Although the use of a bifunctional spacer presents some advantages regarding specificity of modifications during the conjugations, the number of steps, and,

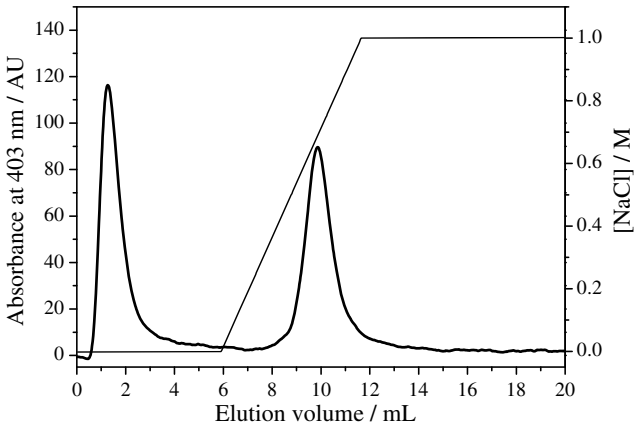


Fig. 3. Chromatogram (bold line) for the separation of LPS-PEG-HRP and unreacted H_2N-PEG-HRP. The thin line represents the change in the NaCl concentration of the eluant. Column: ANX FF (high sub) (1 mL, GE Healthcare). Buffer elution: 1.0 mL min^{-1}.

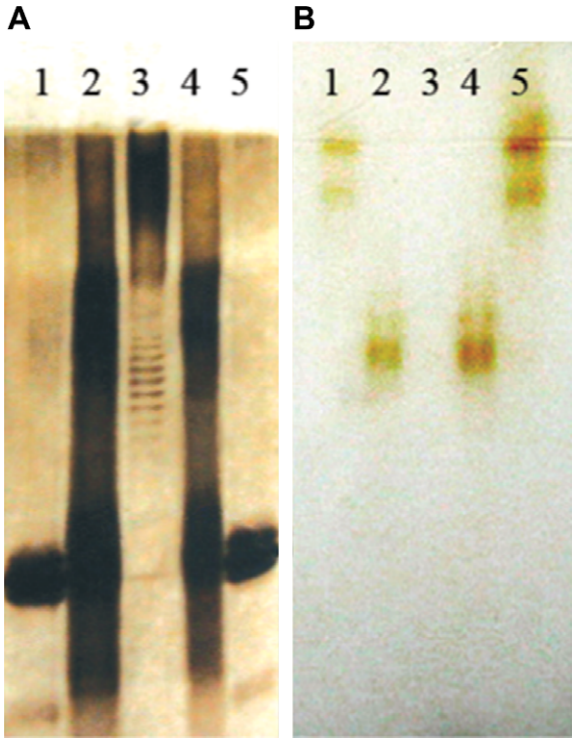


Fig. 4. Electrophoresis of LPS-PEG-HRP, LPS, and HRP under different conditions: (A) Denaturing conditions, developed by silver stain. (B) Nondenaturing conditions, developed by peroxidase activity. (1) HRP, 4 μg; (2) LPS-PEG-HRP, 12 μg; (3) LPS, 6 μg; (4) LPS-PEG-HRP, 6 μg; (5) HRP, 2 μg.

Table 1
Change in peroxidase activity through modification steps

Sample	Condition	Activity (U mg^{-1})
HRP	Biozyme	260
Fmoc-PEG-HRP	After lyophilization	90–110
PEG-HRP	After deprotection	75
PEG-HRP	After dialysis and desalting	25

particularly for this spacer, the solvents involved produce a reduction in the enzymatic activity of the HRP (Table 1). Despite this, a conventional staining procedure can be applied to the conjugate (see Fig. 4B).

Discussion and conclusions

We have shown that the activation of LPS with CNBr in an acetone aqueous solvent is a convenient activation method for further modifications of molecules bearing hydrazine or primary amino groups. The same concept could be extended to hydrazides.

The method allows the preparation of dansyl conjugates with excellent labeling ratios (330 nmol/mg LPS), practically preserving the original endotoxic activity, indicating that the modification is probably taking place in the O-antigen portion of the molecule, far from the lipid A portion. The synthetic pathway described here is very convenient, as a myriad of fluorophores containing hydrazine or primary amino groups may be introduced. The choice of an acetone–water mixture as a solvent works very well, and represents a compromise between two possible scenarios: one is the use of pure acetone, in which neither LPS nor HRP are soluble; the other is the use of an aqueous solution, in which the activated LPS rapidly decomposes, giving inert carbamates [21]. Another important issue is that in this solvent mixture, the temperature for the activation of hydroxyl groups with the *N*-cyanotriethylammonium bromide complex may be kept low, avoiding the very rapid decomposition above −10 °C [28]. When the same protocol as described under Experimental is used, except with water as a solvent, conjugation of LPS with dansyl produces conjugates with 5 nmol dansyl/mg LPS; in the case of HRP, the conjugation was nil. The acetone–water solvent mixture also allows more convenient handling of most of the fluorophores with no loss of LPS biological activity.

Labeling of LPS with a protein is also possible, provided a spacer group is added, which is a common practice in the conjugation of carbohydrate to proteins [29,30]. In this way, conjugation of a protein to LPS allows an excellent labeling ratio (28 nmol/mg LPS) to be obtained, preserving good endotoxic activity (1.1 EU ng^{-1}).

Finally, efficient labeling with amino or hydrazine functional groups allows not only the fine-tuning of a convenient wavelength for fluorescence studies, but also the use of other techniques like staining, luminescence, and amperometry, which, in turn, will improve studies of the different chemical and biological properties of LPS.

Acknowledgments

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