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Electrochemiluminescent disposable cholesterol biosensor based on avidin–biotin assembling with the electroformed luminescent conducting polymer poly(luminol-biotinylated pyrrole)

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HIGHLIGHTS

- Development of one-shot SPE biosensor for cholesterol determination.
- Development of new luminol ECL copolymers with good mechanical properties.
- Synthesis of biotinylated pyrroles by click chemistry.
- Well-arranged self-assembled layers offer good biosensor capabilities.
- Fast reagent immobilization using electrodynamics techniques.

GRAPHICAL ABSTRACT

Diagram of biosensor preparation.

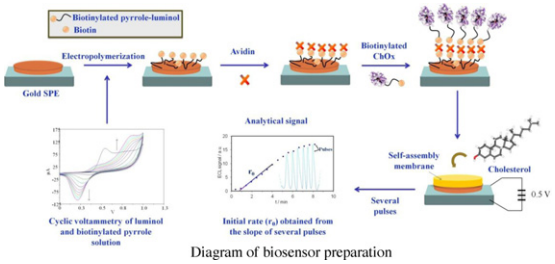

Diagram of biosensor preparation

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ABSTRACT

An electrochemiluminescent cholesterol disposable biosensor has been prepared by the formation of assembled layers on gold screen-printed cells. The detection layer is based on the electro-formation of new luminol copolymers with different synthesized biotinylated pyrroles prepared by click-chemistry, offering a new transduction layer with new electroluminescent properties on biosensors. The electrochemiluminescence (ECL) luminol copolymers are electroformed by cyclic voltammetry (five cycles) at pH 7.0 uses a10^{-3} M biotinylated pyrrole–luminol ratio of 1:10 in PBS buffer. With respect to the recognition layer, cholesterol oxidase was biotinylated by incubation with biotin vinyl sulfone, and immobilized on the copolymer by avidin–biotin interaction. The analytical signal of the biosensor is the ECL enzymatic initial rate working in chronoamperometric mode at 0.5 V excitation potential with 10 s between pulses at pH 9.5. The disposable device offers a cholesterol linear range from 1.5×10^{-5} M to 8.0×10^{-4} M with a limit of detection of 1.47×10^{-5} M and accuracy of 7.9% for 9.0×10^{-5} M and 14.1% for 2.0×10^{-4} M, ($n = 5$). Satisfactory results were obtained for cholesterol determination in serum samples compared to a reference procedure.

1. Introduction

One of the analytes for which monitoring is crucial in clinical analysis in order to control heart diseases, hypertension or arteriosclerosis is cholesterol. This compound can exist in two forms in the blood, free (30%) and esterified (70%). Different biosensors have been described using different methodologies for enzyme immobilization such as covalent binding, entrapment and physical adsorption. Among different options, several solid supports can hold the recognition molecules, such as nanomaterials, conducting polymers, sol–gel, hydrogel and self-assembled monolayers [1]. Conducting polymers offer good capabilities and are largely used as

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transducers for biological interaction [2]. Particularly, polypyrrole is the most commonly employed conducting polymer in sensing applications owing to its biocompatibility, high hydrophilic character and high stability in water [3], with different strategies having been described for the immobilization of biomolecules on polypyrrole [4]. Pyrrole can be electropolymerized in aqueous sulfuric acid conditions [5]. However, it is observed that an increase in the pH (from 1 to 7) produces a progressive passivation of the polypyrrole surface [6] except when using phosphate buffer at neutral pH along with the adequate supporting electrolyte [7].

Additionally, the electropolymerization of biotinylated pyrroles and subsequent attachment of biotinylated enzymes by the avidin–biotin molecular recognition process is a good option for immobilizing enzymes. These molecules need acetonitrile (ACN) and tetrabutylammonium perchlorate to electropolymerize [8], but Rodrígez et al. [9] have shown that those conditions are not necessary because ACN prevents the electro-formation of the polymer and a PBS solution at neutral pH is sufficient. Furthermore, the electropolymerization ability of biotinylated pyrroles is low, hindering the polymer formation. One solution described is copolymerization with free pyrrole [10] or the polypyridinyl complex of ruthenium containing pyrrole groups [11], which improves the process. According to this, it is possible to use monomers with electrochemiluminescent (ECL) properties to prepare copolymerized layers.

Luminol is the most common luminophore for ECL due to its intense emission in the presence of hydrogen peroxide and, consequently, for enzymatic reactions based on oxidase type enzymes. In this paper we use the enzymatically catalyzed oxidation of cholesterol by atmospheric oxygen to produce H_2O_2 which is electro-oxidized along with the luminophore producing an intense ECL emission and providing a procedure with low detections limits.

With respect to cholesterol determination using luminol as monomer, a biosensor was described immobilizing cholesterol oxidase on UltraBind™ membranes working in flow methodology [12]. A recent paper described a cholesterol biosensor based on gold nanoparticles-catalyzed luminol electrogenerated chemiluminescence [13]. Furthermore, luminol, as an aniline derivative, can be electropolymerized on macroelectrodes in a strong acidic medium or under near neutral conditions (pH 6.0) [14]. However, the use of this polyluminol has some drawbacks, such as bad mechanical properties and low reusability [14]. Today, only electrochemical studies on the electropolymerization of luminol in the presence of aniline have been reported [15,16] even by nanowire composite developing [17], with the exception of a paper by Li et al. [18] that uses the poly(luminol-benzidine) polymer on a graphite macroelectrode for ECL hydrogen peroxide determination.

In this work, a cholesterol biosensor based on the copolymerization of luminol with new synthesized biotinylated pyrroles that immobilize cholesterol oxidase by avidin–biotin interaction has been produced. To the best of our knowledge, this is the first time that luminol and biotinylated pyrroles have been electropolymerized together to prepare an electroluminescent copolymer for biosensor development.

2. Experimental

2.1. Chemicals and instrumentation

As monomers for electropolymerization we used luminol (97%), pyrrole (98%), both supplied by Sigma (Sigma–Aldrich Química S.A., Madrid, Spain), and biotinylated pyrroles synthesized by us using biotin (lyophilized powder, ≥99% TLC), propargylamine (98%), divinilsulfone (DVS) and N-biotinyl-3-amino propylammonium trifluoroacetate (**5**) (97% HPLC); cholesterol oxidase (ChOx) from *Streptomyces species* (Sigma, 4.0 mg, 39.0 U mg^{-1}) was biotinylated and immobilized using avidin 5 mg/mL (lyophilized powder, 10–15 U mg^{-1}), all obtained from Sigma. 0.6 M Phosphate buffered saline solution (PBS) pH 7.0 was used for the enzyme preparation and 0.6 M phosphate buffer pH 9.5 for sample preparation, containing in both cases 0.6 M NaCl as the electrolyte. 25 mL of 2×10^{-2} M cholesterol (Sigma) stock solution was prepared weighing 0.19 g cholesterol and 0.23 g NaCl in 6.25 mL of Triton®X-100 leveling off with ethanol. Reverse-osmosis type quality water (Milli-Q Plus 185 from Millipore, Molsheim, France) was used throughout.

Gold, graphite and platinum screen-printed electrodes (SPE) were supplied by Dropsens (Oviedo, Spain). A receptacle was prepared on the disposable SPE cell by covering it with successive layers of plastic white adhesive tape up to 1 mm thick with an 8 mm diameter hole (50 μL volume) in the sensing area.

The ECL emission from the screen-printed cells was measured using a H8529 photomultiplier (PMT) interfaced to a C8855 USB photo-counting unit, both from Hamamatsu (Hamamatsu Technologies K.K., Shizuoka, Japan). The potentiostat used was an Autolab PGSTAT 128N with a FI20 module for crono-coulometric measurements (Metrohm Autolab B.V., Utrecht, The Netherlands). The arrangement used for ECL emission studies has been described elsewhere [19]. Other instrumentation consists of a Crison digital pH-meter (Crison Instruments, Barcelona, Spain) with a combined glass-saturated calomel electrode. Additionally, a Variable Pressure Scanning Electron Microscopy LEO 1430-VP (VPSEM) was used for imaging with increases ranging from 15× to 300,000×. The clinical analysis of the serum samples for cholesterol was made by the Ruiz de Alda Granada Hospital using the Roche Hitachi-912 enzymatic auto-analyzer (Hitachi High-Technologies Europe, Germany).

2.2. Synthesis

2.2.1. Biotinylated pyrroles

Several compounds were synthesized as a previous step before preparing different biotinylated pyrroles (compounds **6**, **7** and **8**). The Supporting Information section contains the spectroscopic data for the different compounds. These compounds were (Fig. 1A):

*2.2.1.1. 1-[2-(ethenylsulfonyl)ethyl]-1H-pyrrole (**1**).* This compound has been reported as a by-product in the synthesis of 1,1′-(sulfonyldi-2,1-ethenediyl)bis-1H-pyrrole. To a mixture of pyrrole (0.85 g, 12.66 mmol) and divinyl sulfone (3.7 mL, 3 equiv.) in anhydrous 1,4-dioxane (50 mL) with KOH (0.08 g) were added. The mixture was magnetically stirred at room temperature for 2 h. The solvent was evaporated under reduced pressure and the resulting crude was purified by column chromatography (ether–hexane 1:4, and then 1:1) yielding **1** (2.02 g, 86.1%).

*2.2.1.2. N-[2-(2-(1H-pyrrole-1-yl)ethylsulfonyl)ethyl]prop-2-yn-1-amine (**2**).* To a solution of vinylsulfone-pyrrole **1** (1.00 g, 5.4 mmol) in THF:2-propanol (1:2, v/v, 75 mL) propargylamine (0.28 g, 5.02 mmol) was added and the reaction mixture was magnetically stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure and the product was purified by column chromatography (EtOAc–hexane 5:1) yielding **2** (1.16 g, 89.5%) as a syrup.

*2.2.1.3. Biotin-azide (**3**).* The synthesis of biotin-azide **3** has been previously reported by the reaction of biotin and 2-azidoethanamine using HATU [2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroium hexafluorophosphate methanaminium] and hidroxybenzotriazole as a coupling reagent in DMF as a solvent with a 68% yield [20]. A solution of biotin

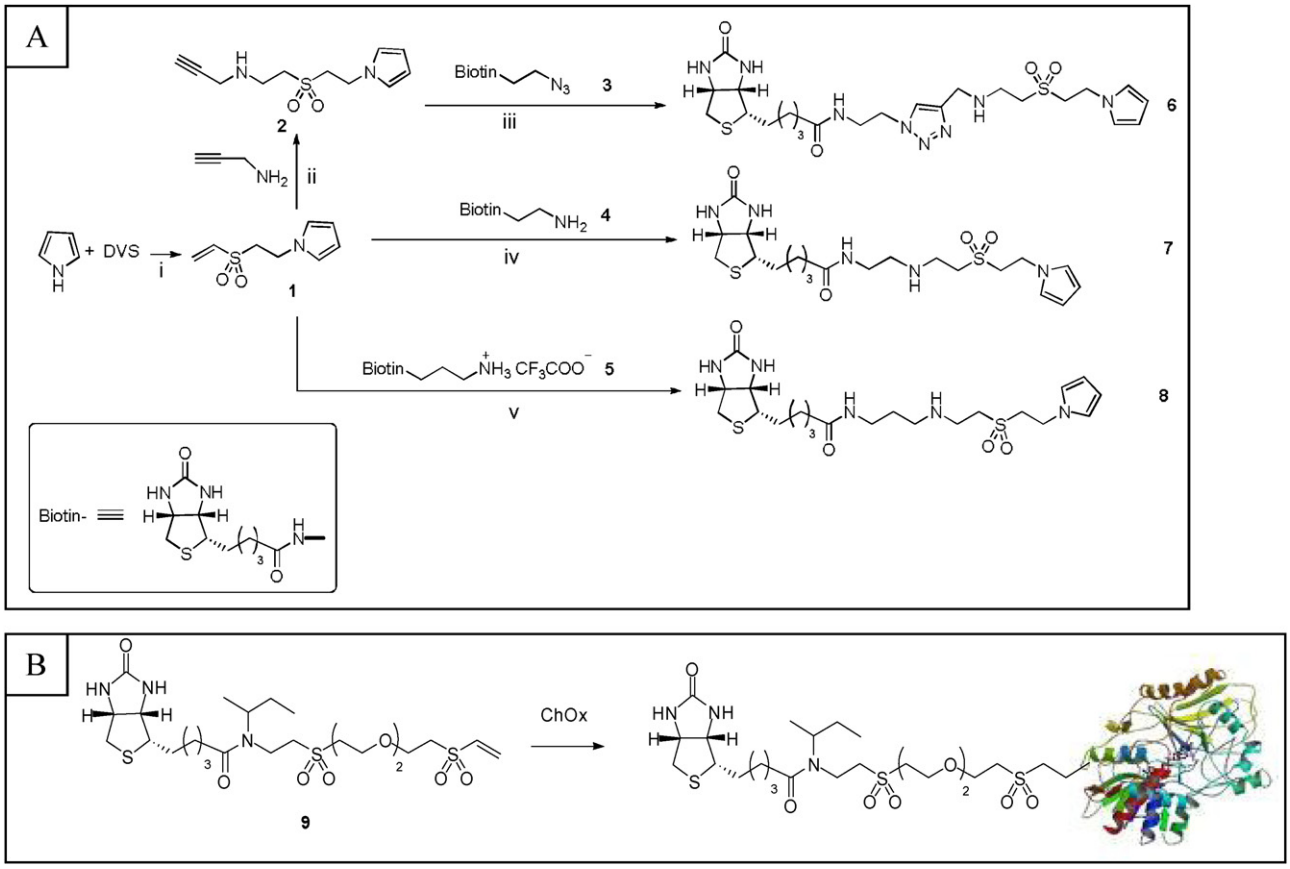

Fig. 1. (A) Synthesis of biotinylated pyrroles. Conditions: (i) KOH, DVS (divinyl sulfone), 1,4-dioxane anh., r.t. 2 h, 86%; (ii) propargylamine, *i*PrOH-THF (2:1), r.t. 24 h, 89%; (iii) 3, $(EtO)_3PCuI$, MeOH, MW irradiation 65 °C 30 min, 74%; (iv) 3, H_2, Pd/C, MeOH, r.t. 12 h after that treatment with 1, r.t. 48 h, 45%; (v) Et_3N, *i*PrOH-THF (2:1), r.t. 24 h, 88%. (B) Biotinylation of ChOx.

(0.30 g, 1.23 mmol) in Cl_2SO (5 mL) was kept at room temperature for 1 h. The reaction mixture was evaporated under vacuum and co-evaporated with anhydrous toluene (3 × 15 mL) to produce biotin acid chloride. The crude product was then dissolved in anhydrous acetonitrile (15 mL) and drop-wise added to a solution of 2-azidoethanamine (0.22 g, 2.56 mmol) and Et_3N (523 μL, 3.68 mmol) in acetonitrile (15 mL). The reaction mixture was kept at room temperature for 4 h. Evaporation of the solvent yielded a crude product that was purified by column chromatography (EtOAc–MeOH 5:1) yielding **3** (0.35 g, 92%).

*2.2.1.4. Biotin-amine (**4**).* Biotin amine **4** was obtained from biotin-azide **3** and was used without purification.

*2.2.1.5. Compound **6**.* To a solution of biotin-azide **3** (0.13 g, 0.417 mmol) in MeOH (15 mL) pyrrole–alkyne **2** (0.12 g, 0.5 mmol) and $(EtO)_3PCuI$ (15 mg) were added as catalyst. The reaction mixture was irradiated at 800 W and 65 °C in a Milestone Star Microwave Labstation for 30 min. Evaporation of the solvent and purification by column chromatography (EtOAc–MeOH, 2:1) gave **6** (0.17 g, 74%).

*2.2.1.6. Compound **7**.* To a solution of biotin-azide **3** (0.15 g, 0.48 mmol) in MeOH (15 mL) Pd/C (15 mg) was added and treated with hydrogen (2 atm) at room temperature for 12 h. After removal of the catalyst by filtering over celite, vinyl sulfone-pyrrole **1** (98 mg, 0.53 mmol) was added. The reaction mixture was kept at room temperature 48 h. Evaporation of the solvent yielded a crude that was purified by column chromatography (EtOAc–MeOH 2:1) giving **7** (0.10 g, 45%).

*2.2.1.7. Compound **8**.* To a solution of **5** (0.16 g, 0.398 mmol) in THF-2-propanol 1:2 (10 mL) vinyl sulfone–pyrrole **1** (0.10 g, 0.54 mmol) and Et_3N (65 μL) was added. The reaction mixture was kept at room temperature 24 h. Evaporation of the solvent yielded a crude that was purified by column chromatography (EtOAc–MeOH 2:1) giving **8** as a foam solid (0.16 g, 88%).

2.2.2. Enzyme biotinylation

ChOx (2.1 mg) in 387 μL phosphate buffer 0.1 M pH 7.0 was incubated with vinyl sulfone biotin (**9**) [21] (25 mM in DMSO, 6.6 μL) at 37 °C for 24 h. After that, the solution of labeled ChOx was used without further purification (Fig. 1B).

2.3. SPE cell pre-treatment

The gold SPE cells were prepared by immersion in 1.0 M of acetic acid applying three voltammetric sweeps between 0.0 and 1.0 V, washing afterwards with 0.2 M PBS buffer pH 7.0.

2.4. Biosensor preparation

The biotinylated pyrrole–luminol copolymers were prepared by electropolymerization using cyclic voltammetry from 0.0 V to 1.0 V, at 0.1 V s^{-1}, with 5 cycles, immersing the gold SPE cells in a 10^{-3} M solution containing both biotinylated pyrrole and luminol each (1:10 ratio) in pH 7.0 PBS. After washing with pH 7.0 PBS, 5 μL excess of avidin was added. After a new washing step with PBS, 5 μL biotinylated ChOx was dropped on the working electrode waiting about 30 min until dry. The adsorbed enzyme, luminol or

biotinylated pyrrole traces were eliminated by washing again with PBS.

2.5. Procedures

2.5.1. For the standards

50 μL volumes of different cholesterol standards prepared from 2×10^{-2} M cholesterol stock solution in pH 9.5 NaCl saline phosphate buffer (0.2 M) were placed on the SPE cell receptacle. Then, the cholesterol biosensor was placed in the holder and covered with the lid that held the PMT. Then, five to six 1 s pulses with 10 s difference between them at 0.5 V were applied.

2.5.2. For the serum samples

300 μL of the serum sample was added to a solution containing 730 μL pH 9.5 phosphate buffer 0.6 M, 186 μL Triton® X-100, 564 μL EtOH and 730 μL 0.6 M NaCl and water up to 3 mL. Then, 0.48 mg of $ZnSO_4$ was added in order to remove any interferences [22]. After that, 50 μL was placed on the receptacle of the SPE cells, working as previously described for standards.

3. Results and discussion

The immobilization of all the required reagents is a key in developing electrochemiluminescent disposable sensors and biosensors. We studied the immobilization of ECL reagents via luminol electrocopolymerization on the working electrode of screen-printed cells. The polymerization of the luminol in the presence of different monomers such as aniline, benzidine and pyrrole derivatives [23] makes it possible to prepare conducting polymers with better electroluminescent and electrochemical properties than polyluminol itself. The incorporation of the bio-recognition element in the biosensor can be performed in different ways, such as crosslinking with glutaraldehyde or entrapment in a cellulose derivative. The preparation of an assembled system containing transduction and recognition steps offers several advantages such as good electrical accessibility, fast response and repeatability. In this case, the assembly is achieved by means of the biotin–avidin affinity system, where the enzyme is anchored to a biotinylated poly(luminol-pyrrole) layer.

3.1. Biotinylated pyrrole synthesis

As a continuation of the contributions made by some of us in the chemistry of vinyl sulfone as an appealing function for bio-conjugation through a Michael-type addition with the amine and thiol groups present in the biomolecules under mild conditions [24], we envisioned the preparation of pyrrole–vinyl sulfone derivative **1** as a key compound for accessing the desired biotinylated pyrrole derivatives (for more details see Supporting Information). Compound **1** is easily prepared by the reaction of pyrrole with divinyl sulfone enough amount with a 86% yield. In the first strategy, the vinyl sulfone group of this compound was exploited by its direct aza-Michael addition with amino-biotin **4**, obtained from azido-biotin **3** (by reduction) or with the commercial amino-biotin **5** yielding the corresponding biotin-pyrrole derivatives **7** and **8** with a 45% and 88% yield, respectively. Alternatively, a two-step strategy was used in order to obtain a compound with a larger spacer between the biotin and the pyrrole moieties. For that, compound **1** was first reacted with propargylamine giving the alkyne–pyrrole **2** in a high yield (89.5%) that was subsequently coupled by CuAAC click-chemistry [25] with azido-biotin **3** leading to the biotin-pyrrole **6** derivative with a 74% yield when performing the reaction using $(EtO)_3PCuI$ as catalyst and under microwave irradiation [26].

3.2. Biotinylated copolymer selection by electrochemical and ECL study

We observed that the pre-treatment of the SPE cells is necessary to remove organic ink constituents and/or contaminants increasing surface roughness or functionalities [27]. Applying three voltammetric sweeps between 0.0 and 1.0 V and using different acid and basic cleaning solutions – (1 M) H_2SO_4, CH_3COOH, NaH_2PO_4, $NaHCO_3$ and NaOH – the best results correspond to use acetic acid (see Fig. S-2D in Supporting Information). Using acetic acid conditions, the electrochemical activity is enhanced by increasing the numbers of chemically reactive sites on the electrode surface [28].

Consecutively, we studied the polymerization of the synthesized biotinylated pyrroles by a potentiodynamic method, first for each monomer separately and later in the presence of luminol in order to prepare the copolymers.

The voltammetric shape of the different biotinylated pyrroles is very similar because they have the same polymer deposit potential (E_{polym}). However, they show different rates of polymerization, measured by the current density j at E_{polym} (Table 1). The highest polymerization rate was observed for compound **6** because the triazole group probably increases the length between the pyrrole and biotin moieties and this effect improves its electropolymerization ability markedly. This effect can benefit the immobilization process because the longer the chain, the higher the coupling of biotin to avidin [29]. Additionally, biotin affects the polymerization process depending on whether the biotin is biotinylated or is free in the solution. In the second case, the electropolymerization of a solution containing biotin and pyrrole, a hindered polymerization effect occurs due to the observed decrease in the current density because biotin is adsorbed on the electrode when it is oxidized, preventing the formation of the polypyrrole oligomers.

In some cases, it has been described that free monomer molecules, inserted between each biotinylated pyrrole, are needed for better polymerization [29], resulting in the formation of a copolymer. This role can be assumed by other polymerizable molecules, such as pyrrole, aniline or aniline derivatives like luminol. We observed that the introduction of luminol increases the growth of the polymer.

With respect to the ECL emission, it was observed that polyluminol alone presents the highest ECL intensity (some 10-fold) although with worse mechanical properties than the luminol containing the copolymers, both pyrrole and biotinylated pyrroles. The highest ECL decrease is observed in poly(luminol-pyrrole) and can be attributed to the absorption of the ECL (λ_{em} = 440 nm) generated by the pyrrole moieties of the polymer (pyrrole absorption [2] occurs at 460 nm and polypyrrole at 440 nm). In the case of biotinylated polymers, the best ECL emission is observed over that of poly(luminol-pyrrole), namely: poly(luminol-**6**), 78%; poly(luminol-**7**), 53%; poly(luminol-**8**),33%, with monomer **6** (absorption at 360 nm) being selected for ECL copolymer formation considering the rate of growth and the ECL emission.

The SPE cell material has a lot of influence on copolymer formation: in platinum SPE cells, a slight polymerization was observed, whereas gold cells offer the best surface and with the polymer growing eight times better than when using graphite, selecting gold SPE as the working cells.

3.3. Optimization of copolymerization conditions

3.3.1. pH influence on copolymerization

The pH exerts a great influence on the polymerization of luminol and compound **6**. It is observed that at pH values higher than luminol pK_a (pK_a 6.30 [14]), a lower electro-formation of polyluminol occurs (E_{pa}: 0.37 V, E_{pc}: 0.54 V at pH 7.0), because the luminol

Table 1
Study of growing and ECL signals of the different formed polymers on a gold SPE cell.

Type	Monomer/s	Electrochemical formation		ECL relative signal/a.u.
		j (μA cm^{-2} cycle^{-1})	E_{polym} (V)	
Polymers	Luminol	5.40	0.21	42650
	Pyrrole	1.30	0.31	0
	Compound **6**	1.05	0.20	0
	Compound **7**	0.35	0.20	0
	Compound **8**	0.70	0.20	0
Copolymers	Poly(pyrrole-luminol)	0.41	0.31	4780
	Poly(luminol-**6**)	2.09	0.20	7820
	Poly(luminol-**7**)	0.70	0.20	6260
	Poly(luminol-**8**)	1.39	0.20	5260
	Pyrrole and biotin	0.80	0.20	0

molecule is in deprotonated form, hindering the electropolymerization, whereas compound **6** is electroformed at different pH values (1.0–7.0). In order to select the best pH for the copolymerization, a pH study at lower and higher than pK_a of luminol was performed. At pH 6.1, in a solution containing luminol and compound **6**, poly(**6**) is formed without luminol contribution (see Supporting Information, Fig. S-1 in which the polymerization is showed by observing the growing peaks each cycle.). In this case, the oxidation of **6** (E_{ox}: 0.53 V) occurs before luminol oxidation (E_{ox}: 0.90 V), launching a fast electropolymerization of **6** instead of luminol copolymerization due to a competition between the formation of the separated polymers (Fig. S-1B). However, at pH 7.0, it can be seen that the copolymer is formed, as Fig. 2 shows. In this case, compound **6** is first oxidized again (0.46 V), now allowing the luminol to be attached to it without oxidation (see first cycle Fig. 2, where no oxidation of the luminol occurs), launching the copolymerization and yielding a polymer containing luminol moieties that agree with the new peaks at 0.44 V (E_{pa}) and 0.27 V (E_{pc}). This is confirmed by SEM and EDX studies (Fig. 3). Additionally, Fig. 2 highlights the presence of the different constituents in the copolymer: pyrrole, biotin and luminol. Once the copolymer is formed on the working electrode, it was electrochemically characterized by cyclic voltammetry using only a solution of pH 7.0 PBS, concluding the non-absorption of the monomers on the formed layer after a washing cycle.

3.3.2. Compound **6**/luminol ratio

To study the optimum monomer ratio for copolymer electroformation considering the ECL output, we changed the ratio, maintaining a constant concentration (1 mM) of **6**. A decrease in the compound **6**/luminol ratio increases the ECL signal because more luminol units are incorporated in the copolymer. The amount of deposited polymer increases as well and 1:10 was selected as the optimum ratio (Fig. S-2A).

3.3.3. Cycle number

An increase in the cycle number in the electropolymerization process (Fig. S-2B) enhances the ECL signal up to 10 cycles, then

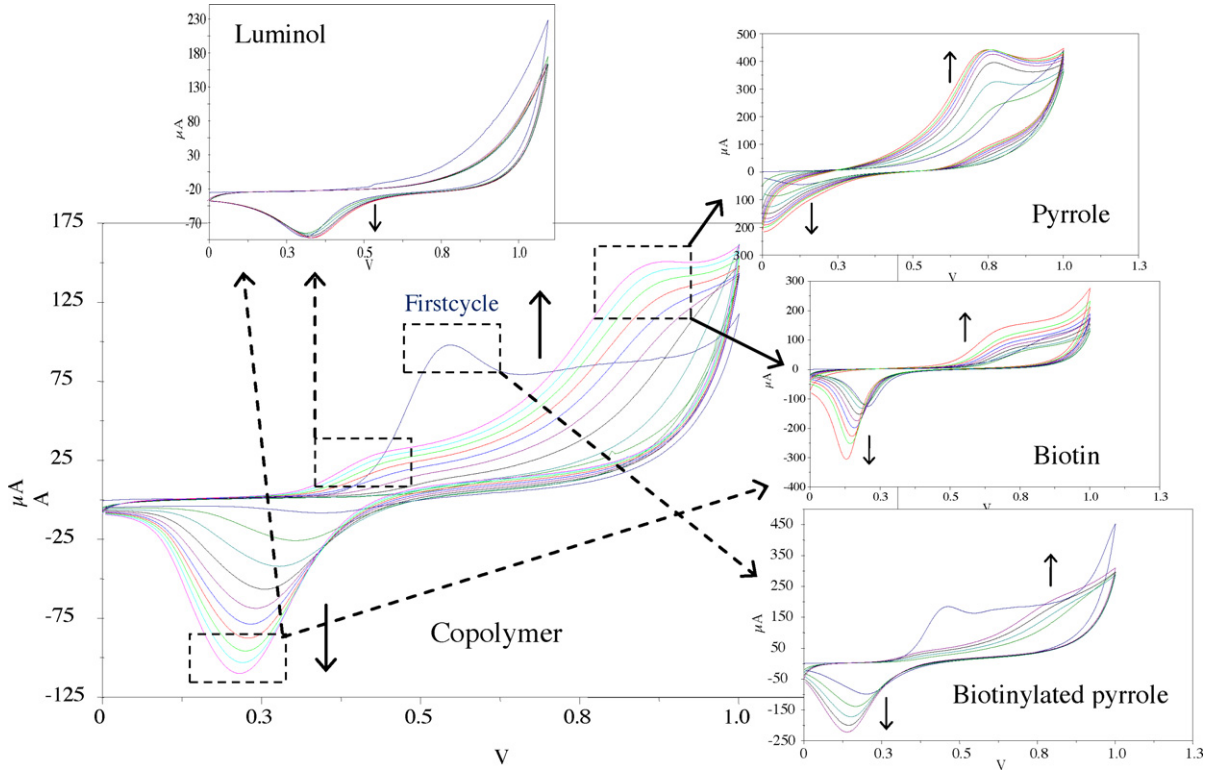

Fig. 2. Cyclic voltammetry of the luminescent copolymer formation. Observe the identification zones of the different monomers which are graphed individually around the cyclic voltammograms of the copolymer. The conditions for the cyclic voltammetries were: 5 cycles in gold LT SPE from 0.0 to 1.0 V, at 0.1 V s^{-1} and solutions of 10^{-3} M in phosphate buffer pH 7.0 for all of them.

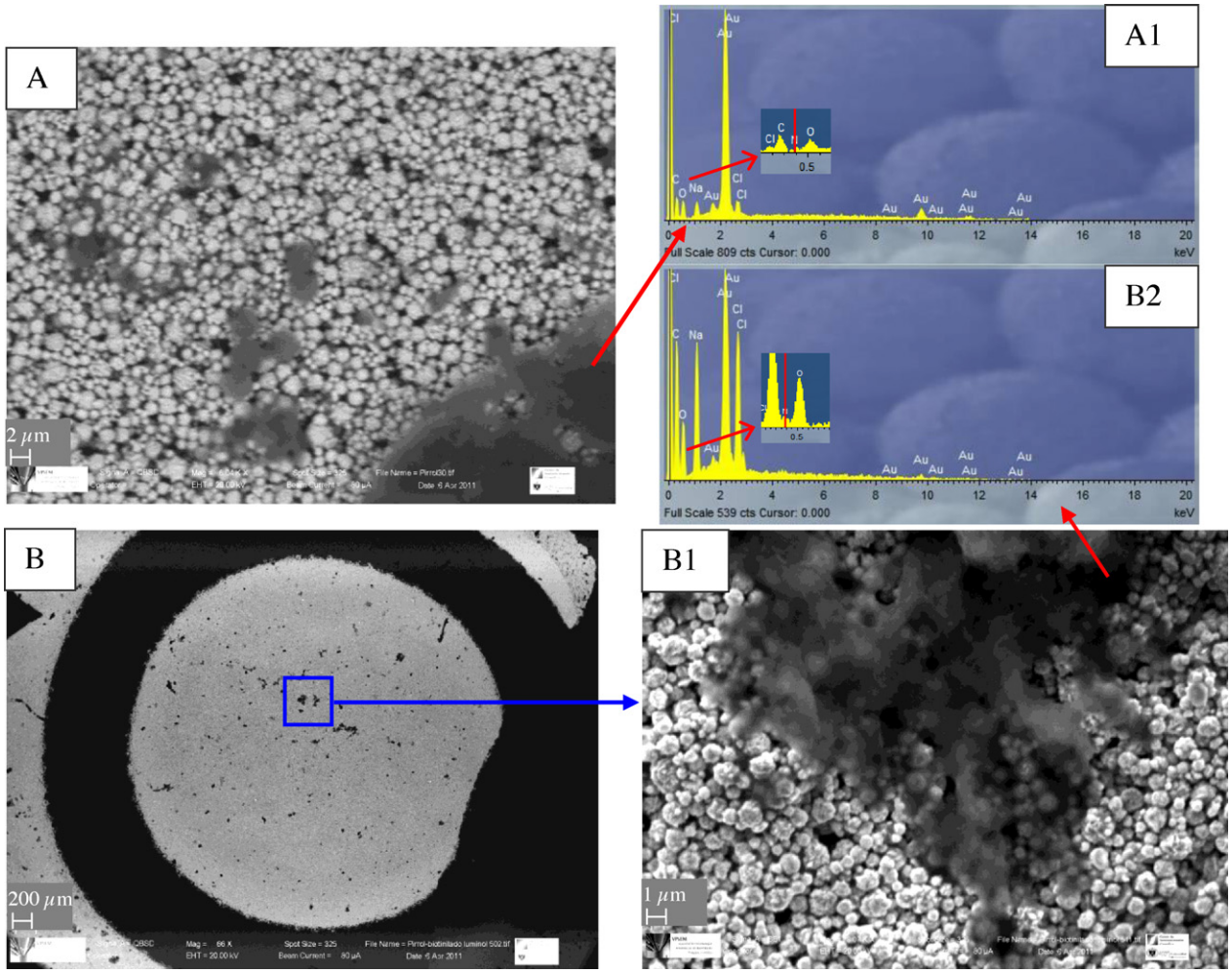

Fig. 3. SEM images and EDX spectra of cholesterol biosensor in gold SPE: (A) polypyrrole; (B) poly(luminol-6). Note the presence of biotin and luminol in the second case as revealed by the EDX spectra due to the increased intensity observed for N, O, and C.

decreasing due to the absorption of the ECL emission because a greater amount of polymer is generated. However, the S/N ratio decreases after 5 cycles, leading to the selection of that number as working conditions.

3.4. Enzyme immobilization

Cholesterol oxidase (ChOx), which catalyzes the atmospheric oxygen oxidation of cholesterol to 5-cholesten-3-ona and H_2O_2 [30], contains in its sequence different amino acids (24 Lys, 4 Cys, 7 His)[31] that are able to react in mild conditions with vinyl sulfone-functionalized reagents via the amine or thiol groups present in their side chain, preserving the biological functionality of the enzyme. In the biotinylation process, typically a supplemented cholesterol concentration (2×10^{-3} M, 6 μL) was included in order to protect the active site of the enzyme, but our results showed that the presence of cholesterol was not suitable because a 99.2% ECL signal decrease was obtained. Thus, the biotinylation was performed without cholesterol. To prepare the self-assembled layers, we studied different concentrations of biotinylated ChOx (from 52.0 IU mL^{-1} to 1040.0 IU mL^{-1}), working with enough avidin concentration, observing that ECL intensity increases up to 520.0 IU mL^{-1} and maintains afterwards, suggesting a saturation of biotinylated pyrrole–luminol copolymer.

3.5. Factors influencing the ECL biosensor

3.5.1. pH

The S/N net ratio ($S/N_{sample} - S/N_{blank\ signal}$) is the most reliable parameter for pH in order to select the best working conditions. The S/N net ratio increases to pH 9.5 (Fig. S-2C), then decreasing as a result of two opposite effects: the optimum enzymatic activity, which is at pH 7.0 [32], and the best conditions for the luminol copolymer oxidation, which starts at basic pH (>8.0) due to the catalytic role of the hydroxyl groups [33]. The selected working pH was 9.5, which is a compromise between the two effects.

3.5.2. Voltage selection

The inclusion of the ChOx enzyme in the polymeric membrane through the biotin–avidin interaction did not result in a change in the excitation potential with respect to that of polyluminol [23] (Fig. S-3)(0.5 V in all cases). However, if the enzyme is not biotinylated and remains in solution, the excitation potential rises to 0.6 V (Fig. S-3D), suggesting that assembling the enzyme in the copolymer facilitates the connectivity of the system.

3.5.3. Analytical signal

The analytical parameter used is the enzymatic conversion obtained by chronoamperometric initial rate estimation (r_0) [34], as the slope from the first 5–6 consecutive pulses for each

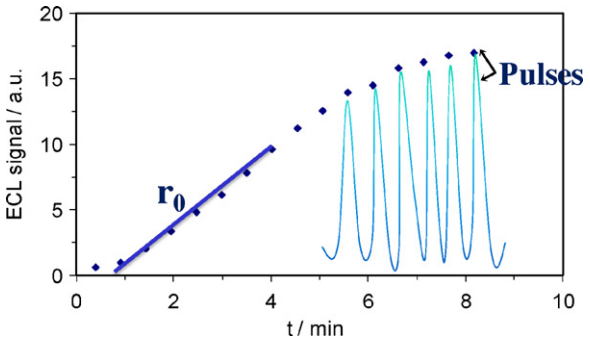

Fig. 4. ECL analytical signal. This signal consists of initial rate estimation (r_0) obtained from the slope of the first 5–6 pulses when 1 s pulses at 0.5 V with 10 s difference between them were applied. Cholesterol concentration 10^{-4} M in 0.2 M saline phosphate buffer pH 9.5.

Table 2
Analytical parameters for cholesterol SPE biosensor.

Parameter	Value	
Range, M	1.5×10^{-5}–8.0×10^{-4}	
Ordinate	29.6 ± 2.3	
Slope	2.7 ± 0.3	
R^2	0.947	
LOD, M	1.47×10^{-5}	
LOQ[a], M	1.50×10^{-5}	
RSD (%) blank ($n = 5$)	11.5	
RSD (%) cholesterol ($n = 5$)	9.0×10^{-5} M	7.9
	2.0×10^{-4} M	14.1

[a] LOQ: limit of quantification.

cholesterol concentration expressed in a.u. min^{-1} (Fig. 4). Conditions such as dwell time and pulse time were optimized at 10 s and 1 s, respectively.

3.5.4. Blank signal and time of measurement

The prepared biosensor shows a low blank contribution to the analytical signal. The use of a self-assembled enzyme hastens the H_2O_2 reaction, and not waiting time is necessary before ECL measurement [34], only 4 min as the analysis time.

3.6. Analytical parameters

The initial rate estimation depends logarithmically on the cholesterol concentration: $r_0 = 2.7 \ln_{(x)} + 29.6$ (Table 2), with a limit of detection (LOD) of 1.47×10^{-5} M using IUPAC standard criteria [35]. The relative standard deviation (RSD) for the blank (0.017 ± 0.002 a.u. min^{-1}) was 11.5% ($n = 5$), a high value due to the low blank signal, and for a cholesterol concentration in the medium level of the range (9.0×10^{-5} M and 2.0×10^{-4} M) was 7.9% and 14.1% ($n = 5$), respectively.

The lifetime of the disposable biosensor was studied protected from light and preserved at 4 °C studying the response of a series of prepared biosensors at 10^{-4} M cholesterol concentration. The lifetime obtained is around 1 month, which is acceptable for this type of biosensor.

Making a comparison regarding the previously developed disposable cholesterol biosensor [36], we obtained a linear range better than the previous biosensor (2.5×10^{-5}–1.0×10^{-4} M), with a similar detection limit (2.3×10^{-5} M) but with a lower repeatability (7.8%), an effect that is due to the necessary additional steps for the preparation of the avidin–biotinylated enzyme complex. With respect to a recent ECL flow biosensor that uses gold nanoparticles [13], that sensor showed better repeatability (4.6%) and low limits of detection as usual.

Table 3
Determination of cholesterol in serum samples by proposed and reference procedures.

Sample	ECL biosensor M	Reference method M	*p*-value (%)	RSD[a] (%)
Serum 1	$(3.4 \pm 0.3) \times 10^{-3}$	$(3.5 \pm 0.1) \times 10^{-3}$	68.2	1.6
Serum 2	$(4.3 \pm 0.5) \times 10^{-3}$	$(3.9 \pm 0.2) \times 10^{-3}$	16.2	7.7
Serum 3	$(2.9 \pm 0.2) \times 10^{-3}$	$(2.8 \pm 0.1) \times 10^{-3}$	59.8	1.8

[a] RSD: % between proposed and the reference method average values.

3.7. Interferences

We studied the influence of typical interferences present in serum samples such as urea and uric acid because both can be oxidized at the electrode at potentials lower than the potential used to start up the ECL. Their common levels in serum samples are: 2.1–7.1 mM for urea and 0.21–0.42 mM for uric acid [37]. When we tested a mixture of 2.5×10^{-4} M in urea and 2.0×10^{-5} M in uric acid, a 10-fold dilution, and 10^{-4} M in cholesterol, a decrease of some thirty-fold (28.9) in the analytical signal was observed.

The greatest contribution of this negative interference is due to uric acid because it can act as a scavenger of radical species such as $OH^{\bullet}$ and electro-oxidized luminol [38], reducing the ECL production. Furthermore, urea increases the signal due to a better electro-oxidation of the luminol [39]. The uric acid interference can be avoided by the addition of $Ba(OH)_2$ or $ZnSO_4$ to the serum samples [40], as described in the Procedure section. The interference produced by urea is eliminated by a blank correction after the addition of $ZnSO_4$.

3.8. Serum sample analysis

The determination of free cholesterol in serum samples is therefore vitally important in the diagnosis of lipid metabolism abnormalities and the prevention of such clinical disorders [41]. Thus, five replicates were measured using the sample treatment described in Section 2. The time required for the analysis was 4 min. The biosensor measurements had been contrasted using a fully automated Roche Hitachi-912 auto-analyzer as the reference method by means of a kinetic assay. The obtained results were compared statistically by the *p*-value parameter. All the values were higher than 5%, that thus there is no statistical difference in the values (Table 3). The comparison of the mean values of both methods, expressed as RSD parameter, shows that in all cases the values are lower than 8%.

4. Conclusions

This paper presents an electrochemiluminescent disposable biosensor using gold screen-printed cells for cholesterol determination in serum samples. The biosensor is prepared by an electro-formation of a biotinylated pyrrole–luminol copolymer by cyclic voltammetry offering improved features such as better adherence and rate of formation than other ECL polymers such as polyluminol. This formed layer presents better emissive characteristics than conventional entrapment by using cellulosic polymers such as Methocel. Avidin has great affinity for biotin, making it possible to assemble the cholesterol oxidase enzyme in a biotinylated form. This biosensor has the peculiarity that no waiting time is necessary before measurement because the incorporated biotin contributes to enhancing the rate of enzyme conversion. To the best of our knowledge, this is the first ECL determination for cholesterol using a copolymer of luminol with biotinylated pyrrole. Drawbacks are related to the precision, which could be improved with mass production techniques which would also cut costs. The presented

cholesterol biosensor offers an easy test for analysis in a disposable format.

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