Talanta 86 (2011) 178–185

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

SPE biosensor for cholesterol in serum samples based on electrochemiluminescent luminol copolymer

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ARTICLE INFO

Article history:
Received 12 June 2011
Received in revised form 16 August 2011
Accepted 28 August 2011
Available online 1 September 2011

Keywords:
Electrochemiluminescence
Disposable sensor
Conducting polymers
Cholesterol
Luminol
Serum samples

ABSTRACT

A poly(luminol–3,3′,5,5′-tetramethylbenzidine) copolymer manufactured by electropolymerization on screen-printed gold electrodes greatly improves the electrochemiluminescence of hydrogen peroxide. Cholesterol oxidase was immobilized on the surface of a poly(luminol–3,3′,5,5′-tetramethylbenzidine) screen-printed cell modified with chitosan to prepare an ECL biosensor for cholesterol. Working under the optimized conditions, the linear dynamic range of cholesterol was 2.4×10^{-5}–1.0×10^{-3} M with a limit of detection of 7.3×10^{-6} M and a precision of 10.3% (5.0×10^{-4} M, $n = 5$) expressed as relative standard deviation. This biosensor was applied to the determination of total cholesterol in serum samples obtaining satisfactory results with respect to the reference procedure. This cholesterol biosensor offers an alternative analytical method with low cost and high speed.

1. Introduction

Cholesterol is an essential lipid found free or esterified in the human organism where it is transported within lipoproteins. It is important to prevent its accumulation in blood because it generates a lipid metabolism disorder and eventually causes arteriosclerosis, cerebral thrombosis and coronary heart diseases. However, deficiencies in cholesterol concentration cause hyperthyroidism, anaemia, malabsorption and wasting syndromes [1]. The normal level of total cholesterol in serum samples is between 5.18×10^{-3} M (men) and 4.89×10^{-3} M (women) [2].

The determination of cholesterol must be done using enzyme-based biosensors because non-enzymatic procedures lack specificity and selectivity [3]. These biosensors are based on different techniques such as spectrophotometry, fluorometry, thermometry, electrochemical methods, and quartz crystal acoustic wave [1]. Recent methods have been based on the use of screen-printed electrodes with an immobilized enzyme with different techniques such as polymeric membranes, composite sol–gel membranes and layer-by-layer technique [4].

The determination of total cholesterol typically needs to hydrolyze the cholesteryl esters using a cholesterol esterase that frees the cholesterol and fatty acids. The free cholesterol can be oxidized in the presence of cholesterol oxidase (ChOx) to produce 4-cholestene-3-one and hydrogen peroxide. The hydrogen peroxide produced can be quantified to determine the total cholesterol typically by means of amperometry [1], chemiluminescence (with peroxidase addition) or electrochemiluminescence [5,6].

Different luminophores for chemiluminescence have been used such as bis-(2,4,6-trichlorophenyl) oxalate (TCPO) [7], lucigenin [6] and luminol [5]. A flow injection analysis was described using a biosensor based on an immobilized cholesterol oxidase amine-modified silica gel via glutaraldehyde activation packed in a column using luminol-ferricyanide as reagents coimmobilized on an anion-exchange column [5].

Electrochemiluminescence (ECL) offers many potential advantages as an analytical technique with respect to chemiluminescence, mainly the possibility of controlling the outbreak of the reaction [8], and has been used also for cholesterol determination. Blum et al. [9] described a flow injection biosensor based on a luminol/hydrogen peroxide reaction induced by a glassy carbon electrode in which the sensing layer contains ChOx immobilized on either UltraBind or immunodyne membrane.

The electrochemical cell used in ECL can be prepared using screen-printing methodology, which makes it possible to miniaturize the system. The immobilization of the reagents on the working electrode in screen-printed cells (SPE) can be achieved in many different ways such as physical adsorption, cross-linking, covalent bonding, entrapment in gels or membranes [10] and electropolymerization. The last technique is based on the formation of polymers by an electro-formation process offering

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doi:10.1016/j.talanta.2011.08.057

reproducible and precise formation of a polymer coating over surfaces, whatever their size and geometry, with an electrochemically controlled thickness [11]. Here an electro-polymerizable luminophore is the key for the preparation of the ECL sensor. The aniline derivative luminol can be electro-deposited using both potentiostatic and potentiodynamic modes on a working electrode of SPE cell forming a polyluminol polymer. Sassolas et al. [12] use polyluminol on SPE graphite cells to prepare a sensor for H_2O_2 and a biosensor for choline by immobilizing the same luminophore as well as choline oxidase in a sol–gel layer [13].

Another possibility is the preparation of a copolymer by selecting a characteristic monomer that can offer good analytical performance or specific immobilization for electrochemiluminescence detection. Li et al. [14] prepared a composite film of poly(luminol–benzidine) on graphite SPE cells for H_2O_2 determination that presents better electrochemiluminescence and analytical characteristics than that of the polyluminol film. In an earlier work, we studied the preparation of electrochemiluminescent copolymers with good mechanical and emissive characteristics for H_2O_2 determination [15]. This study concluded with the selection of the aniline derivative 3,3′,5,5′-tetramethylbenzidine as a co-monomer for luminol polymer formation.

In this work, a disposable analytical device for cholesterol is studied by the electropolymerization of luminol with 3,3′,5,5′-tetramethylbenzidine on a screen-printed gold electrode on which a chitosan membrane containing ChOx is deposited.

2. Experimental

2.1. Chemicals

The monomers for electropolymerization 3,3′,5,5′-tetramethylbenzidine (TMB) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were supplied by Sigma (Sigma–Aldrich Química S.A., Madrid, Spain). Saline buffers were prepared with NaH_2PO_4 0.8 M, pH 6.0, for enzyme and Na_2HPO_4 0.5 M for sample preparation, adjusted to different pH by adding NaOH or HCl, using NaCl 0.25 M as electrolyte. Other solutions, reagents and solvents include cholesterol stock solution of 2×10^{-2} M, cholesterol oxidase (ChOx) from *Streptomyces* species (4.0 mg, 39.0 U mg^{-1}), cholesterol esterase from *Pseudomonas fluorescens* (5 mg, 1.66 U mg^{-1}), chitosan, high molecular weight, Triton® X-100 (1.07 g ml^{-1}), and ethanol absolute quality UV-IR-HPLC. All were obtained from Sigma except ethanol (Panreac Quimica SAU, Barcelona, Spain). The stock solution of cholesterol 2×10^{-2} M was prepared by weighing 0.19 g of cholesterol and 0.23 g of NaCl dissolved in 18.75 ml of ethanol, 6.25 ml of Triton® X-100 and water up to 25 ml. Chitosan was prepared by shaking 1.0 g chitosan for 4 h in 100 ml water containing 1 ml of acetic acid (95.5%) (Panreac). Reverse-osmosis type quality water (Milli-Q Plus185 from Millipore, Molsheim, France) was used throughout.

Screen-printed electrochemical cells (SPE) made of gold (low temperature (LT)) were supplied by Dropsens (Oviedo, Spain). The SPE cells consist of three electrode arrangements on a ceramic support: a round working electrode, a counter electrode, prepared with the same material, and a silver pseudo-reference electrode. Before being used, the electrochemical cells were tested for uniform behaviour. In order to prepare a receptacle on the disposable electrochemical cell, the electrode area was covered 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.

2.2. Enzyme preparations

Cholesterol oxidase was prepared by placing 4.0 mg and 3 ml of 0.1 M phosphate buffer pH 6.0 in an Eppendorf tube and shaking for 2 min. Cholesterol esterase was prepared by dissolving 5 mg in 1 ml 0.8 M phosphate buffer pH 6.0. These two solutions were kept at −20 °C until use.

2.3. Apparatus and software

The ECL emission was measured using a H8529 photomultiplier (PMT) interfaced to a C8855 USB photo-counting unit, both from Hamamatsu (Hamamatsu Technologies K.K., Shizuoka, Japan), connected to a PC. The potentiostats used in this study were an Autolab PGSTAT 128N with FI20 module for crono-coulometric measurements (Metrohm Autolab B.V., Utrecht, The Netherlands) with a connector for screen-printed electrodes supplied by Dropsens and a PS-PC1 model Palmsens potentiostat from Ivium Technologies (Eindhoven, The Netherlands). The arrangement used for ECL emission measurement was described elsewhere [10]. A Crison digital pH-meter with combined glass-saturated calomel electrode (Crison Instruments, Barcelona, Spain) was also used. Additionally, a Variable Pressure Scanning Electron Microscopy LEO 1430-VP (VPSEM) was used for imaging with an increase ranging from 15× to 300,000×. A Roche Hitachi-912 enzymatic auto-analyzer (Hitachi High-Technologies Europe, Germany) was used as a reference method for cholesterol in serum samples.

Software programs used were: Statgraphics Software Package (Manugistics Inc. and Statistical Graphics Corporation, USA), ver. 5.0 (2000) for data treatment, CSW32 v.1.3.3 (2001) and CSW-AIA v. 1.7.3 (2001) (Dataapex Software, Czech Republic) for ECL data treatment, GPES v.4.9 (2007) (Ecochemie, The Netherlands) and PalmSensPC Software v.2.11 (2005) (Palmsens Instruments, The Netherlands) for voltammetric data and Microsoft Office 2003.

2.4. SPE cell pre-treatment

The electrochemical SPE cells were pre-treated before the electro-formation of the copolymer. The treatment consisted of immersing the cells in 0.2 M H_2SO_4 and applying three voltammetric sweeps between −0.2 and 1.0 V by means of a potentiostat. After that, the SPE cells were dried for 10 min at room temperature.

2.5. Biosensor preparation

The electrochemiluminescent copolymer luminol–TMB was prepared in gold LT SPE by means of a cyclic voltammetry working between −0.2 and 1.0 V limits, at a rate of 0.1 V s^{-1} and 5 cycles from a solution containing 10^{-3} M of luminol and TMB (1:1 ratio) in sulfuric acid 0.2 M. After that, 4 μl of chitosan–ChOx (1:3 ratio, v/v) solution was placed on the working electrode and left to dry for about 1 h to form a second layer. The biosensor was washed with 0.2 M of phosphate buffer pH 9.0 to eliminate the adsorbed enzyme, luminol or TMB traces. The prepared cells were stored in the dark at 4 °C until use (see Fig. 1).

2.6. Procedures

2.6.1. Standards

Aqueous standard solutions of different concentrations of cholesterol at pH 9.0 adjusted with 0.2 M phosphate buffer and 0.25 M NaCl were prepared from the stock solution 2×10^{-2} M in Triton X-100 in hydroethanolic media (0.3%, v/v). 50 μl of the standard or sample solution was taken and placed in the receptacle of the SPE cells. The cholesterol biosensor was placed in a black holder and covered with the lid holding the PMT [10]. Then, several pulses

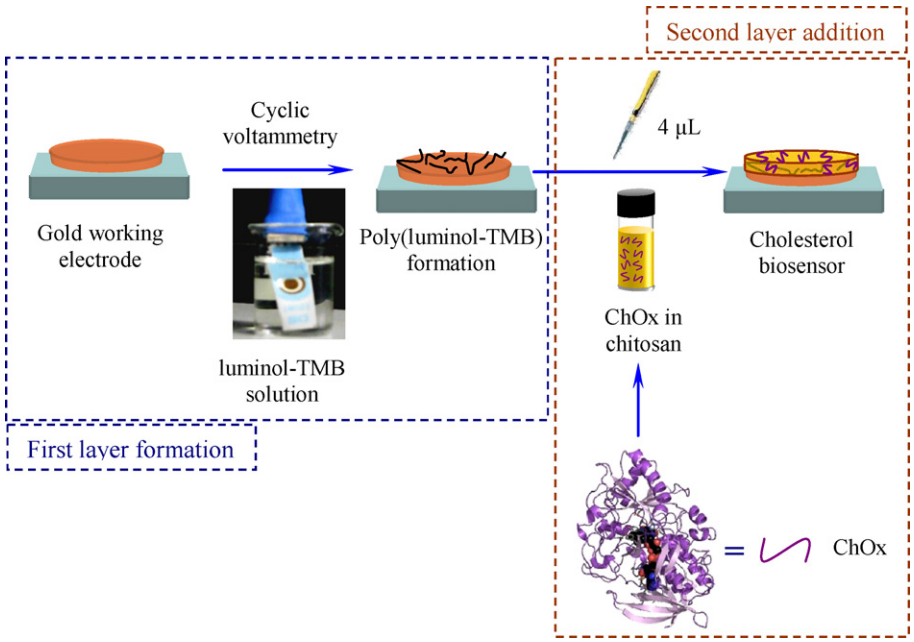

Fig. 1. The two-layer configuration of the cholesterol biosensor. The first layer consists on poly(luminol–TMB) on the gold working electrode of the SPE cell prepared by cyclic voltammetry. The second layer consists on cholesterol oxidase entrapped in chitosan prepared by deposition of a volume of solution.

of 1 s with 10 s difference between them at 0.6 V were applied measuring the ECL emission in the PMT. The analytical signal is defined as the slope, in a.u. min^{-1}, from the measured ECL intensity from six consecutive pulses.

2.6.2. Serum samples

0.1 ml of serum sample was diluted 10-fold in 0.1 M phosphate buffer pH 6.0 with 3.6 μl of 8.3 U ml^{-1} cholesterol esterase. After an incubation period of 5 min at 37 °C, 0.5 ml of this solution was added to 2 ml of 0.2 M phosphate buffer pH 9.0, 2 ml of NaCl 0.25 M and 0.5 ml of water in 5 ml polyethylene tubes containing 10^{-3} M of $ZnSO_4$ ($Ba(OH)_2$ can be used for the same purpose) to remove the interferences [16]. After that, 50 μl was placed in the receptacle of the SPE cells working as previously described for standards.

3. Results and discussion

In this work we study a disposable cholesterol biosensor based on electrochemiluminescent emission coming from luminol chemistry. The common use of reagents in solution, which hinders their use in disposable formats, is avoided by the immobilization of the reagents in the working electrode of an SPE cell. The immobilization of luminol as a polymer paves the way for the creation of ECL biosensors with all the reagents included. The electropolymerization of luminol for analytical purposes has been studied by different authors [12,17] but their use has some drawbacks, such as low adherence and low conductivity [18]. To improve the mechanical and electroluminescent properties of the film, we studied the copolymerization of luminol with different monomers from the family of aniline, benzidine and pyrrol, concluding that copolymerization of 3,3′,5,5′-tetramethylbenzidine (TMB) produces a new material with an interesting mechanical properties and fast growth on SPE cells [15]. Poly(luminol–TMB) copolymer offers a better adherence and ECL intensity than polyluminol. On the other hand, this formed layer presents better emissive characteristics than using conventional entrapments of luminol with cellulosic polymers such as Methocel. Then, the goal of this study is the preparation of a biosensor for cholesterol based on poly(luminol–TMB) as the immobilized luminophore next to cholesterol oxidase on gold SPE cells as well as the optimization of working conditions that are different for the recognition (enzymatic reaction) and transduction (ECL production) steps.

3.1. Biosensor preparation

The preparation and characterization of the new polymer containing luminol moieties is presented first, followed by the presentation of the enzyme immobilization on the SPE cell.

3.1.1. First layer: Electro-formation of the poly(luminol–TMB) copolymer

The electrochemical preparation of the copolymer was performed by a potentiodynamic procedure by cycling with a sufficiently high anodic limit, technique that permits to control the oxidation and the growth of the polymers.

A cyclic voltammetry study of the monomers used working from −0.2 V to 1.0 V in H_2SO_4 0.2 M and 10^{-3} M was conducted, and after the first cycle, regarding the oxidation of the monomers, reduction and oxidation peaks were observed at 0.55 V and 0.69 V, respectively, coming from the formation of the copolymer, the intensity of which increases with each new cycle, as shown in Fig. 2. The electro-formation of the copolymer proceeds via the oxidation of the monomers. In the case of TMB, a monocation radical TMB^+ (blue colour) at 0.25 V is formed followed by diimine TMB^{2+} cations at 0.62 V [19] and then followed by luminol oxidation at 0.9 V. The oxidized species produced reacts by forming a poly(luminol–TMB) blue species on the gold working electrode.

3.1.2. Second layer: Enzyme immobilization

Over electro-formed copolymer, an enzymatic layer was prepared by entrapment in chitosan, an *N*-deacetylated polyelectrolyte derived from the biopolymer chitin.

The morphology of the active surface of the biosensor was studied by SEM. Fig. 3A shows the polycrystalline strands of poly(luminol–TMB) around the working gold electrode which, as shown in a previous work [15], corresponds to the copolymer. The chitosan layer without the enzyme over the gold SPE cell appears as a film covering the entire electrode with a thickness of 2–3 μm

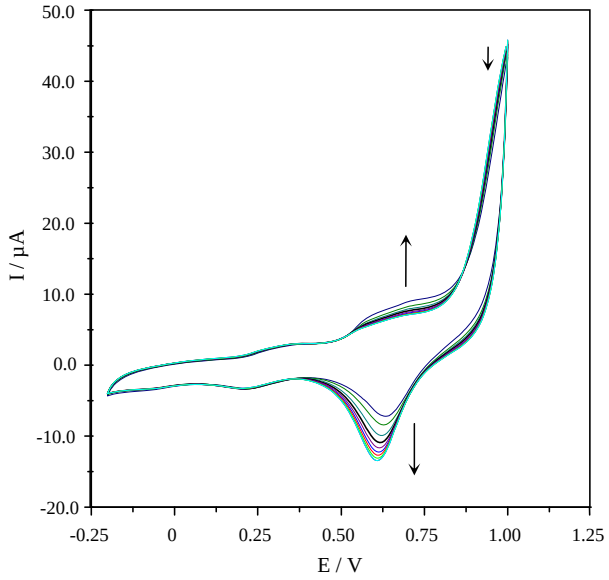

Fig. 2. Potentiodynamic formation of the poly(luminol–TMB) polymer using a solution that contains luminol and TMB monomers. The conditions of copolymerization were 1 mM each monomer in H_2SO_4 0.2 M using gold LT SPE cells for 10 sweeps (0.1 V s^{-1}) from −0.2 to 1.0 V.

according to the side view of the SEM images (Fig. 3C). When ChOx is entrapped in chitosan, a dendritic structure (Fig. 3B on the left) is formed over the copolymer that has a thickness between 20 and 24 μm. In the side view of the SEM image of the biosensor including the two layers (Fig. 3D), it can be seen that the chitosan–ChOx layer generates a bulky structure, unlike chitosan alone.

3.2. Optimization of the composition of the cholesterol biosensor

The main factors that influenced the preparation of the cholesterol biosensor are: (1) the copolymerization conditions, both the ratio of monomers and the number of cyclic sweeps, and (2) the concentrations of enzyme and chitosan used to prepare the enzymatic layer.

3.2.1. Luminol–TMB copolymer ratio

As standard conditions for this optimization study, solutions of 26.0 IU ml^{-1} ChOx and 2×10^{-4} M cholesterol at pH 9.0 with phosphate buffer were used for the ECL measurements. The influence of the luminol–TMB ratio on the electroluminescent characteristics of the copolymer was studied preparing films on SPE cells and varying the concentration of luminol from 0.1 to 10 mM and maintaining a constant TMB concentration at 1 mM. Fig. 4A shows that the optimum luminol–TMB ratio corresponds to 1:1 (1 mM of both luminol:TMB monomers). The analytical signal grows up to 1:1, decreasing drastically afterwards. The increase in the ratio increases the luminol moieties in the copolymer and consequently could enhance the ECL emission, but at the same time it decreases the conductivity of the copolymer, as we already observed in an earlier work [15]. Both effects result in a maximum in the analytical signal.

3.2.2. Cycle number

The number of cycles used has a strong influence on the electroformation of the copolymer. An increase in the cycle number (Fig. 4B), working at a 1:1 monomer ratio, enhances the analytical signal up to 5 cycles, because the amount of the formed poly(luminol–TMB) increases. However, a decrease in the signal appears with more cycles. This could be due to the decreasing of conductivity above mentioned or even to a self-quenching of the ECL emission (emission wavelength at 450 nm) produced by the increased formation of diimine TMB^{2+} cations (absorption wavelength at 450 nm) on the electrode surface [20].

3.2.3. Enzyme immobilization in chitosan

If oxygen is present, the ChOx enzyme can catalyze the oxidation of the cholesterol producing H_2O_2 and 5-cholesten-3-one [21]. This H_2O_2 reacts with the electro-oxidized luminol moieties of copolymer yielding an ECL emission. The polymer selected for the enzyme immobilization was chitosan, due to its good optical properties and the fact that it can be used in ECL because it forms a transparent thin film [22].

To study the immobilization of ChOx in chitosan over the electroformed polymeric film, different solutions in all cases containing 52.0 IU ml^{-1} ChOx and solutions between 0.5 g l^{-1} and 5 g l^{-1}

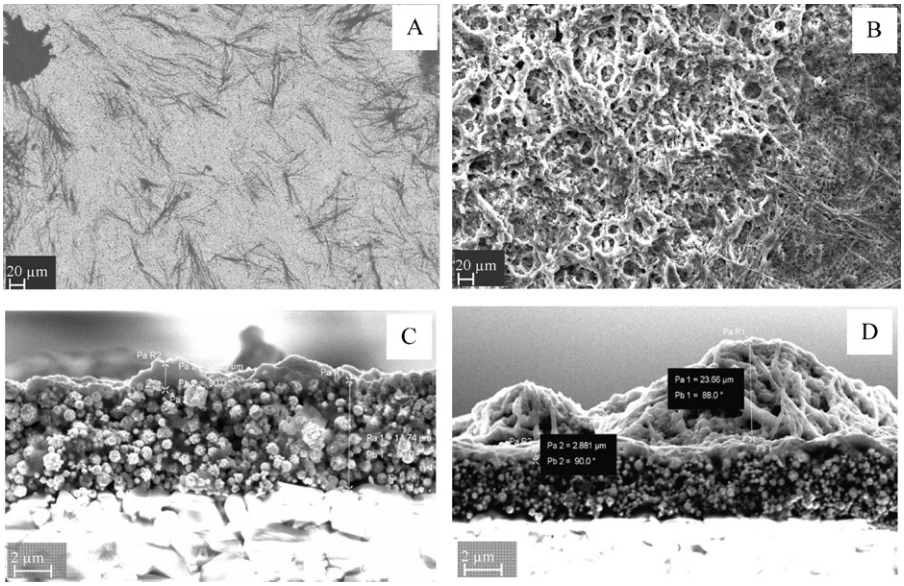

Fig. 3. SEM images of working electrode of cholesterol biosensor. (A) poly(luminol–TMB) strands on gold SPE cell; (B) ChOx into chitosan layer forming a coral-like structure (note the poly(luminol–TMB) strands below chitosan layer to the right of (B); (C) side view of the gold SPE (small beads) and thickness of chitosan layer; (D) side view showing the thickness of the chitosan containing the ChOx layer on SPE.

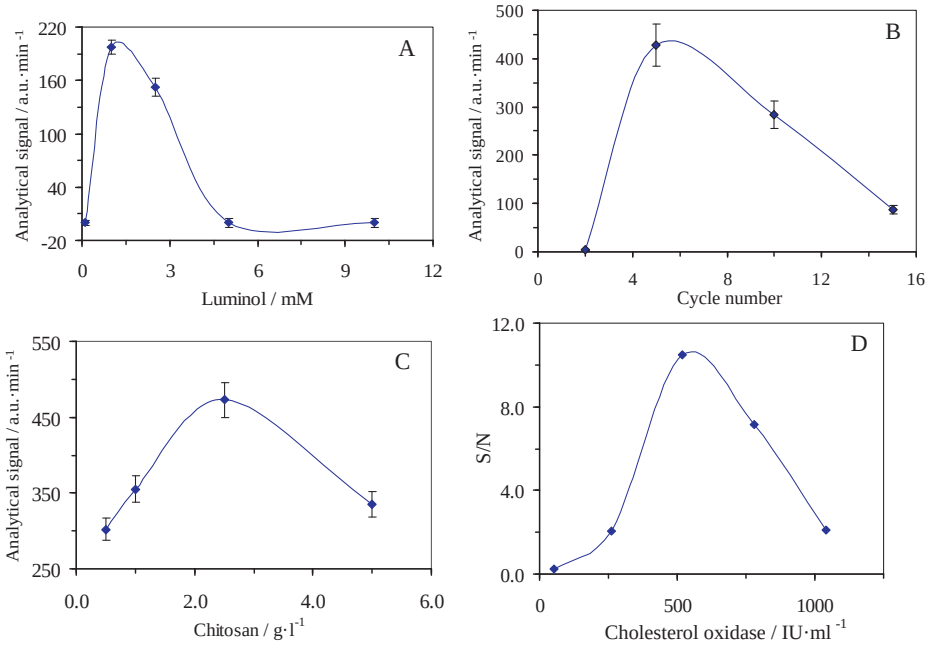

Fig. 4. Optimization of the biosensor components. (A) Luminol:TMB ratio influence, maintaining TMB concentration constant (1 mM); (B) cycle number for the selected 1:1 copolymer ratio; (C) chitosan concentration; (D) S/N response for cholesterol oxidase concentration. Cholesterol concentration used in all experiments was 0.2 mM.

chitosan were used, preparing the biosensing element according to procedure described in the experimental section. In this case, the parameter used for enzyme optimization was the signal-to-noise ratio (S/N) because the standard deviation increases with a chitosan concentration higher than 2.5 g l^{-1} (Fig. 4C). The electrostatic immobilization of negatively charged ChOx molecules [3] in the positive chitosan layer [23] explains the good enzymatic activity with the ECL outcome. Additionally, at higher chitosan concentrations, the membranes become brittle, diminishing the analytical signal and decreasing the repeatability of the biosensor. Therefore, 2.5 g l^{-1} chitosan solution was selected for the membrane preparation.

Subsequently, to optimize the enzymatic charge of the biosensor, a study was carried out immobilizing 52.0–1040.0 IU ml^{-1} ChOx. Fig. 4D shows that an increase in enzyme concentration increases the analytical signal, reaching a maximum at 520.0 IU ml^{-1} and decreasing afterwards. The decrease observed in the two consecutive studies of chitosan and enzyme could be due to the substrate's loss of accessibility to the enzyme, because the other proteins in the vicinity hinder the accessibility of the active site, diminishing the rate of the reaction and decreasing the formation of hydrogen peroxide and consequently, the produced ECL.

3.3. Biosensor measurement conditions

The influence of different influencing factors such as pH, voltage used, waiting time before measurement, blank and analytical signals was established before the characterization of the biosensor.

3.3.1. pH

An increase in pH increases the uncertainty of the replicates in the ECL measurement using the biosensor. For that reason, the S/N ratio was used as the parameter to optimize the working pH in order to minimize this effect. Fig. 5A shows the S/N ratio of the blank and a cholesterol standard. In both cases a maximum is observed at pH 9.0, being the S/N ratio of the cholesterol signal higher (4 times) than the blank signal. With an electroluminescent biosensor, there are two main pH influences: on the enzymatic activity and on the ECL system. The optimum pH for ChOx is 7.0 [24], while the luminol reactivity with H_2O_2 increases with the basicity of the medium (more than 8.0) due to the contribution of hydroxyl groups in catalyzing the ECL reaction [25]. The obtained maximum at 9.0 is a compromise pH value considering both effects and hence was used as the working pH.

3.3.2. Electrode potential optimization

Fig. 5 represents the ECL emission versus voltage for the polyluminol (Fig. 5C) and poly(luminol–TMB) layers (Fig. 5D). In addition to the ECL relative signal (wavy line), both figures include a voltammogram (continuous line) showing the electrochemical oxidation of the two polymers. Different oxidation voltages are observed, namely a shift to the right of the maximum emission voltage for poly(luminol–TMB) polymer (0.6 V) with respect to polyluminol (0.5 V). This variation in the oxidation voltage is due to the presence of TMB moieties in the copolymer whose methyl electron accepting groups hinder the oxidation, shifting the voltage to higher values [26].

3.3.3. Blank signal and waiting time

The blank signal is due to the reaction of the dissolved oxygen present in solution with the oxidized luminol [27] and no contribution due to ChOx or chitosan was observed. The blank S/N ratio grew to pH 9.0, decreasing at a higher pH.

The period of time between the sample addition and the ECL measurement is obviously very important for an enzymatic biosensor, thus the more waiting time, the more H_2O_2 production and ECL outcome. The study of the waiting time (Fig. 5B) shows an optimal analytical signal two minutes from the addition of the sample. The reason that the analytical signal then decreases is attributed to a product inhibition that occurs at high concentrations of H_2O_2. It has been suggested that a second molecule of H_2O_2 can bind to the cholesterol oxidase–H_2O_2 complex to form an inactive ternary complex [28]. Therefore, 2 min was selected as the waiting time before measurement.

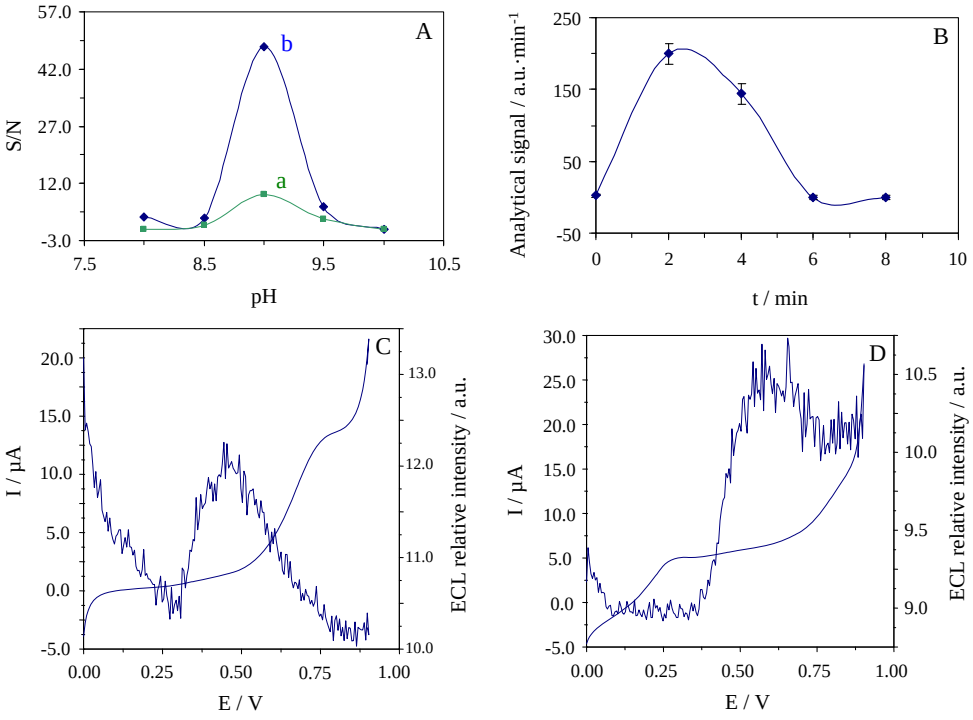

Fig. 5. Experimental ECL measurement conditions. (A) pH influence. In this figure the curve labelled with the letter "a" corresponds to the S/N blank signal and curve "b" corresponds to S/N for cholesterol 0.2 mM; (B) waiting time before measurement; (C) oxidation potential and ECL signal for polyluminol on gold SPE cell; and (D) oxidation potential and ECL signal for poly(luminol–TMB). The electrochemical oxidation is represented by the continuous line and the ECL signal by the wavy line.

3.3.4. Analytical signal

It is well known that luminol emits visible radiation in the presence of hydrogen peroxide in alkaline medium if positive potentials are applied [29,30]. Fig. 6 shows two different forms of analytical data acquisition in order to obtain a calibration function for the biosensor. The consecutive pulses at 0.6 V applied to the SPE cell give rise to a growing ECL signal due to the enzymatic conversion of the cholesterol that produces H_2O_2 (inset Fig. 6). These emission peaks at given cholesterol concentration can be used to obtain the analytical signal in two ways: (1) taking the ECL emission at a fixed time; and (2) taking the enzymatic initial rate estimation from the emission of the first consecutive pulses. Using the ECL intensity at a fixed time (30 s) (selected pulse of the dotted box in the Fig. 6 inset), a linear relationship with cholesterol concentration is observed. Additionally, the initial rate estimated from the slope of consecutive initial pulses gives rise to a linear calibration in a similar way, although in this case the sensitivity and the accuracy (see error bars) are higher than in the previous case. As a conclusion to this study, we chose to use the enzymatic initial rate estimation as the analytical signal (a.u. min^{-1} units).

Conditions like dwell time and pulse time were studied, with the optimal values corresponding to 10 s and 1 s respectively. In order to obtain a short analysis time, we used between 5 and 8 pulses to acquire the analytical signal.

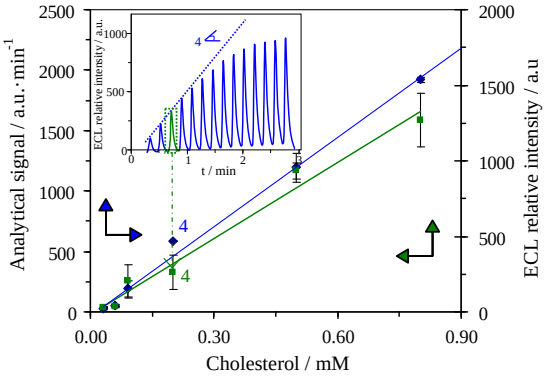

Fig. 6. Selection of the analytical signal. Calibration curves obtained using the ECL intensity (secondary *Y*-axis) at a fixed time (30 s) (see the selected pulse of the green dotted box in the inset) obtaining the lower line and the calibration using the enzymatic initial rate estimation (blue line or upper line) obtained from the slope of successive initial pulses (dotted line in the inset as an example). The inset shows the ECL signal obtained with consecutive pulses of 0.6 V. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Analytical parameters

In order to study the dependence of the cholesterol concentration, different standards from 2.0×10^{-5} M to 1.0×10^{-3} M were measured according to the procedure, obtaining a linear dependence with the initial rate with a limit of detection of 7.3×10^{-6} M using IUPAC standard criteria [31] (Table 1). The relative standard deviation (RSD) ($n = 10$) for the blank was 7.0% and working with a cholesterol concentration in the middle of the range (5.0×10^{-4} M),

Table 1
Analytical parameters for the cholesterol SPE biosensor.

Parameter	Value
Range (M)	2.4×10^{-5}–1.0×10^{-3}
Ordinate	-400 ± 20
Slope	$(2.5 \pm 0.1) \times 10^{7}$
R^2	0.990
LOD (M)	7.3×10^{-6}
LOQ (M)	2.4×10^{-5}
RSD (%) blank ($n = 10$)	7.0
RSD (%) cholesterol ($n = 5$)	9.6 (using 6.0×10^{-5} M) 10.3 (using 5.0×10^{-4} M)

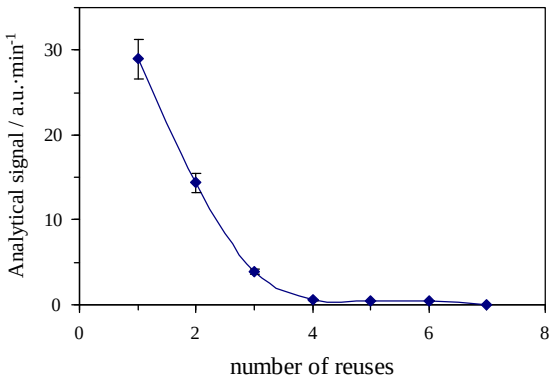

Fig. 7. Repeatability using the same biosensor. It can be seen that a consecutive addition of cholesterol 0.1 mM on the same biosensor, with washing steps, produces a negative exponential decay in the analytical signal.

a RSD of 10.3% was obtained. These and other analytical parameters are included in Table 1.

The reusability of the biosensor was studied. Fig. 7 shows the consecutive use of three biosensors measuring cholesterol 10^{-4} M with a subsequent washing step each time for each one. A negative exponential decay was observed with respect to the analytical signal. This behaviour was observed both when using only a poly(luminol–TMB) layer and only ChOx–chitosan layer with luminol in solution. These results suggest a loss of immobilized reagents on the SPE cell with every washing step, enabling the use of these biosensors in disposable format.

3.5. Interferences

The aim of this work is the determination of cholesterol in human serum samples. First we check the existence of matrix effect through a standard addition study using a set of five sera, observing in general a decreasing in the slope of some 18.4% as average compared to calibration using only phosphate buffer (p-value < 5%, that is, 0.36%).

In order to gain knowledge about potential interferences in human serum that can reduce the analytical signal we study specifically urea, ascorbic acid and uric acid due to the fact that they can be oxidized at the electrode at potentials lower than those used to start up the electrochemiluminescence reaction. The levels of these chemicals in the serum of a typical adult are: 2.1–7.1 mM for urea and 0.21–0.42 mM for uric acid. Ascorbic acid generally is present in plasma and not in serum samples, with a concentration between 2.3×10^{-2} mM and 8.5×10^{-2} mM [32].

Working individually with 2.5×10^{-5} M for urea, 2×10^{-5} M for ascorbic acid or 2×10^{-6} M for uric acid, and 10^{-4} M cholesterol, an increase of 26.8% in the analytical signal when urea is present and a decrease for ascorbic acid (99.3%) and uric acid (103.6%) were observed. The decreasing in the analytical signal in the case of ascorbic acid is due to its lower oxidation potential (0.015 V) [33], followed by the subsequent oxidation by oxygen of the dehydroascorbic acid formed to diketogluconic acid, decreasing the oxygen concentration and hence the analytical signal [34].

The negative interference of uric acid is due to its ability to act as a scavenger of radical species such as $OH^{\bullet}$ and electro-oxidized luminol, as suggested by Radi et al. [35], reducing the ECL production. Urea in their turn increases the ECL signal due to luminol's better electro-oxidation [36].

The interference coming from uric acid can be prevented by the addition of $Ba(OH)_2$ or $ZnSO_4$ to the serum samples [5] as described in the procedure section. The interference coming from urea is eliminated by a blank correction after the addition of $ZnSO_4$.

Table 2
Determination of cholesterol in serum samples by proposed and reference procedures.

Sample	ECL biosensor (M)	Reference method (M)	p-value (%)
Serum 1	$(6.6 \pm 0.8) \times 10^{-3}$	$(6.4 \pm 0.3) \times 10^{-3}$	41.3
Serum 2	$(4.0 \pm 0.1) \times 10^{-3}$	$(4.1 \pm 0.2) \times 10^{-3}$	50.0
Serum 3	$(5.1 \pm 0.3) \times 10^{-3}$	$(5.0 \pm 0.3) \times 10^{-3}$	44.4
Serum 4	$(5.8 \pm 0.2) \times 10^{-3}$	$(6.3 \pm 0.3) \times 10^{-3}$	7.2

However the addition of $ZnSO_4$ followed by blank correction is not enough to eliminate the interferences. Then, a dilution study of serum samples was done. We studied dilutions from 10 to 1000-fold, carrying out standard addition calibrations using different serum samples. In conclusion we find out that a 100-fold dilution eliminates the observed interference. The average slopes of different calibration curves were statistically comparable to the average slopes of different standard additions at this dilution level (p-value > 5%, to be specific, 75.2%), concluding that a 100-fold dilution with the addition of $ZnSO_4$ and a blank correction resolve the problem.

3.6. Applications to serum samples

Determination of cholesterol is needed for the clinical diagnosis of diseases such as coronary heart disease, myocardial infarction and arteriosclerosis [37]. Cholesterol can be analyzed in the human body as free and esterified forms. Cholesterol in serum is partially esterified with long chain fatty acids. For the measurement of total cholesterol, cholesterol esterase is added in order to catalyze the hydrolysis of the esterified molecules obtaining all of the cholesterol molecules in free form [3,38]. In order to prepare a versatile biosensor (for free or total cholesterol), the cholesterol esterase has not been included in the configuration of the biosensor membrane. For that purpose, 0.1 ml of serum sample was diluted 10-fold and incubated for 5 min at 37 °C with 8.3 U ml^{-1} cholesterol esterase to obtain the useful total cholesterol for biosensor measurement. Then, using a 100-fold dilution and five replicates of every sample, the procedure was contrasted using an automatic auto-analyser reference method. The results are presented in Table 2.

4. Conclusions

A biosensor for cholesterol using gold screen-printed cells was successfully developed by the immobilization of all the reagents needed in two layers on the working electrode of the SPE cells, the upper layer consisting of an enzyme in a chitosan membrane and the lower a electrochemiluminescent copolymer of luminol and 3,3′,5,5′-tetramethylbenzidine (TMB). This copolymer offers advantages in immobilization, such as stability and higher ECL signals, by using an electropolymerization technique. The method presents a range of two orders of magnitude obtaining linear calibration and a low detection limit of 7.3×10^{-6} M. The biosensor offers an easy procedure for free or total cholesterol analysis in a disposable format with ECL measurement.

Acknowledgements

We acknowledge financial support from the Ministerio de Ciencia e Innovación, Dirección General de Investigación y Gestión del Plan Nacional de I+D+i (Spain) (Projects CTQ2009-14428-C02-01 and CTQ2009-14428-C02-02) and the Junta de Andalucía (Proyecto de Excelencia P08-FQM-3535). These projects were partially supported by European Regional Development Funds (ERDF). We acknowledge Alicia Aldaya, from Ruiz de Alda Granada Hospital,

for her helpful and kind assistance and Ismael F. Díaz Ortega for his kind assistance.

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