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Disposable luminol copolymer-based biosensor for uric acid in urine

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ABSTRACT

A new electrochemiluminescent (ECL) disposable biosensor for uric acid was manufactured by immobilization in a double-layer design of luminol as a copolymer with 3,3′,5,5′-tetramethylbenzidine (TMB) and the enzyme uricase in chitosan on gold screen-printed cells. The good mechanical and improved electroluminescent characteristics of the new copolymer poly(luminol–TMB) make it possible to determine uric acid by measuring the growing ECL emission with the analyte concentration. The combination of enzymatic selectivity with ECL sensitivity results in a disposable analytical device with a linear range for uric acid from 1.5×10^{-6} to 1.0×10^{-4} M, a limit of detection of 4.4×10^{-7} M and a precision of 13.1% (1.0×10^{-5} M, $n = 10$) as relative standard deviation. Satisfactory results were obtained for uric acid determination in 24 h-urine samples compared to a reference procedure. This uric acid biosensor can be used as a low-cost alternative to conventional methods.

1. Introduction

Uric acid is one of the primary end products of purine metabolism in humans. It is present in biological fluids such as urine and blood serum and many disorders are known to be directly related to variations in uric acid concentration. Examples include gout, arthritis, cardiovascular and renal diseases, neurological and kidney diseases [1–3]. Consequently, uric acid determination is of paramount importance in the diagnosis of diseases caused by disorders of purine biosynthesis and catabolism [4]. The normal level of uric acid in serum is between 0.13 and 0.46 mM (2.18–7.7 mg dL^{-1}), and in urinary excretion between 1.49 and 4.46 mM (25–74 mg dL^{-1}) [5]. Conventional procedures for uric acid determination include spectrophotometry, electroanalysis, chromatography, mainly HPLC, and fluorimetry [4]. All these procedures require conventional instrumentation working in a laboratory environment. However, the current trend towards handheld and portable instrumentation demands new chemistries and devices.

The use of chemiluminescence (CL) for uric acid determination – with a few exceptions – presents a general characteristic: a decrease in the generated emission with concentration, resulting in a negative calibration function. One case is the reaction of luminol and periodate in alkaline media in the presence of uric acid, resulting in an inhibited CL emission, implemented in FIA format and used for its determination in human serum [6] obtaining a limit of detection (LOD) of 1.1×10^{-9} M. Zhao et al. [7] proposed the addition of gold nanoparticles to the running buffer of capillary electrophoresis to catalyze the CL reaction between luminol and hydrogen peroxide, obtaining an LOD value (4.6×10^{-8} M) that is ten times higher with respect to the luminol–periodate system proposed by Song and Hou [6].

A few CL procedures for uric acid are based on the growth of the CL emission. This is the case with the capillary electrophoresis separation of uric acid detected by reaction with luminol and potassium ferricyanide in alkaline medium [8], which obtains an LOD value of 3.5×10^{-7} M, or the use of a specific luminophore for uric acid, as with diperiodatoargentate (III) in alkaline medium [9] (LOD 1.2×10^{-7} M).

In electrogenerated chemiluminescence or electrochemiluminescence (ECL) it is well known that uric acid and some amino acids interfere seriously with the ECL emission, even in low concentrations, of some luminophores such as $Ru(bpy)_3^{2+}$ [10] and luminol [11]. This is the basis of ECL procedures for uric acid based on the inhibition of the $Ru(bpy)_3^{2+}$ emission [11] where different organic and inorganic compounds produce a quenching effect, among them uric acid, which can be determined in the presence of ascorbic acid, as Chen and Zu reported [12]. However, this ECL determination presents a worse LOD (10^{-6} M) than the CL procedures indicated above. As with CL, very few ECL procedures for uric acid are based on the increase of ECL emission. This is the case with the ECL biosensor based on a bis-[3,4,6-trichloro-2-(pentyloxycarbonyl)-phenyl] oxalate on an ITO modified electrode that permits the determination of uric acid in the presence of uricase in solution in real serum samples with an LOD value of 8×10^{-6} M [13]. The LOD values for ECL uric acid determination are almost ten times worse than those

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with CL procedures. The reason for this discrepancy is related to the role of uric acid in ECL production, in that the uric acid prevents the formation of luminol radicals decreasing the ECL signal, as will be discussed later.

In a previous work [14], a biosensor for lactic acid was presented based on a cellulosic polymer Methocel to immobilize all the reagents needed for ECL generation on a graphite screen printed cell. In this work, we extend and perform another immobilization way such as electropolymerization to prepare an electrochemiluminescent copolymer containing luminol [15]. The composition of the screen printed cell (graphite, gold, platinum) is very important for reagents immobilization and for the subsequent ECL outcome, as we study in our previous work about H_2O_2 determination in rainwater [15]. Taking into account these considerations, it is possible to prepare a disposable analytical device for uric acid determination in urine with the advantages of intense ECL emission, short analysis time and elimination of common interferences by immobilizing enzyme uricase chitosan and luminol as the new electroluminescent copolymer.

2. Experimental

2.1. Chemicals

As monomers for electropolymerization we used *o*-dianisine, benzidine dihydrochloride, 3,3′-diaminobenzidine, 3,3′,5,5′-tetramethylbenzidine (TMB), all supplied by Sigma as well as other reagents unless otherwise note (Sigma–Aldrich Química S.A., Madrid, Spain). Saline buffers were prepared in 0.625 M NaCl as electrolyte with 0.5 M Tris (Trizma base 99%) and 0.6 M phosphate (Na_2HPO_4), adjusted to different pH values by adding NaOH or HCl. Stock solutions of 10^{-2} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), from 97% purity reagent, and 10^{-3} M uric acid were used. Uricase from *Bacillus fastidiosus* (5 mg, 14.5 U mg^{-1}) was prepared from 5 mg and 1 mL of 0.1 M Tris buffer pH 8.5 in an eppendorf tube and shaking for 2 min, then aliquoted and preserved at −20 °C until use [16]. 2 mM stock solutions of ascorbic acid and urea were used for interference study (Sigma). Chitosan was prepared by dissolving and shaking for 4 h, 1 g in a 100 mL water containing 1 mL of acetic acid (95.5%, Panreac, Barcelona, Spain). Reverse-osmosis type quality water (Milli-Q Plus185 from Millipore, Molsheim, France) was used throughout.

Gold screen-printed electrochemical cells (SPE) of two types (low temperature, LT, and high temperature, HT) were supplied by Dropsens (Oviedo, Spain). These cells consist of a round working electrode, a counter electrode, both of the same material, and a silver pseudo-reference electrode on a ceramic support. 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. Apparatus and software

The ECL emission from the screen-printed cells was measured using an 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 potentiostat used was an Autolab PGSTAT 128N with FI20 module for chronocoulometric measurements (Metrohm Autolab B.V., Utrecht, The Netherlands) and a connector for the screen-printed electrodes supplied by Dropsens. The arrangement used for ECL studies was described in previous studies [14] and basically consists of a black box that contains two black methacrylate piece holders that fit one piece on the other, one to insert the electrochemical cell in a fixed position and other to contain the PMT. Other instruments consist 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 300k×. A Roche Hitachi-912 enzymatic auto-analyzer (Hitachi High-Technologies Europe, Germany) was used as a reference method for uric acid in 24 h-urine 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.

2.3. SPE cell pre-treatment

The optimized electrochemical pre-treatment of gold LT SPE cells was carried out by immersing the cells in 0.2 M H_2SO_4 and applying three voltammetric sweeps between −0.2 and 1.0 V. After that, the SPE cells were dried for 10 min at room temperature.

2.4. Biosensor preparation

First, the luminol–TMB copolymer was prepared by cyclic voltammetry from −0.2 to 1.0 V limits, at 0.1 V s^{-1}, with 10 cycles using a solution that contains 2 mM of luminol and TMB (1:1 ratio) in 0.2 M H_2SO_4 in a gold LT SPE cell. Then, 4 μL of the chitosan–uricase mixture (1:3 ratio) was dropped onto the working electrode and left to dry for around 1 h at room temperature. The biosensor was washed with 0.2 M of phosphate buffer pH 9.5 in order to eliminate any adsorbed enzyme, luminol or TMB traces. After that, the cells were stored in dark conditions at 4 °C until use.

2.5. Analytical signal

The transitory ECL signal coming from the uric acid biosensor was obtained using consecutive pulses of 0.6 V for 1 s with a dwell time of 10 s. From the ECL intensity of six consecutive pulses, the slope, in a.u. min^{-1}, was obtained and used as the analytical signal.

2.6. Procedures

2.6.1. Standards

Aqueous standard solutions of different concentrations of uric acid at pH 9.5 adjusted with 0.2 M phosphate buffer and 0.25 M NaCl were prepared. 50 μL of the standard or sample solution was taken and placed in the receptacle of the SPE cell. The uric acid-sensitive biosensor was placed in the black holder and covered with the lid holding the PMT, obtaining the analytical signal as indicated above.

2.6.2. Treatment of urine samples

A 24 h-urine sample was diluted 100-fold with 0.2 M phosphate buffer pH 9.5 and NaCl 0.25 M. After that, 50 μL was placed in the receptacle of the SPE cells working as previously described.

3. Results and discussion

This work prepared and characterized a biosensor for uric acid presents the novelty, respect to our previous work [14], of luminol immobilization by electropolymerization technique in order to be used as a disposable ECL biosensor that does not require the addition of any ECL reagents in the solution to be used.

Luminol is an aniline derivative that can be electropolymerized on macroelectrodes [17] or on SPE cells [18] obtaining a poly-

luminol layer that can be used to immobilize the luminophore reagent. However, this polymer presents some drawbacks, such as low adherence and low conductivity [19]. In order to improve the mechanical and electroluminescent properties of the film, a copolymerization study of luminol with different monomers of the family of aniline, benzidine and pyrrol was made previously [15]. From that study it was concluded that benzidine derivatives, and among them especially 3,3′,5,5′-tetramethylbenzidine, were the best option to prepare disposable ECL sensors for hydrogen peroxide that include all the reagents needed for their use. The goal of this study was to prepare an enzymatic ECL biosensor for uric acid that contains both a bioreagent (uricase) and a luminophore (luminol) immobilized on the working electrode of a disposable SPE cell. To this end, different benzidine derivatives were studied as a basis for reagent immobilization.

3.1. ECL SPE biosensor composition

3.1.1. Luminol copolymer selection

In order to select the best copolymer containing luminol for an uric acid ECL determination, a study of the ECL properties of copolymers used in our previous work [15] such as benzidine, *o*-dianisine, 3,3′-diaminobenzidine, 3,3′,5,5′-tetramethylbenzidine with luminol was made by preparing the polymeric film using a solution containing luminol and monomer 10^{-3} M each in H_2SO_4 0.2 M on the working electrode of gold LT SPE cells using 10 sweeps (0.1 V s^{-1}) from −0.2 to 1.0 V. The SPE cells prepared in this way were tested by adding 25 μL of a solution containing uricase 18.6 IU mL^{-1} and 25 μL of 0.5 mM uric acid in 0.2 M phosphate buffer pH 9.5. The reason of this experiment is to check the possible effects of using enzymes and uric acid instead of using only H_2O_2 for ECL emission. Table 1 shows that luminol containing the polymer formed from 3,3′,5,5′-tetramethylbenzidine presents better adherence and higher ECL emission intensity, nearly 26 times higher than using the other monomers. These results agree with our earlier study of a hydrogen peroxide ECL sensor [15]. For that reason, we selected TMB for the luminol copolymerization.

3.1.2. Electro-formation of the poly(luminol–TMB) copolymer

Electrochemical polymerization can be done using three procedures: (1) potentiostatically by using a sufficiently positive polymerization potential (E_{polym}); (2) potentiodynamically by cycling with a sufficiently high anodic limit or (3) galvanostatically by passing a constant anodic current [20]. The second technique is most widely used for polymer preparation and characterization, especially in the case of copolymer formation due to the fact that it offers more information about the process than the other techniques. For that reason, it was chosen to use in this case.

Cyclic voltammetry was used to study the copolymer formation. Working only under acidic conditions (0.2 M H_2SO_4) without monomers, an oxidation peak at 1.0 V was observed that corresponded to surface oxidation of gold by the water, subsequently obtaining a reduction peak at 0.75 V due to the reduction of Au oxides formed in anodic reaction on the working electrode [21]. In the case of a luminol and TMB solution, the cyclic voltammetry of Fig. 1 shows first cycle that indicates the oxidation of TMB (0.70 V) and luminol (0.95 V), observing in the first case the appearance of two cationic species, TMB^+ (blue colour, at 0.30 V) and diimine TMB^{2+} (yellow colour, at 0.62 V) [22] which resulted in the poly(luminol–TMB) blue species formation.

The cyclic voltammetry of Fig. 1 shows the formation of the poly(luminol–TMB) copolymer in a gold SPE cell immersed in a 10^{-3} luminol and TMB monomers solution. The copolymer formed after the first cycle shows a reduction (0.55 V) and an oxidation (0.69 V) peaks. Those peaks increases in intensity due to polymer grew with each new cycle, being noticeable the increases at 0.55 V peak.

Moreover, using only a solution containing H_2SO_4 appears a reduction peak at 0.75 V due to the reduction of gold oxides. But in the presence of monomers (luminol and TMB), this reduction peak (not observed in Fig. 1 due to the presence of monomers) disappeared because the oxidation of monomers avoid the oxidation of gold by water.

Table 1
ECL emissions of uric acid (0.5 mM) of different electropolymerized poly(luminol–benzidine derivatives) films on LT gold SPE cells.

Components	ECL relative intensity/a.u.	%[a]
Poly(luminol–benzidine)	33.9	1.0
Poly(luminol–3,3′,5,5′-tetramethylbenzidine)	3519.0	100.0
Poly(luminol–*o*-dianisine)	135.0	3.8
Poly(luminol–3,3′-diaminobenzidine)	130.1	3.7

[a] This column shows the percent comparison with respect to the best ECL emission obtained using different polymers.

3.1.3. Enzyme immobilization

In order to immobilize the enzyme uricase we first tried trapping it during the poly(luminol–TMB) copolymer preparation, producing in this way only one biosensing layer. Different experiments were performed working at different pH conditions but the copolymer only grew in strong acid conditions (0.2 M H_2SO_4) which denaturalize this protein.

A second approach was to immobilize the enzyme over the electroformed polymer by entrapment in a different polymer. For that purpose, chitosan, an N-deacetylated polyelectrolyte derived from biopolymer chitin, was used with good results.

An SEM study of the prepared SPE biosensor made it possible to differentiate the different layers. Fig. 2 shows a typical SEM image of a biosensor consisting of polycrystalline strands around the working gold electrode and a few bulky structures covered by a chitosan surface (Fig. 2A). In Fig. 2B, the first structure corresponds to a dendritic shape structure of poly(luminol–TMB) as was demonstrated in a previous work by morphological and energy-dispersive X-ray

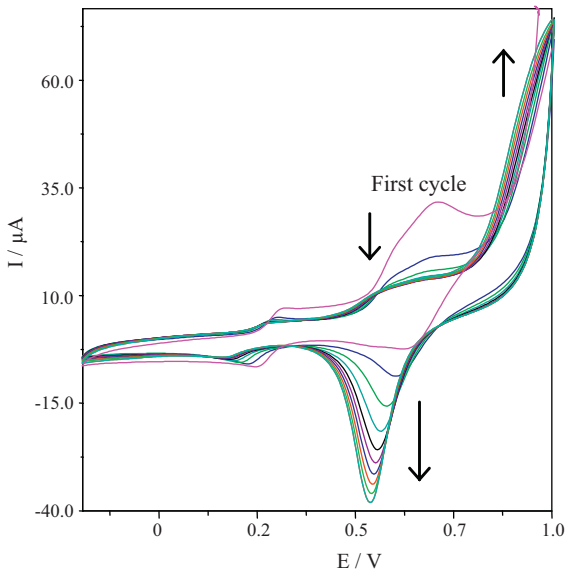

Fig. 1. Potentiodynamic formation of the poly(luminol–TMB) polymer using a solution that contains the monomers by cyclic voltammetry mode. The first cycle indicates the oxidation of the monomers. The conditions of copolymerization were 10^{-3} M each in H_2SO_4 0.2 M using gold LT SPE cells for 10 sweeps (0.1 V s^{-1}) from −0.2 to 1.0 V.

spectroscopy (EDS) microanalysis [15]. The second structure, that presents a bulky grooved form, is assigned to uricase enzyme in chitosan. Additionally, a chitosan layer covers the entire working electrode (Fig. 2C). Regarding to the thickness of the layer, values between 1.0 and 2.6 μm were obtained from side view SEM images (Fig. 2D).

3.2. Optimization of SPE uric acid biosensor composition

The preparation of the uric acid biosensor was influenced by different factors, such as the copolymerization conditions, namely: ratio of monomers, number of cyclic sweeps and concentrations of enzyme and chitosan.

3.2.1. Luminol–TMB copolymer ratio

The electro-formation of the copolymer depends on the luminol–TMB molar ratio. Different concentrations of luminol from 10^{-4} M to 10^{-2} M with a constant TMB concentration at 10^{-3} M were tested in order to prepare the copolymer, measuring the ECL signal produced using 25 μL uricase 18.6 IU mL^{-1} and 25 μL 0.5 mM uric acid. As the optimization parameter, we used the more robust ECL signal-to-noise ratio (S/N) instead of the ECL intensity. Fig. 3A shows that an increase in the luminol–TMB ratio increases the ECL S/N ratio up to a 1:1 ratio, decreasing appreciably afterwards. Cyclic voltammograms obtained at the different luminol–TMB ratios studied reveal very different behaviour depending on the ratio. At the lower ratio (0.1), a blue colour layer is formed, with the two peaks of oxidation (TMB^+ and TMB^{2+}) and reduction of TMB monomer (0.24 V) appearing more clearly in the cyclic voltammetry study (Fig. 3C).

The decrease in the TMB^+ peak current (0.30 V) after each sweep can be ascribed to a decrease in the conductivity of the formed polymer. An increase in the luminol–TMB ratio (10, Fig. 3D) results in a lower amount of blue polymer as well as in a strong diminishing of the TMB^+ peak current (at 0.30 V), in spite of the fact that it has the same TMB concentration as the experiment in Fig. 3C. We suggest that luminol can accelerate the oxidization of TMB^+ into TMB^{2+}, effects observed both in Fig. 1 (1:1 ratio) and Fig. 3D (10:1 ratio), much as Liu et al. propose for alizarin red in the presence of TMB [22]. Furthermore, at a high luminol–TMB ratio, a strong decrease is observed in the oxidation peak current at 0.69 V with each sweep, much higher than at the low ratio, which suggests worse conductivity for the copolymer prepared with a high luminol–TMB ratio. Then, taking into account the conductivity and ECL characteristics, the best luminol–TMB ratio is 1:1.

3.2.2. Cycle number

The cycle number used for copolymer formation is a very important factor. This study was carried out in the same conditions indicated above but at a 1:1 luminol–TMB molar ratio. The amount of poly(luminol–TMB) grew, and consequently did the ECL relative intensity, with the number of cycles up to 10 cycles decreasing substantially the ECL onwards (Fig. 3B). This behaviour can be ascribed to: (1) an ECL self-quenching due to the increased formation of diimine TMB^{2+} cations (absorption wavelength 450 nm and ECL emission centred at 445 nm) with the number of cycles [23] and (2) a decrease in the electrode conductivity with the loading of copolymer, which could produce a decrease in the ECL emissions [24]. 10 cycles were selected for the copolymer preparation.

3.2.3. Enzyme immobilization in chitosan

Uricase is the enzyme that catalyzes the oxidation of uric acid to allantoin and H_2O_2 by oxygen. The reaction of H_2O_2 with electro-oxidized luminol results in an ECL emission. As a polymer for enzyme immobilization, chitosan was selected because it retains the enzymatic activity of the enzyme and offers a stable layer [25] that is compatible with the poly(luminol–TMB) layer. The immobilization of the negatively charged uricase enzyme is based on the electrostatic interaction with positively charged chitosan [26], obtaining a second layer on the poly(luminol–TMB) prepared film.

First, we studied the influence of chitosan on uricase immobilization. Different concentrations of chitosan ranging from 0.5 g L^{-1} to 10 g L^{-1} were used working with a constant and small amount

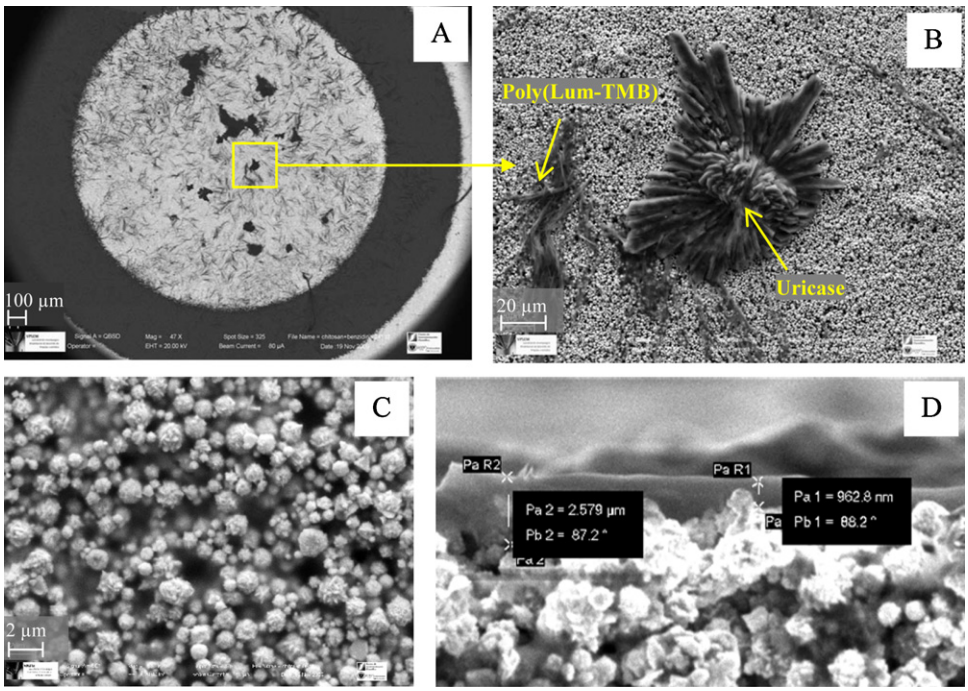

Fig. 2. SEM images of the uric acid gold SPE biosensor. (A) Poly(luminol–TMB) with chitosan and uricase; (B) polyluminol–TMB has a filament shape structure and uricase has a bulky form; (C) shape of chitosan on the gold SPE; (D) thickness of the layer on the SPE (side view).

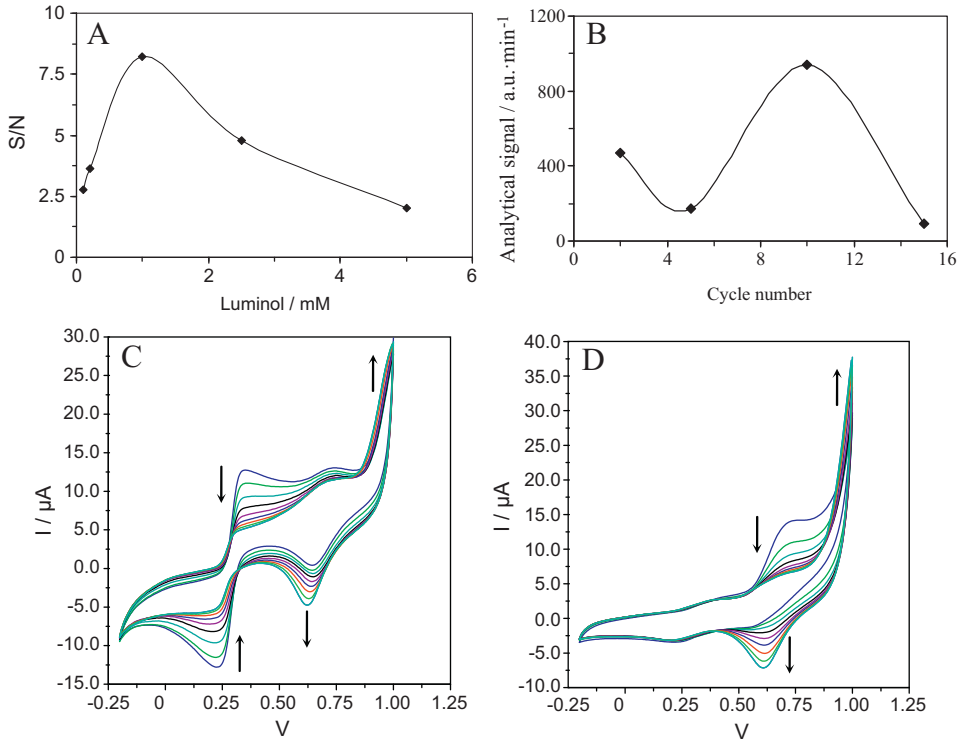

Fig. 3. Preparation of luminol copolymer. (A) optimization of the luminol:TMB ratio maintaining TMB concentration constant (1 mM); (B) optimization of cycle number for the selected 1:1 copolymer ratio; (C) aspect of the cyclic voltammetry using a 0.1:1 ratio; (D) aspect of the cyclic voltammetry for a 10:1 ratio. The conditions of this experiences were in all cases 1 mM of TMB, 0.1 V s^{-1} and H_2SO_4 0.2 M.

of uricase (18.6 IU mL^{-1}). The ECL relative intensity increased with the chitosan concentration used for preparation (Fig. 4A), reaching a maximum at 2.5 g L^{-1}. At higher chitosan concentrations, the ECL signals were nearly constant, although the membranes were brittle. Then, in order to immobilize as much of the enzyme as possible, 2.5 g L^{-1} of the chitosan solution was used.

Next, the amount of enzyme immobilized was optimized, working from 18.6 to 185.6 IU mL^{-1} and 0.5 mM in uric acid. An increase in the enzyme concentration improved the ECL S/N ratio (Fig. 4B) up to 93.0 IU mL^{-1}, then maintaining constant. The optimization at the uric acid concentration of 0.5 mM increased the analytical range, avoiding the inhibition by uric acid observed at 1 mM [27].

The immobilization of the enzyme in chitosan produces a reduction of some 36% in the ECL analytical signal with respect to the solution, due to a reduction in the conductivity occurring in the chitosan layer [28].

3.3. Biosensor measurement conditions

Different studies were performed to optimize the experimental conditions for the ECL biosensor measurement, namely pH, voltage used, waiting time before measurement, blank signal and ECL analytical signal.

3.3.1. pH dependence

The evolution ECL S/N net ratio (S/N_{sample}–$S/N_{blank\ signal}$) with pH shows two maximums: at 9.5 (S/N ratio 2.6) and 11.0 (S/N ratio 4.3) which could be due to the different pH influences on the two reactions: on the enzymatic oxidation of uric acid and on the luminol electro-oxidation, reactions that depend heavily on pH. Under weak alkaline conditions, the enzymatic activity was reinforced with respect to the oxidation of luminol, generating the first maximum at 9.5. The optimum working pH for this enzyme in solution is reported to be 9.0 [27], but its immobilization in a cationic polymer such as chitosan results in a positive displacement in the optimum pH (9.5), according to Goldstein [29,30]. In the case of hard alkaline conditions (pH 11.0) the luminol oxidation was reinforced due to an increased formation of the basic peroxide intermediate of luminol [31]. However, in these alkaline conditions (pH 11.0) a lower precision is obtained for uric acid because a decrease in the enzymatic activity of uricase occurs. Thus, pH 9.5 was selected as the working condition.

3.3.2. Electrode potential optimization

A chronoamperometric study using voltages from 0.2 to 0.9 V was done in order to obtain the best ECL emission intensity from the poly(luminol–TMB) copolymer. The ECL signal increased up to 0.6 V and maintained until 0.7 V, decreasing afterwards (Fig. 4C). This right shift of the maximum emission voltage with respect to the polyluminol immobilized in gold LT electrode, that is 0.5 V [15], is due to the presence of TMB moieties in the copolymer whose electron acceptor methylene groups hinder the oxidation, then shifting the voltage to higher values [20].

3.3.3. Blank signal and waiting time

It is known that luminol exhibits a blank ECL signal in the presence of oxygen due to the oxidation of luminol [32], but we observed in our SPE cells that in the presence of uricase, this blank signal is higher. The reason for the higher signal could be the presence of metals such as copper or iron in the enzyme, producing an extra catalytic effect [27,33]. However, the addition of uric acid dramatically reduced the ECL signal (4.7 times), instead of the expected increase in the signal from the enzymatic formation of H_2O_2 from uric acid, which hinders the potential analytical use of the biosensor. The reason for this behaviour lies in the ability of uric acid to act as a scavenger of radical species such as $OH^{\bullet}$ and electro-oxidized luminol, as was suggested by Radi et al. [34].

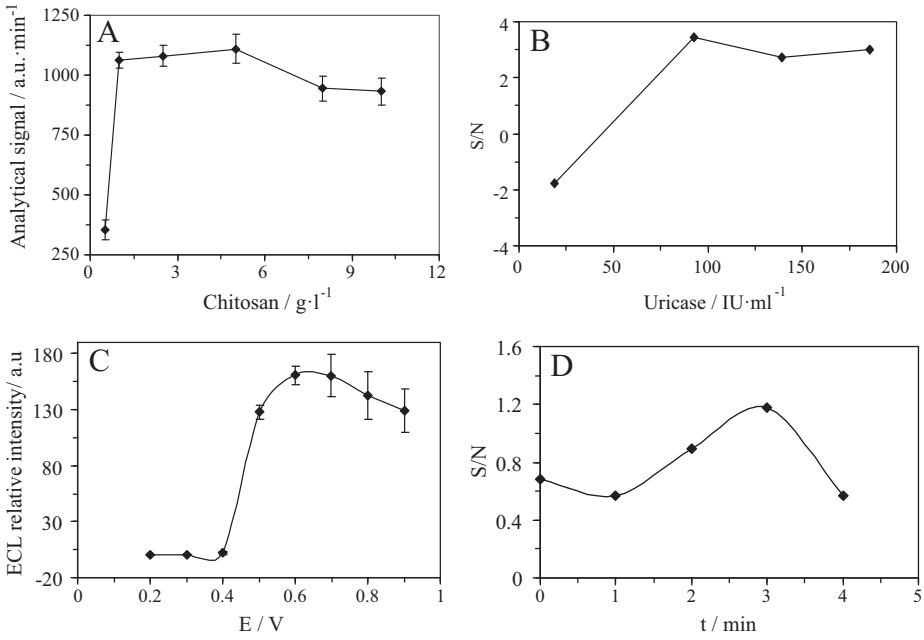

Fig. 4. Optimization of the uricase immobilization and measurement conditions of the biosensor. (A) Influence of chitosan concentration in membrane on analytical signal; (B) effect of the uricase concentration in membrane on S/N ratio; (C) study of excitation voltage on ECL relative intensity in gold SPE cell containing the copolymer; (D) study of the waiting time before measurement on S/N ratio. In all cases, 0.5 mM uric acid was used.

To overcome the problem, we studied the evolution of the analytical signal over time, observing that the blank signals obtained from the SPE cells in the absence and presence of uricase did not change with the waiting time before applying the chronoamperometric pulse. In that case, a potentiostatic mode was used to produce the ECL outcome. It is possible to produce ECL emission using a potentiodynamic mode (cyclic voltammetry), but the sweep time must be very short for a high ECL intensity. In the potentiostatic mode, the potentiostatic step is fast enough to obtain better ECL signal. In potentiostatic mode, the ECL signal grew with the waiting time in the presence of uric acid over the blank signal. The increases in H_2O_2 production over time can explain the experimental data, since one of the proposed mechanisms for ECL from poly(luminol–TMB) sensor is favoured, i.e., (a) oxidation of H_2O_2 by an electro oxidized luminol and (b) electro oxidation of H_2O_2 (0.60 V in our case [15] and 0.65 V in gold electrodes at pH 9.5 as Gerlache et al. report [35]) generating an excited superoxide radical (O_2^{-*}) that reacts with the luminol containing the polymer with an ECL outcome. These two possible concurrent mechanisms are responsible for the higher ECL emission of the poly(luminol–TMB) copolymer compared to luminol in the solution (1.35 times), but in this case the mechanism (a) is not favoured because of the uric acid present. We assume that mechanism (b) is responsible of the ECL production in this case.

Consequently, a study of waiting times from 0 to 5 min was made. Fig. 4D shows the evolution of the net S/N ratio ($S/N_{sample}-S/N_{blank\ signal}$). The signal grew with the waiting time up to 3 min, then decreasing, due to a possible consumption of H_2O_2 by the produced allantoin. This molecule is an active antioxidant that is readily attacked by reactive oxygen species such as H_2O_2 [36]. Therefore, 3 min were selected as the waiting time before the ECL measurement.

3.4. Analytical characterization

For the analytical parameter, we studied the evolution of the ECL signal in chronoamperometric mode: (a) ECL intensity using only one short pulse (<5 s); (b) ECL emission integration using a long pulse (>1 min); and (c) initial rate estimation. Comparing the precision and linearity range (lack-of-fit test), the third parameter offered the best results.

The initial rate was estimated from 5 to 6 successive pulses (Fig. 5) for every uric acid concentration. Conditions such as dwell time and pulse time were optimized obtaining 10 s and 1 s, respectively. The analytical signal is the rate of the ECL emission signal versus time, obtaining a growing tendency due to an enzymatic conversion measured from successive pulses. The slope obtained by this growing is the analytical signal, expressed in a.u. min^{-1}. In the inset of Fig. 5, two points of calibrate were included (slopes 3 and 5) showing how the analytical signal is obtained.

Using five standards from 2.0×10^{-6} M to 1.0×10^{-4} M and ten replicates each, a linear calibration was obtained. Fig. 5 shows the analytical signal against uric acid concentration. It is interesting to note that a linear calibration was obtained in this case instead of the usual logarithmic or double logarithmic behaviour that is typically found in enzymatic methods that use luminol as luminophore [37]. The limit of detection (LOD) obtained using the IUPAC standard criteria was 4.4×10^{-7} M (Table 2). The relative standard deviation (RSD) was 13.1% in the medium level of the range (1.0×10^{-5} M) ($n = 10$) using different biosensors each time.

3.5. Interference study

From the different species present in urine samples such as K^+, NH_4^+, Mg^{2+}, glucose, lactose, urea and ascorbic acid, only two (the last two) act as interferences in electro-analytical and ECL methods [13]. In our case, a study of urea and ascorbic acid was carried out to determine their effects on the ECL emission of the uric acid biosensor.

According to Burtis and Ashwood [38], the normal levels of urea are 428–714 mmol/24 h. We study the influence of urea between 0.2 and 0.8 mM in order to maintain the same ratio uric acid/urea (real ratio 0.428 and 0.71 mM) working at a level of 0.03 mM of uric acid. The results show an increase in the ECL signal when the urea concentration is higher than 0.6 mM (tolerance limit). The reason for this interference is due to a better electro-oxidation of lumi-

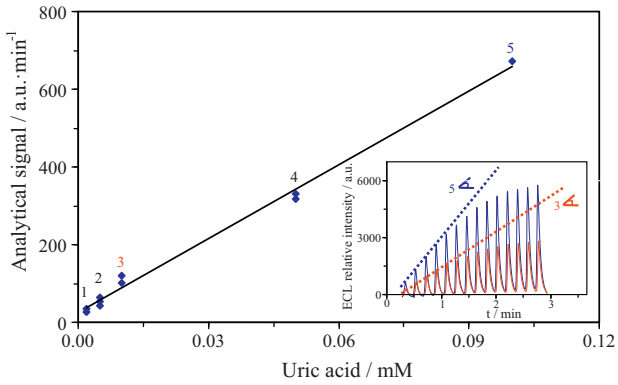

Fig. 5. Linear calibration for the uric acid biosensor. In the inset of the figure is showed the ECL relative signal against time. The analytical signal is the rate (slope) of the ECL emission versus time. The analytical signal is the initial rate obtained from enzymatic conversion measured from successive pulses. In this inset, two points of the calibration were included (slopes 3 and 5).

Table 2
Analytical parameters for uric acid SPE biosensor.

Parameter	Value
Range, M	1.5×10^{-6}–1.0×10^{-4}
Intercept	0.025 ± 0.007
Slope	6300 ± 200
R^2	0.994
LOD, M	4.4×10^{-7}
LOQ[a], M	1.5×10^{-6}
RSD (%) blank ($n = 10$)	4.7
RSD (%) uric acid ($n = 10$)	13.1 (using 1.0×10^{-5} M) 9.3 (using 5.0×10^{-5} M)

[a] LOQ: limit of quantification.

nol in the presence of urea [6]. In the case of ascorbic acid, the normal level is 0.30–0.36 mmol/24 h and using the same considerations than above (0.03 mM in uric acid) the studied concentration should be between 0.0036 mM and 0.0030 mM, but as the interference level in this range is very low, we studied the influence between 0.2 and 1 mM. In this case, a reduction in the ECL signal was observed, with a tolerance limit of 0.2 mM. This inhibition is due to the easier oxidation of ascorbic acid than luminol [39] and the subsequent oxidation of the dehydroascorbic acid formed to diketogluconic acid, decreasing the oxygen present and thus the enzymatic H_2O_2 production [40].

3.6. Application to urine samples

These biosensors were applied for uric acid determination in urine samples. Then, a 24 h-urine sample was taken and diluted 100-fold with 0.2 M phosphate buffer pH 9.5 with 0.25 M of NaCl in order to eliminate the matrix effect. The validation was performed by comparing the results obtained using the biosensors with a conventional method using a clinical auto-analyzer, resulting in *p*-values higher than 5% (Table 3).

Table 3
Determination of uric acid in urine samples by proposed and reference procedures.

Urine Samples	ECL Biosensor, M	Reference procedure, M	RSD[a] %	*p*-Value %
1	$(1.5 \pm 0.2) \times 10^{-3}$	$(1.5 \pm 0.1) \times 10^{-3}$	2.40	63.4
2	$(1.1 \pm 0.1) \times 10^{-3}$	$(9.9 \pm 0.4) \times 10^{-4}$	4.90	29.8
3	$(5.9 \pm 0.7) \times 10^{-4}$	$(6.0 \pm 0.3) \times 10^{-4}$	0.79	34.1
4	$(9.2 \pm 0.8) \times 10^{-4}$	$(9.4 \pm 0.4) \times 10^{-4}$	1.61	50.0
5	$(9.4 \pm 0.4) \times 10^{-4}$	$(9.6 \pm 0.1) \times 10^{-4}$	1.60	50.3

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

4. Conclusions

This paper presents an electrochemiluminescent disposable biosensor using gold screen-printed cells for uric acid determination in urine samples. All the reagents needed for ECL production are immobilized as a double-layer on the working electrode of the SPE cells. A first detection layer is based on the formation of a poly(luminol–TMB) copolymer, which offers improved characteristics such as better adherence and ECL intensity than polyluminol. This formed layer presents better emissive characteristics than the conventional entrapment by using cellulosic polymers as Methocel. A second recognition layer contains the enzyme uricase using chitosan to entrap the protein. To the best of our knowledge, this is one of the very few ECL biosensors for uric acid based on the increase of ECL with analyte concentration using luminol as luminophore and not, as usual, on the inhibition of ECL because inhibition-based methodology generates worse limit of detection and more inaccuracy in the determinations. This is attributed to the higher oxidation potential of poly(luminol–TMB) than luminol and polyluminol, which produces another mechanistic pathway that results in an ECL outcome in the presence of uric acid. The method presents a range of two orders of magnitude with linear calibration and a low detection limit of 4.4×10^{-7} M. The interferences of urea and ascorbic acid were eliminated by a 100-fold dilution that enables their use for uric acid determination in 24 h-urine samples. The presented uric acid biosensor offers an easy test for analysis in a disposable format.

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