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ORIGINAL PAPER

New electrochemiluminescent biosensors combining polyluminol and an enzymatic matrix

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Abstract Performant reagentless electrochemiluminescent (ECL) (bio)sensors have been developed using polymeric luminol as the luminophore. The polyluminol film is obtained by cyclic voltammetry (CV) on a screen-printed electrode either in a commonly used H_2SO_4 medium or under more original near-neutral buffered conditions. ECL responses obtained after performing polymerization either at acidic pH or at pH6 have been compared. It appears that polyluminol formed in near-neutral medium gives the best responses for hydrogen peroxide detection. Polymerization at pH6 by cyclic voltammetry gives a linear range extending from 8×10^{-8} to 1.3×10^{-4}M H_2O_2 concentrations. Based on this performant sensor for hydrogen peroxide detection, an enzymatic biosensor has been developed by associating the polyluminol film with an H_2O_2-producing oxidase. Here, choline oxidase (ChOD) has been chosen as a model enzyme. To develop the biosensor, luminol has been polymerized at pH6 by CV, and then an enzyme-entrapping matrix has been formed on the above modified working electrode. Different biological (chitosan, agarose, and alginate) and chemical (silica gels, photopolymers, or reticulated matrices) gels have been tested. Best performances have been obtained by associating a ChOD-immobilizing photopolymer with the polyluminol film. In this case, choline can be detected with a linear range extending from 8×10^{-8} to 1.3×10^{-4}M.

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Introduction

Electrogenerated chemiluminescence (ECL) of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) is widely used to develop very sensitive biosensors allowing detection of analytes at low concentrations and over a wide linear range [1–3]. The ECL reaction is triggered by the application of a potential on an electrode. In aqueous alkaline solution, the oxidation of luminol in the presence of hydrogen peroxide generates a characteristic blue light emission whose intensity is proportional to H_2O_2 concentration. Hydrogen peroxide can also be envisioned as the product of oxidase-catalyzed reactions, thus extending the detection to other substances.

ECL (bio)sensors generally require the addition of the luminophore in solution. Luminol immobilization on a transducer surface allows to develop simple reagentless sensors. Zhang and Chen previously described luminol polymerization on a glassy carbon electrode in 0.5 M H_2SO_4 [4]. Other groups also formed polyluminol in an acidic medium on conventional electrodes [4–9]. Recently, we first demonstrated that luminol can be polymerized on a screen-printed electrode (SPE) in a near-neutral medium, thus preserving the activity of an enzyme that could be directly entrapped in the polymeric film [10]. The polyluminol film that formed on the electrode surface behaves as a luminophore, like the monomer present in solution, enabling the ECL reaction in the presence of H_2O_2 to proceed.

Based on such a performant sensor for H_2O_2 detection, we have developed an enzymatic biosensor by associating

polyluminol and an oxidase-immobilizing matrix. Choline oxidase has been chosen as a model enzyme.

To develop biosensors for choline detection, we have tested a bilayer configuration associating polyluminol with a ChOD-immobilizing matrix. Luminol has been first polymerized at pH6 onto the SPE. Then, choline oxidase has been immobilized in a matrix formed on the modified screen-printed electrode. In the presence of choline, hydrogen peroxide is produced by the oxidase-catalyzed reaction. When an appropriate potential is applied on the electrode, the oxidized polyluminol reacts with H_2O_2, producing light the intensity of which is proportional to choline concentration in a definite range.

Different immobilization techniques have been reported in the literature to develop choline biosensors. In our group, ChOD has commonly been immobilized on DEAE (diethylaminoethyl)–Sepharose beads that were entrapped in a photopolymer [11–16]. Cross-linking between ChOD, BSA, and glutaraldehyde has also been reported [17–26]. Choline oxidase has also been immobilized by adsorption [27–29], via avidin–biotin reaction [30] or by covalent immobilization [31–33]. Entrapment in a silica gel has also been reported [34, 35].

In the literature dealing with polyluminol, only one biosensor coupling glucose oxidase and polyluminol was described, the polymer being formed in a very acidic medium [7]. Glutaraldehyde was used as a cross-linking agent in this case, and quite high glucose concentrations could be detected (linear range 10^{-5}–10^{-3}M). In our work, choline oxidase has been chosen as a model enzyme, and immobilization procedures have been tested, most of them being used for the first time in a polyluminol-based chemiluminescent biosensor. Adsorption represents the easiest way to immobilize enzymes but it is well-known that weak interactions (hydrogen bonds, Van der Waals...) are involved in this case so that enzyme leaching is possible. Entrapment has been chosen to obtain high enzyme activity in mild conditions on the electrode. Direct coating of the working electrode with a mixture of enzyme and pre-polymer is easy to perform, and it does not need complex reaction processes like for covalent linkage for instance. In this work, different chemical (three photopolymers, silica matrices, and a reticulated gel) and biological (alginate, chitosan, and agarose) gels have been tested and compared for the design of performant ECL reagentless biosensors, allowing the detection of oxidase-substrate compounds. The chosen biological matrices have not previously been used to immobilize choline oxidase. They represent new immobilization supports for this enzyme. It is also the first time that an enzyme is pre-immobilized on positively charged beads before being entrapped within a biological matrix. An original process has also been used in order to form thin alginate films.

Experimental

Reagents

Hydrogen peroxide, veronal (sodium diethylmalonylurea), sulfuric acid, potassium dihydrogen phosphate were obtained from Prolabo. Potassium chloride was supplied by BDH. Luminol (3-aminophthalhydrazide), sodium bicarbonate, albumin from bovine serum (BSA), choline oxidase from *Alcaligenes* species, and choline were purchased from Sigma. Tetramethoxysilane (TMOS), tetraethoxysilane (TEOS), chitosan, and alginate were obtained by Fluka. Nafion and glutaraldehyde were supplied by Aldrich. DEAE–Sepharose (average particle size 45–165 μm) fast flow was obtained from Pharmacia. Agarose was obtained from Euromedex. Poly(vinyl alcohol)-bearing styrylpyridinium groups (PVA-SbQs) with different characteristics have been used. PVA-SbQ A was purchased from Polysciences (SbQ content of 1.025%, solid content of 13.3), while PVA-SbQ B (SbQ content of 1.06%, solid content of 11.1, polymerization degree of 2,300, saponification degree of 88, pH6.2) and C (SbQ content of 2.94%, solid content of 14.48, polymerization degree of 500, saponification degree of 88, pH6.3) were from Toyo Gosei Kogyo.

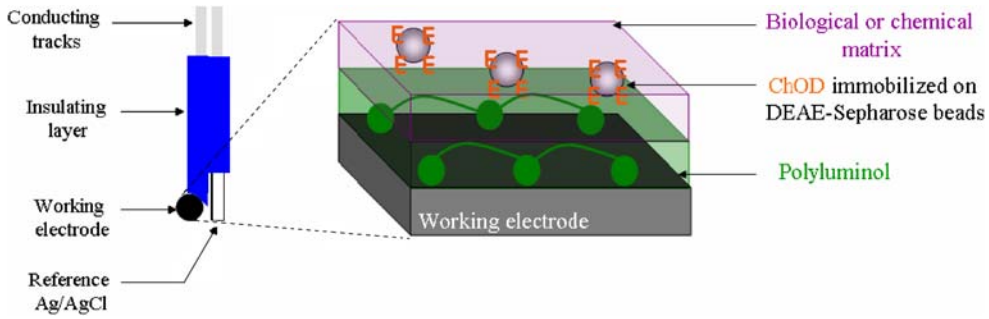
All buffers and aqueous solutions were prepared with double-distilled demineralized water. A 5.5 mmol L^{-1} luminol stock solution was prepared in 10^{-2}M KOH.

Electrode fabrication

Screen-printed electrodes comprise a carbon working electrode (area, 19.6 mm^2) and a printed Ag/AgCl reference electrode (Scheme 1), and they are mass-fabricated in-house by a multi-stage screen-printing process using a DEK 248 screen-printing machine (DEK, UK). A polyester monofilament fiber screen (DEK, UK) characterized by a mesh size of 260 counts per inch and a thickness of 13 μm was used to print the different inks onto a plastic sheet. The basal tracks were printed first using a silver-based ink (Electrodag 418 SS, Acheson, USA) and dried 15 min at 100°C. In the second step, the carbon paste (Electrodag PF-407A, Acheson, USA) was printed onto the basal tracks and cured for 15 min at 100°C. Then, the Ag/AgCl paste (Electrodag 6037 SS, Acheson, USA) was printed onto one of the basal tracks and dried at 100°C. Finally, an insulating ink was deposited (Minico M7000, Acheson, USA), which was cured for 20 min at 100°C.

Electrode pre-treatment

An electrochemical pre-treatment has been applied to SPEs in order to remove organic ink constituents or contami-



Scheme 1 Choline biosensor configuration. A two-electrode system is used with a carbon working electrode and a printed Ag/AgCl reference electrode. Luminol is polymerized on the working electrode by cyclic voltammetry, thus forming the polyluminol layer. Then, a biological or chemical matrix is formed, giving the enzymatic layer, including choline oxidase immobilized on DEAE–Sepharose beads via electrostatic interactions

nants, to increase surface roughness or functionalities, and thus to improve ECL detection performances [36, 37].

Electrochemical pre-treatments of SPEs were performed using a Voltalab PGZ 301 potentiostat (Radiometer Analytical, Villeurbanne, France). An auxiliary platinum electrode and a SPE connected to the potentiostat were placed in 1 M $NaHCO_3$. Pre-treatments were carried out by applying an anodic potential of 1.3 V vs. Ag/AgCl until a total charge of 1.2 C. This procedure has been shown to represent the optimized screen-printed electrode pre-treatment conditions to use before performing ECL measurements [38].

Luminol polymerization and electrochemical characterization

The electrochemical formation of polyluminol films was performed using a Voltalab PGZ 301 potentiostat. An auxiliary platinum electrode and a screen-printed two-electrode system connected to the potentiostat were placed in a medium containing luminol. Soluble oxygen was not removed from the monomer solution prior to polymerization which was performed by cyclic voltammetry:

1. in 0.1 M phosphate/0.1 M KCl pH6 containing 1 mM of luminol. Polyluminol films were formed by ten scans, in the potential range from 0 to +600 mV vs. Ag/AgCl, at a scan rate of 100 mV s^{-1}.
2. in 0.5 M H_2SO_4 containing 1 mM of luminol. Luminol was polymerized in the potential range from 0 to +900 mV vs. Ag/AgCl with a scan rate of 100 mV s^{-1} (scan number: 10).

Before and after luminol polymerization, electrochemical characterization was performed in 0.1 M phosphate/0.1 M KCl buffer pH6. For this purpose, the applied potential was varied between 0 and +600 mV vs. Ag/AgCl at a scan rate of 100 mV s^{-1}.

Enzyme immobilization

Biosensor configuration

In this work, a ChOD-immobilizing matrix was associated to a polymeric luminol film to develop a polyluminol-based biosensor allowing choline detection. A bilayer configuration was developed (Scheme 1). Luminol was first polymerized at pH6 by cyclic voltammetry onto a SPE. Then, ChOD-immobilizing beads were entrapped in a membrane formed on the above modified working electrode.

Enzyme immobilization on DEAE–Sepharose beads

ChOD exhibits a low p*I* (p*I*=4.5) so that it is negatively charged in a 30 mM veronal–HCl pH9 alkaline buffer. The enzyme was thus immobilized on anion-exchanger beads by electrostatic interactions. DEAE–Sepharose (100 µL) was packed from ethanol slurry into a plastic column and equilibrated with 10 mL of 30 mM veronal–HCl, pH9. Thereafter, the gel was loaded with 10 mg mL^{-1} solution of ChOD (200 µL). Enzyme immobilized on DEAE-beads was then stored at +4°C in the same buffer.

ChOD-immobilizing bead entrapment

The enzymatic activity deposited in photopolymers, silica matrices, or biological (chitosan, agarose, and alginate) gels was 0.16 µmol min^{-1}. The enzymatic activity deposited in a membrane obtained by cross-linking between glutaraldehyde, BSA, and ChOD was 2.7 µmol min^{-1}.

1. Biological matrices

   (a) Alginate membrane

Alginates are derived from brown seaweed extracts and are composed of 1,4-linked β-D-mannuronic acid and α-D-

guluronic acid. Sodium alginate is a monovalent salt that forms a gel when dispersions of alginate and calcium ions are in contact with each other. Contrary to more classical experimental procedures involving deposition of a sodium alginate solution followed by immersion in a calcium-rich medium [39], it can be noticed that an original protocol has been used here in order to form thin alginate films. The influence of several parameters has been studied (data not shown) before defining the following operational mode.

A 1% (*w*/*v*) alginate solution was prepared by dissolving sodium alginate powder in 30 mM veronal–HCl buffer pH 9. ChOD-immobilizing beads (10 μL) were mixed with 60 μL of sodium alginate; 10 μL of this solution was deposited onto the working electrode that was let dry under ambient air. Alginate membrane was formed by adding 30 mM calcium chloride (10 μL) on the dried surface.

(b) Chitosan membrane

Chitosan is a *N*-deacetylated derivative of chitin, a natural biopolymer found in the exoskeleton of crustaceans or insects and in fungal cell wall.

Chitosan is insoluble in water, but the presence of amino groups renders it soluble in acidic solutions below pH about 6.5. A 1% (*w*/*v*) chitosan solution was prepared by dissolving chitosan flake in 2% (*v*/*v*) acetic acid. ChOD-immobilizing beads (10 μL) were mixed with 50 μL of 1% chitosan solution and 10 μL of 30 mM veronal–HCl buffer pH9; 10 μL of this mixture was deposited onto the working electrode and let dry under ambient air.

(c) Agarose membrane

A 1% (*w*/*v*) agarose solution was prepared by heating 1 g of agarose powder in 100 mL of 30 mM veronal–HCl buffer pH9. Melted agarose (60 μL) was mixed with 10 μL of ChOD-immobilizing beads. The final mixture (10 μL) was deposited on the working electrode and dried under ambient air. The agarose gel formed when temperature decreased.

2. Chemical matrices

(a) PVA-SbQ photopolymers

ChOD-immobilizing beads were entrapped in PVA-SbQs photo-cross-linked matrix. Three photo-cross-linkable polymers differing in structure (especially SbQ content) were used. A mixture of PVA-SbQ A, B, or C (10 μL), ChOD-immobilizing beads (17 μL), and 0.1 M phosphate/0.1 M KCl buffer pH6 (93 μL) was prepared; 10 μL of this mixture was deposited on the working electrode. Then, photopolymerization was performed for 30 min under a 60 W tungsten lamplight. The distance between modified SPE and lamp was 20 cm.

(b) Silica matrices

Two precursors have been tested: TMOS and TEOS.

The silica sol was prepared by mixing 0.5 mL of precursor, 0.6 mL of H_2O, 50 μL of 0.1 M HCl, and 0.25 mL of Nafion. This stock solution was stored at +4°C. Nafion was added as it is known that the presence of this polymer within a sol–gel-derived silica matrix prevents cracking [40]. The sensing layer was prepared by deposition of 10 μL of a mixture consisting of 10 μL of sol, 16 μL of ChOD-immobilizing beads, and 84 μL of 0.1 M phosphate/0.1 M KCl buffer pH7.

(c) Cross-linking

ChOD was immobilized onto the polyluminol-modified working electrode by cross-linking the enzyme with BSA through glutaraldehyde. 50 μL of 10% BSA (*w*/*v*) prepared in veronal buffer, 50 μL of ChOD (1 mg of ChOD in 50 μL of veronal buffer) and 20 μL of 5% glutaraldehyde solution (prepared in veronal buffer) were mixed. Then, 10 μL of this solution was deposited on polyluminol film. Membrane was air-dried at room temperature.

Experimental ECL system

The experimental ECL system has been described elsewhere [10, 12]. Briefly, a potentiostat (PRGE, Tacussel-Radiometer, Villeurbanne, France) was used to apply a fixed potential of +450 mV vs. Ag/AgCl. The electrode was placed in a glass cuvette protected from ambient light and containing 2.5 mL of 30 mM veronal–HCl buffer, pH9, under stirring. A liquid core optical fiber (L.O.T. Oriel, Courtaboeuf, France; core diameter 5 mm, overall diameter 7 mm), connected to the photomultiplier tube of a luminometer (Biolumat LB 9500, Berthold, Pforzheim, Germany), faced the electrode. Electrochemiluminescence measurements, expressed in arbitrary units (au), were registered on a graphic recorder (Servotrace, Sefram, Saint-Etienne, France). In the present work, luminol was polymerized on the graphite working electrode. The ECL reaction was initiated by injection of definite volumes of H_2O_2 or choline in 30 mM veronal–HCl buffer, pH9, delivered through a peristaltic pump (0.5 mm tube diameter, 4.3 mL min^{-1} flow rate).

Determination of choline oxidase activity

The activity of choline oxidase deposited in the matrix associated to polyluminol onto screen-printed electrodes has been estimated using an electrochemical method.

The measurement of oxidase activity was adapted from [41]. A platinum anode poised at a fixed potential of +650 mV vs. Ag/AgCl enables H_2O_2 detection. The electrode was immersed in 15 mL of 30 mM veronal pH 9. The concentration of choline was chosen equal to 11 Km, i.e. about 10 mM (the Km value was close to

0.9 mM). Choline was injected into the buffer prior to the enzyme in order to control the electrochemical interference generated by the analyte. Then, the enzyme was added into the buffer. H_2O_2 enzymatically produced vs. time is related to the oxidase activity. After electrode calibration with known concentrations of H_2O_2, the current variation due to the enzyme could be expressed in µmol H_2O_2 min^{-1}.

Results and discussion

Luminol polymerization by cyclic voltammetry

Figure 1 shows the consecutive cyclic voltammograms of polyluminol deposition in 0.1 M phosphate/0.1 M KCl pH6 (Fig. 1a) or in sulfuric acid medium (Fig. 1b). Whatever the polymerization medium used, the same general features could be obtained. The peak A results from the oxidation of luminol monomer. In the subsequent sweep cycles, the oxidation current of this peak decreases, whereas peaks B and C appear and gradually increase. The single redox couple is attributed to the oxidized (C) and reduced (B) forms of polyluminol, and its increasing intensity during consecutive scans clearly indicates the growth of the polyluminol film on pre-treated SPE. A special behavior can be observed at pH1.5. After the classical increases of intensity during the first scans, a decrease is observed. It

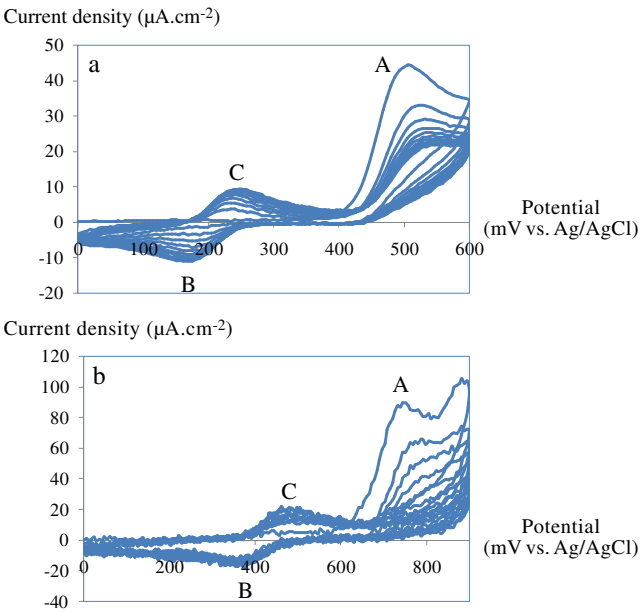


Fig. 1 Cyclic voltammogram of luminol polymerization process. CV was performed in **a** 0.1 M phosphate/0.1 M KCl pH6 or **b** 0.5 M H_2SO_4 containing 1 mM luminol on pre-treated SPE. The number of scans was ten, and the scan rate was 100 mV s^{-1}. *A* Peak of luminol oxidation, *B* reduced form of polyluminol, *C* oxidized form of polyluminol

can be related to differences in the kinetics of polyluminol film surface covering depending on the polymerization medium. As electrodeposition is favored at pH1.5, the polyluminol film more rapidly covers the electrode surface, thus forming a thick film with increased insulating properties, whereas the process needs more scans to be performed at pH6.

Cyclic voltammetry appears as a visual polymerization process that allows the polymer formation to be followed in real time. The efficiency of polyluminol electrogeneration can thus be directly checked by using this electrochemical mode. It clearly appears that luminol can be successfully polymerized either in 0.5 M sulfuric acid or in 0.1 M phosphate/0.1 M KCl pH6 buffer by cyclic voltammetry performed on electrochemically treated SPEs.

Sensor for H_2O_2 detection

We have developed a sensor for H_2O_2 detection based on luminol polymerized either in an acidic solution or a more original near-neutral medium. ECL performances of these systems can be compared, when luminol is electrogenerated in 0.5 M H_2SO_4 or in 0.1 M phosphate/0.1 M KCl pH6 buffer (Fig. 2b). The sensor based on luminol polymerized in a near-neutral medium by cyclic voltammetry is characterized by a sensitivity of 3×10^7 au M^{-1} and a linear range extending from 8×10^{-8} to 5.3×10^{-4} M H_2O_2 (Fig. 2b, curve A, inset, log(signal)=0.865 (log [H_2O_2])+6.791, R^2= 0.994), whereas with luminol polymerized in 0.5 M H_2SO_4, sensitivity is drastically lower (4.6×10^4au M^{-1}) and the responses to H_2O_2 are linear between 8×10^{-6} and 1.3×10^{-3} M (Fig. 2b, curve B, inset, log(signal)=0.833 (log [H_2O_2])+ 4.936, R^2=0.993). Sensors based on luminol polymerized by cyclic voltammetry in a buffer at pH6 instead of 0.5 M H_2SO_4 are characterized by a noticeable improvement in both sensitivity (3×10^7au M^{-1} at pH6; 4.6×10^4au M^{-1} in H_2SO_4) and concentration detection range. Figure 2a shows typical dynamic responses obtained under near-neutral polymerization conditions. The steady-state signal shows a response time of ca. 1 min, after polymerization either at pH6 (Fig. 2a) or at pH1.5 (data not shown). It can be underlined that using a near-neutral buffer as the polymerization medium presents two main advantages. Better responses for H_2O_2 detection are obtained, and the mild conditions are also more suitable for activity preservation of an enzyme that could be directly entrapped in the polymeric film.

Biosensor for choline detection

The performant sensor for H_2O_2 detection can also be used to detect many other oxidase-substrate compounds. In this work, ChOD has been chosen as a model enzyme. A

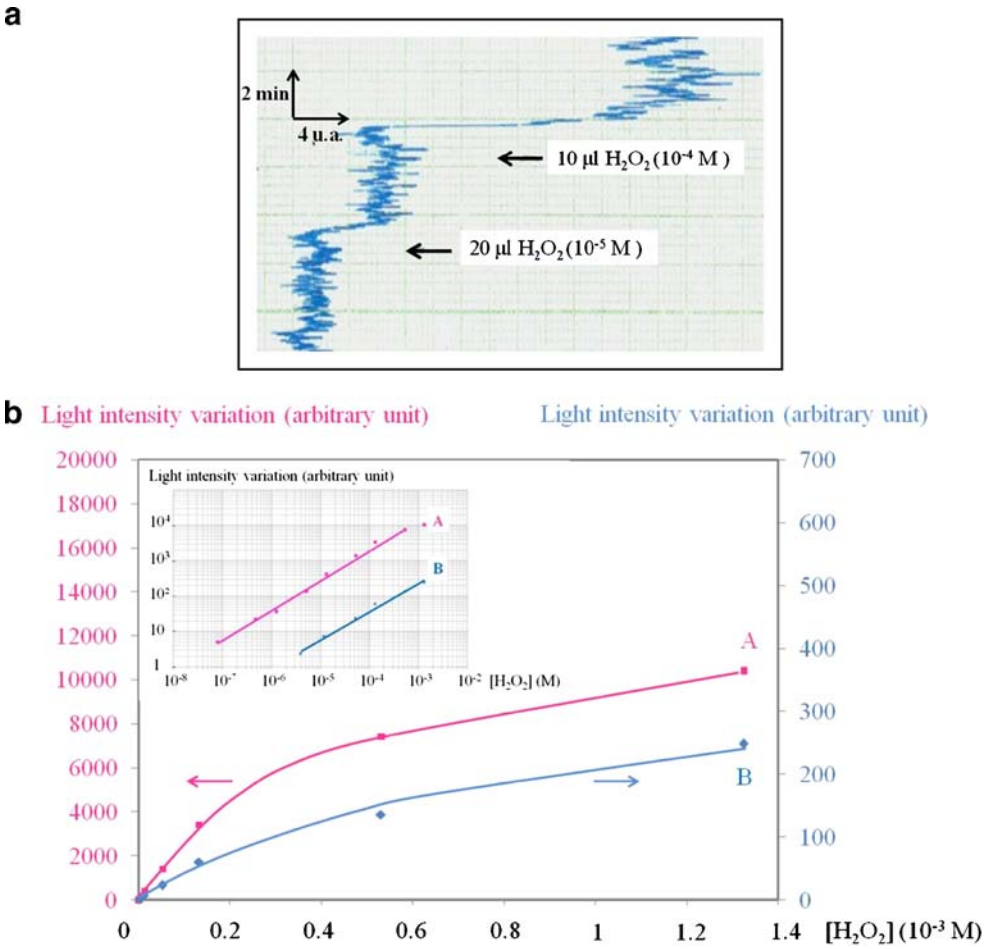


Fig. 2 Hydrogen peroxide sensor. **a** Dynamic responses to H_2O_2 injections of a sensor based on luminol polymerized at pH6. **b** Calibration curves (direct and log–log (*inset*) representations) for the detection of hydrogen peroxide based on luminol polymerized by cyclic voltammetry (ten scans, potential sweep from 0 to +600 mV vs. Ag/AgCl, 100 mV s^{-1}) in (*A*) 0.1 M phosphate/0.1 M KCl pH 6 or (*B*) 0.5 M H_2SO_4 containing 1 mM luminol

biosensor for choline detection has been developed by association of the polyluminol film with a ChOD-immobilizing biological or chemical matrix. We tried to include the enzyme in the polymerization medium during polyluminol formation. Unfortunately, it was not possible to immobilize enough choline oxidase activity under our experimental conditions and very low responses were obtained. Another way of immobilizing the enzyme has been found. Luminol has been polymerized by cyclic voltammetry at pH6 onto electrochemically treated SPE, and then the ChOD-binding beads have been incorporated in a gel formed on the modified surface (Scheme 1). Several chemical or biological immobilization matrices have been tested: photopolymers, reticulated matrices, silica, alginate, agarose, and chitosan gels. In order to be able to compare ECL performances obtained with the different matrices, the deposited enzymatic activity was always the same, except for the cross-linking membrane, where the deposited activity was higher.

Table 1 Influence of chemical polymers (sol–gel silica precursors, types of PVA-SbQ, glutaraldehyde cross-linking) on ECL detection of choline

Matrices	Precursors	Linear range for choline (M)	ECL response to 10^{-4}M choline (au)	Equations (y = log(signal), x = log[choline])	R^2
Sol–gel	TMOS	4×10^{-7}–1.3×10^{-4}	700	$y = 0.909x + 6.515$	0.992
	TEOS	7×10^{-7}–1.3×10^{-4}	500	$y = 0.946x + 6.486$	0.993
PVA-SbQ	A	4×10^{-7}–5×10^{-4}	800	$y = 0.949x + 6.73$	0.988
	B	8×10^{-8}–1.3×10^{-4}	1,000	$y = 0.779x + 6.066$	0.995
	C	7×10^{-7}–1.3×10^{-4}	500	$y = 0.964x + 6.545$	0.992
Cross-linking		8×10^{-7}–1.3×10^{-4}	400	$y = 0.936x + 6.392$	0.996

Luminol polymerization on pre-treated SPEs was performed by cyclic voltammetry and silica matrices, PVA-SbQ, or glutaraldehyde-based membranes entrapping ChOD-charged beads were formed above the polymeric film

Chemical matrices

Different chemical matrices have been tested: silica matrices based on two precursors (TMOS or TEOS), photopolymers based on three precursors with different characteristics (polymerization and saponification degree, SbQ, and solid content), and reticulated matrices.

Table 1 presents ECL performances obtained with different sol–gel precursors. It appears that the best responses for H_2O_2 detection were obtained with TMOS. In this case, the linear range extended from 4×10^{-7} to 1.3×10^{-4}M of choline (log(signal)=0.909 (log[choline])+6.515, R^2=0.992; Table 1 and Fig. 3b, curve B).

Other chemical matrices have been tested as oxidase-immobilizing structures to be coupled with a polyluminol layer. Photopolymers are commonly used to develop enzymatic biosensors. Table 1 presents ECL performances obtained with different PVA-SbQs. It appears that the best responses for H_2O_2 detection were obtained with PVA-SbQ B that is characterized by quite a high polymerization degree (2,300) and a SbQ content close to 1%. The best results were obtained with a low SbQ content and a degree of polymerization higher than 1,700, in agreement with Ichimura and Watanabe [42] and Leca et al. [43]. Using PVA-SbQ B, the linear range extended from 8×10^{-8} to 1.3×10^{-4}M of choline (log(signal)=0.779 (log[choline])+6.066, R^2=0.995; Table 1 and Fig. 3b, curve A). It can be underlined that choline was detected with the same linear concentration range than that obtained for H_2O_2 sensor (Fig. 2b, curve A). These results indicate that the hydrogen peroxide produced by the enzyme is directly detected via the polyluminol film, the sensor for H_2O_2 detection being not limiting. The close contact between choline oxidase and polyluminol allows hydrogen peroxide to be generated and in turn to trigger ECL reaction. Figure 3a shows dynamic responses to choline obtained with a biosensor associating polyluminol film and PVA-SbQ polymer. The response time is shown to be close to 1 min that is quite the same as the one noticed for H_2O_2 detection (Fig. 2a). The presence of the photopolymer does not induce noticeable diffusional constraints. Finally, photopolymers give rise to better responses than silica matrices obtained either from TMOS or TEOS precursors. Moreover, they do not suffer from "cracking" like silica gels do.

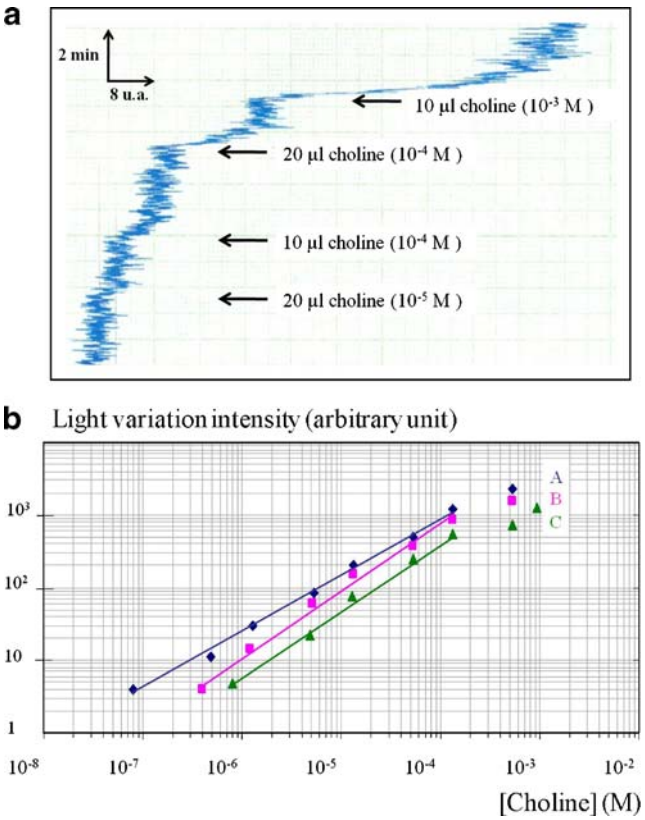


Fig. 3 Choline biosensor based on chemical polymers. **a** Dynamic responses to choline injections of a biosensor associating polyluminol and PVA-SbQ B polymer. **b** Calibration curves (log–log representation) for choline detection with biosensors based on polyluminol associated with (*A*) PVA-SbQ B, (*B*) silica matrix from TMOS, or (*C*) cross-linking membrane. Luminol polymerizations were achieved in 0.1 M phosphate/0.1 M KCl buffer pH6 containing 1 mM luminol by cyclic voltammetry (ten scans, between 0 and 600 mV vs. Ag/AgCl, 100 mV s^{-1})

During photopolymerization, polyluminol film is exposed together with the PVA-SbQ layer under light during 30 min. Fortunately, this light exposition does not appear to be detrimental for polyluminol luminescence. These results are in agreement with those reported in [15] with ECL biochips using luminol immobilized in a PVA-SbQ matrix. Light exposition does not appear to noticeably quench luminophore luminescence, for monomeric luminol as well as for polymeric luminol. Photopolymers are easy to prepare, and they seem to represent good candidates compared to brittle silica matrices.

Immobilization of the enzyme by cross-linking with glutaraldehyde and BSA is often achieved to develop choline biosensors [17–26]. However, glutaraldehyde is known to decrease enzyme activity so that it appears necessary to deposit a high activity. Thus, we have deposited an activity equal to 2.7 µmol min^{-1}. Choline could be detected with a linear range extending from 8×10^{-7} to 1.3×10^{-4}M (log(signal)=0.936 (log[choline])+6.392, R^2=0.996; Table 1 and Fig. 3b, curve C). Although the deposited enzyme activity was higher, the choline detection performances were lower than those obtained with silica matrices or photopolymers. Nevertheless, the performances are better (lower detected concentrations and wider linear range) than those previously reported with a polyluminol-based glucose biosensor that used glutaraldehyde for enzyme immobilization [7].

Table 2 Influence of biological polymers (chitosan, agarose, or alginate) on ECL detection of choline

Matrices	Linear range for choline (M)	ECL response to 10^{-4}M choline (au)	Equations (y = log(signal), x = log[choline])	R^2
Chitosan	4×10^{-7}–1.3×10^{-4}	600	$y = 0.792x + 5.966$	0.983
Agarose	8×10^{-7}–1.3×10^{-4}	150	$y = 0.836x + 5.62$	0.989
Alginate	4×10^{-6}–1.3×10^{-4}	100	$y = 0.928x + 5.801$	0.993

Luminol polymerization on pre-treated SPEs was performed by cyclic voltammetry, and biological polymer-based membranes entrapping ChOD-charged beads were formed above the polyluminol film

Biological matrices

Different biological matrices immobilizing ChOD have also been associated with a polyluminol film in order to develop a choline biosensor. To our knowledge, this is the first time that choline biosensors are developed using chitosan, agarose, or alginate matrices as ChOD immobilization matrices. Linear ranges and ECL responses to 10^{-4}M choline obtained with chitosan, agarose, or alginate as the ChOD-immobilizing matrix are presented in Table 2 and Fig. 4b. The biosensor comprising an alginate gel allowed choline detection between 4×10^{-6} and 1.3×10^{-4}M (log (signal)=0.928 (log[choline])+5.801, R^2=0.993; Table 2 and Fig. 4b, curve C). When an agarose gel was associated with polyluminol, better performances were obtained, and the linear range for choline extended from 8×10^{-7} to 1.3×10^{-4}M (log(signal)=0.836 log([choline])+5.62, R^2=0.989; Table 2 and Fig. 4b, curve B). The biosensor based on a chitosan matrix gave the best linear range, extending from 4×10^{-7} to 1.3×10^{-4}M (response to 10^{-4}M choline was 600 arbitrary units) (log(signal)=0.792 (log[choline])+5.966, R^2=0.983; Table 2 and Fig. 4b, curve A) close to performances obtained with TMOS-based silica gels (Table 1 and Fig. 3b, curve B). Figure 4a shows dynamic responses to choline obtained with a biosensor associating polyluminol film and a chitosan membrane. The response time was close to 1 min that is quite the same as the one previously noticed for H_2O_2 detection without enzyme (Fig. 2a) or for choline detection using PVA-SbQ-entrapped ChOD (Fig. 3a). In all cases, operational stability was seven to eight assays. The relative standard deviation (RSD) was 8.8% (n=8). Such results appear to be correct, keeping in mind that our devices are disposable.

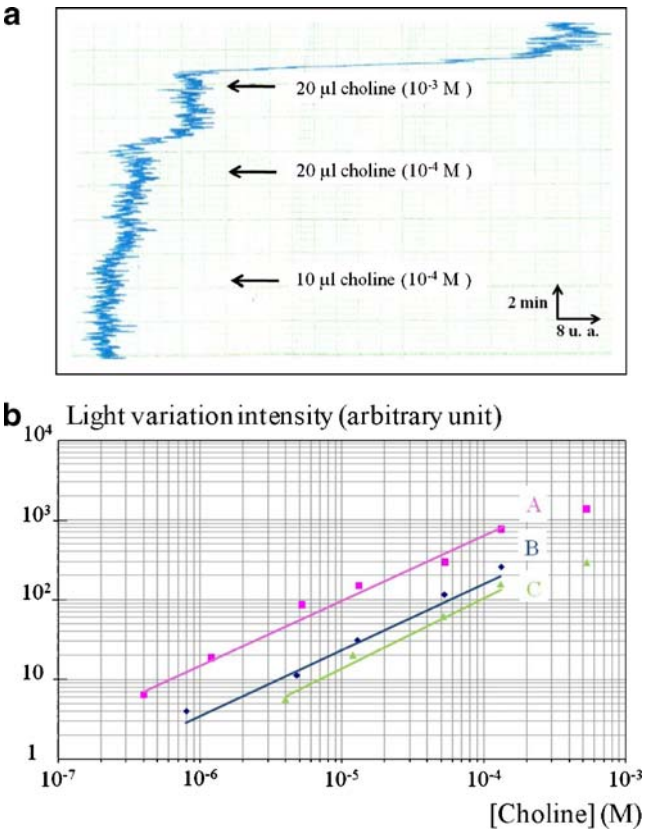


Fig. 4 Choline biosensor based on biological polymers. **a** Dynamic responses to choline injections of a biosensor associating polyluminol and chitosan polymer. **b** Calibration curves (log–log representation) for choline detection with biosensors based on polyluminol associated with (*A*) chitosan, (*B*) agarose, or (*C*) alginate gel. Luminol polymerizations were achieved in 0.1 M phosphate/0.1 M KCl buffer pH6 containing 1 mM luminol by cyclic voltammetry (ten scans, between 0 and 600 mV vs. Ag/AgCl, 100 mV s^{-1})

Finally, comparing chemical and biological matrices, the photopolymer B associated to a polyluminol film gives better responses for choline detection than the chitosan gel. Even if it was not so obvious at first glance, the photopolymer B appears as the best ChOD-immobilizing matrix to couple with polyluminol. Enzyme entrapment in a photopolymer presents advantages: This immobilization technique is easy and rapid, and contrary to silica gel, the PVA-SbQ matrix does not suffer from cracking.

Comparison with other choline biosensors

Table 3 is a compilation of the best performant biosensors for choline detection reported in the literature. Biosensors showing detection limit higher than 10^{-6}M are not presented in this table [15, 18, 20, 23, 29, 31, 32, 44].

Numerous amperometric biosensors for choline detection have been developed. Recent biosensors allow choline detection from around 10^{-7}M [21, 22, 35, 45]. Best

Table 3 Performances of choline biosensors obtained in this work and in other researches

Detection mode	Immobilization technique of ChOD	Linearity range (M)	Detection limit (M)	References
Amperometry	Entrapment in a silica gel	Up to 1.6×10^{-3}	5×10^{-7}	[34]
		5×10^{-6}–10^{-4}	10^{-7}	[35]
	Adsorption	5×10^{-7}–10^{-4}	5×10^{-7}	[27, 28]
	Adsorption/Dialysis membrane	5×10^{-7}–7×10^{-5}	10^{-7}	[45]
	Cross-linking	5×10^{-5}–2×10^{-3}	5×10^{-7}	[17]
		Up to10^{-4}	1.2×10^{-7}	[21]
		Up to 10^{-4}	3.3×10^{-7}	[22]
		10^{-6}–2×10^{-3}	7×10^{-7}	[26]
	Covalence	5×10^{-8}–2×10^{-5}	5×10^{-8}	[33]
	PVA-SbQ	Up to 10^{-4}M	2.5×10^{-9}	[43]
CL	Affinity	4×10^{-8}–10^{-4}	5×10^{-9}	[30]
	DEAE– Sepharose beads/PVA-SbQ	1.4×10^{-9}–4×10^{-5}	2×10^{-9}	[14]
ECL	DEAE– Sepharose beads/PVA-SbQ	4×10^{-8}–1.2×10^{-4}	4×10^{-8}	[11, 16]
		2×10^{-8}–10^{-4}	ND	[12]
		8×10^{-8}–1.3×10^{-4}	2×10^{-7}	In this work

performances were obtained with biosensors developed in our laboratory in 1990 [33] and in 1995 [43]. Choline oxidase was immobilized either on a chemically pre-activated Immunodyne membrane [33] or in PVA-SbQ [43], and the detection limit was as low as 5×10^{-8}M [33] or 2.5×10^{-9}M [43] (Table 3).

Optical biosensors appear more performant than the amperometric ones as they allow detection of lower choline concentrations (Table 3). Our group and Yao's team developed chemiluminescent or electrochemiluminescent biosensors for choline detection. In all cases, luminol was used in solution. Best performances were obtained with the chemiluminescent biosensor presented in [14]. In this work, ChOD was immobilized on DEAE–Sepharose beads, which were entrapped in a PVA-SbQ photopolymer. It is interesting to notice that such a photopolymer appears well adapted to choline oxidase entrapment, which can be coupled not only to amperometric [43] but also to optical detection, using luminol in solution [11, 12, 14, 16] or immobilized as shown here.

The choline biosensor described in this paper based on photopolymer-immobilizing ChOD associated with polyluminol gives high performances with a linear range extending from 8×10^{-8} to 1.3×10^{-4}M. Detection limit was 2×10^{-7}M (signal-to-noise ratio=3). Compared to other chemiluminescent or electrochemiluminescent biosensors, the main advantage of our system is that the luminophore is immobilized on the screen-printed electrode, so that our biosensor appears as a simple, reagentless, and disposable device. It must be kept in mind that our devices still need to be optimized.

Conclusion

Performant reagentless ECL (bio)sensors for choline have been developed using polymeric luminol as the luminophore instead of luminol in solution. Contrary to other groups that use an acidic solution to polymerize luminol, polymeric film was electrogenerated by cyclic voltammetry on a screen-printed electrode under near-neutral buffered conditions. H_2O_2 sensor based on luminol polymerized at pH6 has been shown to give better responses than the ones obtained by polymerization at pH1.5. A linear range from 8×10^{-8} to 1.3×10^{-4}M H_2O_2 was observed, whereas the only optical polyluminol-based biosensor previously reported in the literature showed a linear range between 10^{-5} and 10^{-3}M [7].

Based on the performant sensor for H_2O_2 detection, a bilayer biosensor for choline detection has been developed by associating polyluminol with a matrix immobilizing choline oxidase. Among chemical and biological matrices tested, best performances were obtained with a ChOD-immobilizing photopolymer associated with polyluminol. In this case, choline could be successfully detected in the same linear concentration range as for H_2O_2. Our system is as performant as ECL biosensors previously developed in our lab [12], and it presents the supplementary advantage to be easy-to-use and reagentless since luminophore is immobilized on the electrode surface.

Such a performant choline detection system should be further improved by optimizing sensing layer composition in terms of polymer and enzyme loadings. Moreover, monolayer enzymatic biosensors based on a "diffuse and polymerize"

process [38] could also be envisioned. The reagentless and disposable optical biosensors based on polymeric luminol electrochemiluminescence could be applicable to the detection of many other oxidase-substrate compounds.

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