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One-shot lactate chemiluminescent biosensor

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ABSTRACT

A new chemiluminescence-based one-shot biosensor for lactate is described. The lactate recognition system is based on lactate oxidase (LOx) and the transduction system consists of luminol, peroxidase from *Arthromyces ramosus* (ARP) and metallic aluminum, all immobilized in a polyion complex membrane. The measurement of the chemiluminescence in a luminometer when 1 mL of sample is injected into a conventional cell containing the disposable sensing membrane makes it possible to determine lactate. The compositions of the membrane and reaction conditions have been optimized to obtain adequate sensitivity. The one-shot biosensor responds to lactate rapidly, with the typical CL acquisition time being 2 min, with a linearized logarithmic dependence whose dynamic range was from 5×10^{-5} to 4×10^{-3} with a detection limit of 9.2×10^{-6} M and a sensor-to-sensor reproducibility (relative standard deviation, R.S.D.) of 5.5% at the medium level of the range. The performance of the chemiluminescent one-shot biosensor was tested for the analysis of lactate in yoghurt, validating the results against an enzymatic reference procedure. The proposed method is quick, inexpensive and sensitive and uses conventional CL instrumentation.

1. Introduction

L(+)-Lactate is produced in the anaerobic metabolism of glucose and its determination is of interest in clinical analysis, sports medicine and food analysis. The measurement of lactate is routinely performed with liquid chromatography [1], spectrophotometry [2] and amperometry, mainly with enzymatic electrodes [3]. Lactate analysis is needed in different fields such as food, sports medicine and health. In foodstuffs, lactate is produced by bacterial fermentation and is an essential component related to the manufacture of cheese, yoghurt, milk, and more, making monitoring lactate an important quality control parameter.

The rapid evaluation of blood lactate levels that are needed to determine the optimal workload for athletes, soldiers or workers in the field or to evaluate patients or animals in a point of care unit, can be performed with one-shot sensors [4]. Different types of one-shot sensors have been described for lactate and some of them are on the market, especially the fitness market, for use with portable handheld monitors, small benchtop monitors and freestanding monitors [5].

Most of the one-shot sensors described for lactate are based on enzymatic dry chemistries, on the usual basis of lactate oxidase (LOx) [6] and lactate dehydrogenase [7] with photometric [8] or amperometric transduction [9,10]. The chemiluminescent (CL) transduction [11] commonly based on enzymatic schemes, mainly lactate oxidase/peroxidase/luminol [12] and lactate dehydrogenase/NADH/bioluminescent enzymes [13] have been implemented in different types of flow [14,15] or fiber optic formats [12]. However, chemiluminescent detection has been used to a much lesser extent in one-shot sensors despite its advantages of high sensitivity and simple instrumentation, because of the more complex immobilization of

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all the chemistries needed and the need of time control of CL measurement.

Chemiluminescent one-shot sensors described up to date are mainly enzymatic or immunological and the CL measurement is carried out by a luminometer [16,17], a portable scanning luminometer [17] or different types of photographic films, especially instant photographic film [18–20], with a densitometer or a scanner [21] or even including the photographic film in the analytical element as a photoresponsive layer [22].

Different enzymatic chemiluminescent one-shot sensors have been described that typically are multilayered elements containing the immobilized reagents. These reagents can be located: (1) in one reagent layer as the described glucose sensor containing luminol, glucose oxidase and peroxidase in a mixture of gelatine and 1,2-bis(vinylsulfonylacetamido)ethane [23] or encapsulated in osmosensitive microcapsules to improve their lifetime; and (2) in several layers, as is the case with a described glucose sensor with enzymes in a micro-fiber glass disk and luminol in a cellophane membrane studied by Carter et al. [18].

One example of a non-enzymatic one-shot sensor has been proposed for hydrogen peroxide in rainwater based on a membrane of hydroxyethyl cellulose containing cobalt chloride as a catalyst and a surfactant [24].

Different heterogeneous and homogeneous chemiluminescent one-shot immunoassays have been described including nucleic acid hybridization assays, immunochemical assays such as sandwich assays, competitive binding assays and direct binding assays.

In the case of this heterogeneous assays the separation of unbound label compounds as well as interfering sample components from the reaction zone prior to the CL measurement can be accomplished by washing and removing the washing liquid with an adjacent absorbent layer. The application of a solution detection chemistry (luminol, hydrogen peroxide, surfactants, enhancers) triggers the CL reaction [16,17,25–27]. Alternatively, after washing the unreacted label from the membrane, a piece of paper wetted with CL chemistry can be placed next to this membrane to unleash CL emission [28]. In the case of homogeneous formats, a three-zone sensor is proposed. The second zone traps the unreacted labeled ligand coming from the first zone. The third zone contains the chemistry necessary to trigger the CL response [29].

The use of these one-shot chemiluminescent sensors is widespread and examples have been cited for hydrogen peroxide [24], hypochlorite [30], glucose [18,23], theophylline [29], the herbicide 2,4-dichlorophenoxyacetic acid [28], the herbicides atrazine, terbythylazine and ametryn [16], 4-*n*-nonylphenol [17], human chorionic gonadotropin [27], and DNA hybridization assays [19,26], among others.

The aim of this study was to develop and characterize an one-shot chemiluminescent biosensor for lactate, selecting yoghurt as an interesting and representative application, based on the generation of hydrogen peroxide by the couple lactate oxidase and peroxidase in the presence of metallic aluminum and subsequent reaction with luminol, which includes all the reagents and processes needed for the assay in one single membrane.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical-reagent grade. Reverse-osmosis type quality water (Milli-Q Plus185 from Millipore S.A.S 67120, Molsheim, France) was used throughout. L-(+)-Lactate 10^{-2} mol L^{-1} stock solutions in water were prepared using L-(+)-lactate lithium salt 97% from Sigma (Sigma–Aldrich Química S.A., Madrid, Spain) which was standardized by iodimetry. Solutions of lower concentrations were prepared by dilution with water. Other solutions used were Tris 0.1 M buffer from Trizma base 99% (Sigma) which were adjusted to different pH by adding NaOH or HCl.

The CL reaction were carried out by the following reagents: 10^{-2} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) stock solution from 97% (Sigma), lactate oxidase (LOx) from *Pediococcus species*, 39 IU mg^{-1}, from Sigma, peroxidase from *Arthromyces ramosus* (ARP), 758 IU mg^{-1}, from Fluka (Fluka, Madrid, Spain) and powder aluminum metal (<75 μm particle size) from Sigma.

For membrane making we used D4 and D6 polyurethane hydrogel, 60 g L^{-1}, from Tyndall–Plains–Hunter Ltd. (Ringo, NY, USA); polyvinyl triacetate, cellulose triacetate, the polycation poly-L-lysine (PLL) hydrobromide (MW 84,000 g mol^{-1}) and polyanion poly(sodium 4-styrenesulfonate) (PSS) (MW 70,000 g mol^{-1}) 30 wt% (density 1.16 g mL^{-1}) solution in water, all supplied by Sigma. As support for membranes we used sheets of Mylar-type polyester (Goodfellow, Cambridge, UK), both transparent and also opaque colored sheets and a metallized plastic mirror. Additional chemicals were tetrahydrofuran (THF), as a solvent, and the anion-exchange resins Sephadex QAE-25 and DEAE, and IRA 400 chloride form, all supplied from Sigma.

2.2. Enzyme preparation

The LOx solution was prepared taking 1.40 mg into a 1 mL of pH 8.8 Tris buffer 0.1 M and conserved in Eppendorf tubes (60 μL) at −20 °C. For ARP preparation, 0.22 mg was taking in 100 μL of pH 8.8 Tris buffer 0.1 M and conserved in Eppendorf tubes at −20 °C.

2.3. Apparatus and software

CL emission was measured using a CL-1 Camspec luminometer equipped with an external chamber for CL measurement using standard UV–visible cells, previously described [30]. This accessory consists of a metallic rectangular box with a built-on cell holder. The 12 mm wide cell holder is made of a matte black painted iron block that closes with a metallic lid to prevent external light from entering. The PMT is next to the cell and a switch makes the measurement possible. The cell lid has a central hole for sample introduction by means of a 6 cm PVC tube painted black. The whole accessory is shut with a black metallic cover that has a hole similar to that of the cell lid. To check the accessory, a LED-based test system was used. The luminometer was interfaced to a Pentium III microcomputer via an INT7—24 bit A/D Integration board for luminescence

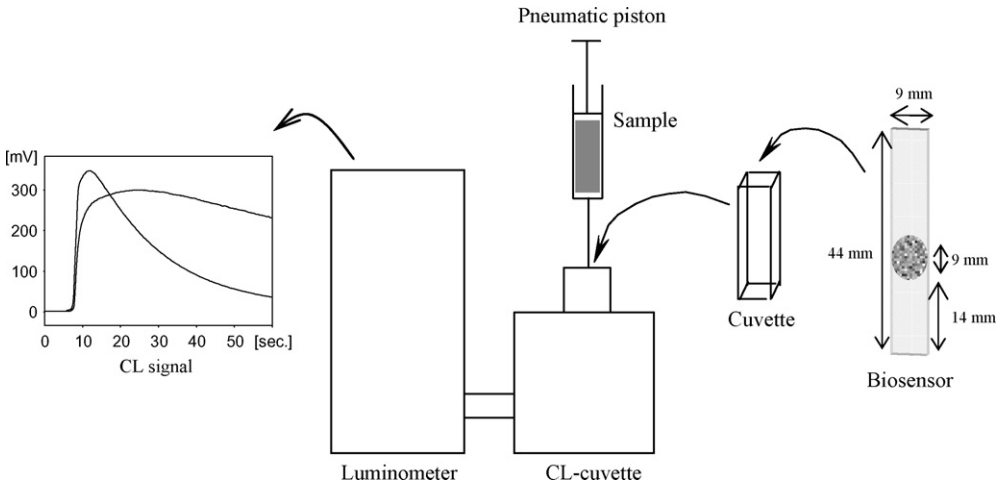

Fig. 1 – Instrumental setup for lactate determination.

acquisition and subsequent manipulation of data. The introduction of the sample in the luminometer was made by means of a conventional syringe of different volume with a double-acting pneumatic system designed by us (Fig. 1) to improve the reproducibility of the measurements.

Other apparatus and laboratory materials consist of a laboratory-made spin-on device [31], a Crison digital pH-meter (Crison Instruments, Barcelona, Spain) with combined glass-saturated calomel electrode and Hellma OS and QS 100 cells with 1, 2, 5 and 10 mm light path.

Software programs used for the treatment of the data were: CSW32 software package supplied by DataApex Ltd. v 1.2.5 (2001) (Prague, Czech Republic) used for the acquisition and manipulation of the luminescence data; Statgraphics software package (Manugistics Inc. and Statistical Graphics Corporation, USA, 1992), ver4.0 (1993).

2.4. *Membrane preparation*

The lactate-sensitive membranes were produced from a cocktail containing all the chemicals on a polyester substrate using a spin-coating technique. For the preparation of the cocktail the following procedure was used: to 3.50 mg of luminol were added 200 μL of 2×10^{-2} M NaOH in a 3 mL glass vial and this was ultrasonically treated for 15 min. Then, 1753 μL of pH 8.8 Tris buffer 0.1 M were added to the vial and shaken just 1 min and consecutively 1.68 mg of PLL, 9 μL of ARP peroxidase (15,500 IU L^{-1}) and 57 μL of LOx (710 IU L^{-1}) were added and shaken a minute after each addition. Finally we mixed 28.6 μL of an ultrasonicated solution for 5 min containing 10^{-4} M PSS and a mixture of dispersed powdered aluminum 2.96×10^{-3} M. The vial containing all chemicals was shaken for 2 min before use.

The disposable biosensors were cast by placing 30 μL of the cocktail prepared previously by a micropipette on a 9.0 mm × 44 mm × 0.5 mm dust-free thick transparent polyester sheet using a homemade spin-coater [31] rotating at 90 rpm. After spinning for 1 min, the membrane was removed from the spin coater and dried at room temperature for 4 h and then stored in refrigerator at 4 °C. The sensing area of the disposable biosensor is a 9 mm of diameter translucent circular film with a calculated thickness about 6 μm, considering the membrane as a cylinder and the density of the chemicals.

2.5. *Procedures*

2.5.1. *Procedure for samples and standards*

An aqueous standard solutions of lactate containing between 5×10^{-5} and 4×10^{-3} M in lactate with the pH adjusted to 8.8 with Tris buffer 0.1 M, was taken in a 1 mL syringe. A lactate-sensitive biosensor was placed on a 5 mm cell in front off the PMT of the cell holder of the luminometer external chamber. After closing the external chamber with the two lids, the sample was injected into the cell through the PVC tube by means of the syringe driven by a pneumatic piston, then measuring the CL emission without wavelength discrimination.

2.5.2. *Reference procedure*

As a reference procedure we used the enzymatic bioanalysis Boehringer Mannheim UV-Kit method from r-Biopharm AG (ATOM S.A., Barcelona, Spain) [32] for the determination of L-lactate in foodstuffs and other materials. The sample preparation used consists of mixing 2 g of yoghurt with 98 mL of water with an electric mixer, filtering and using 0.10 mL of the filtrate for the assay.

2.5.3. *Treatment of samples*

For the analysis of yoghurt samples, an adequate amount (typically 2 g) was weighed, mixed and homogenized with 200 mL of water and then filtered the solids through a conventional filter of paper (0.217 mm thick with 20–25 μm pore size) (Albet, Valencia, Spain). Then the procedure previously described was applied.

3. Results and discussions

3.1. *Immobilization of the reagents for the recognition chemistry*

The first step for the study of this one-shot chemiluminescent biosensor for lactate based on the atmospheric oxidation

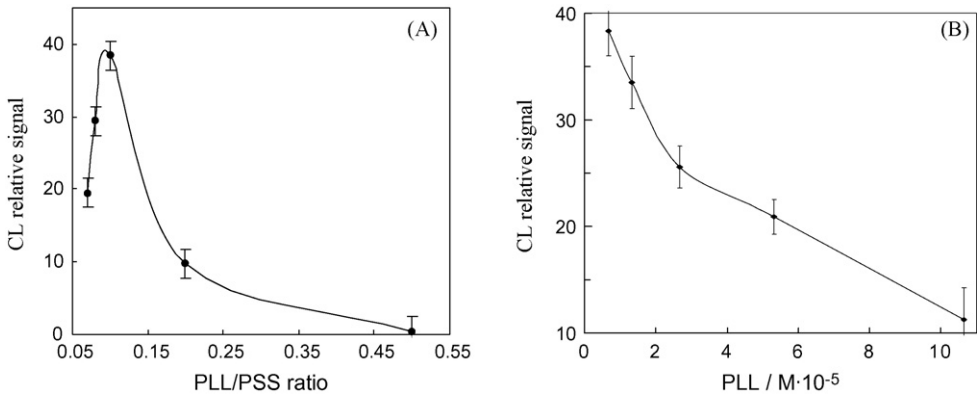

Fig. 2 – Influence of the composition of polyion complex membranes on light intensity. (A) PLL/PSS molar ratio in forming complex polyion membranes; (B) influence of PLL concentration at 0.1 PLL/PSS ratio. Each point was replicates five times with new biosensor each.

of lactate catalyzed by LOx and the subsequent reaction of hydrogen peroxide with luminol in basic medium catalyzed by peroxidase is to prepare a membrane containing all of the reagents needed to make the lactate recognition possible.

To include all these reagents in a membrane, we tested different immobilization systems; namely, (1) entrapment using D-4 and D-6 polyurethane, PVA and cellulose acetate; and (2) ion-exchange using polyion complex layer. The relative CL signals obtained were very different: PVA (0.4%), cellulose acetate (1.3%), D6 (0.1%), D4 (0.8%), and polyion complex (100%). The best results were obtained using a polyion complex membrane formed by reacting a linear polycation, such as poly-L-lysine, in the presence of anionic LOx, with a linear polyanion, such as poly-4-styrenesulfonate [33].

We initially tested two possible membrane arrangements: (1) a two membrane configuration with one membrane containing the lactate recognition chemistry – LOx – and the other containing the CL transduction – ARP and luminol –; and (2) only one membrane containing all reagents. Although it has been described that enzyme compartmentalization improves the performance of the sensor [34], we did not succeed preparing a two layer configuration efficiently using the polyion complex. Consequently, we included all the reagents in a single membrane.

The peroxidase selected was *A. ramosus* instead of horseradish peroxidase (HRP), due to its faster catalysis [35] because ARP binds luminol in specific sites [36].

In all cases the membranes were prepared from solutions of polymers and reagents in a solvent which are cast on a support with a spin-coating technique. We tested different types of solid supports for the sensing layer: transparent colorless and different opaque colored polyester and metallized plastic mirror in order to increase the CL signal, but the results were very similar in all cases. Then, we chose the transparent polyester strip as a convenient support.

We observed that signal produced by the sensing membrane using the polyion complex is two times higher than the same reaction in solution using the same reagents and experimental setup.

3.2. *Optimization of lactate one-shot biosensor composition*

Once the membrane materials had been selected, we studied different factors related to the membrane making, including the concentration of chemicals and membrane polymers. All these factors were studied univariantly. In all cases the parameter to optimize was the CL relative signal, that is, the CL signal for lactate subtracting the CL background produced in the absence of lactate.

The first factor to consider when preparing the membranes is the pH of the cocktail used to make the membranes. As the pH for maximum enzymatic activity of LOx (pH 7.5 [37]) and ARP (pH 9.5 [38]) are quite different, an 8.8 pH buffer is used first for the subsequent study as a compromise for optimum enzymatic activity and stability. The optimization of the working pH is studied in Section 3.3 below. The initial enzyme concentrations used according to the literature were: 15,500 IU L^{-1} for ARP [37], and 140 IU L^{-1} for LOx [39].

3.2.1. *Polyion complex layer composition*

The mixture of PSS and PLL produce a white precipitate that forms a membrane by evaporation. To find the best composition we studied the molar ratio PLL to PSS. Two solutions containing LOx, ARP, with luminol along with PLL in one of them, and PSS in the other were used, preparing different membranes on a Mylar polyester support by mixing different volumes, casting with a spin-on device system, and measuring the CL emission at 4×10^{-4} M lactate (Fig. 2A). The best PLL/PSS ratio is 0.1 which can be related to: (1) the electric charge of the membrane; thus, the PLL must be in excess to electrostatically interact with all the anionic reagents – LOx, ARP and luminol – and at the same time offer a positively charged membrane to allow the permeation of lactate; and (2) the thickness of the membrane which increases with the PLL/PSS ratio slowing the entrance of the lactate and hence decreasing the signal. A lower ratio than 0.1 produces brittle membranes and worse responses.

To study the dependence of the thickness of the membrane (between about 5 and 100 μm), a number of biosensors

were prepared with the optimum PLL/PSS ratio (0.1) but using different polymer concentrations. It was observed that the higher the polymer concentration, and thus a higher thickness, the lower the CL relative signal is (Fig. 2B). We selected 1.33×10^{-5} M in PLL and 9.52×10^{-7} M in PSS solutions for the preparation of the sensing membranes; due to the increase in the signal (13%) and the speed of the response (32%) observed with respect to the maximum values shown in Fig. 2A, concentrations less than the above cannot be used because they produce brittle membranes that dissolve in contact with any solution.

3.2.2. *LOx and ARP influence*

To optimize the concentration of both enzymes, we prepared one-shot biosensors using a constant ARP concentration, at a usual value of 15,500 IU L^{-1} [35], and modified the LOx concentration between 140 and 1000 IU L^{-1}. Then, and using the optimum LOx concentration found, we studied the ARP influence between 1500 and 110,000 IU L^{-1}. Taking into account the K_m constants (K_m ARP: 7×10^{-6} M [35], K_m LOx: 9.3×10^{-3} M [38]) and the concentration of both enzymes ([ARP] > [LOx]), we confirmed, considering the Michaelis–Menten equation, that LOx becomes the limiting factor, producing a more sensitive lactate biosensor. Fig. 3A shows the results obtained for both enzymes. The CL increases with LOx concentrations up to 710 IU L^{-1}. Then, the CL relative value reaches a plateau due to an excess in enzyme concentration with respect to the substrate. In the case of ARP, a maximum in the CL relative signal is observed which then decreases as a consequence of the contribution of the blank signal, which increases with ARP concentration, decreasing the CL relative signal, because of a reaction between luminol and ARP [40]. The optimum enzymatic charge in the membrane corresponds to 0.021 IU LOx and 0.46 IU ARP.

3.2.3. *Luminol dependence*

The CL relative signal increases with luminol concentration (Fig. 3B) up to 10^{-3} M, decreasing then slightly. This fact has been described for CL in solution [37,41] and justified by Misra and Squatrito [42] as due to the accumulation of products or dismutation of reactive intermediates rather than the depletion of reagents.

3.2.4. *Aluminum role*

We observed that the incorporation of powdered aluminum metal (Al) into the membrane increases the CL relative signal. Thus, the use of a small Al amounts in the membrane induces the magnitude enhancement in the CL relative signal more than two orders and reduces the experimental errors without increasing the blank signals. To optimize the Al content we prepared different biosensors containing dispersed Al particles (diameter <75 μm) ranging from 5.9×10^{-5} to 4.7×10^{-4} M. An increase in CL relative signal with Al concentration is observed up to 1.2×10^{-4} M, decreasing then (Fig. 3C). Thus, this concentration was used afterwards, which meant a molality of 1.82×10^{-6} m in Al in the dry thin sensing membrane. At Al concentrations in molarity higher than 1.2×10^{-4} M, the CL emission slows down. In order to clarify this phenomenon, Fig. 4 has been included, which shows only the CL emission of Al at higher concentrations (the blanks are not represented

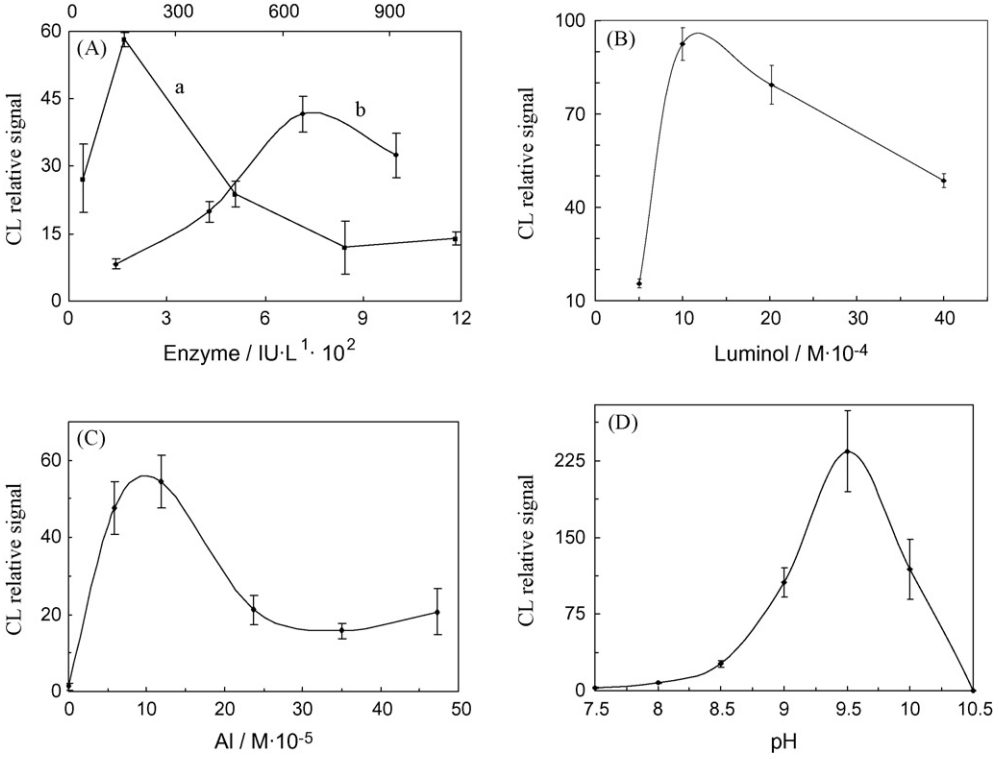

Fig. 3 – Effect of enzymatic contribution to CL relative emission signal. (A) The ARP curve (a) reaches a maximum at 15,500 IU L^{-1} decreasing then. The LOx curve (b) increases with concentration given a plateau at 710 IU L^{-1}. (B) Luminol dependence. (C) Aluminum dependence. (D) pH dependence. Each point was replicates five times with new biosensor each.

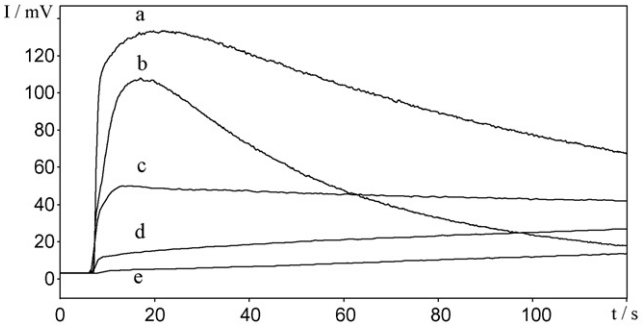

Fig. 4 – Effect of Al concentration in membrane on CL emission at pH 8.8. (a) 1.2×10^{-4} M Al; (b) 10^{-3} M Al; (c) 10^{-2} M Al; (d) 10^{-1} M Al; (e) membrane prepared on an Al plate.

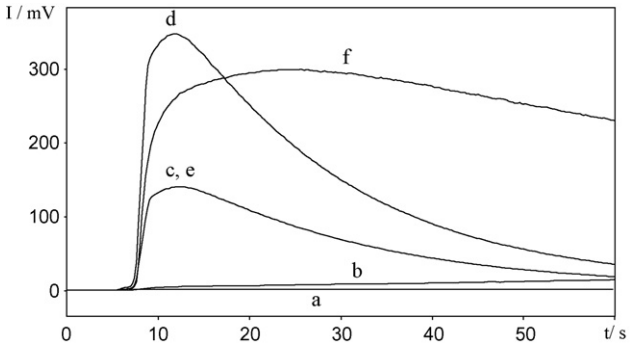

Fig. 5 – Effect of membrane composition on CL emission. (a) Al and luminol plus buffer; (b) Al and luminol plus lactate; (c) ARP and luminol plus buffer; (d) ARP and luminol plus lactate; (e) ARP, luminol and Al plus buffer; (f) ARP, luminol and Al plus lactate. All the membranes include LOx (710 IU L^{-1}). In all cases the concentrations were: Al 1.2×10^{-4} M; ARP 15,500 IU L^{-1}; luminol 10^{-3} M and lactate 6×10^{-4} M.

but they decrease the same way as the lactate signals). It is easy to see that there is a change in the emission pattern with a progressive decrease in CL emission (Fig. 4, curves a–e) suggesting a major contribution of one of the three reactions that take place in the medium (lactate to pyruvate by LOx; luminol–ARP–H_2O_2; luminol–Al–H_2O_2). The reaction that is intensified is the slow luminol–Al–H_2O_2 reaction, as described by Kulmala et al. [43], which is explained in more detail later in the paragraph on the CL mechanism.

3.2.5. *Biosensor preparation*

The composition of the sensing membrane discussed above was studied preparing the biosensors with 20 μL of cocktail in a spin-on-device system. The use of different volumes (10–40 μL) of optimized cocktail means an increase of some 23% in the CL signal and change the relative standard deviation (R.S.D.) values from 12.4% (10 μL) to 3.0% (30 μL). The R.S.D. is reduced some 75%, thus increasing the precision. 30 μL was therefore chosen to prepare the sensing membrane because the use of higher volumes exceeds the size of the plastic support used.

3.3. *Optimization of experimental conditions*

The pH dependence (Fig. 3D) shows maximum CL emission at 9.5 working at a constant lactate concentration (10^{-4} M). However, the variation in the CL relative signal with the lactate concentration is very low at this pH because a small discrimination between different lactate concentrations is observed. Additionally, the R.S.D. at this pH is high (25%). The use of a lower pH gives lower CL relative signals but better sensitivity for lactate, with the optimum pH being at 8.8 if we take into account the contribution of the standard deviation of the CL signal (see error bars in Fig. 3D). The selected pH was 8.8 considering sensitivity and the S/N ratio and was adjusted with Tris buffer due to better lactate discrimination. The explanation for the higher signal observed at pH 9.5 is due to the higher enzymatic activity of ARP at this pH [28,44]. Additionally at this pH, the production of H_2O_2 catalyzed by LOx is very low, but the efficiency of the luminol–ARP reaction is very high because of the enzyme and also because of the luminol, whose reactivity increases with pH up to pH 11 [37].

The dependence of Tris buffer concentration in the membrane was studied between 10^{-4} and 0.1 M, showing a decreasing in the CL relative signal with the precision worsening when the buffer concentration increases. This decrease was described by Sakharov [45] working with peroxidases like ARP and HRP in solution. As a compromise solution for higher signal and precision, we chose 10^{-2} M buffer. Similarly, the same effect is observed when salts, such as NaCl, increase their concentration from 10^{-4} to 0.1 M. Both behaviors could be explained by an osmotic pressure effect; that is, at a high concentration of salts in the sample solution, the polyion membrane lowers the lactate entrance, generating a weaker CL emission.

To study influence of the sample volume, we used four optical cells (1, 2, 5, 10 mm) with the biosensor membrane placed on the side far away from the PMT, and injecting sample volumes of 0.3, 0.7, 1.0, and 2.0 mL, respectively, in such a way that in all cases the CL biosensor was covered by the solution. The CL relative signal rose up to a volume of 1 mL staying constant at higher volumes, because the increases in distance between the membrane and the PMT cancel the effect. Thus, we selected a 5 mm path width cell for the rest of the study. In all cases and to increase the precision of the injection of the sample, we use a pneumatically operated automatic system (2 bar) to inject the sample into the CL cuvette by means of a conventional syringe [30].

The reuse of the same membrane with new lactate solution provides decreasing CL readings of some 25% each time, for which reason the membrane is only appropriate for one-shot use.

3.4. *Characteristics of CL signal*

The injection of a blank solution containing only pH 8.8 Tris buffer in the cuvette housing the disposable membrane, generates an initial CL signal (<5 s) that decreases over time to disappear (Fig. 5e). In the case of lactate containing solution, the initial signal is typically more intense, although

with low precision, and maintains over time up to some 30 s decreasing then slowly (Fig. 5f). In order to obtain the best CL relative signal, that is the CL lactate signal (Fig. 5f) subtracting the CL background produced in the absence of the analyte (blank) (Fig. 5e), we studied its evolution over time in the presence and absence of lactate showing maximum at 30 s, although the acquisition at 2 min improves the precision.

3.5. CL mechanism

When the lactate membrane (containing luminol–LOx–ARP) comes into contact with the pH 8.8 buffer, an initial CL emission is observed that decays to a steady state signal (Fig. 5c). This blank could be explained by the reaction of luminol with ARP due to traces of oxidants such as Compound I in the ARP and/or peroxides in the luminol [40,46].

The introduction of lactate in the membrane produces the oxidation of the lactate to pyruvate because of the oxygen catalyzed by LOx produces H_2O_2 which slowly reacts with luminol with concomitant CL emission (Fig. 5d). The use of peroxidases such as ARP catalyzes the luminol oxidation through a three-step pathway [37] that concludes in the formation of an excited 3-aminophthalate dianion which finally produces CL, enhancing the light emission [47].

The inclusion of powdered metallic aluminum in the sensing membrane improves the CL relative signal [48]. Therefore, in the presence of ARP and metallic Al, a fast CL emission occurs that is maintained over time (Fig. 5f). This growth in CL emission by Al could be related to the more efficient turnover of ARP through the reactive intermediate Compound I production by radicals generated from H_2O_2 by Al. Fig. 5e shows the effect of Al on the blank. The introduction of Al into the membrane does not increase the blank signal because Al only reacts in the presence of H_2O_2, confirming the mechanistic role of Al.

In the absence of ARP but in the presence of Al, the CL blank emission does not work at pH 8.8 [43] (Fig. 5a); even though with the H_2O_2 formed by LOx at the expense of the lactate, only a slight emission occurs that cannot be compared with CL in the presence of ARP (Fig. 5b).

3.6. Analytical parameters

The analytical function was obtained by means of a calibration set composed of five standards with five replicates of each standard between 5×10^{-5} and 4×10^{-3} M and using 30 s and also 2 min (Fig. 6, curves g and h) as acquisition time. The observed dependence between the CL relative signal and lactate concentration in both cases (30 s and 2 min) is a logarithmic curve that was linearized using: $I = A \times \log[\text{lactate}] + b$. The sensitivity is higher by a factor of 2.5 when an acquisition time of 30 s is used (Table 1).

This observed analytical function is not common with lactate sensors. Generally, a linear analytical function for lactate is reported in FIA or SIA procedures [18,49,50] and a log–log dependence is described in fiber optical sensors with immobilized enzymes [34,51].

As the calibration function is logarithmic, the detection limits were obtained using the standard criteria [52]

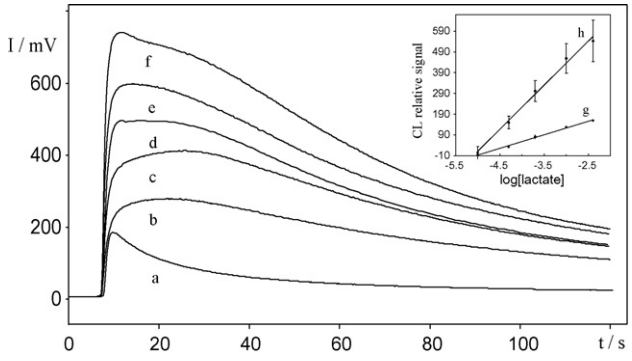

Fig. 6 – Calibration curves. CL measurement curves from different lactate concentrations: (a) blank; (b) 2×10^{-4} M; (c) 6×10^{-4} M; (d) 8×10^{-4} M; (e) 10^{-3} M; (f) 2×10^{-3} M; (g) linearization graph at 30 s representing CL relative signal ($y_{lactate} - y_{blank}$) vs. lactate concentration logarithm; (h) linearization graph at 2 min representing CL relative signal ($y_{lactate} - y_{blank}$) vs. lactate concentration logarithm.

$DL = y_b + 3s_b$, where y_b is the average blank signal and s_b is the standard deviation of blank, which was determined from 10 blanks (R.S.D. 5%). The detection limits found were 9.2×10^{-6} and 3.5×10^{-5} M for 2 min and 30 s acquisition time, respectively, due to different precisions obtained in both cases.

The repeatability, as relative standard deviation, obtained using 10 different one-shot biosensors at different lactate concentrations was around some 3–8% at 2 min and 10–15% at 30 s. Taking into account the precision figures, we prefered to use 2 min as acquisition time for this lactate one-shot biosensor.

The lifetime of the one-shot biosensor, keep away from the light and protected at 4 °C, was studied by using a series of prepared biosensors and regularly checking their response to an analyte concentration of 4×10^{-4} M. The lifetime obtained was over 1 month (average signal 33.8 ± 2.4; decreasing at 22 days: 1.0%; at 30 days: 10.1%; at 35 days: 45.6%, at 45 days: 95.1%), which is acceptable for this type of biosensor and coincides with the long-term stability study of amperometric lactate biosensors based on LOx immobilized in the same membrane used here [33]. The cost of the biosensor considering only reagents and support to make the membrane is around 0.06 € unit^{-1}.

The performance characteristics of the biosensor developed here and different sensors for determining lactate are compared in Table 2.

Table 1 – Analytical parameters

Parameter	Value t 30 s	Value t 2 min
Linear range (M)	5×10^{-5}–4×10^{-3}	5×10^{-5}–4×10^{-3}
Ordinate	953.1	320.1
Slope	168.3	65.4
r	0.971	0.998
Detection limit (M)	3.5×10^{-5}	9.2×10^{-6}
R.S.D. (%) blank	10.4	5.0
R.S.D. (%) lactate (8×10^{-4} M)	12.3	5.5

Table 2 – Comparison of lactate sensor characteristics

Sensor	Range (M)	DL (M)	Precision (%)	Application	Reference
CL FIA	10^{-4}–1.5×10^{-3}	1.2×10^{-5}	1.9	Yoghurt	[39]
Amperometric electrode	3×10^{-4}–10^{-3}	10^{-6}	2.0	Serum and milk	[33]
CL FIA fiberoptic	8×10^{-5}–2.5×10^{-2}	4×10^{-5}	1.7	Dairy products	[34]
Amperometric screen printed	10^{-6}–5×10^{-4}	5×10^{-7}	2.5	Dairy products and clinical analysis	[6]
CL FIA biosensor	10^{-6}–10^{-3}	2×10^{-7}	1.3	Plasma and milk samples	[53]
Amperometric screen printed	7.5×10^{-5}–10^{-3}	5×10^{-5}	7.5	Wine	[9]
Enzymatic O_2 electrode	–	8.6×10^{-6}	5	Dairy products and serum samples	[54]
Amperometric FIA	10^{-5}–1.8×10^{-3}	10^{-6}	10	Dairy products	[55]
CL FIA	10^{-7}–10^{-3}	8×10^{-8}	0.24	Food and drinks	[15]
One-shot CL	5×10^{-5}–4×10^{-3}	9.2×10^{-6}	5.5	Yoghurt	This paper

Table 3 – Determination of lactate in yoghurt by proposed and reference procedures

Yoghurt sample	CL biosensor ($g\,L^{-1}$)	Reference ($g\,L^{-1}$)	M.C.P.* (%)	P_{val} (%)
1	0.77 ± 0.05	0.79 ± 0.03	96.8	22.1
2	0.81 ± 0.05	0.82 ± 0.01	99.8	97.6
3	0.79 ± 0.04	0.78 ± 0.04	102.8	80.8

* M.C.P.: methods comparison percent.

3.7. *Applications*

The feasibility of the described biosensor was checked by analyzing lactate in different yoghurt samples. The coexisting substances in yoghourt checked as possible interferents were acetic acid, citric acid, ascorbic acid, uric acid and glucose. The concentrations of the different compounds in the diluted yogurt samples are roughly: glucose 5×10^{-5} M; ascorbic acid 2.5×10^{-6} M; uric acid 1.5×10^{-5} M with similar concentrations of the rest of the acids. None of them interfere with the CL response at 10^{-4} M of lactate. The selectivity of the enzymatic reaction along with the permselectivity of the polyion complex layer justifies these results.

The only treatment needed is dilution with Tris buffer obtaining a white solution, and filtration through a conventional Albet filter paper with a 20–25 μm pore size to separate the proteins. A small volume of this filtrate transparent solution, about 2 mL, is necessary to make the analysis. This simple treatment is possible considering the permselective characteristics of polyionic membrane used, with a cut-off around 10^3 D [33]. The data in Table 3 shows that the lactate content obtained using the proposed one-shot biosensor was in good statistical agreement with the data obtained with the manual enzymatic method (Boehringer-Mannheim kit) (including the dilution factor). The results obtained comparing both methods were between 95 and 105%.

4. Conclusions

A chemiluminescent one-shot biosensor has been developed using an enzymatic recognition system based on LOx and a transduction system based on luminol, ARP and metallic aluminum for lactate, immobilized in a plastic support by a polyion complex membrane prepared from PLL and PSS. This permselective membrane is suitable for the preparation of transparent disposable membranes for one-shot lactate biosensors with the characteristics of selectivity and quick response. The one-shot biosensor described here makes it possible to monitor lactate in most practical situations. Examples include lactate control in yoghurt, with advantages in reducing contamination, cost reduction and reagent wastage, especially with luminol which is typically not immobilized in chemiluminescent sensors. It is environmentally friendly and has an estimated lifetime of at least 1 month when protected from light in normal conditions.

The drawbacks are related to precision, typically between 5 and 12% depending on the acquisition time used, but this could be improved with mass production techniques, which could also cut costs. The use of different mass production techniques and the development of small, hand-held and battery-operated instruments that will make decentralised analyses possible are now under investigation.

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REFERENCES

[1] J.B. Ewaschuk, G.A. Zello, J.M. Naylor, D.R. Brocks, J. Chromatogr. B 781 (2002) 39.
[2] S. Benthin, J. Nielsen, J. Villadsen, Anal. Chim. Acta 247 (1991) 45.
[3] D. Compagnone, D. Moscone, G. Palleschi, Recent Res. Dev. Pure Appl. Anal. Chem. 1 (1998) 73.
[4] L.F. Capitán-Vallvey, in: C.A. Grimes, E.C. Dickey, M.V. Pishko (Eds.), Encyclopedia of Sensors, 1st ed., The Pennsylvania State University, 2005.
[5] D.C. Klonoff, Diabetes Technol. Ther. 5 (2003) 929.
[6] N.G. Patel, A. Erlenkotter, K. Cammann, G.C. Chemnitius, Sens. Actuators B 67 (2000) 134.

[7] S.D. Sprules, J.P. Hart, R. Pittson, S.A. Wring, Electroanalysis 8 (1996) 539.
[8] J.R. Braig, Diagnostic optical biosensor for combined enzyme-spectroscopic analysis of a biological fluid, PCT Int. Appl. 2006127724 (2006).
[9] A. Avramescu, T. Noguer, M. Avramescu, J.L. Marty, Anal. Chim. Acta 458 (2002) 203.
[10] J.M. Kauffmann, Ann. Pharm. Fr. 60 (2002) 28.
[11] H.Y. Aboul-Enein, R.I. Stefan, J.F. Van Staden, Crit. Rev. Anal. Chem. 29 (1999) 323.
[12] C.A. Marquette, A. Degiuli, L.J. Blum, Appl. Biochem. Biotechnol. 89 (2000) 107.
[13] S. Girotti, S. Ghini, G. Carrea, R. Bovara, A. Roda, R. Budini, Anal. Chim. Acta 255 (1991) 259.
[14] H. Nakamura, Y. Murakami, K. Yokoyama, E. Tamiya, I. Karube, M. Suda, S. Uchiyama, Anal. Chem. 73 (2001) 373.
[15] A. Hemmi, K. Yagiuda, N. Funazaki, S. Ito, Y. Asano, T. Imato, K. Hayashi, I. Karube, Anal. Chim. Acta 316 (1995) 323.
[16] M.Y. Rubtsova, J.V. Samsonova, A.A. Ezhov, A.M. Egorov, Biocatalysis-2000: Fundamental & Applications, 2000. Vol. 41, No. 6. Supplement, pg 33. Moscow State University, Chemistry Department, ISSN: 05799384.
[17] J. Samsonova, V.M.Y. Rubtsova, M. Franek, Anal. Bioanal. Chem. 375 (2003) 1017.
[18] T.J.N. Carter, T.P. Whitehead, L.J. Kricka, Talanta 29 (1982) 529.
[19] L.J. Kricka, X. Ji, Anal. Sci. 7 (1991) 1501.
[20] A. Hasegawa, Chemiluminescent analytical test strip and holder, JP 08184595 (1994).
[21] O.S. Khalil, T.F. Zurek, K.R. Genger, P.J. Curtis, Y.H. Jou, S.M. Cotter, Method and apparatus for heterogeneous chemiluminescence assay, US 5,089,424 (1992).
[22] P.O. Vogelhut, Chemiluminescent analytical device, US 4,231,754 (1980).
[23] S. Furuya, T. Imai, N. Hiratsuka, T. Ikeda, A. Kondo, Integral multilayered analysis elements and their use in body fluid analysis for clinical diagnosis, JP 62103542 (1986).
[24] A. Tahirovic, A. Copra, E. Omanovic-Miklicanin, K. Kalcher, Talanta 72 (2007) 1378.
[25] T.R. Kissel, A.E. Friedman, S.A. Fingar, Chemiluminescent reagent and assay using a substituted acetanilide for light generation, US 5,705,357 (1998).
[26] H. Akhavan-Tafti, K. Sugioka, Y. Sugioka, L.V. Reddy, Chemiluminescent detection methods using dual enzyme-labeled binding partners, US 5,843,666 (1998).
[27] R.T. Belly, M.M. Kopcienski, C. Erdrich, M.A. Kildal, Format for immunoassay in thin film, US 5,958,339 (1999).
[28] M.Y. Rubtsova, G.V. Kovba, A.M. Egorov, Biosens. Bioelectron. 13 (1998) 75.
[29] L.A. Liotta, B.C. Christiansen, A.R. Day, T. Harlacher, K. Paweletz, Light-emitting immunoassay, US 5,942,407 (1997).
[30] J. Ballesta Claver, M.C. Valencia Miron, L.F. Capitán-Vallvey, Anal. Chim. Acta 522 (2004) 267.
[31] K. Seiler, W. Simon, Anal. Chim. Acta 266 (1992) 73.
[32] Boehringer Mannheim. Boehringer Mannheim L-lactate kit, 2006.
[33] F. Mizutani, S. Yabuki, Y. Hirata, Anal. Chim. Acta 314 (1995) 233.
[34] A. Berger, L.J. Blum, Enzyme Microb. Technol. 16 (1994) 979.
[35] T. Nakayama, T. Amachi, J. Mol. Catal. B: Enzyme 6 (1999) 185.
[36] M. Tanaka, K. Ishimori, I. Morishima, Biochemistry 38 (1999) 10463.
[37] B.B. Kim, V.V. Pisarev, A.M. Egorov, Anal. Biochem. 199 (1991) 1.
[38] J. Gu, P. Xu, Y. Qu, J. Mol. Catal. B: Enzyme 18 (2002) 299.
[39] P.B. Martelli, B.F. Reis, A.N. Araujo, M. Conceicao, B.S.M. Montenegro, Talanta 54 (2001) 879.
[40] E. Lissi, C. Pascual, M.D. Del Castillo, Free Radic. Biol. Med. 16 (1994) 833.
[41] Y. Huang, W. Liu, J. Pharm. Biomed. Anal. 38 (2005) 537.
[42] H.P. Misra, P.M. Squatrito, Arch. Biochem. Biophys. 215 (1982) 59.
[43] S. Kulmala, C. Matachescu, A. Kulmala, D. Papkovsky, M. Hakansson, H. Ketamo, P. Canty, Anal. Chim. Acta 453 (2002) 253.
[44] J. Kulys, K. Krikstopaitis, S. Ebdrup, A.H. Pedersen, P. Schneider, J. Mol. Catal. B: Enzyme 2 (1996) 93.
[45] I.Yu. Sakharov, Biochemistry 66 (2008) 515.
[46] C. Pascual, M.D. Del Castillo, C. Romay, Anal. Lett. 25 (1992) 837.
[47] M. Nakamura, S. Nakamura, Free Radic. Biol. Med. 24 (1998) 537.
[48] M.J.R. Previte, C.D. Geddes, J. Fluor. 17 (2007) 279.
[49] A. Roda, S. Girotti, B. Grigolo, S. Ghini, G. Carrea, R. Bovara, I. Zini, R. Grimaldi, Biosens. Bioelectron. 6 (1991) 21.
[50] R.W. Min, J. Nielsen, J. Villadsen, Anal. Chim. Acta 312 (1995) 149.
[51] L.J. Blum, S.M. Gautier, A. Berger, P.E. Michel, P.R. Coulet, Sens. Actuators B 29 (1995) 1.
[52] N.J. Miller, J.C. Miller, Estadística y Quimiometría para Química Analítica, 4th ed., Prentice Hall, Madrid, 2002.
[53] F. Wu, Y. Huang, C. Huang, Biosens. Bioelectron. 21 (2005) 518.
[54] M.M.F. Choi, Food Chem. 92 (2005) 575.
[55] F. Ghamouss, S. Ledru, N. Ruille, F. Lantier, M. Boujtita, Anal. Chim. Acta 570 (2006) 158.