Disposable electrochemiluminescent biosensor for lactate determination in saliva

J. Ballesta Claver, M. C. Valencia Mirón and L. F. Capitán-Vallvey*

Received 8th December 2008, Accepted 7th April 2009
First published as an Advance Article on the web 22nd April 2009
DOI: 10.1039/b821922b

An electrochemiluminescence-based disposable biosensor for lactate is characterized. The lactate recognition system is based on lactate oxidase (LOx) and the transduction system consists of luminol. All the needed reagents, luminol, LOx, BSA, electrolyte and buffer have been immobilized by a Methocel™ membrane placed on the working electrode of the screen-printed electrochemical cell. The measurement of the electrochemiluminescence (ECL) is made possible *via* a photocounting head when 50 μl of sample is placed into the screen-printed cell with a circular container containing the disposable sensing membrane. The compositions of the membrane and reaction conditions have been optimized to obtain adequate sensitivity. The disposable biosensor responds to lactate after 20 s when two 1 s pulses at 0.5 V are applied to obtain the analytical parameter, the ECL initial rate. The linearized double logarithmic dependence for lactate shows a dynamic range from 10^{-5} to 5×10^{-4} M with a detection limit of 5×10^{-6} M and a sensor-to-sensor repeatability, as relative standard deviation, RSD, of 3.30% at the medium level of the range. The ECL disposable biosensor was applied to the analysis of lactate in human saliva as an alternative procedure for obtaining the lactate level in a non-invasive way. Interferences coming from components of saliva were studied and eliminated in a simple way that was easy to handle. The procedure was validated for use in human saliva, comparing the results against an enzymatic reference procedure. The proposed method is quick, inexpensive, selective and sensitive and uses conventional ECL instrumentation.

1. Introduction

The development of non-invasive procedures for the measurement of metabolites in matrices other than blood is very interesting, particularly for people who need to frequently control parameters such as glycemia and lactate, and for those who have problems with blood extraction.[1]

Lactic acid appears when the aerobic metabolism of glucose to produce energy, such as ATP molecules, switches to anaerobic metabolism. When lactic acid lowers the blood pH, it can damage muscles, and thus the lactate level needs to be monitored in critical-care patients, primarily to prevent heart attacks. Lactate analysis is also used in diabetes control, food analysis and sports medicine to help athletes tailor their training. The determination of lactate is generally carried out by invasive techniques using blood as the sample. Of the different sampling sites and matrices for non-invasive sensing, saliva is especially interesting for non-invasive lactate analysis due to its high correlation to blood lactate; typically a 1 : 4 saliva/blood ratio,[2] with the lactic acid level in human saliva being around 2×10^{-4} M.[1]

The evaluation of blood lactate levels can be performed using different types of monitors, from portable handheld to small benchtop or freestanding monitors[3] using a variety of methods, including enzymatic and biosensor methods based on lactate oxidase and/or lactate dehydrogenase immobilization and electrochemical and spectrophotometric techniques.[4,5]

Sensors for lactate have been developed over the last two decades, mostly amperometric ones, based on the change in concentration of a redox-active reactant or product *via* an analyte-specific enzyme reaction. Different enzyme biosensors for lactate have been described, mainly, cytochrome b2, lactate monooxygenase and lactate oxidase immobilized on different types of polymers,[6] conducting polymers[7] or nanomaterials.[8] Different types of screen-printed amperometric disposable sensors have been described for lactate and some of them are on the market, especially the fitness market.[9]

Chemiluminescence (CL), recognized as a useful and versatile technique with low detection limits, has been used for sensing lactate based on enzymatic systems, chiefly lactate oxidase/peroxidase/luminol[10] and lactate dehydrogenase/NADH/bioluminescent enzymes[11] implemented in different types of flow[12,13] or fiber optic formats.[10] Nevertheless, chemiluminescent detection has rarely been used in disposable sensors despite its advantages of sensitivity and simple instrumentation, because of the need to immobilize all the chemistries required and the time control to trigger the sensing reactions upon contact with the sample.

Different types of enzymatic or immunological sensors in disposable format based on chemiluminescence measurement using a luminometer,[14] portable scanning luminometer[15] or different types of photographic films[16] have been proposed for organic and inorganic analytes.[17]

Electrochemiluminescence (ECL), the luminescence produced by the relaxation of excited molecules coming from an

Solid Phase Spectrometry Research Group, Department of Analytical Chemistry, Faculty of Sciences, Campus Fuentenueva, University of Granada, Granada, E-18071, Spain. E-mail: lcapitan@ugr.es

electrochemically initiated reaction, is a very convenient technique that combines the sensitivity of light-emitting reactions with easy control of the reaction. In this way, ECL has been used for detection in chromatographic systems and electrophoresis and in sensors, mainly in flow and fiber optic format[18–20] but rarely in disposable format. Blum and co-workers have described[21,22] a screen-printed multiparametric biosensor, including lactate, based on ECL detection of enzymatically produced hydrogen peroxide that is an interesting approximation to disposable devices. The immobilization of peroxidases in a photopolymer that includes a dispersed anion exchanger containing luminol makes it possible to capture spatially defined ECL signals using a CCD camera.

The aim of this study was to develop a disposable electrochemiluminescent biosensor for lactate based on the generation of hydrogen peroxide by lactate oxidase and the subsequent reaction with electrochemically oxidized luminol, based on commercial screen-printed electrochemical cells with reagents immobilized in a Methocel™ membrane on the graphite working electrode and conventional PMT detectors. Additionally, we are interested in its application to the determination of lactate in saliva as a very convenient matrix for non-invasive monitoring of this metabolite.

2. Experimental

2.1. Chemicals

L-(+)-Lactate 10^{-2} mol l^{-1} stock solutions in pH 9.0 phosphate buffer 0.2 M were prepared using L-(+)-lactate lithium salt 97% from Sigma (Sigma-Aldrich Química S.A., Madrid, Spain). Solutions of lower concentrations were prepared by dilution. Buffers, made with Tris 0.5 M (Trizma base 99%) and phosphate 0.5 M (Na_2HPO_4) and adjusted to different pH values by adding NaOH or HCl were prepared. Other chemicals were NaCl, $CaCl_2$, KCl, KSCN, NH_4Cl, citric acid, uric acid, ascorbic acid and sodium carboxymethyl cellulose (CMC). All of these compounds were supplied by Sigma.

The ECL reaction was carried out by the following reagents: 10^{-2} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) stock solution from 97% luminol, lactate oxidase (LOx) from *Pediococcus* species, 39 IU mg^{-1} and 96% albumin from bovine serum (BSA) powder, all supplied by Sigma. To make the membrane, we used poly(vinyl chloride), glutaraldehyde solution (grade I, 25% w/v in water), cellulose triacetate (CTA), poly-L-lysine hydrobromide (MW 84 000 g mol^{-1}) and poly(sodium 4-styrenesulfonate) (MW 70 000 g mol^{-1}) 30 wt% solution in water, all supplied by Sigma, and Methocel™ 90HG (hydroxypropylmethyl cellulose) purchased from Fluka. Additional chemicals were the solvent tetrahydrofuran (THF); the plasticizer *o*-nitrophenyl octyl ether (NPOE), and the quaternary ammonium salt Aliquat 336, all supplied by Sigma. All chemicals used were of analytical-reagent grade. Reverse-osmosis-type quality water (Milli-Q Plus185 from Millipore, Molsheim, France) was used throughout.

2.2. Enzyme preparation

The LOx solution was prepared by placing 1.40 mg into 1 ml of pH 7.0 phosphate buffer 0.1 M and conserved in Eppendorf tubes (60 μl) at −20 °C.

2.3. Artificial saliva preparation

An artificial saliva was prepared containing 5 mM of NaCl, 1 mM of $CaCl_2$, 15 mM of KCl, 1 mM of citric acid, 5 mM of uric acid, 1 mM of ascorbic acid, 0.2 mM of lactate, 1.1 mM of KSCN, 4 mM of NH_4Cl and 10 g l^{-1} of sodium carboxymethyl cellulose (CMC) in water.[23,24]

2.4. Apparatus and software

ECL emission was measured using an H8529 photomultiplier (PMT) interfaced by a C8855 USB photocounting unit, both from Hamamatsu Technologies, connected to a personal computer. The potentiostat used was a PS-PC1 model Palmsens from Ivium Technologies (Eindhoven, Netherlands) with a connector for screen-printed electrodes.

We designed a two-piece holder made of black polystyrene, one piece to hold the PMT and another where the screen-printed electrodes are placed as close as possible to the PMT. The holder was placed in a customized black box with vents for wire connections to the potentiostat and computer (Fig. 1).

Other apparatus and laboratory materials consisted of a Crison digital pH-meter (Crison Instruments, Barcelona, Spain) with combined glass-saturated calomel electrode, and a customized controlled temperature chamber (−20 °C to 80 °C). Software programs used were: CSW32 software package from DataApex Ltd. ver. 1.2.5 (2001) (Prague, Czech Republic) for the acquisition and manipulation of luminescence records; Hamamatsu Labview program (Hamamatsu Photonics, Hamamatsu, Japan) for the acquisition of luminescence signals; Palmsens-PC program for potentiostat control (Palmsens Instruments BV, Netherlands) and a Statgraphics software package (Manugistics Inc. and Statistical Graphics Corporation, USA), ver. 5.0 (2000) for data treatment.

2.5. Screen-printed electrochemical cells

Screen-printed electrochemical cells were provided by Palmsens Instruments BV and consist of a round-shaped graphite working electrode, a graphite counter electrode and a silver pseudo-reference electrode on a 10 mm × 40 mm × 0.5 mm plastic support. Before being used, the electrochemical cells were tested for uniform behavior. In order to prepare a receptacle on the disposable electrochemical cell we covered the electrode area

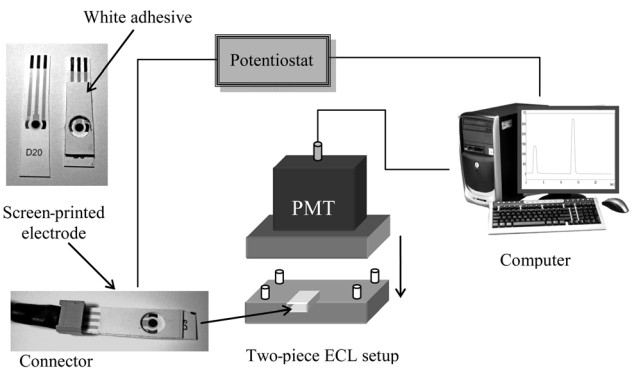

Fig. 1 Configuration of the instrumental setup for lactate determination.

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 (see Fig. 1).

2.6. Disposable biosensor preparation

The lactate-sensitive membranes were made using a solution prepared in a 3 ml glass vial with 0.51 mg of luminol, 2.5 mg of BSA, 12.5 mg of Methocel, 50 μl of 12.5 IU ml^{-1} LOx and 200 μl of pH 8.5 phosphate buffer. This membrane solution was shaken and ultrasonicated for 10 min and then stored in a refrigerator at 4 °C.

The disposable biosensors were cast by placing 5 μl of the prepared membrane solution on the working electrode of the electrochemical cell. After this, the biosensors were dried at room temperature for 1 h and then stored in a refrigerator at 4 °C until use.

2.7. Procedures

2.7.1. Standards. Aqueous standard solutions of lactate containing between 10^{-5} and 5×10^{-4} M in working buffer, consisting of pH 9.0 phosphate buffer 0.2 M containing 0.25 M of NaCl, were prepared. 50 μl of standard or sample solution were taken and deposited in the receptacle of the screen-printed cell. The lactate-sensitive biosensor was placed in the holder previously described and covered with the lid that held the PMT. Then, two pulses of 1 s with 10 s of difference between them at 0.5 V were applied measuring the ECL emission in the PMT without wavelength discrimination.

2.7.2. Saliva. 50 μl of prepared saliva (see 'Treatment of saliva samples') was placed in the receptacle of the cell and 0.4 V was applied for 4 s. 6 s later, two pulses of 1 s with 10 s of difference between them at 0.5 V were performed measuring the ECL signal as indicated above. For the determination of lactate in saliva, it was necessary to process an internal blank[25] for every sample. This blank consisted of a measurement using the same protocol with the biosensors prepared without enzyme.

2.7.3. Reference. As a reference procedure, we used the enzymatic bioanalysis Boehringer Mannheim UV-Kit method from R-Biopharm AG (ATOM S.A., Barcelona, Spain).[26] It was necessary to introduce a centrifugation (10 minutes at 1000 rpm) and a filtration step (0.40 μm cellulose acetate filter) for the determination of L-lactate in saliva because the kit only indicates general directions for organic samples.

2.8. Treatment of saliva samples

Saliva samples coming from healthy volunteers who brushed their teeth and rinsed their mouths to minimize the influence of oral microbiological flora and nutrition were collected.[27] These samples were frozen after collection (−20 °C) to arrest the bacterial metabolism.[1] For the analysis of lactate in human saliva, typically 500 μl of supernatant liquid, after thawing the sample, was mixed with 2.0 ml of working buffer. Then, the solution was filtered through a 0.4 μm cellulose acetate filter and used for the test. In the case of the reference procedure, centrifugation was needed, after thawing, using the same sample preparation but using water to dilute the sample.

3. Results and discussion

3.1. Reagent immobilizations for ECL lactate sensing

In this study, we are interested in the preparation of a disposable electrochemiluminescent biosensor for lactate based on the atmospheric oxidation of lactate catalyzed by LOx producing hydrogen peroxide that reacts with electrochemically oxidized luminol in a basic medium, generating ECL emission. For this, we needed to immobilize all of the reagents needed to make the lactate recognition and transduction possible.

To immobilize the reagents, LOx and luminol, we tried different methods. For LOx we used entrapment with Methocel,[28] ion-exchange with a polyion complex layer made with poly-L-lysine and polystyrenesulfonate,[29] encapsulation with cellulose triacetate (CTA) and cross-linking with glutaraldehyde, both in solution or vapor combined to BSA.[30,31] The luminol immobilization was studied by entrapment with Methocel, Nafion or PVC with NPOE as plasticizer and Aliquat 336 as an ion-pair formation agent, and encapsulation with CTA.

Using the above immobilization systems, we studied two membrane arrangements: monolayer and bilayer, the latter to overcome compatibility problems between the reagents. Of the different bilayer configurations tested, only two arrangements offered some results: (a) a Nafion upper layer containing luminol and a lower layer with LOx cross-linked with glutaraldehyde (relative ECL signal 0.5%); and (b) a polyion complex membrane with LOx in the upper layer and a PVC lower layer with luminol (0.8%). Only three different types of monolayers work with lactate: (a) PVC-based membrane (0.4%); (b) CTA membrane (1.3%); and (c) Methocel membrane (100%).

Immobilization by cross-linking with glutaraldehyde cannot be used for luminol inclusion due to a Schiff base formation that hinders the luminol oxidation and consequent ECL emission.[32] Membranes containing hydrophobic polymers such as PVC or CTA give poor results due to low enzymatic activity and low lactate uptake. The polyion complex membrane could be used for this immobilization[14] but we obtained low adherence on the graphite working electrode and brittle membranes. Methocel-based membranes offered the best results considering adherence, good electrical properties and their hydrophilic character. Their reuse is limited due to the loss of 30% of the ECL signal with each new use, but this is not a problem if the biosensors are used in disposable format. Consequently, we selected Methocel as the entrapment polymer for the preparation of the monolayer ECL biosensor.

3.2. Optimization of lactate biosensor composition

Once the membrane material had been selected, we studied different factors related to making the membrane, including the concentration of chemicals and membrane polymers. All these factors were studied univariantly.

3.2.1. Layer composition

Methocel. To optimize the Methocel concentration, we prepared membranes using polymer concentrations ranging from

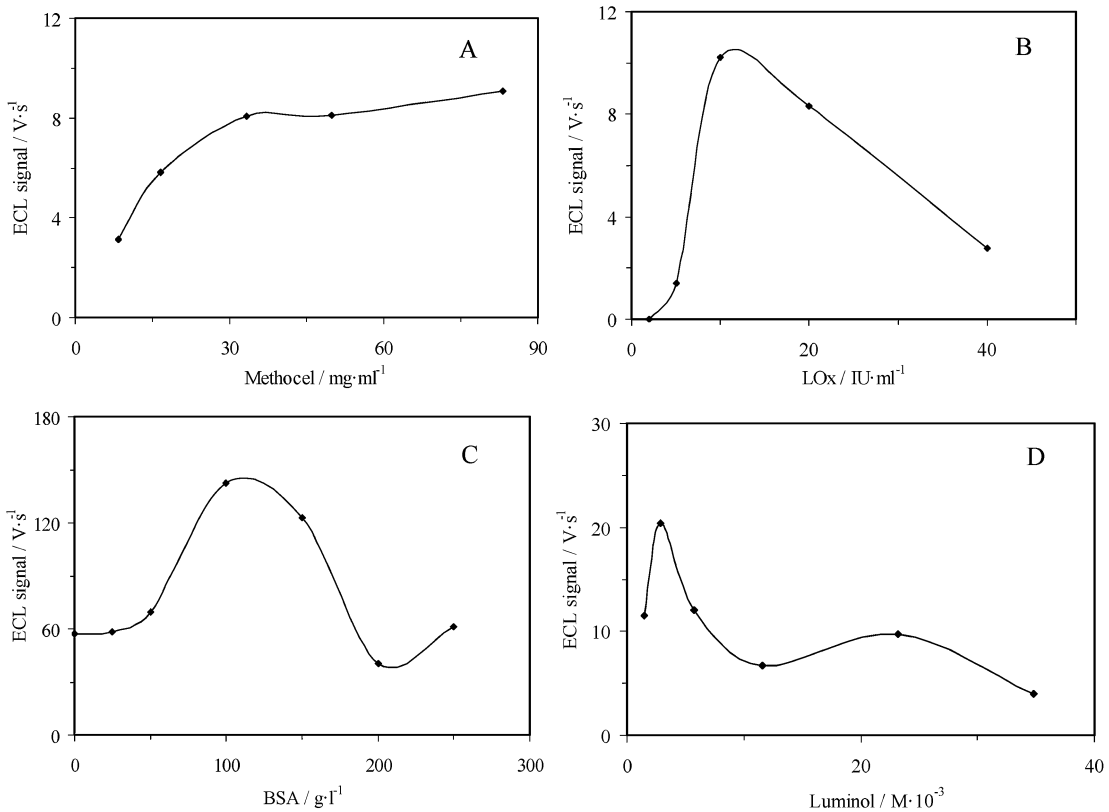

Fig. 2 Optimization of membrane composition. (A) The ECL signal increases with Methocel reaching a maximum at 49.9 mg ml^{-1}. (B) The LOx concentration produces a maximum at 12.5 IU ml^{-1}. (C) BSA dependence shows a maximum at 100 g l^{-1}. (D) Luminol dependence. In all cases 10^{-4} M of lactate concentration was used.

8.32 to 83.2 mg ml^{-1}. The ECL signal increases as the Methocel concentration goes up to 49.9 mg ml^{-1}, reaching a plateau at higher concentrations (Fig. 2A). This is due to the fact that Methocel, an aqueous hydroxypropylmethyl cellulose polymer, contributes to the enhancement of the electric properties of the membrane.[28,33]

Membrane solution pH. An important factor to consider for membrane preparation is the solution pH. The pH of maximum enzymatic activity of LOx is 7.5,[34] but since luminol needs a basic medium for solution and for ECL emission, an 8.5 pH buffer was selected[35] as a compromise for optimum enzymatic activity and stability.[36] The optimization of the working pH is studied in Section 3.4 below.

LOx enzyme. To optimize the concentration of the enzyme, we prepared disposable biosensors modifying the LOx concentration between 3.1 and 40.0 IU ml^{-1} obtaining a maximum at 12.5 IU ml^{-1} (Fig. 2B), then decreasing, probably due to an absorption of generated ECL emission by an excess of the enzyme. This absorption is possible considering that the absorption maximum of LOx is 450 nm and the ECL emission is centered on 425 nm.[37] The optimum enzymatic charge in the membrane corresponds to 0.062 IU LOx.

BSA protein. The usual limited stability of enzymes in the membranes can be overcome using proteins, like BSA, as stabilizers.[38,39] To study BSA influence, different membrane solutions with BSA concentrations from 25 to 250 g l^{-1} were prepared and the corresponding biosensors showed a maximum ECL signal against lactate at 100 g l^{-1} BSA (Fig. 2C). It has been suggested that basic groups carrying lone pair electrons from BSA interact with protons of the amino and hydrazide groups of luminol[40] stimulating the intramolecular chemically initiated electron-exchange luminescence (CIEEL) mechanism of luminol[41] and contributing to increase the signal. A further increase of BSA in the membrane decreases the ECL signal. Consequently, a concentration of 100 g l^{-1} BSA was chosen to make the membrane, which means 90 times of BSA in mass with respect to LOx in membrane, as is usual with BSA–enzyme immobilization.[38]

Luminol. The ECL signal increases with the luminol concentration in the membrane (Fig. 2D) up to 2.9×10^{-3} M, then decreasing slightly. This fact has been described for luminol in solution[42,43] and justified by Misra and Squatrito[44] as due to the accumulation of products or the dismutation of reactive intermediates rather than the depletion of reagents.

3.2.2. Membrane solution volume. The influence of the membrane solution volume used in making the membrane was studied preparing biosensors with volumes from 5 to 25 μl on the working electrode. The results showed a progressive decrease in the ECL relative signal reaching around 84% when the volume

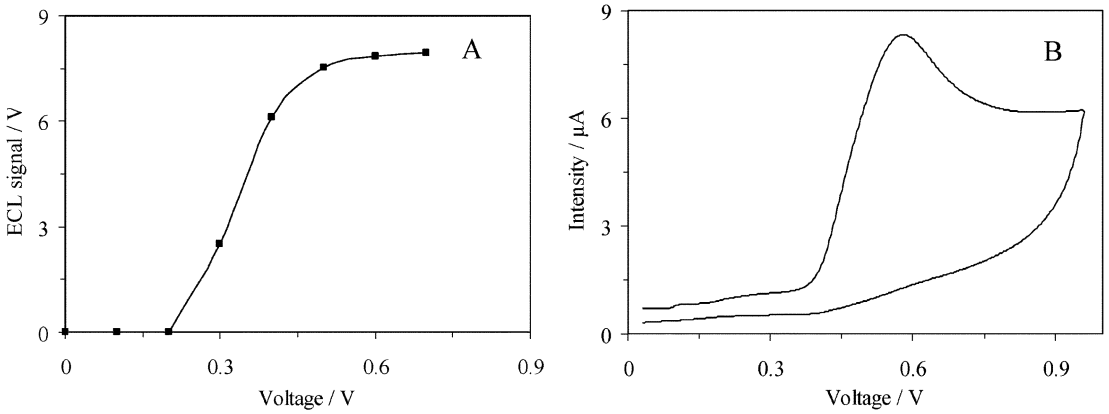

Fig. 3 (A) Dependence of ECL signal on voltage applied during 1 s. (B) Cyclic voltammogram of luminol.

increased to 25 μl. Thus, 5 μl was chosen as the optimal value. The reason for this behavior could be related to the increase in the thickness of the membrane which reduces the uptake of lactate. Higher volumes spread the membrane solution over all three electrodes, drastically reducing the ECL emission.

3.3. Electrochemiluminescent conditions

3.3.1. Voltage selection. Luminol is electrochemically oxidized and produces light emission in basic solutions at positive potentials when oxygen or hydrogen peroxide is in the medium. In this case, the cyclic voltammetry of luminol immobilized in Methocel on the working electrode shows an electrooxidation signal at 0.6 V (Fig. 3B). However, working in chronoamperometric mode, the ECL signal starts at a fixed potential of 0.3 V[45] and the intensity increases steeply as the potential increases, up to 0.5 V and continuing in this way (Fig. 3A) selecting 0.5 V as the optimum potential considering the uric acid interference (see Section 3.7: Applications). One advantage of the use of graphite electrodes is that the oxidation potential of hydrogen peroxide is more positive (typically 1.12 V at pH 7.4) than that of luminol, unlike that which occurs with other electrodes like platinum.[46]

3.3.2. ECL signal. The ECL is conventionally initiated using different transient-state electrochemical techniques such as cyclic voltammetry, normal pulse voltammetry, differential pulse voltammetry and chronoamperometry.[47] In this case, since the ECL signal varies with time due to enzymatically catalyzed hydrogen peroxide generation, is important to know the emission pattern with time to select the measurement conditions. For a constant amount of lactate, the ECL signal coming from successive pulses at the same voltage grows with time, reaching a plateau due to the enzymatic kinetic and luminol consumption (Fig. 4).

We studied different possible analytical parameters working in chronoamperometric mode: (a) ECL intensity using a short pulse (<5 s); (b) ECL emission integration, using a long time pulse (≥1 min); and (c) initial rate estimation. Comparing precision and linearity range (lack of fit test), the third parameter offered the best results.

The initial rate for different lactate concentrations, which obey a Michaelis–Menten kinetic, can be estimated more easily using a two point simplification, as shown in Fig. 4. In this way, it is not necessary to obtain the initial reaction rate from the ECL profiles measured using equal consecutive pulses, but using intensities at two pulses ($v_{ECL} = \Delta I_{ECL}/\Delta t$), in V s^{-1} units. Since a background ECL emission exists, the analytical signal v_{ECL} was corrected for the blank as $v_{standard} - v_{blank}$.

3.3.3. Electrochemical parameters. The electrochemical parameters studied were: (a) duration of the pulse; and (b) time interval between consecutive pulses. In the first case, we studied three different pulse durations (1, 2 and 3 s) observing that when the time of pulse increases, the analytical signal v_{ECL} decreases (some 76.4% from 1 to 3 s). This is due to fact that the longer the time pulse, the higher the consumption of generated hydrogen peroxide is and the higher the ECL emission is, but the difference in intensity with the next pulse is lower. For that reason, we used 1 s as the optimum time for the pulse. For case (b), different time intervals between consecutive pulses for ECL acquisition were studied (10, 30, 50 s). The results obtained (100% for 10 s; 83.1% for 30 s and 42.1% for 50 s) show that 10 s gives the best signal due to the higher luminol absorption on the working electrode when the pulses are nearest.[48]

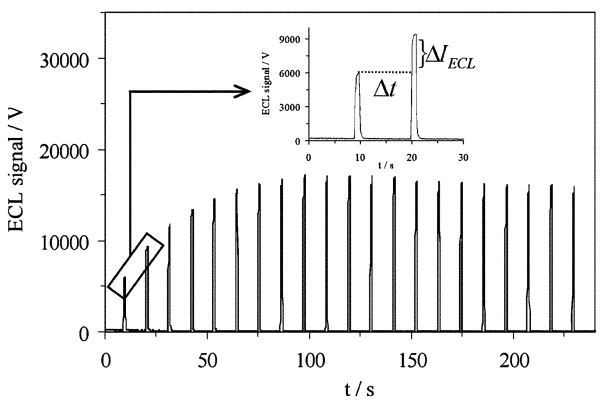

Fig. 4 Set of consecutive pulses of 1 s at 0.5 V each 10 s. The inset shows an enlargement of the two first pulses used for analytical signal acquisition. The experimental conditions were pH 9.0 phosphate buffer 0.2 M, 0.25 M NaCl and 2×10^{-3} M of luminol.

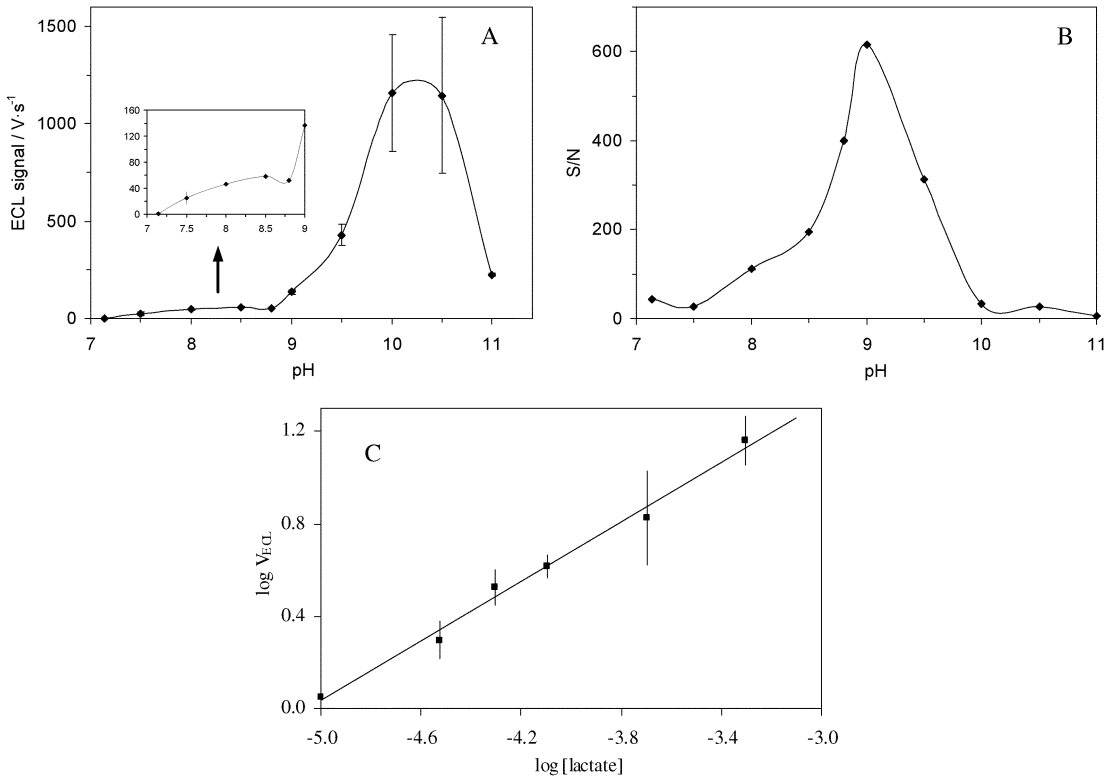

Fig. 5 The pH optimization for lactate biosensor. (A) A maximum is shown at pH 10.25 working at a constant lactate concentration (10^{-4} M) although with poor repeatability when pH increases because the blank signal increases. (B) The S/N representation is presented obtaining the maximum at pH 9.0, which was selected. (C) Log–log calibration curve for lactate using five replicates for concentration.

3.4. Optimization of experimental conditions

3.4.1. pH influence. The pH influence was studied between 7.0 and 11.0, working at a constant lactate concentration (10^{-4} M) and using five replicates for each pH. Fig. 5A shows two different ECL emissions maxima, first at pH 8.5 and second at 10.25 but with lower precision, a behavior similar to that observed by Chen and co-workers,[48] but if the signal-to-noise ratio (S/N) is represented, only one maximum appears at pH 9.0 (Fig. 5B). If the pH is greater than 9.0, the S/N decreases because the blank signal and its standard deviation grow strongly with pH. For this reason, we selected pH 9.0 as the working pH. The first ECL maximum at 8.5 is ascribed to the compromise between the enzymatic activity of LOx (maximum at 7.5) and the reactivity of luminol, which increases with pH up to 10.5.[49] The dramatic subsequent increase in ECL intensity with pH is due to the increase in the concentration of the basic peroxide intermediate of luminol. The decrease in ECL emission at a pH higher than 10.5 is justified by the strong decrease in quantum yield of 3-aminophthalate at a pH above 11.0.[50]

3.4.2. Buffer and electrolyte contribution. pH 9.0 phosphate and Tris were studied as buffer solutions, observing that a Tris buffer means a decrease of around 30% in the ECL signal with respect to the phosphate buffer, because Tris buffer is highly electro-active and known to lower the electro-oxidation of luminol. The dependence of the selected phosphate buffer concentration was studied between 0.05 and 0.8 M, showing a maximum at 0.2 M and a gradual decrease at higher phosphate buffer concentrations. This behavior could be due to an osmotic pressure effect that lowers the lactate entrance in the membrane, decreasing the ECL emission. Additionally, the presence of an electrolyte is necessary to increase the conductivity of the membrane and the peak resolution.[51] Different concentrations of NaCl from 0.12 to 2.0 M were prepared, obtaining a maximum at 0.25 M.

3.4.3. Volume of sample. The sample volume placed in the receptacle of the biosensor was studied, showing that the ECL signal grows when the volume increases. An increase from 10 to 50 µl means an increase of 71.9% in the ECL, because an increase in the sample volume means an increase in the amount of lactate and consequently in the ECL signal. Higher volumes (60 µl) cause the signal to decrease, probably due to the dispersion caused by the lens shape of the deposited drop. We selected 50 µl, the maximum capacity of the receptacle prepared on the biosensor, as the sample volume.

3.4.4. Temperature. This parameter affects both enzymatic reaction[34] and luminol reaction,[52] being more pronounced for the first. The ECL signal of this biosensor increases with temperature an average of 2.6 V s^{-1} per Celsius degree. The selected working temperature was room temperature (20–25 °C) because of the easier conditions for routine analysis and sufficient sensitivity for lactate determination.

3.5. ECL mechanism

In alkaline medium, luminol is electrochemically oxidized to diazaquinone at 0.5 V on the screen-printed graphite electrode and

reacts with hydrogen peroxide produced by atmospheric lactate oxidation catalyzed by LOx giving a luminescent emission.[21,53] If the medium is more alkaline, a higher emission is obtained, due to an increase in luminol deprotonation,[46,54,55] as shown in Fig. 5A. In the absence of lactate, a background ECL signal is observed that starts to grow from pH 9.0 up to 10.5 (the background ECL signal increases 206.3 V s^{-1} per pH unit). This background signal is due to the reaction of the dissolved oxygen present in the solution with the oxidized luminol and also to the more efficient production of excited 3-aminophthalate with pH.[49] Thus, the quantification of lactate must be performed in weakly alkaline conditions because the increases in the background produce lower precision; namely, from 4.5% of relative standard deviation (RSD) at pH 9.0 to 34.8% at pH 10.5, justifying the selected working pH of 9.0.

3.6. Analytical parameters

The analytical function was obtained by means of a calibration set composed of 6 standards with 5 replicates, each between 10^{-5} and 4×10^{-3} M at pH 9.0. The logarithmic behavior was linearized using a double logarithmic curve: $\log v_{ECL} = a\log$ [lactate] + b (Fig. 5C). This analytical function is commonly used with ECL enzymatic biosensors.[21]

Since the calibration function is logarithmic, the limit of the detection (LOD) is obtained using the standard criteria:[56] LOD = $y_b + 3s_b$, where y_b is the average blank signal and s_b is the standard deviation of the blank, which is determined from ten blanks from different biosensors (RSD 5.13%). The LOD found using these criteria is 5×10^{-6} M.

The repeatability, expressed as RSD of lactate concentration, was studied in two ways: (a) using a set of 10 different screen-printed biosensors working at three lactate concentrations within the analytical range (RSD 3–5%); and (b) using the same screen-printed cell 10 times prepared anew after each measurement and working at 10^{-4} M lactate concentration (RSD 3.68%) (Table 1). Thus, the use of different biosensors produces a precision quite similar to that obtained using the same screen-printed cell.

The lifetime of the disposable biosensor was studied protected from light and preserved at 4 °C using a series of prepared biosensors and regularly checking their response to a lactate concentration of 10^{-4} M. The signal grows up to 5 days after the biosensors are prepared, as can be observed in the Shewhart diagram (Fig. 6). The lifetime obtained is around 2 months, which is acceptable for this type of biosensor and coincides with the long-term stability study of an amperometric lactate biosensor based on LOx by Mizutani *et al.*[29] The cost of the biosensor considering reagents, screen-printed cells and support for membrane making is around 1.06 €/unit.

A comparative study of different sensors is presented in Table 2, showing that the disposable biosensor described has a good precision, although a shorter lineal range when compared to other sensors.

Table 1 Lactate biosensor analytical parameters

Parameter	Value	
Linear range/M	10^{-5} to 5×10^{-4}	
Ordinate	4.12 ± 0.04	
Slope	0.91 ± 0.04	
r^2	0.992	
LOD/M	5×10^{-6}	
RSD (%) blank ($n = 10$)[a]	5.13	
RSD (%) lactate ($n = 5$)[a]	5×10^{-5} M	3.91
	2×10^{-4} M	3.30
	5×10^{-4} M	4.20

[a] Number of replicates.

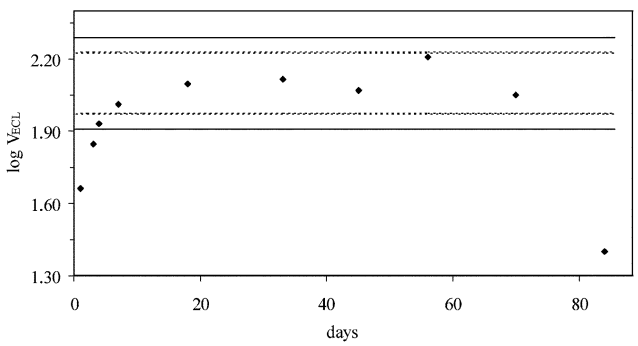

Fig. 6 Shewhart diagram for lifetime of disposable lactate biosensor.

3.7. Applications

The feasibility of the described biosensor was checked by analyzing lactate in human saliva. Saliva is a complex mixture of parotid, submandibular, sublingual, and minor gland secretions, mixed bacteria, sloughed epithelial cells, crevicular fluid, inorganic and organic compounds as lactic acid.[1] First, we tested the potential interference of whole saliva in lactate analysis by measuring a 10^{-4} M lactate solution in the presence of saliva. We observed a decrease in the ECL signal, although this decrease depended on the dilution of the saliva (55.4% to 1 : 2 dilution, 27.3% to 1 : 4, 25.7% to 1 : 8, and 18.8% to 1 : 12), which suggests a matrix effect that could be reduced with dilution. Additionally,

Table 2 Comparison of lactate enzymatic-based biosensor characteristics

Sensor	Range/M	LOD/M	Precision (%)	Application	Ref.
Amperometric screen-printed	10^{-6} to 5×10^{-4}	5×10^{-7}	2.5	Dairy products, blood and serum	64
Amperometric screen-printed	10^{-4} to 10^{-3}	5×10^{-5}	10.8	Wine samples	65
Amperometric screen-printed	10^{-7} to 1.8×10^{-6}	10^{-7}	3.0	Dairy products	66
Amperometric micromachined sensor	10^{-5} to 5×10^{-3}	—	5–12	Blood and saliva	27
CL disposable biosensor	5×10^{-5} to 4×10^{-3}	9.2×10^{-6}	5.5	Yoghurt	14
ECL biosensing chip	2×10^{-6} to 2×10^{-4}	2×10^{-6}	<8.0	Serum	22
ECL screen-printed	3×10^{-7} to 10^{-4}	3×10^{-7}	4.4	—	53
ECL disposable biosensor	10^{-5} to 5×10^{-4}	5×10^{-6}	3.9–4.2	Saliva	This paper

calibration curves in the presence (standard addition) and absence of saliva showed different slopes (a difference of 59.8%), confirming the matrix effect.

To overcome the matrix effect, we studied the different alternatives indicated below. We always used saliva coming from a healthy subject in fasting conditions whose teeth were brushed and mouth rinsed 30 min before sampling.[27]

In the first instance, we studied some procedures to eliminate proteins, epithelial cells and biomolecules and/or extract lactate. To do this, we compared the ECL signal of saliva with 10^{-4} M added lactate with the ECL signal of a standard lactate 10^{-4} M. Saliva was diluted 1 : 4 with working buffer in all cases. Centrifugation is a common method because it stops the bacterial action and removes both the cells and the turbidity[1] but in this case it produced a diminution in the ECL signal of 14.3%. Other methods were used obtaining the following decrease in percentages: heating in a water bath (51.3%); extraction with diethyl ether and hexane (10.2% for diethyl ether and 55.8% for hexane); C_{18} solid phase extraction (32.4%) and filtration through syringe filters (0.25 μm: 25.7%, and 0.40 μm: 12.6%). As can be seen, none of these procedures produce a partial effective elimination of interferences.[57,58]

To study possible interferents, we prepared artificial saliva[23] checking that the interference in lactate determination was similar to that of human saliva. Then we studied the interference of the different artificial saliva components (Table 3), with uric acid and ascorbic acid being the main interferents. Both acids are the most common interferents in amperometric biosensors for lactate.[59] The usual concentration of these acids in saliva is 5×10^{-3} M for uric acid and 10^{-3} M for ascorbic acid and their oxidation potentials in the electrochemical cells in our working conditions was 0.62 V and 0.11 V, respectively (Fig. 7A), values similar to those reported by John.[60]

Table 3 Contribution of different saliva components to ECL signal

Components[a]	% Decrease in ECL signal
$CaCl_2$	0.0
KCl	0.0
NH_4Cl	0.3
Lactate	0.0
Citric acid	2.5
Ascorbic acid	98.3
Uric acid	98.9
KSCN	6.8
CMC[b]	0.0

[a] Tested at the concentrations of artificial saliva. [b] CMC: carboxymethyl cellulose.

Ascorbic acid interferes by reducing the H_2O_2 generated from lactate. As a possible way to eliminate this interference, we tried prior oxidation on the screen-printed graphite electrode, a procedure described for the amperometric determination of uric acid in the presence of ascorbic acid.[61,62] We observed that by applying 0.4 V for 4 s before ECL measurement, ascorbic acid is oxidized and its interference disappears at its usual concentration in saliva. Additionally, if we compared the double logarithmic calibration function for lactate both with and without a constant amount of saliva, applying the previous 0.4 V only for the saliva sample, we observed parallel linear functions (slopes with a similarity of 96.9%); *i.e.* there is still another interference, due to uric acid, but there is no matrix effect (Fig. 7B). Uric acid is electrochemically oxidized[63] at the same time as luminol and this effect interferes with the measurement.

To estimate the uric acid effect, different concentrations of uric acid were added to saliva samples, showing a decrease in the ECL signal when the uric acid increases. From the inspection of lactate calibration functions with different added uric acid concentrations ($\log v_{ECL} = 0.94 \log$ [lactate] + 5.89 for no addition; $\log v_{ECL} = 0.89 \log$ [lactate] + 4.64 for 2×10^{-4} M; $\log v_{ECL} = 0.92 \log$ [lactate] + 3.62 for 5×10^{-3} M) (Fig. 7B) we concluded that uric acid only affects the ordinate of the calibration and does not contribute to the matrix effect.

To correct the error from the presence of uric acid, we used the internal blank procedure[25] for every saliva sample. This blank (v_{blank}) was obtained by means of a similar biosensor without enzyme.

In brief, it is possible to analyze saliva for lactate by means of the proposed ECL biosensor using a 1 : 4 dilution with working buffer, filtration through a 0.40 μm cellulose filter, followed by pre-treatment on the same biosensor applying 0.4 V for 4 s and finally a blank correction of saliva.

To verify the reliability of the proposed procedure, we analyzed saliva from three different people, validating the results

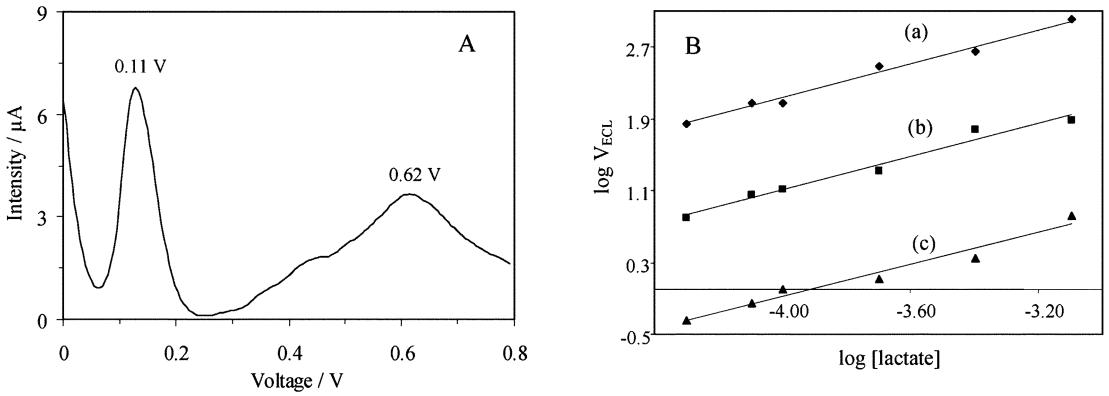

Fig. 7 (A) Voltammogram from a solution of ascorbic acid (10^{-3} M) and uric acid (5×10^{-3} M) at pH 9.0 phosphate buffer 0.2 M and 0.25 M NaCl. (B) Effect of different uric acid concentrations on lactate calibration curve: (a) no addition; (b) 2×10^{-4} M; and (c) 5×10^{-3} M.

Table 4 Determination of lactate in saliva by proposed and reference procedures

Saliva sample	ECL biosensor/M	Reference/M	MCP[a] (%)	P_{val} (%)
1[b]	$(3.10 \pm 0.25) \times 10^{-4}$	$(3.11 \pm 0.13) \times 10^{-4}$	99.6	94.6
2[c]	$(6.9 \pm 1.5) \times 10^{-4}$	$(8.39 \pm 0.79) \times 10^{-4}$	82.2	35.9
3[b]	$(3.37 \pm 0.01) \times 10^{-4}$	$(3.39 \pm 0.35) \times 10^{-4}$	99.4	94.3

[a] MCP: methods comparison percent. [b] Male. [c] Female.

against a manual enzymatic spectrophotometric method (Boehringer Mannheim kit) used as a reference procedure (Table 4).

4. Conclusions

An electrochemiluminescent disposable biosensor for lactate in saliva has been developed using an enzymatic recognition system based on lactate oxidase and a transduction system based on luminol. We succeeded in preparing and optimizing a very simple disposable optical sensor through the immobilization of all the reagents needed in a Methocel™ cellulose membrane on commercial screen-printed graphite electrochemical cells. This is environmentally friendly and has an estimated lifetime of at least two months when protected from light in normal conditions. Good repeatability, quick measurement (20 s) and versatility make it possible to use the sensor to monitor lactate in saliva samples as routine analysis with a sample treatment compatible with a disposable format. To the best to our knowledge, this is the first ECL biosensor used to monitor lactate in saliva and, thus, could be considered as a non-invasive analysis alternative to the usual ways of determining blood lactate. Currently, we are working on designing and tuning optical portable instrumentation for biosensors based on screen-printed electrodes using ECL as an analytical technique.

Acknowledgements

We acknowledge financial support from *Ministerio de Educación y Ciencia, Dirección General de Enseñanza Superior* (Spain) (Projects CTQ2005-09060-CO2-01, CTQ2005-09060-CO2-02 and P06-FQM-01467).

References

1 G. G. Guilbault, G. Palleschi and G. Lubrano, *Biosensors Bioelectron.*, 1995, **10**, 379–392.
2 R. V. T. Santos, A. L. R. Almeida, E. C. Caperuto, J. Martins and L. F. B. P. Costa Rosa, *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 2006, **145**, 114–117.
3 D. C. Klonoff, *Diabetes Tech. Therapeut.*, 2003, **5**, 929–931.
4 B. Detry, W. Nullens, M. L. Cao, A. Frans, A. Robert and T. Clerbaux, *Eur. Resp. J.*, 1998, **11**, 183–187.
5 Suman and A. Kumar, *Sens. Trans. J.*, 2008, **96**, 18–31.
6 K. Yoda, K. Shimazaki and Y. Ueda, *Ann. N.Y. Acad. Sci.*, 1998, **864**, 600–604.
7 M. Gerard, A. Chaubey and B. D. Malhotra, *Biosensors Bioelectron.*, 2002, **17**, 345–359.
8 X. Cui, C. M. Li, J. Zang and S. Yu, *Biosensors Bioelectron.*, 2007, **22**, 3288–3292.
9 Medizin, *Overview: Lactate Analysers*, http://medizin.li/lactate/, 2008.
10 C. A. Marquette, A. Degiuli and L. J. Blum, *Appl. Biochem. Biotechnol.*, 2000, **89**, 107–115.
11 S. Girotti, S. Ghini, G. Carrea, R. Bovara, A. Roda and R. Budini, *Anal. Chim. Acta*, 1991, **255**, 259–268.
12 H. Nakamura, Y. Murakami, K. Yokoyama, E. Tamiya, I. Karube, M. Suda and S. Uchiyama, *Anal. Chem.*, 2001, **73**, 373–378.
13 A. Hemmi, K. Yagiuda, N. Funazaki, S. Ito, Y. Asano, T. Imato, K. Hayashi and I. Karube, *Anal. Chim. Acta*, 1995, **316**, 323–327.
14 J. Ballesta-Claver, M. C. Valencia-Miron and L. F. Capitan-Vallvey, *Anal. Chim. Acta*, 2008, **629**, 136–144.
15 J. V. Samsonova, M. Y. Rubtsova and M. Franek, *Anal. Bioanal. Chem.*, 2003, **375**, 1017–1019.
16 A. Hasegawa, *Chemiluminescent analytical test strip and holder*, JP 08184595, JP, 1994.
17 L. F. Capitan-Vallvey, in *Encyclopedia of Sensors*, ed. C. A. Grimes, E. C. Dickey and M. V. Pishko, The Pennsylvania State University, Pennsylvania, USA, 1st edn, 2005, pp. 55–93.
18 M. M. Richter, *Chem. Rev.*, 2004, **104**, 3003–3036.
19 *Electrogenerated Chemiluminescence*, ed. A. J. Bard, Marcel Dekker, New York, 2004.
20 W. Miao, *Chem. Rev. (Washington, D. C.)*, 2008, **108**, 2506–2553.
21 C. A. Marquette and L. J. Blum, *Sens. Actuators, B*, 2003, **B90**, 112–117.
22 C. A. Marquette, A. Degiuli and L. J. Blum, *Biosensors Bioelectron.*, 2003, **19**, 433–439.
23 J. Y. Gal, Y. Fovet and M. dib-Yadzi, *Talanta*, 2001, **53**, 1103–1115.
24 D. J. Oh, J. Y. Lee, Y. K. Kim and H. S. Kho, *Int. J. Oral Maxillofac. Surg.*, 2008, **37**, 1027–1031.
25 G. Ramis Ramos and M. C. Garcia Alvarez-Coque, *Quimiometria*, Sintesis S.A., Madrid, 2000.
26 Boehringer Mannheim, Boehringer Mannheim L-lactate kit, 2006.
27 C. G. J. Schabmueller, D. Loppow, G. Piechotta, B. Schuetze, J. Albers and R. Hintsche, *Biosensors Bioelectron.*, 2006, **21**, 1770–1776.
28 I. Palchetti, S. Majid, A. Kicela, G. Marrazza and M. Mascini, *Int. J. Environ. Anal. Chem.*, 2003, **83**, 701–711.
29 F. Mizutani, S. Yabuki and Y. Hirata, *Anal. Chim. Acta*, 1995, **314**, 233–239.
30 C. Chouteau, S. Dzyadevych, C. Durrieu and J. M. Chovelon, *Biosensors Bioelectron.*, 2005, **21**, 273–281.
31 E. V. Gogol, G. A. Evtugyn, J. L. Marty, H. C. Budnikov and V. G. Winter, *Talanta*, 2000, **53**, 379–389.
32 N. M. Rao, K. Hool and T. A. Nieman, *Anal. Chim. Acta*, 1992, **266**, 279–286.
33 I. Palchetti, S. Laschi and M. Mascini, *Anal. Chim. Acta*, 2005, **530**, 61–67.
34 J. Gu, P. Xu and Y. Qu, *J. Mol. Catal. B: Enzym.*, 2002, **18**, 299–305.
35 G. P. Jirka, A. F. Martin and T. A. Nieman, *Anal. Chim. Acta*, 1993, **284**, 345–349.
36 M. M. J. Oosthuizen, M. E. Engelbrecht, H. Lambrechts, D. Greyling and R. D. Levy, *J. Biolumin. Chemilumin.*, 1997, **12**, 277–284.
37 K. Yorita, T. Matsuoka, H. Misaki and V. Massey, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 13039–13044.
38 B. Lillis, C. Grogan, H. Berney and W. A. Lane, *Sens. Actuators, B*, 2000, **68**, 109–114.
39 O. M. Schuvailo, O. O. Soldatkin, A. Lefebvre, R. Cespuglio and A. P. Soldatkin, *Anal. Chim. Acta*, 2006, **573–574**, 110–116.
40 H. Karatani, *Bull. Chem. Soc. Jpn.*, 1987, **60**, 2023–2029.
41 G. B. Schuster, *Acc. Chem. Res.*, 1979, **12**, 366–373.
42 B. B. Kim, V. V. Pisarev and A. M. Egorov, *Anal. Biochem.*, 1991, **199**, 1–6.
43 Y. Huang and W. Liu, *J. Pharm. Biomed. Anal.*, 2005, **38**, 537–542.
44 H. P. Misra and P. M. Squatrito, *Arch. Biochem. Biophys.*, 1982, **215**, 59–65.
45 L. Zhu, Y. Li, F. Tian, B. Xu and G. Zhu, *Sens. Actuators, B*, 2002, **84**, 265–270.
46 S. Sakura, *Anal. Chim. Acta*, 1992, **262**, 49–57.
47 H. X. Yu and H. Cui, *J. Electroanal. Chem.*, 2005, **580**, 1–8.
48 Y. Su, J. Wang and G. Chen, *Anal. Chim. Acta*, 2005, **551**, 79–84.
49 K. E. Haapakka and J. J. Kankare, *Anal. Chim. Acta*, 1982, **138**, 263–275.
50 J. Lee and H. H. Seliger, *Photochem. Photobiol.*, 1972, **15**, 227–237.
51 W. Wang, H. Cui, Z. X. Deng, Y. P. Dong and J. Z. Guo, *J. Electroanal. Chem.*, 2008, **612**, 277–287.

52 Z. Lin, J. Sun, J. Chen, L. Guo and G. Chen, *Electrochim. Acta*, 2007, **53**, 1708–1712.
53 B. P. Corgier, C. A. Marquette and L. J. Blum, *Anal. Chim. Acta*, 2005, **538**, 1–7.
54 K. A. Fahnrich, M. Pravda and G. G. Guilbault, *Talanta*, 2001, **54**, 531–559.
55 G. Salgado, J. Navarrete, C. Bustos, C. Sanchez and R. Ugarte, *Quim. Nova*, 2006, **29**, 381–384.
56 N. J. Miller and J. C. Miller, *Estadística y Quimiometría para Química Analítica*, Prentice Hall, Madrid, 4th edn, 2002.
57 F. Karaburun, PhD Thesis, Middle East Technical University, 2004.
58 G. Kyuchoukov, D. Yankov, J. Albet and J. Molinier, *Industrial & Engineering Chemistry Research*, 2005, **44**, 5733–5739.
59 G. Palleschi, M. H. Faridnia, G. J. Lubrano and G. G. Guilbault, *Anal. Chim. Acta*, 1991, **245**, 151–157.
60 S. A. John, *J. Electroanal. Chem.*, 2005, **579**, 249–256.
61 J. Wu, J. Suls and W. Sansen, *Electrochem. Commun.*, 2000, **2**, 90–93.
62 J. M. Zen and C. T. Hsu, *Talanta*, 1998, **46**, 1363–1369.
63 J. Marsh and G. Dryhurst, *J. Electroanal. Chem.*, 1979, **95**, 81–90.
64 N. G. Patel, A. Erlenkotter, K. Cammann and G. C. Chemnitius, *Sens. Actuators, B*, 2000, **67**, 134–141.
65 A. Avramescu, T. Noguer, V. Magearu and J. L. Marty, *Anal. Chim. Acta*, 2001, **433**, 81–88.
66 F. Ghamouss, S. Ledru, N. Ruillq, F. Lantier and M. Boujtita, *Anal. Chim. Acta*, 2006, **570**, 158–164.

