Anal. Chem. **2010,** *82,* 5568–5572

Amperometric Biosensor for Direct Blood Lactate Detection

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An amperometric sensor for lactate quantification is presented. The developed biosensor requires only 0.2 U of lactate oxidase, which is immobilized in a mucin/albumin hydrogel matrix. By protecting the platinum surface with a Nafion membrane, typical interference related to negatively charged species such as ascorbic acid has been minimized to practically undetectable levels. Electrochemical properties associated with the Nafion membrane are assessed as a function of Nafion concentration. In a phosphate buffer solution of pH 7.0, linear dependence of the catalytic current upon lactate bulk concentration was obtained between 2 and ~1000 μM. A detection limit of 0.8 μM can be calculated considering 3 times the standard deviation of the blank signal divided by the sensitivity of the sensor. The lactate biosensor presents remarkable operational stability and sensitivity (0.537 ± 0.007) mA.M^{-1}, where the error is the standard deviation of the slope calculated from the linear regression of the calibration curve of a fresh biosensor. In this regard, the sensor keeps practically the same sensitivity for 5 months, while the linear range decreases until an upper value of 0.8 mM is reached. Assays performed with whole blood samples spiked with 100 μM lactate gave (89 ± 6)% of recovery.

The quantification of L-lactate by amperometric biosensors is gaining importance not only for clinical purposes,[1−4] but also for food and wine quality assessment.[5] Physiological lactate levels are related to the status of anaerobic metabolism associated with muscle contraction.[6−8] Under resting conditions, healthy persons have lactate concentrations between 0.6 and 2 mM, but during strong physical activity the value of this parameter can rise up to 20 or 30 mM. Lactate quantification is particularly important in sports medicine, since athletes have to stop their physical activity when they reach their lactate threshold.[9,10] After this limit, the concentration of lactate rises exponentially and the athlete may have metabolic disorders and injured tissues.[7]

Several pathologic conditions can also increase the lactate production, such as the case of patients with cardiac disease and diabetes. Elevation of resting blood lactate concentration is not only associated with survival risk,[1] but it can also be used as an indicator of the patient oxygen supply.[2] Accordingly, rapid determination of lactate is particularly important in special care units.

Lactate oxidase (LOD) is widely used in lactate biosensors because of its simple reaction and easy biosensor design configuration. LOD catalyzes the oxidation of lactate to pyruvate. In presence of dissolved O_2, the enzyme can be reoxidized, releasing H_2O_2. This last product can be oxidized at the electrode surface restoring the former concentration of O_2 and giving a current proportional to the amount of dissolved lactate. Chemical and electrochemical reactions involved in a LOD sensor can be summarized by the following reactions:

$$\text{L-lactate} + \text{LOD}_{ox} \rightarrow \text{pyruvate} + \text{LOD}_{red} \quad (1)$$

$$\text{LOD}_{red} + O_2 \rightarrow \text{LOD}_{ox} + H_2O_2 \quad (2)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

Despite biosensors' specificity, they often suffer from some limitations, such as long response time, short stability, and poor reproducibility. Stability is of great importance for the success of these devices as analytical instruments.[11,12] LOD is not a very robust enzyme. As a consequence, it is usually found that its catalytic properties decay rather quickly when it is removed from its natural matrix and attached to a surface.[13] In a previous study, we showed that a mucin/albumin matrix is a very good option for immobilizing this enzyme.[14] The best performance of this LOD sensor was found when LOD is cross-linked within a matrix composed of a 70/30 mucin/albumin mass ratio. Since this matrix forms a hydrogel, LOD can be immobilized without suffering

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10.1021/ac1004426
Published on Web 06/02/2010

major structural changes and loss of activity. This matrix was entrapped between two polycarbonate membranes and fixed to the surface of a Pt electrode. Under optimal conditions, the sensor showed a 3 orders of magnitude linear range, excellent sensor-to-sensor reproducibility, and remarkable sensitivity.[14] A major problem to be solved was the inefficient elimination of interferences. In this regard, not only H_2O_2 can react at the electrode surface, but other electroactive compounds such as ascorbate, urate, and cysteine may also react. These compounds are commonly found in blood samples, and all of them are in some way charged under physiological conditions. Extensive efforts have been devoted to minimize these interferences[4,6,15] using perm-selective coatings,[16] and intermediary redox reactions[17,18] multilayers of polymers[19] and different strategies involving Nafion[13,20,21] have been employed with differing degrees of success.

Nafion is a sulfonated fluoropolymer with a hydrophobic backbone of polytetrafluoroethylene attached to hydrophilic side chains that end on sulfonic groups. As the membrane takes up water, side chains swell to accommodate the solvent, while the membrane backbone acts against this effect until they reach an equilibrium state. The hydrophobic/hydrophilic properties of nafion membranes vary depending on its concentration and the kind of solvent used to prepare the film.[22] It is known that Nafion can exclude interfering electrochemical active substances from the electrode surface.[20] Nevertheless, in all cases, a significant loss of sensitivity has been reported, irrespective of that nafion is mixed with the matrix[21] or placed on the sensor surface.[13,20]

The aim of the present study is to develop a lactate biosensor that shows negligible signal change in the presence of typical interfering species. Our approach involves the use of a nafion membrane between the platinum electrode and the mucin/albumin hydrogel, where the enzyme has been immobilized. Since the enzymatic product H_2O_2 is uncharged at pH 7, it is able to pass through the nafion membrane while other charged species are excluded. Under optimal conditions, the interference of ascorbic acid is negligible, considering its usual concentration in blood, but the sensor keeps good sensitivity to lactate when it is exposed to untreated blood samples.

EXPERIMENTAL PART

Reagents. All solutions were prepared with ultra pure water (18 MΩ·cm^{-1}) from a Millipore Milli-Q system. The base electrolyte solution (0.1 M) consisted of 0.05 M HK_2PO_4/0.05 M H_2KPO_4 (Merck, Germany). This solution was renewed weekly, and small amounts of H_2SO_4 (Baker, U.S.A.) or KOH (Merck, Germany) were used to fix it at pH 7.0. Stock solutions of 0.1 M lactate (Sigma, U.S.A.) and 5% (v/v) glutaraldehyde (Backer, U.S.A.) were prepared in the base electrolyte. Nafion (Aldrich, U.S.A.) was received as a solution 5% (w/w); the solvent was a mixture of aliphatic alcohols of low molecular weight and water. A total amount of 100 U LOD from Pediococus species (Sigma, U.S.A.) was dissolved in 1000 μL of the base electrolyte, then the solution was separated into aliquots of 20 μL and stored at −20 °C. Thus, every aliquot bore 2 U of LOD. Mucin (Sigma, U.S.A.) was mortared and stored as dry powder at 4 °C. Bovine serum albumin (Sigma, U.S.A.) was used as received. All solutions were stored at 4 °C. All reagents were of analytical grade and used as received. Polycarbonate membranes of 0.05 μm pore size were cut in discs of 6 mm diameter (Millipore, U.S.A.). Blood samples were provided by the Hospital Privado SRL in vials with walls protected with heparin and analyzed immediately after their reception. An aliquot of 950 μL whole blood was spiked with 50 μL of 0.1 M lactate solution to perform a recovery assay of the sensor.

Apparatus. All electrochemical experiments were performed with an Autolab PGSTAT 30 Electrochemical Analyzer (Eco Chemie, The Netherlands). The measurements were carried out using a conventional three-electrode system. A Pt wire was the counter electrode, a Ag|AgCl|KCl (3 M) (CH Instruments, U.S.A.) was the reference electrode, and a 2 mm diameter Pt disk (CH Instruments, U.S.A.) was the working electrode. Amperometric detection was carried out under batch conditions, and the solution was stirred with a magnetic stir bar at 120 rpm during the whole electrochemical experiment.

Nafion Membrane Preparation. Solutions of Nafion of different concentration were prepared. The undiluted solution of Nafion was designed as Nafion 100%. Other dilutions with concentrations ranging from 1% (v/v) to 75% (v/v) were prepared by dissolving aliquots of Nafion 100% into absolute ethanol. To prepare a Nafion membrane, an aliquot of 20 μL Nafion was dispensed on the surface of a platinum electrode and left to dry in a hood of filtered air. It is extremely important to avoid dust contamination to get reproducible membranes.

Preparation of the Enzymatic Matrix. A total mass of 6.0 mg composed by 70/30 mucin/albumin was dissolved in 40 μL of base electrolyte. Proteins were mixed for 5 min and then transferred into a vial containing 2 U of LOD. The resulting 60 μL LOD-matrix system was mixed for extra 5 min and stored at 4 °C.

Construction of the Enzymatic Electrode. A 6 μL aliquot of the LOD−matrix system was mixed with 3 μL of glutaraldehyde and entrapped between two membranes of polycarbonate. The resulting enzymatic matrix was placed on the Pt−Nafion modified working electrode and fixed with an open cap (see Figure 1). After waiting 5 min, buffer solution was used to gently rinse the biosensor and eliminate glutaraldehyde molecules that did not react with the polymeric matrix.

RESULTS AND DISCUSSION

The idea of using Nafion as a negatively charged polymer that blocks interferences and increases the specificity of sensors is well-known.[13,20,22] However, in the case of biosensors, the presence of this kind of polymer can also compromise the stability of the enzyme or hinder the electrochemical reaction of the enzymatic product. To study the influence of Nafion membranes on the analytical response of a lactate biosensor, the electrochemical

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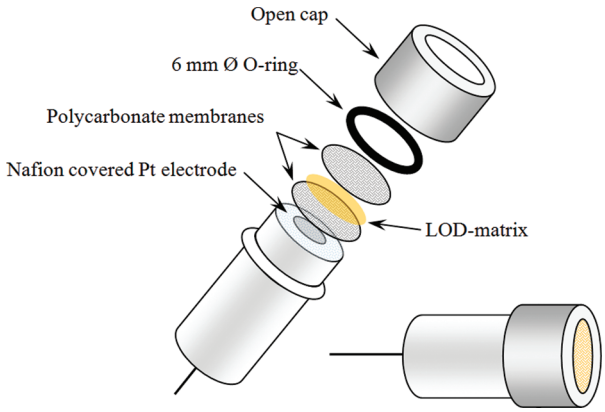

Figure 1. Scheme showing the construction of the lactate biosensor.

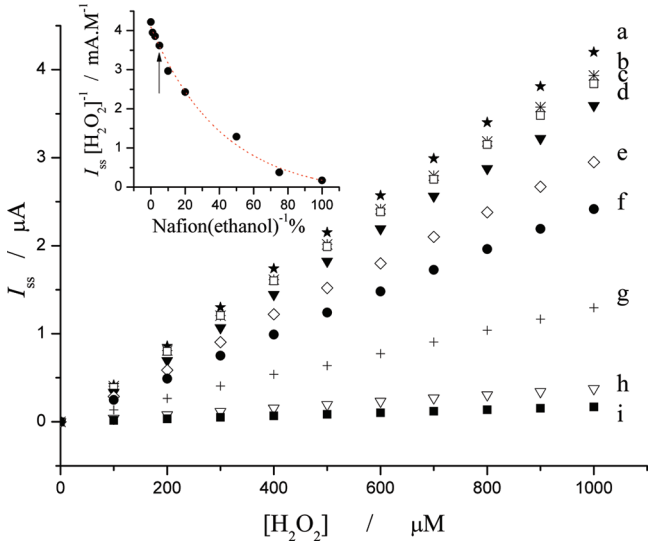

Figure 2. Calibration curves corresponding to the oxidation of H_2O_2 on a Pt electrode covered with Nafion membranes of different concentration. Nafion/ethanol% (v/v): (b) 1, (c) 2.5, (d) 5, (e) 10, (f) 20, (g) 50, (h) 75, and (i) 100. Curve a belongs to a bare Pt electrode. E = 0.65 V, pH = 7.0. Inset: Dependence of the electrode sensitivity to H_2O_2 on the Nafion concentration.

oxidation of H_2O_2 was analyzed when the electrode surface was coated with films prepared from different solutions of Nafion. Since H_2O_2 is the product of the enzymatic reaction, it should be the unique electroactive species that reaches the electrode surface.

Figure 2 shows a set of calibration curves corresponding to the oxidation of H_2O_2 on Pt electrodes covered with different membranes of Nafion. Curve a corresponds to the values of steady state limiting current (I_{ss}) when H_2O_2 is oxidized on a bare Pt electrode. The inset shows the dependence of the sensitivity of modified electrodes on the concentration of Nafion used for the construction of each membrane. Although the sensitivity decreases as the concentration of Nafion increases, all curves exhibit linear behavior. When compared with the bare Pt surface, the signal decreases more than 90% for membranes prepared with Nafion/ethanol > 75%, whereas the sensitivity is higher than 50% if Nafion/ethanol is <20%. This effect is much more drastic when the electrochemical reaction of negatively charged species such as ascorbate is studied

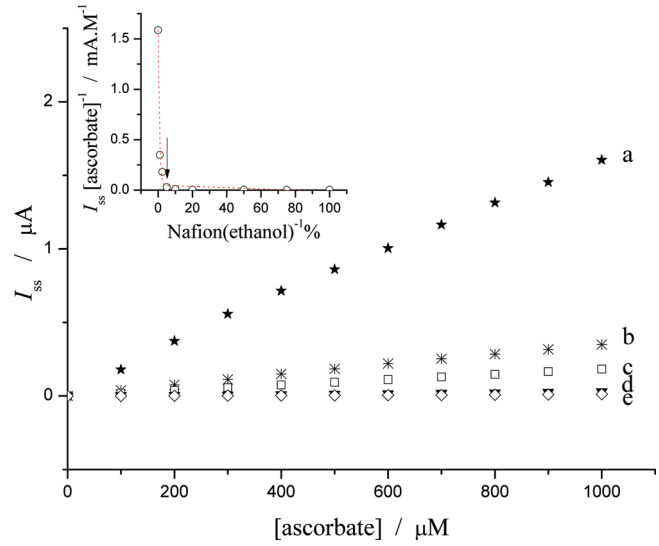

Figure 3. Calibration curves corresponding to the oxidation of ascorbate on a Pt electrode covered with Nafion membranes of different concentration. Nafion/ethanol% (v/v): (b) 1, (c) 2.5, (d) 5, (e) 10, (f) 20, (g) 50, (h) 75, and (i) 100. Curve a belongs to a bare Pt electrode. E = 0.65 V, pH = 7.0. Inset: Dependence of the electrode sensitivity to ascorbate on the Nafion concentration.

(Figure 3). When the Pt surface is protected with a membrane prepared from solutions of Nafion/ethanol > 5%, the electrochemical signal of ascorbate ions is lower than 4% of that expected from a bare electrode. From the comparison of Figures 2 and 3, it can be observed that the selectivity properties of Nafion membranes depend on the concentration of Nafion used for the membrane construction.

From the above results, the best selectivity is achieved when the surface is modified with solutions of 5% Nafion/ethanol. Accordingly, the enzymatic electrodes were prepared following the protocol indicated at the experimental section utilizing this Nafion concentration. Figure 4 shows chronoamperometric profiles of lactate biosensors prepared in absence and presence of the Nafion membrane, Figure 4A and B, respectively. The curves in part a show the response of the biosensor when 100 μM lactate is added; the curves in part b correspond to transients recorded after the addition of 100 μM ascorbic acid. Both electrodes require a preconditioning step of 2000 s at 0.65 V to get a low and stable signal of background current. This step is necessary only for freshly prepared electrodes, since it becomes significantly shorter for subsequent calibration curves (around 300 s). Chronoamperometry was also used for the construction of calibration curves at different potentials (not shown). The optimum response was found at E = 0.65 V.

The electrochemical response of the biosensor without Nafion reaches steady state around 90 s after the injection of 100 μM lactate (curve a of Figure 4A). Under these conditions, the value found for I_{ss} = (74 ± 3) nA. A similar experiment in presence of a Nafion membrane prepared from a 5% Nafion/ethanol solution gives a value of I_{ss} = (63 ± 1) nA after the addition of lactate. The current rises with a similar slope as in the absence of the Nafion membrane, but only 50 s is required to reach steady state in this case. This relatively fast response of the sensor might be related to a better adhesion of polycarbonate to electrode surfaces modified with Nafion. Concerning the

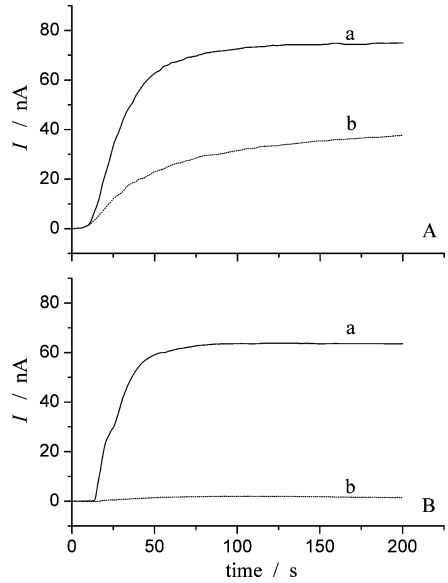

Figure 4. Chronoamperometric profiles of lactate biosensors when exposed to solutions of (a) 100 μM lactate and (b) 100 μM ascorbic acid. (A) Sensor without a Nafion membrane. (B) Sensor protected with a membrane prepared from a 5% Nafion/ethanol solution. E = 0.65 V, pH = 7.0.

chronoamperometric signal of ascorbate, these anions diffuse slower than H_2O_2 through the polycarbonate membrane, and the system requires around 5 min to reach a steady state current. As expected, a remarkable diminution of the oxidation current of ascorbate is achieved when the electrode is protected with the film of Nafion, curve b of Figure 4B. The I_{ss} corresponding to the addition of 100 μM ascorbate is only (1.7 ± 0.2) nA, more than 96% lower than that of a sensor without the Nafion membrane.

As was shown in a previous work, the biosensor has relatively good operational stability, around 30 days, without the Nafion membrane.[13] In that case, the sensor was prepared utilizing 0.1 U of LOD instead of 0.2 U. Figure 5 shows calibration curves corresponding to a biosensor protected with a Nafion membrane. From the data related to day 1, it is possible to calculate the sensitivity of the sensor as (0.537 ± 0.007) mA M^{-1}, where the error corresponds to the standard deviation of the slope of this calibration curve. After the construction of a calibration curve, the biosensor was rinsed with buffer and stored in the fridge at 4 °C. The presence of the Nafion membrane and the use of a higher concentration of enzyme result in the outstanding operational stability of the sensor. After 5 months, the sensor exhibits linear response for 2 μM < [lactate] < 800 μM and an average sensitivity of (0.51 ± 0.03) mA M^{-1}. Moreover, the sensitivity of the sensor is 30% of the initial response a year after its assembly. The detection limit of the sensor is 0.8 μM, calculated as 3 times the standard deviation of the blank signal divided by the sensitivity of the sensor. However, it was possible to assess only samples with lactate concentration higher than 2 μM due additional noise associated with the sample injection process.

The recovery of added lactate was also calculated according the suggested protocol.[23] Figure 6 shows chronoamperometric profiles of a lactate biosensor when 100 μL of (a) blood, (b) 5 mM lactate in buffer solution, and (c) blood spiked with 5 mM

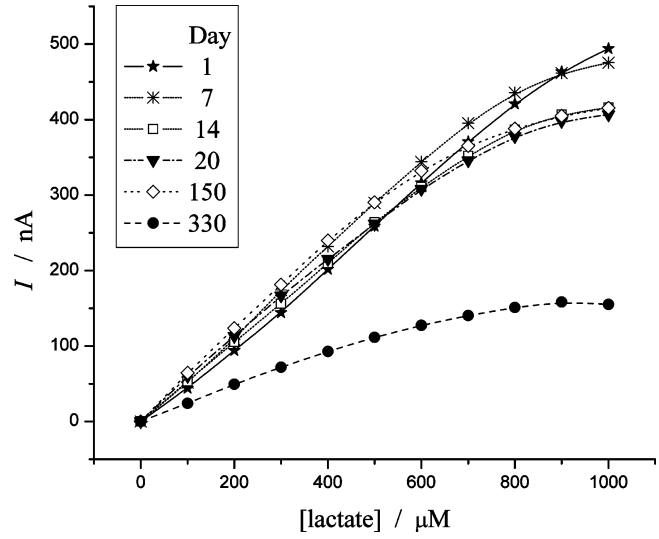

Figure 5. Calibration curves of a lactate biosensor exposed to different concentrations of lactate. Sensor protected with a membrane prepared from a 5% Nafion/ethanol solution. E = 0.65 V, pH = 7.0.

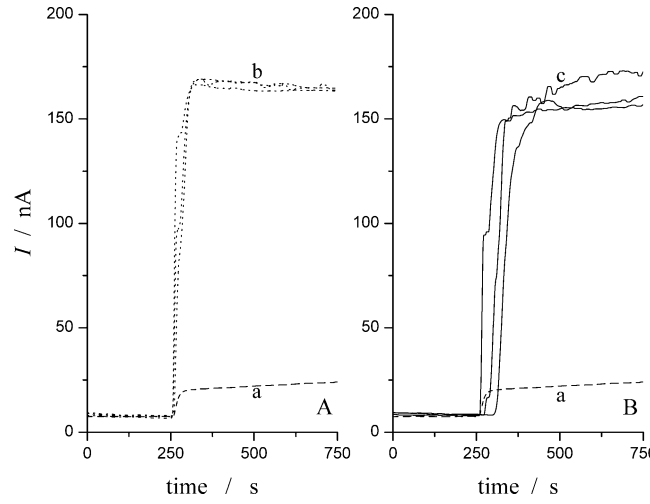

Figure 6. Chronoamperometric profiles for a lactate biosensor when samples of (a) 100 μL of blood, (b) 5 mM lactate dissolved in 100 μL background electrolyte, and (c) 100 μL of blood spiked with 5 mM lactate are independently added to a chamber containing 2 mL background electrolyte. Biosensor protected with a membrane prepared from a 5% Nafion/ethanol solution. E = 0.65 V, pH = 7.0.

lactate are added to an electrochemical chamber with 2 mL of base electrolyte pH 7.0. In this way, every sample is diluted 21 times when it is exposed to the biosensor. The current increases (16.1 ± 0.1) nA after the addition of the sample of whole blood. According to the calibration curve of this sensor, the expected concentration in blood would be (0.77 ± 0.01) mM, which is in agreement with a value for the basal lactate level of a healthy person.[3] The average values of solutions b and c are (156 ± 1) nA and (155 ± 7) nA, respectively. Thus, when the sensor is exposed to samples of whole blood, its response will be (89 ± 6)% of the signal expected from a buffer solution with the same lactate concentration.

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CONCLUSIONS

The development and performance of an amperometric lactate biosensor has been described. The signal related to typical interfering species has been significantly reduced by placing a Nafion membrane between the Pt electrode and the sensing membrane. The membranes prepared from the more concentrated solutions of Nafion stop larger amounts of interfering species. However, this sort of membranes also hinders the passage of the enzymatic product and lowers the sensitivity of the electrode. In this regard, the signal corresponding to ascorbic acid diminishes 96% when a membrane is prepared from a 5% Nafion/ethanol dilution, but a decrease of only 15% is observed on the signal of H_2O_2.

The proposed biosensor requires only 0.2 U of lactate oxidase and keeps its sensitivity practically unchanged for 5 months. The use of the Nafion membrane increased more than 5 times the operational stability of the biosensor. During this time, linear response is observed for solutions with lactate concentrations that range from 2 to ~1000 μM. Due to the high stability of the sensor, it is possible to use it for the direct assessment of lactate in whole blood samples. In this regard, once the biosensor has been assembled, it would be necessary to calibrate it only daily or weekly, depending on the desired accuracy. When human blood samples were spiked with a standard solution of lactate, a recovery assay of (89 ± 6)% was obtained for the sensor. Accordingly, the proposed sensor would be suitable for lactate quantification in untreated blood samples.

ACKNOWLEDGMENT

This work was funded by FONCYT, CONICET, and Secyt-UNC. F. Garay and A. Baruzzi are permanent research fellows of CONICET.

Received for review February 18, 2010. Accepted May 17, 2010.

AC1004426