Full Paper

Amperometric Biosensor Based on Immobilization of Oxalate Oxidase in a Mucin/Chitosan Matrix

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Abstract
An amperometric electrode for oxalate determination immobilizing the oxalate oxidase in a mucin/chitosan (muc/chit) gel with glutaraldehyde as crosslinking agent is presented. The effect of muc/chit weight ratio and volume percent (vol.%) of glutaraldehyde was studied. A very low dynamic response was observed in the case of 100% chitosan with 5 vol.% crosslinking agent. The addition of mucin to chitosan for enzyme immobilization resulted in a biosensor with much better performance, concerning to dynamic response, sensitivity, and stability, with 75% of the initial response after two months. The ratio muc/chit 70/30 was considered optimum for the immobilization. A slight crosslinking and the incorporation of mucin largely influences the swelling and diffusion of the analyte; a direct effect of these properties on the calibration slope was found; the hydrophilic environment for the biomolecule also favor the enzymatic activity through a higher enzyme-substrate interaction.

Keywords: Biosensor, Chitosan, Electrochemistry, Hydrogels, Oxalate

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1. Introduction

Enzyme based biosensors have proved to be an excellent option for metabolite determination due to the advantages of enzyme specificity, the analytical precision as well as simplicity of use. In these devices the enzyme is immobilized in an electrode using different kinds of supports, including polymeric matrices. Although immobilization improves stability and facilitates that the enzyme can be reused [1–4], some considerations with respect to the immobilization conditions deserve to be done, as they have important analytical consequences due to the strong effect on the global enzymatic reaction [5]. On one hand, conformation changes as a result of interactions between enzyme and matrix may modify enzyme K_m and V_{max}; on the other hand, the enzymatic reaction takes place in a layer separated from the bulk of the analyte solution through which substrates and products need to be transported by diffusion. This "internal diffusion" is slower than that through bulk solution because of steric barriers and the high solids content of the enzyme layer. The microenvironment which surrounds an immobilized enzyme can act not only as a barrier for the free diffusion of molecules but it also may attract or repel substrate or product to its surface concentrating or depleting them in the immediate vicinity of the enzyme. The charge of the polymer obviously influence the partition of a charged substrate with direct consequences on the response, and therefore on the sensitivity of the biosensor [6, 7].

This dependence of the reaction rate on the local microenvironment clearly indicates that these biosensors can be improved by modifying the enzyme immobilization procedure.

Although it is recognized that there is no universal support for all enzymes and their applications, a number of desirable characteristics should be common to any material considered for immobilizing enzymes. They include: high affinity to proteins, availability of reactive functional groups for direct reactions with enzymes and for chemical modifications, hydrophilicity, mechanical stability and rigidity, regenerability, and ease of preparation [4].

In a previous paper we proposed an amperometric electrode based on the immobilization of oxalate oxidase in a 70/30 weight ratio mucin/carbopol gel for oxalate sensing, with quite high stability and much longer linear range, although a lower sensitivity, than with albumin as polymer support [8].

Oxalate is a metabolite excreted by kidney that produces oxidant stress and death of renal cells at pathological concentrations [9]. Many methods have been recommended for oxalate determination in clinical laboratory analyses but some of them are time-consuming and expensive (as chromatographic and spectrophotometric) while some others need a chemically pretreated sample [10, 11]. These

drawbacks may be overcome using oxalate biosensors, many of which have been developed in the last years with different performances [12, 13].

Mucin, mainly composed of glycoproteins, is able to form viscoelastic gels with more than 95% water content [14] giving an excellent environment for enzymes. Although electrodes prepared with 100% mucin showed a long linear range, they had little sensitivity and fair stability. We found great advantages of mixing this polymer with carbopol, very well-known for its mucoadhesive properties. The sensitivity and stability were improved and we also demonstrated that the interactions between mucin and carbopol molecules produce a notable gel strengthening reflected on the viscoelastic behavior and rheologic synergism [15]; this physicochemical property rendered in the high stability of the electrode.

In the present paper we substitute carbopol, a negatively charged polymer, by a polycation, chitosan, for immobilizing the oxalate oxidase. Among the many carriers that have been considered and studied for immobilizing enzymes, organic or inorganic, natural or synthetic, chitin and chitosan exhibit most of the above characteristics [16]. Chitosan, the principal derivative of chitin, is a natural polyaminosaccharide. It is insoluble in water, but the presence of amino groups renders it soluble in dilute solutions of most organic acids, below pH about 6.5, and makes chitosan a cationic polyelectrolyte, one of the few found in nature [16]. This basicity grants chitosan singular properties: adhesion to negatively charged surfaces, aggregation with polyanionic compounds, and chelation with heavy metal ions. Both the solubility in acidic solutions and aggregation with polyanions impart chitosan excellent gel-forming properties with important applications [17, 18]. Different authors have reported biosensors based on chitosan [19–22] with improved stability. We investigated the effect of mixing mucin with chitosan for immobilizing the enzyme in the analytical performance of an oxalate biosensor.

The analytical performance of an amperometric electrode for oxalate determination, prepared immobilizing the enzyme in different mucin/chitosan gels, is presented. The effect of polymer composition and crosslinking agent concentration on swelling and diffusion properties is analyzed and these properties are related to the analytical behavior.

2. Experimental

2.1. Instrumentation

A cell designed for oxygen detection was adapted and used for electrochemical detection of the hydrogen peroxide formed during the enzymatic reaction. This cell includes a 0.6 mm diameter Pt disk as working electrode, an outer silver ring (Ag/AgCl) as a reference electrode and a Pt as counter electrode.

The working electrode was polarized at +0.650 V (versus Ag/AgCl) for the oxidation of hydrogen peroxide. An Autolab (Ecochemie) electrochemical analyzer was used as the polarizing source.

2.2. Reagents

Mucin (muc) (porcine stomach Type III, partially purified), oxalate oxidase (ODD) enzyme and chitosan (chit) (low molecular weight, 75–85% deacetylated) were obtained from Sigma Ltd; potassium oxalate from Anedra; polycarbonate membranes (pore diameter 0.05 μm) were purchased from Millipore, Ireland. Glutaraldehyde (25 vol.%) was purchased from Mallinckrodt Baker, USA. The pH 2.85 buffer solution was prepared using 0.5905 g per 100 mL succinic acid (Anedra) in purified water (Milli Q system).

2.3. Procedures

For the preparation of the electrodes, 3.0 μL of solution containing enzyme (5.38 U/mL) were mixed with 3.0 μL of the following solutions which were prepared dissolving: 0.4 g of mucin (100%), different weight mucin/chitosan (90/10, 70/30, 50/50 or 30/70) ratios or chitosan (100%) in 4.1 mL of 2 vol.% acetic acid (pH 2.8). These compositions were named as follows: muc100, muc/chit 90/10, muc/chit 70/30, muc/chit 50/50, muc/chit 30/70, and chit100, respectively. Then, each of these resultant solutions was mixed with 1.3 μL of aqueous glutaraldehyde solutions of different concentrations (0.5, 3.0 or 5.0 vol.%) and immediately applied to a piece of internal membrane (polycarbonate 0.05 μm pore size). After that, a piece of 1 cm^2 of the external membrane (polycarbonate 0.05 μm pore size) was placed onto the matrix and this sandwich was pressed between two microscope slides at room temperature for 10 min. The resulting laminates were exhaustively washed with buffer solution (pH 2.85) to remove the excess of glutaraldehyde. Then, the washed laminates were fixed over the Pt working electrode.

Calibration curves of the oxalate electrodes prepared using the matrixes described above were plotted from the current-time curves obtained by addition of 10 μL of 0.05 M potassium oxalate to 5.0 mL of buffer solution.

The time required to reach 95% of the steady state in the current-time curves was considered as a measure of the speed of the response (t_{95}). Stability was evaluated by performing several calibration curves on different days after the preparation of the following matrix for immobilization: muc100, muc/carbopol 70/30, muc/chit 70/30 cross-linked with 5.0 vol.% glutaraldehyde and chit100, muc/chit 70/30 cross-linked with 0.5 vol.% glutaraldehyde. When not used the electrodes were stored at 4 °C in succinic buffer (pH 2.85).

For diffusion measurements, the following gels: muc/chit 90/10, muc/chit 70/30, muc/chit 50/50, muc/chit 30/70, and chit100 (all of them cross-linked with 5.0 vol.% glutaralde-

hyde) were introduced between two polycarbonate membranes without the presence of the enzyme. Diffusion through chit100 cross-linked with 0.5 vol.% was also measured. The enzyme was excluded from these experiments in order to observe exclusively the effect of the matrix in the diffusion of the analyte without interacting with the enzyme. Additionally the enzyme was present in a very low concentration inside the matrix of the amperometric electrodes. In each experiment, oxalate was allowed to diffuse from a stirred 20 mM oxalate solution buffered (in succinic acid) at pH 2.85 through the sandwich placed on an oxalate enzymatic electrode, used for continuous detection of the oxalate that crosses each different matrix. The electrode used for the detection was prepared immobilizing the enzyme in the muc/chit 70/30 (cross-linked with 0.5 vol.% glutaraldehyde) as described above. The time from which the current begins to increase (τ_0) and the slope of the curve were considered as the measure of the diffusion rate.

The swelling indexes of muc/chit 70/30, muc/chit 50/50 and muc/chit 30/70 (all of them cross-linked with 5.0 vol.% glutaraldehyde) and muc/chit 70/30 (cross-linked with 3.0 vol.% glutaraldehyde), were gravimetrically determined. For these measurements each gel (approximately 0.1 g) was allowed to swell into a vessel, in 50 mL of the buffer (pH 2.85) for 96 h, weighted and then dried at room temperature until constant weight.

The degree of hydration is defined as: % Swelling = $[(M - Mo)\ 100/Mo]$, where M and Mo are the weights of the wet and the dry matrix, respectively.

3. Results and Discussion

3.1. Effect of Matrix Composition on the Calibration Curves

The current-time response of electrodes prepared immobilizing the oxalate oxidase in chitosan, when oxalate samples were added to a buffer solution, is shown in Figure 1. Almost four hours were necessary to reach the stationary current condition using 5.0 vol.% glutaraldehyde for crosslinking. This amount of crosslinking agent was used in order to compare the results with those previously obtained [8]. The behavior changed drastically with 0.5 vol.% glutaraldehyde but still the dynamic response was very slow.

The incorporation of mucin in the matrix produced important changes in the response of the electrode. Rate response, linear range, and slope were significantly enhanced as shown in Figure 2. Figure 2a shows the current–time profiles when oxalate oxidase was cross-linked with different muc/chit ratios using 5.0 vol.% glutaraldehyde as crosslinking agent. The presence of mucin in the matrix clearly favors the reaction rate independently of the mucin proportion. The high signal/noise ratio observed in these curves is an important property of the muc/chit matrix-based electrodes which made possible to reach a detection limit of 0.1 μM.

The oxalate calibration curves performed with these electrodes are shown in Figure 2b. Curve 1 of this figure corresponds to the calibration curve of a biosensor prepared with the enzyme immobilized in muc100; it has a very wide linear range, and concentrations as high as 1000 μM can be measured although with a very low sensitivity. Curve 6 corresponds to a biosensor prepared with chit 100 (in this case with glutaraldehyde 0.5%); a high slope but in a very small range of linearity was observed. With biosensors prepared combining mucin and chitosan different results are obtained. Comparing with muc100, as the proportion of chitosan in the matrix was increased the current also did (curves 2, 3, 4 and 5), but progressively the curves become nonlinear resulting in a further decrease in the current values for concentrations above 300 μM. The effect of composition on the slopes was measured at a level of oxalate higher than 300 μM, that is, in the clinical relevance range. The slope of the curves clearly depends on the percentage of mucin into the support. The maximum in the slope value was found in the range muc/chit 70/30 and muc/chit 50/50 as indicated in Figure 2c. The ratio 70/30 was considered the optimum for immobilizing oxalate oxidase in a muc/chit matrix regarding both analytical properties, sensitivity and linear range. In the inset of figure 2b the calibration curve performed with muc/carbopol 70/30 matrix (results of [8]) was included for comparison with the results obtained with muc/chit 70/30 matrix.

3.2. Effect of Matrix Composition on the Diffusion of Oxalate and Swelling

Diffusion experiments were done with the different matrices in the absence of enzyme to analyze the relationship between the analytical response and the diffusion rate; the results are shown in Figure 3A. The experiment begins ($t = 0$) when oxalate is introduced into the diffusion cell. The diffusion of oxalate through chit100 is very slow, with τ_0

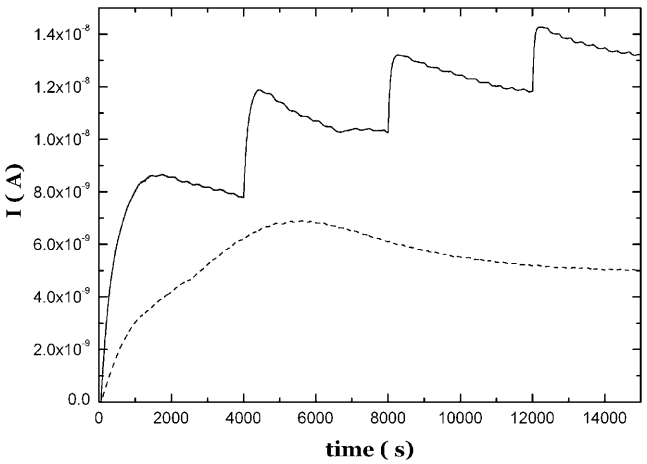

Fig. 1. Current–time profiles obtained from additions of oxalate to electrodes containing oxalate oxidase cross-linked with chit100 and 5 vol.% glutaraldehyde (----) or 0.5 vol.% glutaraldhyde (—).

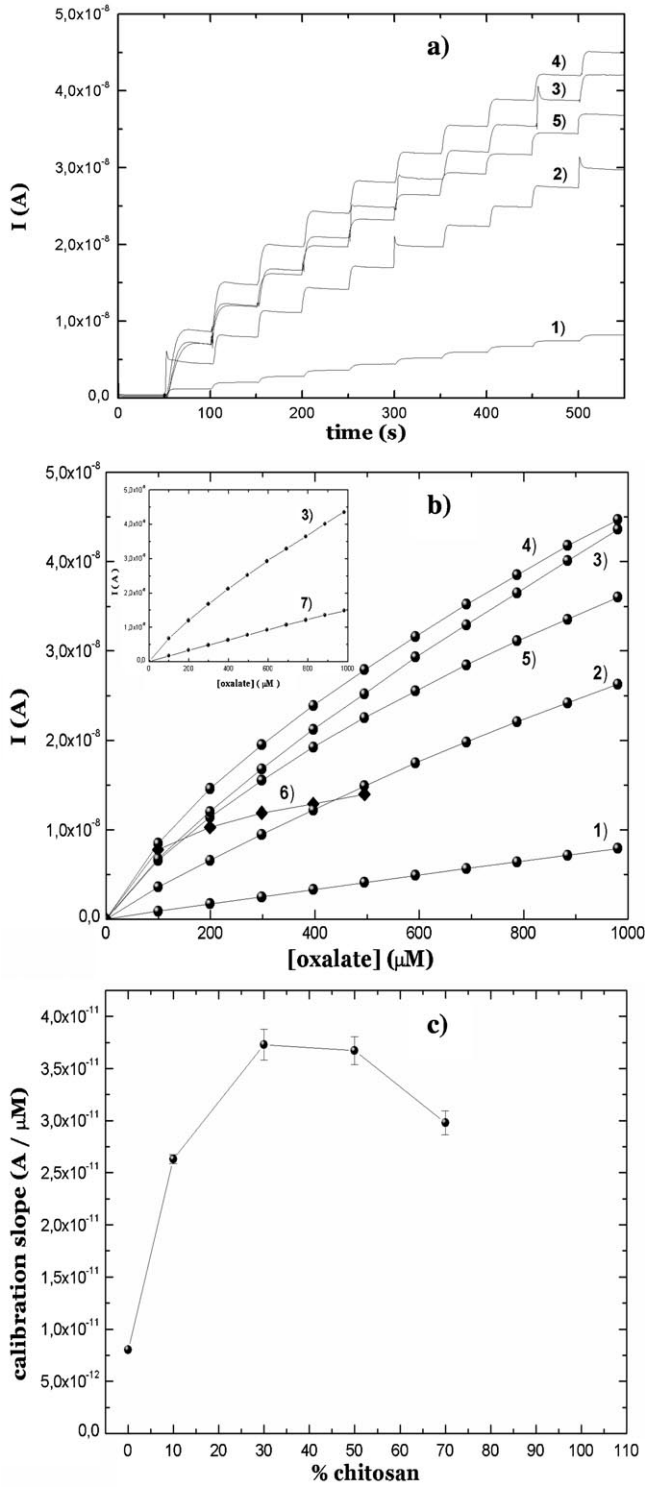

Fig. 2. a) Effect of mucin presence in the matrix composition on the current–time profiles obtained from additions of oxalate to electrodes containing oxalate oxidase cross-linked with: 1) Muc100; 2) muc/chit 90/10; 3) muc/chit 70/30; 4) muc/chit 50/50; 5) muc/chit 30/70); 5 vol.% glutaraldehyde in all cases. b) Calibration curves of enzymatic electrodes for oxalato determination corresponding to the matrices: 1) Muc100; 2) muc/chit 90/10; 3) muc/chit 70/30; 4) muc/chit 50/50; 5) muc/chit 30/70; 5 vol.% glutaraldehyde in all cases 6) chit100; 0.5 vol.% glutaraldehyde. Inset: 3) muc/chit 70/30; 7) muc/carbopol 70/30; 5 vol.% glutaraldehyde in both cases. c) Effect of the matrix composition on the slope of the calibration curves.

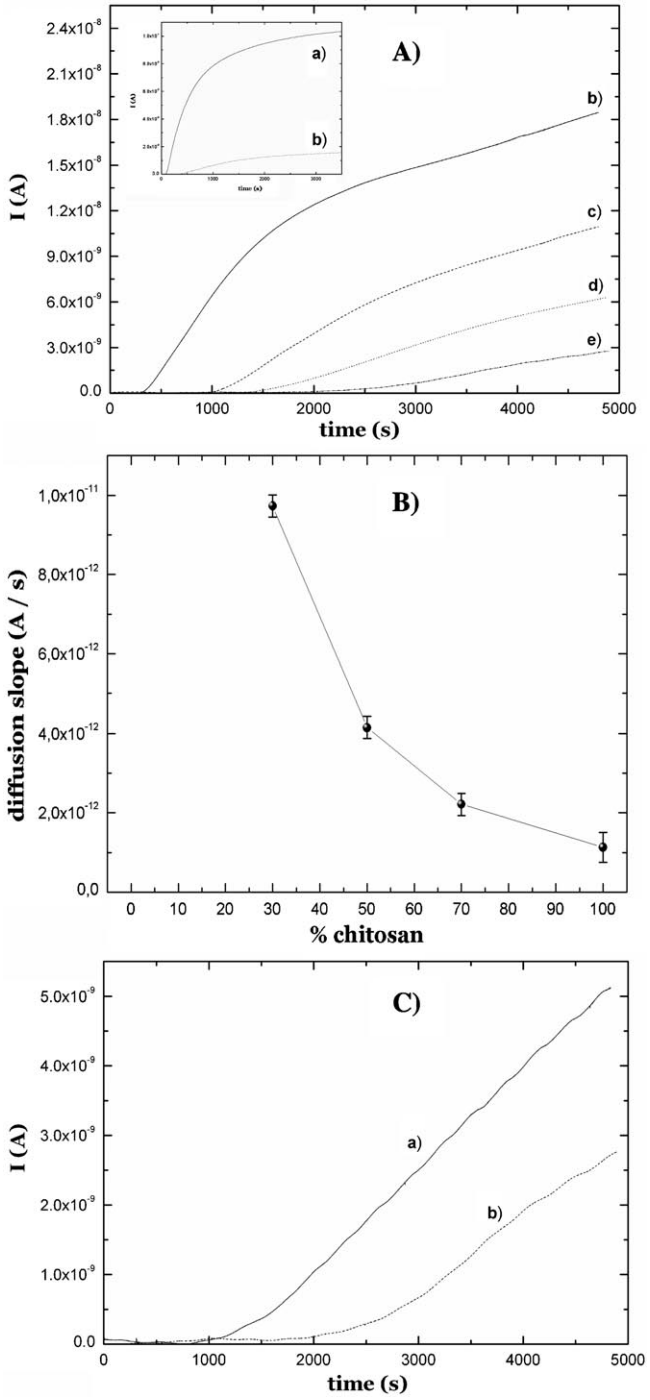

Fig. 3. A) Oxalate diffusion curves measured as described in the text, through: a) muc/chit 90/10; b) muc/chit 70/30; c) muc/chit 50/50; d) muc/chit 30/70; e) chit100. Crosslinking agent: 5 vol.% glutaraldehyde in all cases. B) Effect of the matrix composition on the slope of the diffusion curves. C) Effect of crosslinking density on the oxalate diffusion curves through chit100 cross-linked with a) 0.5 vol.% glutaraldehyde and b) 5 vol.% glutaraldehyde.

around 2000 s and a very small slope. As the amount of mucin in the matrix increases, so does the current slope, decreasing τ_0 at the same time; a high diffusion rate was observed for muc/chit 90/10 but for muc100 the experiment was not possible to be performed because the matrix did not

gellify properly. The decrease in diffusion slope as the amount of chitosan increases is depicted in Figure 3B.

The effect of the crosslinking density in the chit100 matrix on the diffusion rate was also analyzed (Fig. 3C). Low amounts of glutaraldehyde favors the diffusion of oxalate. This result clearly indicates the influence of the facility of substrate to reach the enzyme active sites, on the rate of analytical response. As stated above, it was not possible to perform a calibration curve using chitosan cross-linked with 5.0 vol.% glutaraldehyde. Considering that at low pH values the swelling of chitosan containing matrices is an important property that may influence the microenvironment of the enzyme, swelling indexes of the muc/chit matrices were also measured to analyze the effect of the muc/chit ratio. The results are shown in Figure 4. The swelling largely increases with the amount of mucin and with the decrease in concentration of glutaraldehyde.

3.3. Effect of Matrix Composition on the Stability of the Electrode

In Figure 5 the stability of the electrodes is analyzed. For a better comparison a standardized sensitivity, calculated by dividing the daily slopes by the first day value, was plotted. The muc/chit 70/30 matrix prepared with 5.0 vol.% glutaraldehyde resulted very stable maintaining 75% of the initial activity after 50 days. The activity of a matrix prepared with 0.5 vol.% glutaraldehyde showed an activity of 50% on the day 45st. These electrode performances were significantly better than the response of the muc/carbopol 70/30 matrix [8]. As seen in Figure 5, the electrodes prepared with chit100 or muc100 showed poor stability.

The performance of amperometric electrodes for oxalate measurements, prepared immobilizing the enzyme with mucin and chitosan cross-linked with glutaraldehyde was studied. When only chitosan is used for the immobilization,

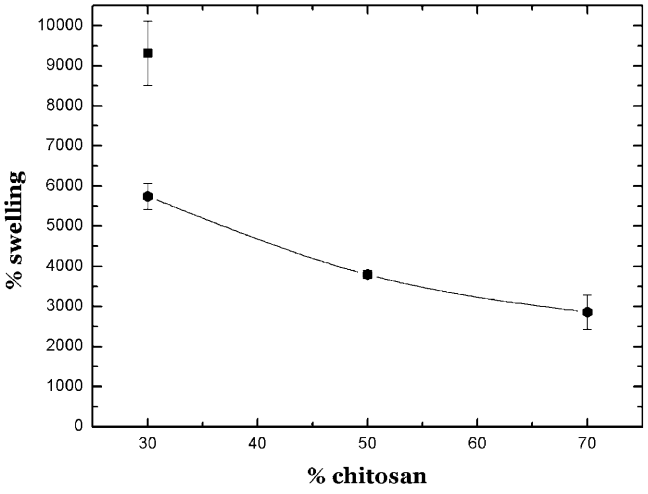

Fig. 4. Effect of the matrix composition on the swelling index; 5 vol.% glutaraldehyde (hexagon), 3 vol.% glutaraldehyde (square).

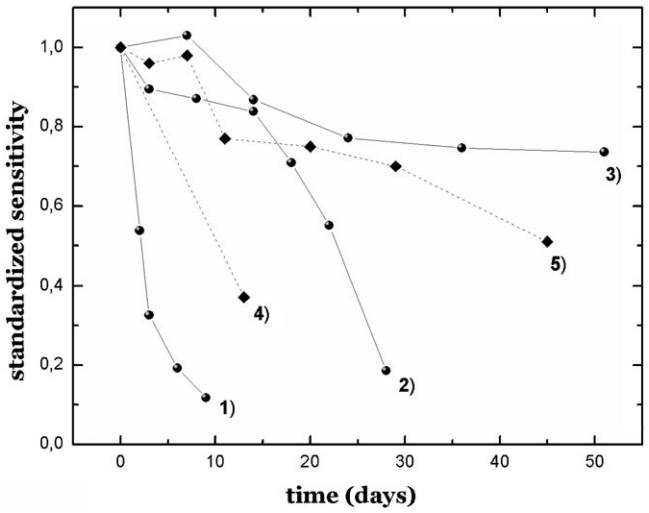

Fig. 5. Comparison of the stability of the electrodes. Standardized stability was calculated dividing the slope of the calibration curve by that obtained the first day after preparing the electrode. Matrix composition: 1) Muc100; 2) muc/carbopol 70/30 (with comparative purposes); 3) muc/chit 70/30 with 5 vol.% glutaraldehyde in all cases; 4) chit100 with 0.5 vol.% glutaraldehyde; 5) muc/chit 70/30 with 0.5 vol.% glutaraldehyde.

a very slow response is found, especially with high crosslinking density. The addition of mucin to chitosan for enzyme immobilization resulted in a biosensor with much better performance, concerning to dynamic response, stability, sensitivity (at concentrations higher than 100 μM) and linear range.

The high density of crosslinking in chit100 is possibly the responsible in the slowness of the observed response. On one side, swelling and diffusion mainly depend on the crosslinking density lightly cross-linked hydrophilic polymers form hydrogels with important amount of free water molecules. As the crosslinking density increases, the water content, the swelling capacity, the mesh size of the network and consequently the diffusion rate decrease. On the other side, high crosslinking density may induce loss of enzymatic activity since the polymeric network may obstruct the active sites and distortion of the tertiary structure of the enzyme [23].

Not only the crosslinking density, but also the incorporation of another component largely influences the swelling and diffusion of the analyte in the matrix. The addition of a hydrophilic polymer increases water uptake and swelling. Hybrid polymeric networks [23] formed between chitosan and a hydrophilic polymer can present a higher versatility than hydrogels formed only by chitosan cross-linked with itself. The covalent crosslinking between chitosan chains can be perturbed decreasing crosslinking density and making available more amino groups producing a marked dependence of swelling with pH. In this case, the presence of mucin, a hydrophilic polymer with a high molecular weight and spaced amino groups, offers the possibility of forming a more open cross-linked structure, markedly increasing the

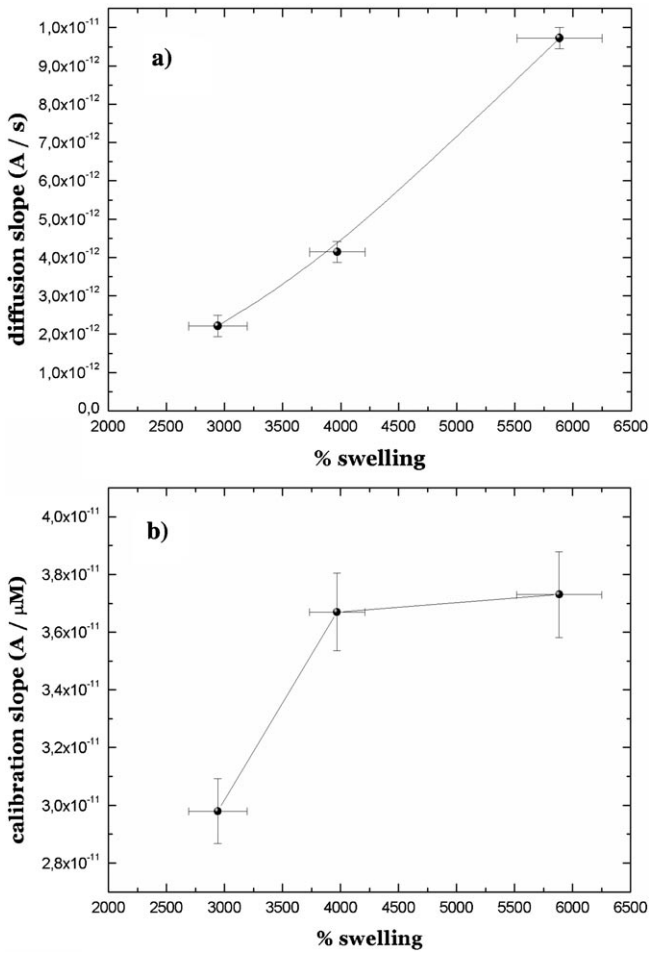

Fig. 6. a) Relationship between diffusion rate of oxalate through the matrices and the swelling percentage. The increasing values of diffusion rate and swelling correspond to muc/chit 30/70; 50/50 and 70/30 respectively. b) Dependence of the slope of the calibration curves with the swelling percentage of the matrices. The increasing values of slope and swelling correspond to muc/chit 30/70; 50/50 and 70/30 respectively.

diffusion of the analyte as shown in Figure 3A. This facility of oxalate to move through matrices with important amount of mucin is closely related to the fact that these matrices are highly swollen, as it was shown in Figure 4. As expected then, Figure 6a shows an almost linear relationship between diffusion rate and swelling.

According also to these considerations, a direct relationship between diffusion rate or swelling and calibration slope could be expected; this in fact was reached except for matrices with high amounts of mucin (muc 90 and muc 100), leading to the results in Figure 6b (see also the maximum in Fig. 2c). Electrodes prepared with muc/chit 90/10, and muc100, as shown in Figure 2, exhibit very low sensitivity in spite of a high diffusion rate of the analyte. A low load of enzyme due to a non efficient crosslinking with mucin under these conditions might be the reason of the decrease in the slope.

About the immobilization of the enzyme, mild coupling conditions are essential, in order to avoid the chemical modification of amino acid residues near the active site, as well as any change in the tertiary structure that may affect the activity. A slight crosslinking and a more hydrophilic environment for the biomolecule favor the enzymatic activity through a higher enzyme-substrate interaction. The strong electrostatic interaction between a polyanion and a polycation, mucin and chitosan respectively, probably contributed to obtain more stable electrodes with low quantity of glutaraldehyde. Nevertheless, if the amount of mucin is too high, although diffusion is enhanced, the charge of cross-linked enzyme in the matrix could decrease leading to the maximum in sensitivity.

The positive charge of chitosan at the pH of enzymatic activity may also be an important factor that influences the analytical behavior. Partition of the analyte, enhanced when the polymer has opposite charge could yield also in the high sensitivity of these electrodes.

4. Conclusions

An excellent matrix for immobilizing oxalate oxidase by combining optimum amounts of mucin, chitosan and glutaraldehyde has been obtained. Both, the increase in the amount of mucin and the decrease in crosslinking density produce important changes in the chemical and physicochemical properties of the matrix which, in this case, markedly improves the electrode performance. We have demonstrated that the kinetic and the analytical behavior of the electrodes are caused by structural, diffusion limitation-related and microenvironmental factors. Additionally the high signal/noise ratio of these electrodes proves to be an excellent performance at very low concentrations of oxalate making them potentially useful for this analyte in blood samples.

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