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Comparative behavior of glucose oxidase and oxalate oxidase immobilized in mucin/chitosan hydrogels for biosensors applications

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ABSTRACT

The analytical response of amperometric glucose electrodes was explained in the framework of the properties of hydrogels with different muc/chit ratio at pH = 7.00 used as immobilization matrix for glucose oxidase. The continuous increase in the sensitivity of the electrodes with the amount of chitosan was attributed to a more efficient crosslinking without affecting the enzyme activity. The results were compared with those obtained using the same matrices to immobilize Oxalate Oxidase at pH = 2.85 where it has the highest activity. A maximum sensitivity at muc/chit 70/30 ratio for these oxalate electrodes was observed. Decrease in permeability of negatively charged oxalate with the chitosan content in the matrices, in spite of the high partition coefficient, explains the analytical differences with glucose electrodes. Swelling and viscoelasticity of the hydrogels allowed to asses the importance of a hydrophilic environment for the enzyme in both cases.

1. Introduction

The possibility of immobilizing biorecognition materials, particularly enzymes, in different supporting matrices, has drastically increased the popularity of enzymatic methods, because it has given place to the generation of devices known as biosensors, which allow the biological material to be reused in successive analytical measurements, without substantially affecting its activity. This characteristic, added to its analytical advantages, construction simplicity and the ability to be used directly in different biological matrices are responsible for the present wide use of biosensors, which is now extending to new biorecognition elements used in dosage of antigens, antibodies and DNA among others [1].

Currently, the enzymatic amperometric electrodes are certainly the most widely used biosensors. In these devices the enzyme is immobilized in different supports where the enzymatic reaction will take place once the analyte has reached the enzymatic site by diffusion [2]. Oxidases, the enzymes most commonly used in these electrodes, have oxygen as natural co-substrate which is converted to hydrogen peroxide during the enzymatic reaction [3]; the electrochemical oxidation of hydrogen peroxide results in an electric current which is a measure of the substrate's analytical concentration [4].

In spite of the advantages mentioned above, the immobilization of the enzyme can have a strong impact on its catalytic activity, because the interactions between the support and the enzyme may produce conformational changes in the recognition element, changing the kinetic parameters, K_m and V_{max} [5,6]. In addition, the internal diffusion of substrate is much slower than in the solution due to steric barriers and the quite solid nature of the enzymatic matrix. The surrounding of the enzyme can be not only a barrier but also may attract or repel substrate or reaction product due to electrostatic interactions, either increasing or decreasing its concentration near the recognition element [7–9]. Electric charges of enzymatic support as well as of the analyte, may then greatly influence the biosensor's response [10,11].

Immobilization procedures as well as the properties of the enzymatic support have to be considered because, as explained, the analytical performance of these devices is directly related to the microenvironment, which in turn has great influence on the efficiency of the enzymatic reaction [7,12–19].

Nevertheless, in spite of the importance of the immobilization step, it is normal practice to use trial and error to find optimal conditions for a chosen enzyme, thus ensuring the highest possible retention activity [1].

This empirical method has led us to focus our work, in the last years, on the systematic study of the physicochemical properties of polymeric matrices used to immobilize enzymes trying to relate them to the sensors performance.

Hydrogels are very attractive materials due to their high water content [20–23] which additionally makes them biocompatible for "*in vivo*" sensing [24]. Gel forming properties, hydrophilicity,

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antibacterial properties and remarkable affinity to proteins are unique characteristics of chitosan and carbopol, materials that we have chosen for immobilizing enzymes.

Crosslinking of many enzymes to chitosan with different applications, not only biosensing, has been reported; one of the most used crosslinking agents is glutaraldehyde, due not only to its reliability but also to the availability of amino groups in enzymes and in chitosan [1]. These benefits have been used by Yao et al who developed a novel biosensor based on glucose oxidase trapped in chitosan films crosslinked with glutaraldehyde on a Pt electrode [25].

In the first paper of this series, we developed a sensor for oxalate immobilizing the enzyme oxalate oxidase at pH = 2.85, value of maximum activity, in a mucin/carbopol matrix [26,27]. Carbopols, polymers of polyacrylic acid, very well known by their mucoadhesive properties, are neutral at pH below 6; the presence of carbopol was very successful and improved the stability and sensitivity of the sensor obtained with mucin [26]. Chitosan, a polycation at acidic pH values (pKa = 6), has similar mucoadhesive properties and great crosslinking capacity. As it posseses different chemical and biological properties, mainly due to the amino groups that make it soluble in aqueous acidic media, acquiring positive charge we decided to use it instead of carbopol. Considering that oxalate is negatively charged at pH = 2.85 we expected a greater partition coefficient in chitosan than in carbopol, and therefore a higher analyte concentration with the subsequent benefit for the response of the sensor [26–29]. This effect was indeed obtained and sensitivity and stability were improved, although the response rate decreased drastically at high chitosan concentrations [28,29]. We decided to continue these studies using this excellent matrix (muc/chit 70/30) to immobilize another enzyme which has a maximum activity pH above chitosan pKa. We chose glucose oxidase that has maximum activity at pH = 7.00 and a neutral substrate, glucose. These differences, pH of the matrix, and the substrate charge led to different analytical behavior of the sensors.

In the present paper we immobilize Glucose Oxidase (GOX) in matrices with different ratio muc/chit mixtures at pH = 7.00 and related the response of the resulting biosensors to the properties of the polymeric matrices. The results are compared with those obtained using the same matrices at pH = 2.85 where Oxalate Oxidase (ODD) has its highest activity. To the differences in the properties of the matrices produced by the changes in pH we must add the different nature of the analytes, considering that at pH = 2.85 glucose is neutral and oxalate is an anion.

To perform this comparative analysis we evaluated on one side, swelling and viscoelasticity of muc/chit matrices at pH = 7.00 and pH = 2.85 and on the other, the partition coefficient and the permeability of glucose and oxalate at both pH values respectively; finally we analyzed the influence of these properties on the response of these hydrogel based biosensors.

2. Experimental section

2.1. Materials

Mucin (muc) (porcine stomach Type III, partially purified), glucose oxidase (GOX) enzyme, oxalate oxidase (ODD) enzyme and chitosan (chit) (low molecular weight, 75–85 % deacetylated) were obtained from Sigma Ltd; anhydrous glucose from Baker; potassium oxalate from Anedra; potassium sulphate from Mallinckrodt; potassium chloride from Merck and 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 solution was prepared using 0.5905g per 100 mL succinic acid (Anedra) in purified water (Milli Q system). The pH = 7.00 phosphate buffer was prepared using anhydrous dibasic sodium phosphate from Cicarelli and anhydrous monobasic sodium phosphate from Anedra.

2.2. Methods

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 wire 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.

2.2.1. Construction of electrodes

The glucose electrodes were assembled crosslinking 6.0 μL of a mixture containing the polymer and the enzyme with 1.30 μL of glutaraldehyde 5.0% v/v. The mixture of polymer and enzyme was prepared by dissolving: 0.2000 g of mucin (100%), different weight ratios of mucin/chitosan (90/10, 70/30, 50/50, 30/70, 20/80) or chitosan (100%) in 2.050 mL of acetic acid 2% v/v (pH = 2.80). These compositions were named as follows: muc100, muc/chit 90/10, muc/chit 70/30, muc/chit 50/50, muc/chit 30/70, muc/chit 20/80 and chit100, respectively. Then, each of these resultant solutions was mixed with 2.050 mL of a solution of glucose oxidase in phosphate buffer at pH = 7.00 (6,668 U/mL). The enzyme concentration for each electrode was 20 U, an amount generally used in the construction of biosensors for glucose.

The crosslinking reaction was carried out at room temperature for 10 min pressing the polymer matrix enzyme between two polycarbonate membranes forming a “sandwich”. The resulting enzyme layer was washed with phosphate buffer at pH = 7.00 to remove excess of glutaraldehyde and placed on the Pt working electrode.

The oxalate electrodes were assembled as described in previous paper [28].

2.2.2. Calibration curves

Calibration curves of the different glucose electrodes were plotted from current-time curves obtained by addition of 10.0 μL of 1 M glucose to 5.0 mL of phosphate buffer solution at pH = 7.00. The performance of electrode prepared with chit100 was also determined in the following conditions: phosphate buffer solution at pH = 7.00 containing 50 mM potassium sulphate and phosphate buffer solution at pH = 7.00 containing 50 mM potassium chloride.

Chronoamperometry was the electrochemical technique used to record the electrical response.

The oxalate calibration curves were obtained by addition of 10.0 μL of 0.05 M potassium oxalate to 5.0 mL of succinic acid solution at pH = 2.85 as described in previous paper [28].

2.2.3. Study of the physicochemical properties

The experimental procedure used to measure permeability and partition coefficient was the same for both analytes, oxalate at pH = 2.85 and glucose at pH = 7.00.

Here we present the experimental procedure followed at pH = 7.00. In the case of oxalate, the experiments were performed as previously detailed [28,29].

2.2.3.1. Permeability (P). Glucose permeability measurements was performed in the muc/chit 70/30, 50/50, 30/70, 20/80 and chit100 hydrogels; all of them crosslinked with glutaraldehyde 5.0% v/v in absence of enzyme. These polymeric matrices were placed between a polycarbonate membrane in direct contact with the glucose

solution and with an enzymatic electrode that allows continuous and indirect detection of glucose by chronoamperometry. This biosensor was constructed by immobilizing glucose oxidase in muc/chit 70/30 crosslinked with glutaraldehyde 0.5% v/v. Them, the diffusion cell was assembled with the sandwich containing the polymeric matrix, the biosensor, and a counter electrode; the experimental procedure was done as following:

(a) The diffusion cell was filled with a phosphate buffer;
(b) Electric current was recorded until a baseline was obtained (constant current);
(c) Glucose solution was added to the stirred phosphate buffer beginning of permeability experiment;
(d) Glucose diffuse through the matrix and it is detected by enzymatic electrode;
(e) The electrochemical response was followed by chronoamperometry.

In each experiment, glucose concentration was 20 mM at pH = 7.00.

The time at which the current begins to increase, called "τ_0", or the slopes of current-time graphic were taken as a measure of the permeability, considering that they are inversely proportional. The current signal was normalized by the signal of the enzymatic electrodes used in each experiment considering that glucose and oxalate electrodes work at different concentrations ranges; in this way the comparison of the physicochemical properties of the matrix was possible.

2.2.3.2. Partition coefficient (K_d). For the partition coefficient measurements, two portions of each matrix, muc/chit: 30/70, 50/50, 70/30, 80/20; and chit100 crosslinked with glutaraldehyde 5.0% v/v were placed in separate beakers with 40.0 mL of 5 mM glucose solution. After 72 h, the glucose concentration of the supernatant was determined using a biosensor for glucose. The content of glucose inside the hydrogels was calculated from the difference between the mass of glucose in the initial solution (before immersing the hydrogel) and the final solution at equilibrium (after 72 h).

After equilibration the matrices were extracted from the solution and its volume was measured. The Principle of Archimedes was used to determinate the volume of the hydrogels. Hydrogels were suspended in heptane (δ_{Hep} = 0.68 g/mL) and the thrust each hydrogel was measured. Thrust is the force applied to submerged hydrogel; it has opposite direction to the weight and it is the weight of the heptane volume displaced by the immersed hydrogel. Then:

$$\vec{E} = M_{Hept}g = V_{Hept}\delta_{Hept}g = V_{hydrogel}\delta_{Hept}g \quad (1)$$

M_{Hept} is the mass of displaced heptane, g is the acceleration of gravity, V_{Hept} is the displaced heptane volume, δ_{Hept} is the density of heptane and $V_{hydrogel}$ is the volume of the polymer sample.

The volume of the hydrogels was calculated to apply the following equation:

$$V_{hydrogel} = V_{Hept} = M_{Hept}/\delta_{Hept} \quad (2)$$

Subsequently, the mass of glucose into each the hydrogels (in mol) was related to its respective volume obtained (L) and the molar concentration within the hydrogels was calculated. The partition coefficient (K_d) was calculated from Equation (3) [30]:

$$K_d = \frac{C_m}{C_s} \quad (3)$$

Here, C_m and C_s are the concentrations of glucose within hydrogel and the solution at equilibrium, respectively.

2.2.3.3. Swelling index of hydrogels (S). Swelling indexes of the muc/chit matrices: 70/30, 50/50, 30/70, 20/80 and chit100 crosslinked with glutaraldehyde 5.0% v/v were measured. Each hydrogel was placed in 40.0 mL of phosphate buffer at pH = 7.00 for 72 h; them, they were dried at room temperature until constant weight. The polymer weight was measured in the swollen and dry states. The degree of hydration was obtained with the following equation [31–33]:

$$\%\text{Hinchamiento} = \frac{M - M_0}{M_0} * 100 \quad (4)$$

where M and M_0 are the weights of the polymer matrices in the swollen and dry states, respectively.

2.2.3.4. Rheology of hydrogels. The rheological study was applied to the following samples: muc100, muc/chit: 70/30, 50/50, 30/70 and chit100 crosslinked with glutaraldehyde 5.0% v/v. The hydrogel samples were placed in phosphate buffer at pH = 7.00 during 72 h until the swelling equilibrium was reached. All measurements were performed in duplicate with Anton Paar rheometer at controlled temperature (25 °C) using a plate geometry of 24.984 mm (PP25) and gap position of 1 mm between plates.

The rheological characterization was performed by measuring the elastic and viscous modulus of the samples with oscillatory court flow of small amplitude. For this purpose, two types of tests were applied to each hydrogel. The first test was an amplitude sweep or Dynamic Strain Sweep (DSS); a constant frequency oscillation (in this case 10 s^{-1}) was applied to the sample and the amplitude of the oscillation, deformation or strain "γ", was varied from 0.01% to 30.00%. This preliminary test was done to determine the linear viscoelastic range of each material, in other words, the amplitude range in which the measurement modules are constant and not dependent on the applied wave amplitude. Working in the linear range ensures that the measured strain is a sinusoidal wave and the equations used to calculate the modules are correct.

Once selected the value of strain, a second test called Dynamic Frequency Sweep (DFS) was applied to each sample. A frequency sweep between 1 and 100 s^{-1} at constant amplitude strain (γ = 2.00%) was applied to analyze the dependence of the elastic (G′) and viscous (G″) modulus with frequency.

2.3. Results

In Fig. 1a the current-time responses of amperometric biosensors for glucose determination are shown. These electrodes were prepared immobilizing glucose oxidase in matrices with different muc/chit ratios using 5.0% v/v glutaraldehyde as crosslinking agent. The changes in the current values observed every 50 s, when a stationary condition is reached, correspond to successive aggregates of glucose standard solution. The corresponding calibration curves, obtained with the stationary current values at each final concentration value are plotted in Fig. 1b. The slope of the curves, direct measure of the sensitivity of the electrodes, clearly depends on the proportion of both polymers in the matrix; as the amount of chitosan increases also does the slope of the curves. But on the other hand the presence of mucin contributes to increase the linear range of the curves, an important analytical parameter to be considered in a biosensor; consequently plots obtained with high percentage of mucin have a wide linear range but very low sensitivity.

It is very important to point out that the dynamic response of these electrodes, that is the time to reach the stationary current after each glucose addition, is quite short and almost the same in all the glucose electrodes prepared; in other words it is independent of the muc/chit ratio in the hydrogel.

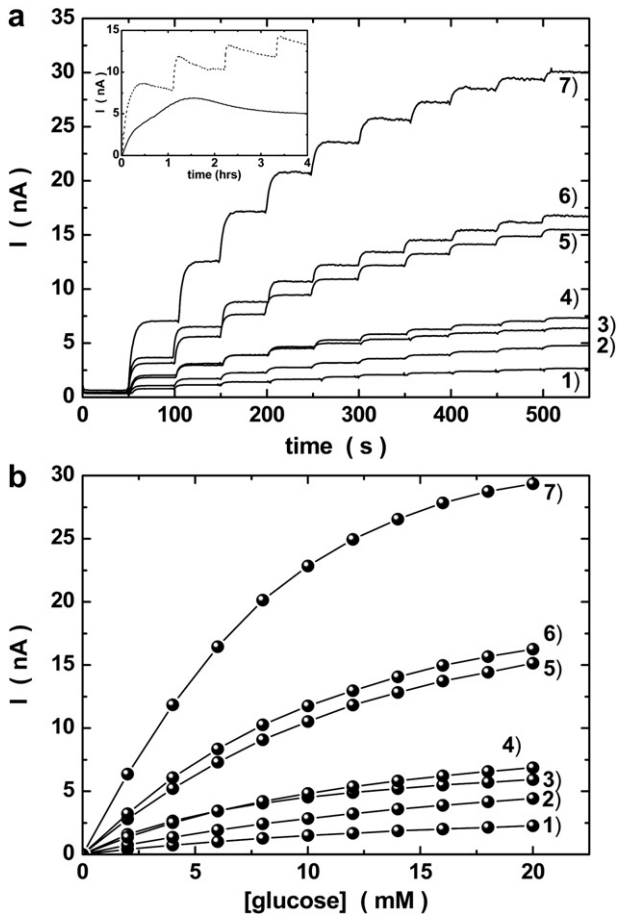

Fig. 1. a Current-time profiles obtained from additions of glucose to electrodes containing glucose oxidase crosslinked with: 1) muc100; 2) muc/chit 90/10; 3) muc/chit 70/30; 4) muc/chit 50/50; 5) muc/chit 30/70; 6) muc/chit 20/80; 7) chit100 and glutaraldehyde 5.0% v/v in all cases. Inset: Current-time profiles of oxalate electrodes containing oxalate oxidase crosslinked with: chit100 and glutaraldehyde 5.0% v/v (——); chit100 and glutaraldehyde 0.5% v/v (- - - -). b Calibration curves of enzymatic electrodes for glucose determination corresponding to the polymeric matrices: 1) muc100; 2) muc/chit 90/10; 3) muc/chit 70/30; 4) muc/chit 50/50; 5) muc/chit 30/70; 6) muc/chit 20/80; 7) chit100; glutaraldehyde 5.0% v/v in all cases.

A different situation is found in the case of oxalate [28,29], where the effect of the polymer ratio has a strong influence on the time response. Electrodes prepared immobilizing oxalate oxidase in chitosan respond in an extremely slow way, even if the crosslinking density is decreased using glutaraldehyde 0.5% v/v instead of 5.0% v/v. This effect is shown in the inset of Fig. 1a. Nevertheless, when a small amount of mucin was added to the matrix, surprisingly the dynamic response improves, reaching values quite similar to those observed for glucose electrodes [28].

Fig. 2 summarizes the analytical results obtained for glucose when the composition of the matrix is changed. In the same figure the corresponding results for oxalate are included for comparison [28]. The dependence of the slopes of the calibration curves on the proportion of chitosan is different in both cases. The oxalate electrode shows a maximum slope at muc/chit 70/30 ratio, while a continuous increase is observed in the slope of the calibration curves with the amount of chitosan, for glucose electrodes.

These results show a marked effect of the change in matrix composition in the response of both, glucose and oxalate sensors; but one can also note that for each value of matrix composition, there are differences between the behavior of glucose and oxalate sensors. Obviously, the different values of pH in the immobilization matrix, pH = 7.00 for glucose sensors and pH = 2.85 for oxalate,

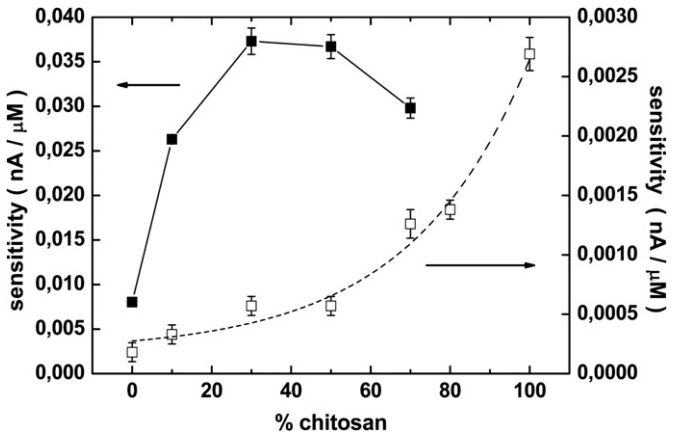

Fig. 2. Effect of the hydrogel composition on the sensitivity (slope of the calibration curves) of oxalate electrodes (full square) and glucose electrodes (empty square).

affect the microenvironment of the respective enzymes and therefore their activity.

On the other hand the different chemical nature of the analytes, that is glucose and oxalate, and their interaction with the surroundings may also affect the success of the enzymatic reaction. In other words, the polymeric matrix certainly influences not only the enzymatic activity but also the availability of substrate for the enzyme, being both factors closely related to the efficiency of the enzyme–substrate interaction and therefore to the analytical response of this type of biosensor.

In the following sections we will show results related to the analyte and matrix properties as well as the interaction between each other under pH conditions of maximum enzymatic activity.

2.3.1. Analyte availability in the hydrogels

Availability means that not only a high concentration of the substrate, the analyte in this case, in the surroundings of the enzyme is necessary; but it should also have freedom to move inside the hydrogel and reach the active site of the enzyme. Partition coefficient and permeability of glucose at pH = 7.00 in the different matrices was therefore evaluated and compared with the corresponding values for oxalate at pH = 2.85.

2.3.1.1. Partition coefficient (K_d). Concentrations of glucose inside the hydrogel were measured at pH = 7.00. Fig. 3 shows the effect of

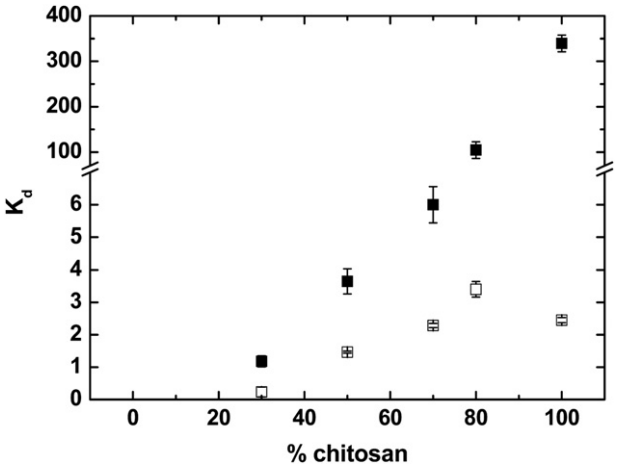

Fig. 3. Effect of the hydrogel composition on: oxalate K_d in succinic acid solution at pH = 2.85 (full square) and glucose K_d in phosphate buffer at pH = 7.00 (empty square).

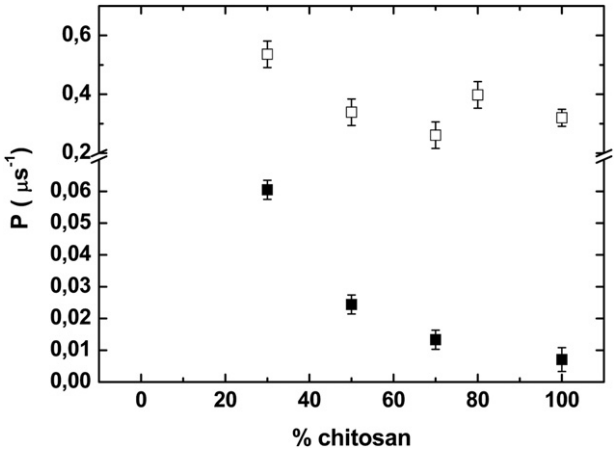

Fig. 4. Effect of the hydrogel composition on: oxalate permeability in succinic acid solution at pH = 2.85 (full square) and glucose permeability in phosphate buffer at pH = 7.00 (empty square).

the matrix composition on K_d. As glucose is a neutral substrate, there is only a slight increase of concentration up to only three times the external glucose concentration. The result corresponding to oxalate anion is also included in the figure. In this case, its concentration increases as does the proportion of chitosan, reaching a $K_d = 400$ for chit100.

2.3.1.2. Permeability (P). Analysis of glucose permeability through the hydrogels containing different percentages of chitosan was performed at pH = 7.00. The results are shown in Fig. 4. Glucose permeability is almost constant except at low chitosan percentage, where it is slightly higher. On the other hand, as the content of chitosan in the gel increases, the permeability of oxalate at pH = 2.85 decreases. These values are also included in the figure. An important point to remark is that oxalate permeability is at least one order of magnitude lower than the corresponding values for glucose in the whole matrix composition range.

2.3.2. Hydrogel properties and enzymatic activity

Good activity of the immobilized enzyme is essential for the proper functioning of the biosensors. The immobilization process is an important factor considering that it could produce conformational changes in the enzymatic structure leading to a loss of activity. A hydrophilic environment for this type of enzyme certainly favors its activity and consequently the sensor performance. Thus, evaluation of the enzyme surroundings in the hydrogel may give helpful information to understand the differences in the analytical behavior of the sensors. Rheology and swelling analysis of the different matrices were then done at both pH values in absence of enzyme and of substrates.

2.3.2.1. Rheology of hydrogels. Rheology analysis provides information on viscoelasticity and polymeric architecture which combined with swelling studies give a good insight of the enzyme environment in the hydrogel. Fig. 5 show how the elastic (G′) and viscous (G″) modules vary respectively with frequency (ω) in the hydrogels crosslinked with glutaraldehyde 5.0% v/v for different muc/chit ratios at pH = 7.00. As shown in the figure, the G′ values are higher than G″ throughout all the frequency range studied, which corresponds to the gel-like behavior of materials; the low variability of G′ with frequency is also characteristic of covalently crosslinked polymer networks. Fig. 6 show how the modulus G′ ($\omega = 1$ Hz) changes with the composition of the polymer matrix at pH = 2.85 and pH = 7.00. In both cases, as the percentage of chitosan increases so does the G′ value, although it is not linear, showing rheological synergism in muc/chit mixtures crosslinked with glutaraldehyde. This refers to the physical interactions associated with the formation of molecular entanglements between mucin and chitosan chains in addition to the covalent crosslinking; this produces greater stress with the applied strain, in other words, at intermediate compositions these materials present a more elastic behavior than expected.

2.3.2.2. Swelling index of hydrogels (S). Swelling studies give information about the degree of hydration of the hydrogels and indirectly of the hydrophilic environment surrounding the enzyme. Fig. 7 shows that as the content of chitosan increases in the polymeric matrix at both pH values, producing an increase in the crosslinking density due to the amount of amino groups, the swelling index decreases. In the same figure we can see that swelling increases with the mucin content in the matrix, especially at pH = 2.85. This behavior can be associated with the decrease of elasticity in the network as the mucin content increases together with electrostatic repulsion within the hydrogel. The increase in G′, which coincides (concomitant) with the decrease in swelling with the amount of chitosan, shows the relationship between both these properties.

2.4. Discussion

Most enzymatic amperometric electrodes are devices where the analyte concentration is indirectly measured through the electrooxidation or electroreduction current of some product of the

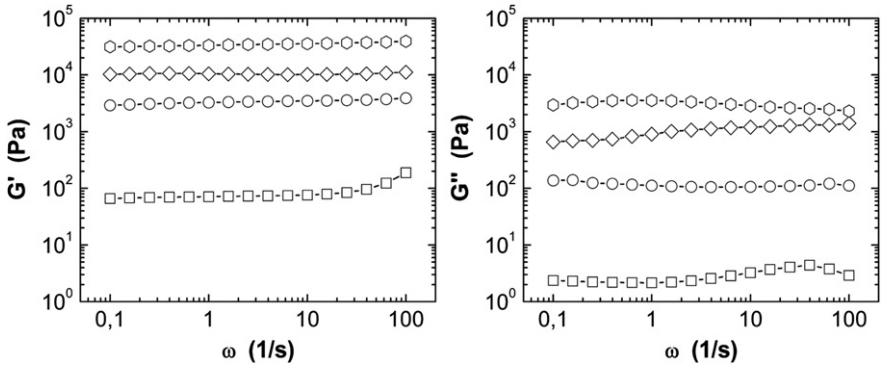

Fig. 5. Viscoelastic behavior corresponding to the polymeric matrices: muc/chit 70/30 (square); muc/chit 50/50 (circle); muc/chit 30/70 (diamond); chit100 (hexagon) crosslinked with glutaraldehyde 5.0% v/v in all cases (at pH = 7.00).

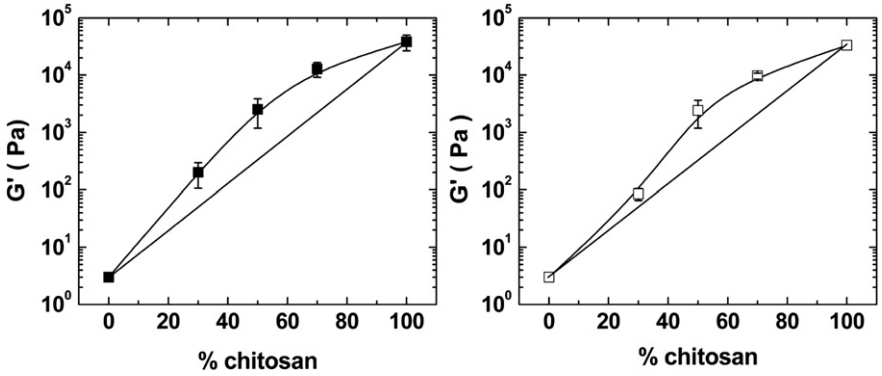

Fig. 6. Effect of the hydrogel composition on elastic (G′) modulus at pH = 2.85 (full square) and pH = 7.00 (empty square).

enzymatic reaction. When the enzyme is an oxidase, and this is the case of the oxalate and glucose biosensors considered in this work, H_2O_2 is the electroactive product. This means that all the analytical properties of the biosensor will result from the efficiency of the enzymatic reaction, in other words of the amount of H_2O_2 generated and its further oxidation on the transducer, commonly a platinum electrode at a predetermined electric potential.

This makes several characteristics of the enzyme, of the substrate, and of the media where they react, namely the polymeric matrix, impact on the biosensor performance.

Both the slope of the calibration curve, i.e. the sensitivity of the electrode, and its stability depend largely on an efficient binding of the enzyme with the matrix, which means that it should maintain its activity as long as possible. The enzyme is covalently crosslinked with glutaraldehyde via amino groups of amino acids; in the case of the matrix, chitosan amino groups are mainly those involved in the binding, indicating that as the amount of this polymer increases, the immobilization is greater.

In the case of glucose electrodes the increase in the slope of the calibration curves with the chitosan proportion is an indication of higher enzyme activity as result of a more efficient crosslinking without affecting the enzyme sites.

On the contrary, in the case of oxalate, the maximum observed in Fig. 2 indicates on one side that at high percentage of chitosan although the immobilization increases, there is an important decrease in the activity of the enzyme. On the other, a great amount of mucin, this is low muc/chit ratios, is not favorable for the performance. In this case the enzyme is not properly kept inside the matrix, resulting in very low sensitivity. Remember that the protein portion of this glycoprotein, i.e. amino acids necessary for a better crosslinking, is at the core surrounded by carbohydrates.

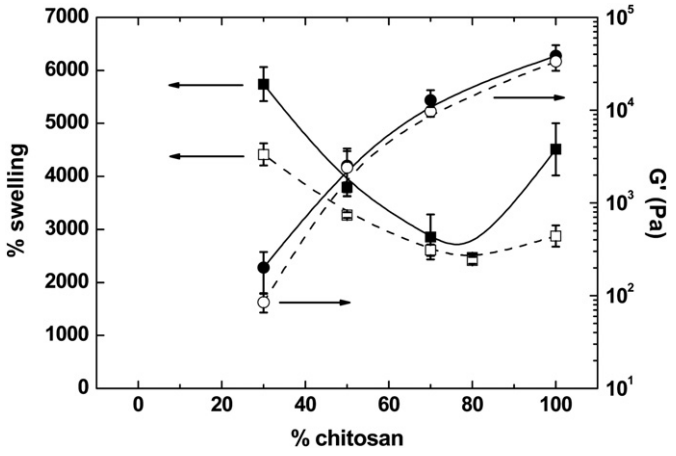

Fig. 7. Comparison of the effect of the hydrogel composition on swelling percentage and elastic (G′) modulus at pH = 2.85 (full square) and pH = 7.00 (empty square).

Swelling and viscoelasticity analysis indicate that at both pH values, 2.85 and 7.00, it is possible to ensure the enzymes a good hydrophilic environment.

Concerning the substrate, both, concentration and the ability of moving into the matrix to reach the enzymatic site, are expected to affect the efficiency of the enzyme–substrate interaction. This is why we measured the partition coefficient and permeability of both substrates at the pH values corresponding to maximum enzyme activity in each case, i.e. 2.85 and 7.00 for each muc/chit ratio used in this study.

Partition coefficient showed great differences between the behaviors of both substrates. On one hand, inside the matrix the concentration of acid oxalate anion, negatively charged at pH = 2.85, is much higher than the glucose concentration at pH = 7.00 at all matrix compositions. On the other hand, the concentration of both analytes increases with the content of chitosan, especially oxalate anion above muc/chit 20/80, reaching values of K_d two orders of magnitude higher than those of glucose.

The ability of the analyte to reach the active site is fundamental for the efficiency of the enzymatic reaction in a biosensor. The strong decrease in permeability of oxalate acid with the chitosan percentage is an indication that the movement of these ions inside the matrix is strongly inhibited. In spite of the high concentration of oxalate anion this decrease is a consequence of the electrostatic attraction between the negative ions and the positively charged amino groups of the polymeric network.

Table 1
Summary of the behavior of GOX and ODD immobilized in mucin/chitosan hydrogels.

Properties[a]	Biosensor	
	Glucose	Oxalate
Sensitivity	Increases	Maximum at 70/30 muc/chit ratio
Enzyme-matrix crosslinking	Increases	Increases
Availability of substrate	Good. Permeability is constant and high	Decreases due to decrease in permeability
Hydrophilic environment surrounding the enzyme	Good. Swelling decreases and G′ increases	Good. Swelling decreases and G′ increases

[a] Comparison of the effect of increasing the chitosan content in the matrices for oxalate oxidase and glucose oxidase on the sensitivity of the electrodes, enzyme-matrix crosslinking, availability of substrate and hydrophilicity of the media.

There is almost no influence of composition on the value of glucose permeability and at the same time it is always much higher than the values obtained for oxalate anion. Certainly this behavior can be accounted for by the absence of charge on the molecule and the fact that at pH = 7.00 the amount of positively charged amino groups is much less than at pH = 2.85.

When taking these considerations into account, the analytical behavior of both biosensors and the differences between them, shown in Fig. 2, can easily be understood. The absence or low amount of charges on the polymer and the neutrality of the analyte indicate that the analytical behavior of the glucose electrode is mainly determined by the increasing capability of immobilizing the enzyme without important loss of its activity. On the contrary, the strong decrease in permeability, this is in ability of the analyte of moving inside the matrix, together with a possible inhibition of the enzyme due to a high crosslinking density between enzyme and polymer, determine the maximum observed in the slope of the oxalate biosensor.

3. Conclusion

The results presented in this paper demonstrate the importance of considering factors such as the chemical nature of the substrate and physicochemical properties of the hydrogel in the design of biosensors that use hydrogels as immobilization support. The efficiency of the enzyme–substrate interaction determines the amount of electroactive species and therefore the magnitude of the analytical response.

In Table 1 a comparison of the effect on availability of substrate, hydrophilicity of the media, and sensitivity of the electrodes with the content of chitosan in the matrices is presented for oxalate and glucose.

The right combination of polymers as immobilization support for the enzyme is a powerful tool for the development of these analytical devices. Studies related to permeability, partition coefficient, swelling index, viscoelasticity among others are a better option than the trial and error methods frequently used.

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