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Physicochemical properties of a mucin/chitosan matrix used for the development of an oxalate biosensor

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ABSTRACT

Immobilization conditions of biomolecules play an important role in a biosensor performance. In this paper the response of amperometric electrodes for oxalate is related with some physicochemical properties of the matrices where oxalate oxidase was immobilized. Swelling indexes, partition coefficients and permeability of oxalate in mucin/chitosan matrices in a pH = 2.85 succinic acid solution were measured. These properties were also determined in the 70/30 mucin/chitosan matrix in solutions containing succinic acid and sulfate or chloride anions at the same pH value. The results indicate that the sensitivity of the biosensors is mainly determined by the swelling index of the matrix. High slopes of the calibration curves of the electrodes were obtained with highly swollen matrices indicating the importance of a hydrophilic environment for a good enzymatic response.

1. Introduction

Hydrogels are water-swollen polymeric structures with a three-dimensional network and physical integrity, containing chemical or physical crosslinks. They are used in numerous biomedical applications including biosensors, ophthalmological devices, biomembranes and carriers for controlled delivery of drugs or proteins [1].

The hydrogels swelling behavior constitutes one of the most important characteristics in evaluating the ability of polymeric gel to function in a particular application. It describes the capacity of the hydrogels to absorb a liquid fluid at equilibrium and it is a function of the network structure, crosslinking density, hydrophilicity and degree of ionization of the functional groups as well as the morphology of the network. Additionally, in solute transport through hydrogel, size, shape and solute ionization affect its diffusion through the network [2,3]. In general terms, the transport in polymeric matrices can be described in terms of chemical and/or frictional features. Effects of chemical interactions include the retardation of solute diffusion due to attractive forces. In some cases, chemical interactions dominate the diffusion process, whereas in other cases they are almost negligible. In contrast, solute diffusion will always be influenced by frictional effects, which include solvent effects, steric hindrance, hydrodynamic effects, etc. [4,5].

In enzymatic biosensors, the enzyme is immobilized in different supports including polymeric matrices where the enzymatic reaction takes place. Through this layer substrates and products need to be transported by "internal diffusion" which is slower than that through bulk solution because of steric barriers and high solid content of the layer. The microenvironment that surrounds the immobilized enzyme can act as a barrier for the free diffusion of molecules but it also may attract or repel the substrate or the product to its surface concentrating or depleting them in the immediate vicinity of the enzyme. The charge of the polymer obviously influences the partition of a charged substrate with direct consequences on the response, and therefore on the sensitivity of the biosensor [6–8].

In a previous paper [9] we reported the preparation of an amperometric biosensor for oxalate determination based on the immobilization of the oxalate oxidase in a mucin/chitosan gel at pH = 2.8 corresponding to the value of maximum enzymatic activity. In that paper, different proportions of mucin and chitosan were crosslinked with glutaraldehyde and used for the immobilization of the enzyme. Mucin, a hydrophilic polymer mainly composed of glycoproteins, is able to form viscoelastic gels with more than 95% water content giving an excellent environment for enzymes [10,11]. Chitosan, the principal derivative of chitin, is a natural

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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 [12]. We observed that although chitosan is widely used for biomedical applications in drug delivery [13] and in biosensors to immobilize biomolecules [14–16], when the enzyme was immobilized in chitosan, an electrode with a very slow response, useless for analytical applications was yielded. The presence of mucin, strongly increased the rate response and the electric current resulting in a higher calibration slope of the electrodes than that obtained only with chitosan as matrix for the immobilization. The effect of the polymer composition and crosslinking agent concentration on the analytical response was evaluated and related with changes in the swelling and the permeability of the matrices [17]. An enhancement of the matrix swelling was observed as the amount of mucin increased, which was accompanied by an increase in oxalate permeability. Nevertheless, the effect on the analytical performance of the sensor decreased for higher amounts of mucin. It was probable that the low crosslinking density caused a decrease in the enzyme charge. As consequence of both effects, a maximum in the sensitivity of the calibration curves of the electrodes was observed for 70/30 mucin/chitosan ratio. In the analysis, the effect of the positive charge of chitosan at the pH of enzymatic activity (pH = 2.85), was not considered; nevertheless partition of the negatively charged analyte, oxalate, in this positively charged matrix is a factor that could strongly influence the performance of these electrodes [18]. In the present paper, we measured the partition of oxalate and evaluated the influence of this and other properties of the immobilization support on the electrode activity. The effect of sulfate and chloride anions on the electrochemical response and on the physicochemical properties was considered.

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 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. Reagents

Mucin (muc) (porcine stomach Type III, partially purified, M-1778), oxalate oxidase (ODD, from Barley seedlings, O-4127) enzyme and chitosan (chit) (low molecular weight, 75–85% deacetylated, 448869) were obtained from Sigma Ltd.; potassium oxalate from Anedra; potassium sulfate 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 buffer solution was prepared using 0.5905 g per 100 mL succinic acid (Anedra) in purified water (Milli Q system). In some experiments potassium sulfate or potassium chloride were added to the solution to reach 50 mM final concentration.

2.3. Procedures

For the preparation of the electrodes, 3.0 μL of solution containing the enzyme (5.38 U/mL) was mixed with 3.0 μL of the following solutions prepared dissolving: 0.4000 g of different weight mucin/chitosan (70/30, 50/50 or 30/70) ratios in 4.1 mL of 2 vol.% acetic acid (pH = 2.85). These compositions were named as follows: muc/chit 70/30, muc/chit 50/50 and muc/chit 30/70. Then, each of these resultant solutions was mixed with 1.3 μL of aqueous glutaraldehyde solutions of concentration 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, permeability and partition coefficient of oxalate and swelling indexes of the muc/chit 70/30 matrix were determined in the following conditions: (1) succinic acid pH = 2.85, (2) succinic acid pH = 2.85 containing 50 mM potassium sulfate and (3) succinic acid pH = 2.85 containing 50 mM potassium chloride. These properties were measured also in muc/chit 50/50 and muc/chit 30/70 only in succinic acid pH = 2.85.

Calibration curves were plotted from the current–time curves obtained by addition of 10 μL of 0.05 M potassium oxalate to 5.0 mL of solution. The time required to reach 95% of the steady state in the current–time curves was as considered as a measure of the speed of the response (t 95).

For permeability measurements, the following gels: muc/chit 70/30, muc/chit 50/50 and muc/chit 30/70 (all of them crosslinked with 5.0 vol.% glutaraldehyde) were introduced between two polycarbonate membranes without the presence of the enzyme. The enzyme was excluded from these experiments in order to observe exclusively the effect of the matrix in the permeability of the analyte without interacting with the enzyme. In each experiment, oxalate was allowed to diffuse from a stirred 20 mM oxalate solution (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 (crosslinked 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 permeability.

For the measurement of the partition coefficient, portions of matrices muc/chit: 30/70; 50/50 and 70/30, by duplicate, were immersed in distinct solutions (40 mL) containing potassium oxalate 0.5 mM. After 96 h, the concentration of the oxalate in each supernatant was determined by capillary electrophoresis. The oxalate content into the matrices was calculated from the differences found in the quantity of the initial and final solutions.

After equilibration the matrices were removed from the solutions and the volumes were determined from Eq. (1) [2] in order to relate the quantity of oxalate into the samples with its volume.

$$Vs = \frac{W_{a,s} - W_{h,s}}{\rho_h} \quad (1)$$

Here, Vs is the volume of the sample in the swollen state, $W_{a,s}$ is the weight of the swollen sample in air, $W_{h,s}$ is the weight of the swollen sample in heptanes (a non-solvent), and ρ_h is the density of heptane. To obtain accurate weight measurements in heptane, the samples were placed in a stainless steel mesh basket which was suspended in heptane.

Once the volumes were measured, the quantity of oxalate into the samples (in mol) was related with the volume (in mL) to calculate the molar concentration.

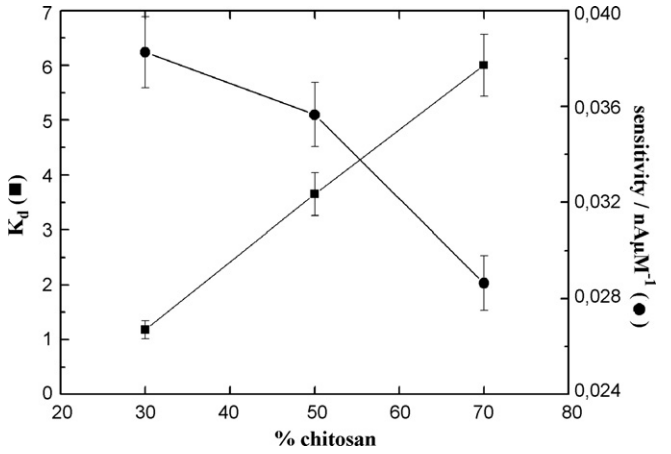

Fig. 1. Effect of the matrix composition on the slope of the calibration curves and on oxalate K_d in succinic acid pH = 2.85.

Then, the partition coefficient (K_d) was calculated from Eq. (2) [2]:

$$K_d = \frac{C_m}{C_s} \quad (2)$$

Here, C_m and C_s are the drug concentration in the sample and the drug concentration in the solution at equilibrium, respectively.

The swelling indexes of muc/chit 70/30, muc/chit 50/50 and muc/chit 30/70 (all of them crosslinked with 5.0 vol.% glutaraldehyde) were gravimetrically determined. For these measurements each gel 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 Eq. (3) [4]:

$$\%\text{Swelling} = \frac{M - M_0}{M_0} \times 100 \quad (3)$$

Here, M and M_0 are the weights of the wet and the dry matrix, respectively.

3. Results

3.1. Effect of matrix and solution compositions on the partition coefficient (K_d) of oxalate

Oxalate partition in the different matrices was measured at pH = 2.85 and, according to the distribution diagram at this pH, the acid oxalate anion ($HC_2O_4^-$), with a negative charge, is the main specie. Fig. 1 shows the effect of the matrix composition on the partition coefficient of acid oxalate. The coefficient increases almost five times when the muc/chit ratio is changed from 70/30 to 30/70, this is, when the amount of chitosan is increased. This result is expectable considering that the intake of anions should increase with the proportion of chitosan this is, with the amount of positive charges inside the matrix, due to electrostatic interaction. Although the electric current that flows through these electrodes strongly depends on the analyte amount inside the matrix, the sensitivity of the sensor decreases with the amount of chitosan in spite of this increment of oxalate concentration. This effect, shown also in the figure, is an indication that other factors, not only the charge, are influencing the enzymatic response.

The performance of the electrode prepared with the muc/chit 70/30 matrix for the immobilization of the enzyme that resulted with the highest slope, was evaluated in solutions of succinic acid at pH = 2.85 containing potassium sulfate or potassium chloride. As shown in Fig. 2 the calibration slope markedly decreased in the

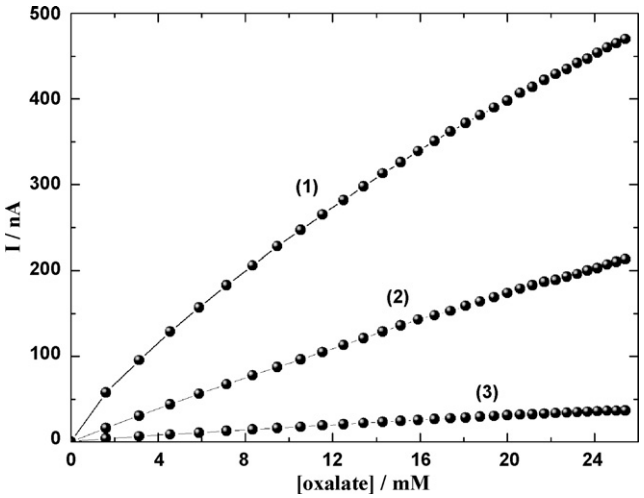

Fig. 2. Calibration curves of enzymatic electrodes for oxalate determination corresponding to the muc/chit 70/30 matrix crosslinked with 5 vol.% glutaraldehyde in (1) succinic acid pH = 2.85; (2) succinic acid pH = 2.85 containing 50 mM potassium sulfate and (3) succinic acid pH = 2.85 containing 50 mM potassium chloride.

presence of these ions especially in the case of chloride. It was then necessary to evaluate the effect of these ions on the activity of the enzyme in solution. Fig. 3 shows that sulfate ions did not introduce any difference in the enzymatic response in solution but the presence of chloride affected it indicating some specific interaction between the enzyme and this anion in solution. The important fact that the enzymatic response diminished in the matrix but not in solution, effect especially observed in the case of sulfate, is a clear indication that electrostatic interactions are influencing the response of the biosensor probably affecting the physicochemical properties of the matrix which in turn could affect either, the accessibility of the analyte to the enzyme, the enzyme activity itself or both.

Considering these observations, partition coefficients (K_d) of oxalate were measured with the muc/chit 70/30 matrix imbibed in succinic acid at pH = 2.85 (1) and in succinic acid with (2) sulfate or (3) chloride. In Fig. 4a and b the decrease in the slope and in the partition coefficient was found. It is possible to observe different response in the presence and in the absence of these anions; it is evident also, and according to these results, that an increase of

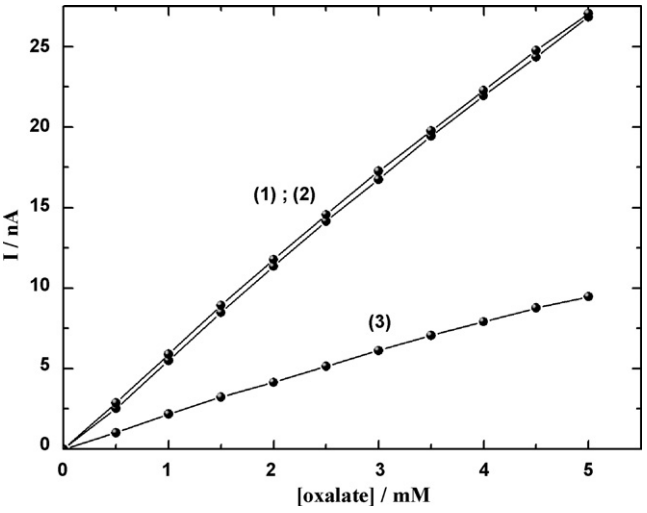

Fig. 3. Calibration curves for oxalate determination obtained with oxalate oxidase in (1) succinic acid pH = 2.85; (2) succinic acid pH = 2.85 containing 50 mM potassium sulfate and (3) succinic acid pH = 2.85 containing 50 mM potassium chloride.

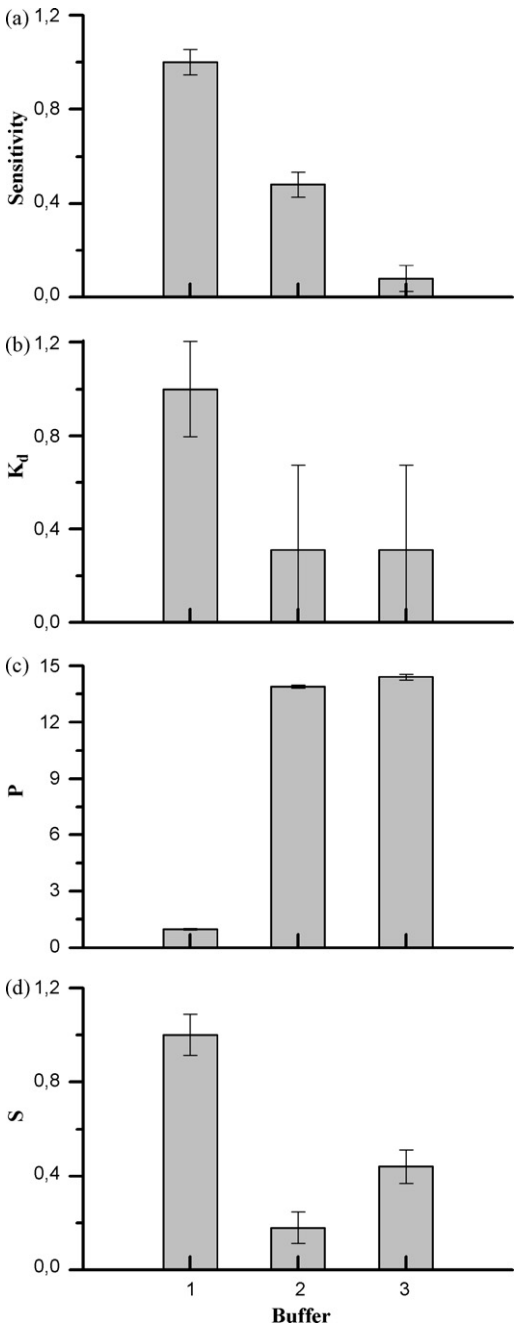

Fig. 4. Effect of the solution composition on: (a) slope of the calibration curves (sensitivity); (b) oxalate K_d; (c) permeability (P) and (d) swelling (S). (1) Succinic acid pH = 2.85; (2) succinic acid pH = 2.85 containing 50 mM potassium sulfate and (3) succinic acid pH = 2.85 containing 50 mM potassium chloride. For a better comparison all parameters have been normalized with respect to the muc/chit 70/30 matrix in succinic acid pH = 2.85 (bar 1) whose value has been considered unity.

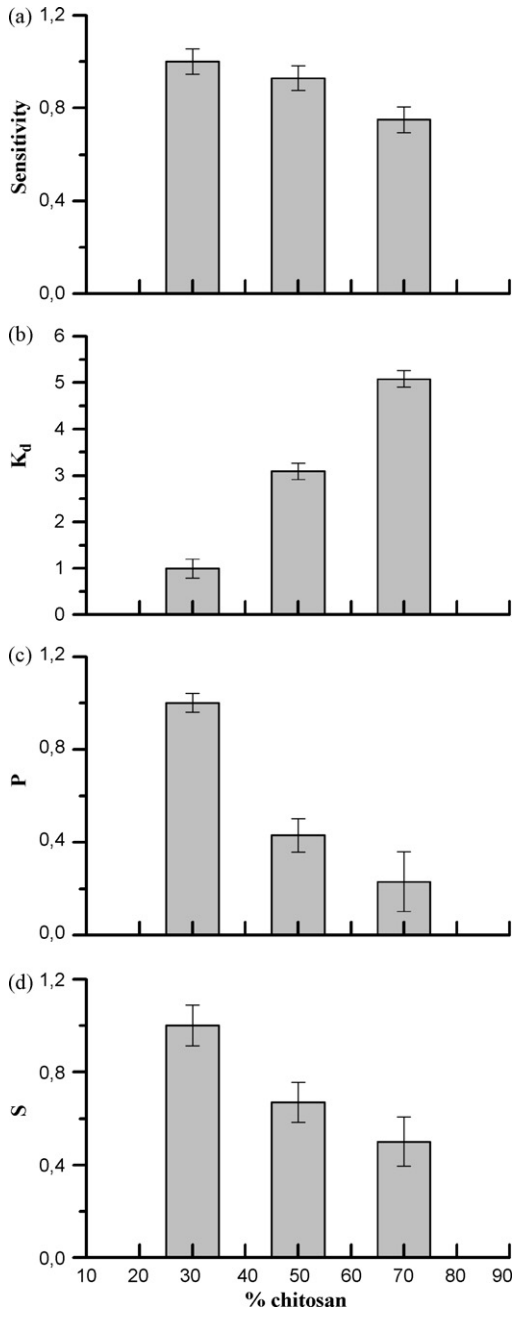

Fig. 5. Effect of the matrix composition on: (a) slope of the calibration curves (sensitivity); (b) oxalate K_d, (c) permeability (P) and (d) swelling (S) in succinic acid pH = 2.85. For a better comparison all parameters have been normalized with respect to the muc/chit 70/30 matrix in succinic acid pH = 2.85 (bar 1) whose value has been considered unity.

the analyte concentration inside the matrix is not enough condition to obtain a better response of the electrode. In fact, the electric current that flows in an amperometric biosensor is a global measure of the extent of chemical reaction between oxalate oxidase and oxalate inside the matrix; all conditions that favor the activity of the immobilized enzyme will result in a higher sensitivity, that is, in a higher slope of the calibration curve. It seems then, that the amount of active enzyme in the matrix largely depends on the conditions of immobilization which are given by the composition and the hydrophilicity of the matrix, the crosslinking density, the charge of the polymeric chains and the interdependence of all these properties that could affect it.

3.2. Effect of matrix and solution compositions on permeability and swelling

With the purpose of understanding the relation between the matrix behavior and the sensor response, the permeability and the swelling were measured in the muc/chit 70/30 matrix in presence of sulfate or chloride anions. An increase of almost one order of magnitude in the permeability and a strong decrease in the swelling were produced by the addition of these anions (Fig. 4c and d).

Besides, with a comparative aim and to facilitate the analysis, Fig. 5 resumes the effect of the matrices composition on their physicochemical properties and on the calibration curves. A higher proportion of chitosan produced a decrease in the swelling and in

the permeability of oxalate, effect which can be assigned to restrictions in the movement of the ions due to the higher crosslinking density.

4. Discussion

The results show that although the increase in positive charges of the matrices enhanced the intake of oxalate, there is a decrease in the swelling and in the permeability with the amount of chitosan [8]. The addition of salts produces interesting changes on these properties, decreasing K_d and swelling but markedly enhancing the permeability. These effects can certainly be due to a shielding of the polymeric charges which play an important role in these phenomena. Contractile force of the network and electrostatic interaction are combined in theoretical models to understand swelling of ionic gels and the influence of pH, salt concentration and ionic strength [19]. The Donnan potential pulls the anions into the positively charged matrix and the cations into the solution. The interaction with the fixed ions of the matrix strongly affects the electrolyte sorption. Association between the ions and the ionic groups of the matrix localizes the ion, reducing the Donnan potential and the exclusion of the electrolyte. The charge of these counterions has a strong influence on the ion distribution inside and outside the matrix. This effect is purely electrostatic and is much stronger than the specific effects of the swelling pressure by interactions. So, the matrix will prefer the ion of higher charge. Oxalate acid, has one negative charge at pH = 2.85 and therefore, in the presence of sulfate, the partition coefficient of oxalate will decrease. On the other hand, the matrix will prefer the ion with smaller solvated volume, as is the case of chloride [20].

A special consideration to the permeability has to be done. This is a process which depends on both, diffusion rate and partition of solute [2,8]. It is know that the diffusion of the solutes within the gels is a complex process that takes place in the regions filled with water in the space delimited by the polymer. Many factors can disturb these regions and can produce a retarding effect on the solute movement. Among these factors, the existence of charged groups in the polymer may bind the solute molecule. Besides, the polymer chain mobility is also an important factor which may govern the solute motion [18]. The experimental conditions certainly affect permeability especially when both solute and polymer are charged.

In our case, when the proportion of chitosan in the matrix increases, the oxalate permeability decreases probably due to the increase in positive charges into the network and also in the crosslinking density. This is consistent with the increase in K_d. On the other hand, as a consequence of the stronger electrostatic interaction between sulfate or chloride ions and the fixed groups of the polymer, the permeability of oxalate is highly enhanced as the amount of "free" amino groups are reduced and the oxalate ion is more mobile within the network. It is interesting to point out that in spite of these opposite tendencies, in both situations the sensitivity of the electrodes diminishes.

It can be inferred from the above results that the swelling of the matrices plays an important role in the performance of a biosensor (Fig. 4d). The swelling increases as the fixed and mobile charges tend to be surrounded by the solvent; dilution of these charges due to osmotic pressure difference and repulsive interactions contribute to increase the swelling. A high density of crosslinks in the matrices reduces the ability to swell; also, strong association between fixed groups and counterions decrease the swelling because the osmotic pressure and the tendency to form solvation shells are reduced. In this analysis, it is important to distinguish between the effects produced by hydration water and free water. The volume of free water is not necessarily a measure of the swelling [20]. The swelling can increase although the amount of free water decreases. For example, when the size of the hydrated ion is large, the swelling increases but it produces a swelling pressure that pushes the free water out of the matrix [20]. The opposite effect, in which the amount of free water increases and the swelling decreases, could be also possible [20]. This effect could be produced by the sulfate when it interacts with the fixed charges of the chains, producing the increase in free water, and a decrease in hydration water due to the screen of fixed charges, contributing to explain the increase in the solute permeability previously mentioned.

5. Conclusion

The electrochemical response of an enzymatic biosensor depends in a crucial way on the efficiency of the enzyme substrate interaction. This interaction in turn depends on the concentration of the substrate, in this case oxalate, in the neighborhood of the enzyme, and of the amount of active enzyme in the matrix. In those cases, when the biomolecule is immobilized in the polymer, the physicochemical properties of the matrix play an important role in the performance. The results of this paper show that in spite of the increase in K_d with the amount of chitosan, the enzyme losses activity due to the decrease in the swelling which affects its hydrophilic environment. The presence of some anions produced a similar effect on the swelling and in consequence in the activity of the sensor.

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