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Viscoelastic and swelling properties of glucose oxidase loaded polyacrylamide hydrogels and the evaluation of their properties as glucose sensors

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Abstract

Here we report the immobilization of glucose oxidase in polyacrylamide hydrogels carried out by aqueous crosslinking copolymerization of acrylamide and *N*,*N*′ methylene bisacrylamide in the presence of the enzyme. The swelling and viscoelastic properties of the hydrogels were evaluated as a function of the content of crosslinker of the polymer chains and enzyme concentration. Amperometric measurements were also carried out to evaluate the system as a glucose biosensor.

Keywords: Polyacrylamide hydrogels; Viscoelastic properties; Glucose biosensor

1. Introduction

Polymer gels are a unique class of macromolecular networks that contain a large fraction of solvent within their structure [1–3]. The ability of polymer gels to undergo substantial swelling and collapsing, up to 1000 times in volume [4], as a function of their environment is one of the most notable properties of these materials [5]. The phenomenon of gel volume transitions, which can be induced by temperature, pH, or ionic strength, among other stimuli, has prompted researchers to investigate gels as potential actuators, artificial muscles, sensors, controllable membranes for separations and modulators for delivery of drugs [6–11]. Gels are particularly appropriate for biomedical applications because of their ability to simulate biological tissues [12].

Gels are usually formed by free radical polymerisation of monomers in the presence of a difunctional crosslinking agent [13–14]. They can be made either in bulk or in nano or microparticles. The bulk gels are easy to handle and to study, but have very slow swelling rate, while the gel nanoparticles act quickly to an external stimulus, but are too small for some applications [15].

In recent years, polyacrylamide-based hydrogels have received considerable attention because of their use in many applications [16–19]: specific sorbents, support carrier in biomedical engineering, aggregating agents, soil improvement agents, polymer processing or improving textiles, paper strengthening agent, adhesive, paints, oil salvaging agents, etc. The kinetics of network formation in free-radical copolymerisation of acrylamide and *N*,*N*′-methylene-bis-acrylamide in aqueous solution has been extensively studied and it has been reported that the polyacrylamide network exhibits inhomogeneous crosslink distribution [20]. Partially hydrolysed polyacrylamide gels exhibit volumetric phase transition by changing the temperature, pH, and solvent composition [21]. More studies on polyacrylamide gels have focused in the gelation conditions, swelling properties, phase transitions in swollen gels and their applications. However, there are few studies that deal with the determination of the structure of polyacrylamide gels and its derivation from viscoelastic measurements [22].

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Polyacrylamide gels have been extensively used for the matrix of electrophoresis and to encapsulate drugs, enzymes and proteins for application in drug delivery systems and biosensors [23–24]. For example, the immobilisation of enzymes into polymer gels allows their stabilisation and the retention of the enzymatic activity for long periods of time. Nevertheless, the modifications induced by the enzyme in the molecular structure and in the viscoelastic and swelling properties of the gel have been little studied [25].

In the present study, we have investigated the viscoelastic and the swelling properties of polyacrylamide gels and polyacrylamide gels with entrapped glucose oxidase (GOx) with the aim to prepare a biosensor. The principal objective is to study the effect of the introduction of GOx on the molecular structure and properties of the gels. This objective was performed using two methods: (i) swelling experiments and, (ii) viscoelastic measurements. The swelling experiments of polymer gels provides a good approach to determination of the average molecular weight of the network chains in the gel, and thus, the effect of GOx on the structure of the gel. Likewise, the viscoelastic experiments permit to determine the shear modulus, G, which can be related to the molecular weight of the network chains using the theory of rubber elasticity, and hence to study the effect of the introduction of GOx in the gel structure. Finally, amperometric measurements were carried out to evaluate the GOx loaded polyacrylamide gels as glucose sensor.

2. Experimental section

2.1. Sample preparation

2.1.1. Materials

The acrylamide (AAm) (Panreac), the initiator ammonium persulfate (Fluka), the crosslinker *N*,*N*′ methylene bisacrylamide (Bis) (Aldrich), the accelerator *N*,*N*,*N*′,*N*′ tetramethylethylendiamine (TEMED) (Bio-Rad), the D+ glucose (Merck) and the enzyme (EC 1.1.3.4.) GO_x from *Aspergillus niger* (Sigma) were used as received.

2.1.2. Preparation of pure gels

PAAm gels were prepared by crosslinking copolymerization of AAm with a small amount of Bis in aqueous solution. The polymerization was initiated by the ammonium persulfate/TEMED redox system and carried out at room temperature. At a constant monomer concentration of 0.25 g/mL, gels with different amounts of crosslinker were prepared. 25 g of acryl amide, 0.626 g of ammonium persulfate, 220 μL of TEMED and appropriate amounts of Bis (see Table 1) were dissolved in 100 mL of deionized water (milli-Q grade). The molar percentage of crosslinker with respect to monomer thus achieved ranged from 1.5% to 8%. Solutions were poured out into Petri dishes and allow to react at room temperature for 24 h. When gelation is achieved cylindrically shaped specimens of 20 mm in diameter and approximately 2 mm in height were cut.

2.1.3. Preparation of gels with entrapped GO_x

The encapsulation of GO_x was carried out following the previous procedure slightly modified. To prevent enzyme denaturation, we have used phosphate buffer pH 6 as aqueous phase to solubilise the enzyme and the temperature was controlled and kept below 35 °C. 12.5 g of AAm, 0.132 g of ammonium persulfate, 110 μL of TEMED and the appropriate amounts of Bis to achieve molar percentages of crosslinker to monomer ranging from 2% to 5% (see Table 2) were dissolved in 50 mL of phosphate buffer pH = 6, poured out into Petri dishes and allow to react for 24 h. Cylindrical specimens of 20 mm in diameter and 2 mm in height were cut after completion of the reaction.

2.2. Methods

2.2.1. Swelling measurements

The cylindrical specimens of the prepared gels were immersed in deionized water and kept there until equilibrium is attained at room temperature. The relative degree of swelling was determined by weighting the specimens at different times until constant weight is obtained. The absolute degree of swelling at equilibrium was determined by thermogravimetric analysis performed in a Perkin–Elmer TGA apparatus. Pieces of the gel swollen to equilibrium were introduced in the TGA oven and maintained at 100 °C until water is completely removed. The measurement of sample weight before and after water evaporation allows to determine the degree of swelling.

2.2.2. Rheological measurements

Dynamic viscoelastic measurements were performed in a TA Instruments AR1000 Rheometer, using the parallel plate shear mode to measure the storage modulus, G', the loss modulus, G', and the loss tangent, $\tan\delta$. To avoid the influence of aging and the kinetics of gelation on the G'

Table 1
Conditions for the preparation of pure PAAm gels

Content of crosslinker (%)	1.5	2	3	4	5	7	8
N, N′ methylene bisacrylamide (g)	0.8140	1.0854	1.6265	2.1738	2.7134	3.7988	4.3322

Table 2
Conditions for the preparation of PAAm gels with entrapped GO_x

Content of crosslinker (%)	2	3.2	4	5
N, N′ methylene bisacrylamide (g)	0.543	0.866	1.084	1.357
GO_x (g)	1.15	2.50	2.50	2.50

modulus, the measurements for all samples were performed 24 h after the gels were prepared (initial gels). Gels after swelled to equilibrium were also measured (swollen to equilibrium gels). The operating conditions were the following: temperature sweep between 10 and 100 °C, temperature scan rate 2 and 10 °C/min, plate diameter 20 mm, frequency 1 Hz, and torque 50 μNm. The linear viscoelastic region was located with the aid of a torque sweep. Frequency scans from 100 to 0.1 Hz in isothermal conditions and torque 50 μNm were also carried out. To avoid the evaporation of solvent in the course of the rheological measurements, a solvent trap from TA Instruments was used.

2.2.3. Amperometric measurements

The amperometric measurements at constant potential were carried out in a Metrohm Polarecord potentiostat E-506. Electrochemical measurements were performed in 0.1 M phosphate buffer in a three-electrode cell with a platinum electrode as working electrode, an Ag/AgCl reference electrode an a platinum counter electrode.

3. Results

3.1. Swelling experiments

Fig. 1 depicts the evolution of the swelling degree (defined as the inverse of the polymer volume fraction, v_2) with time for pure PAAm gels of different content of crosslinker. From this figure it can be observed that: (i) the freshly prepared gels ($t=0$ min) swell when immersed in an excess of water, at a rate which is dependent on the content of crosslinker of the samples: the time needed to achieved the equilibrium of swelling diminish as the content of crosslinker increases; (ii) the equilibrium swelling degree decreases as crosslinking degree increases; and (iii) the difference between the swelling degree in the equilibrium of swelling and in the initial state, ΔQ (see the inset of Fig. 1) has a low value (i.e., the degree of swelling in the initial state is similar to that in the equilibrium of swelling) for all the samples and is dependent on the content of crosslinker (it decreases with the increase of the content of crosslinker).

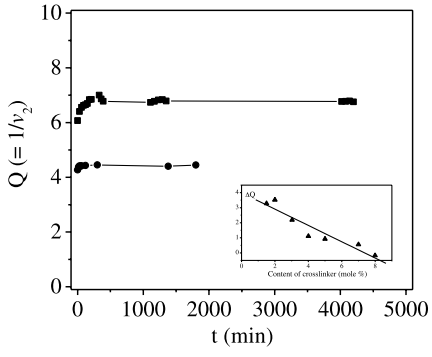

Fig. 1. Evolution of the swelling degree, Q, with time for PAAm gels of content of crosslinker 3% (■) and 8%(●). Inset: Evolution of the difference between the swelling degree in the equilibrium state and in the initial state ($\Delta Q=Q_s-Q_r$) as a function of the content of crosslinker for PAAM gels.

3.2. Rheological experiments

A gel should be characterized by an elastic modulus at zero frequency. Therefore, the viscoelastic characterization of crosslinked PAAm-water systems can be performed from frequency scans as presented in Fig. 2. This figure shows the evolution of storage modulus and loss tangent with frequency for a PAAm gel in the initial state (content of crosslinker 8%) at different temperatures.

As it will be seen later, the quantity required when the results are interpreted in terms of rubber elasticity theory is the equilibrium shear modulus. Nevertheless, here the shear modulus has been obtained by dynamic measurements. Strictly speaking, the frequency dependent storage modulus G' and the loss modulus G'' were measured. However, the values of the loss tangent were always below 0.02 (see Fig. 2), which implies that $G'' \ll G'$. In addition, no systematic frequency dependence was observed in the range studied. All these results indicate that the storage modulus can be used in place of the equilibrium modulus [22]. From now on, no distinction will be made in this paper between the equilibrium moduli and the storage moduli determined at 1 Hz (obtained at this frequency for all the samples and experiments for the sake of simplicity).

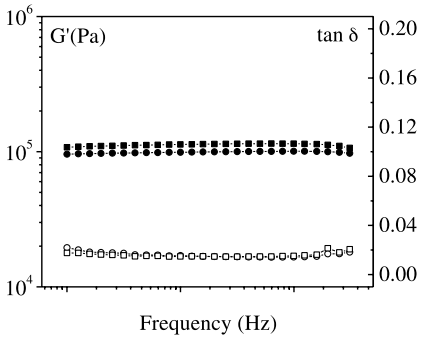

Fig. 2. Storage modulus (full symbols) and tan δ (open symbols) as a function of frequency for a PAAm gel of content of crosslinker 8% at 10 °C (circles) and 25 °C (squares).

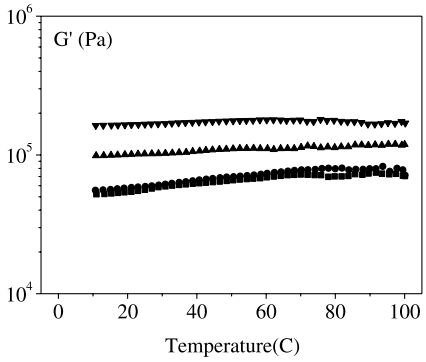

Fig. 3. Storage modulus as a function of temperature for PAAm gels of content of crosslinker: 2 (■), 3 (●), 7 (▲) and 8% (▼) obtained at a heating rate of 10 °C/min.

In Fig. 3 the curves corresponding to the evolution of storage modulus versus temperature for PAAm gels in the initial state of different degrees of crosslinking are represented. Two conclusions can be drawn from this figure: (i) the storage modulus increases with temperature and follows a behavior of the type $G \sim kT$, characteristic of chemical gels whose elasticity is of entropic origin and (ii) the storage modulus increases with the content of crosslinker of the gels.

Fig. 4 shows the variation of the equilibrium modulus with the content of crosslinker for PAAm hydrogels in the initial state and in the equilibrium of swelling. In both series, the shear modulus increases with the content of crosslinker. In addition, there are no virtual differences in the modulus for the two series of data within the experimental error. This is due to the small differences between the initial state and the equilibrium state with regard to the swelling degree (see Fig. 1).

The effect of GOx in the viscoelastic properties of PAAm hydrogels can be observed in Fig. 5. Fig. 5(a) shows the evolution of the storage modulus with temperature for PAAm gels of different content of crosslinker loaded with GOx (concentration 50 mg/mL) in the initial state. The same overall behavior as for pure PAAm gels in the initial state can be observed: the storage modulus increases with temperature following a law of the type $G' \sim kT$, and also

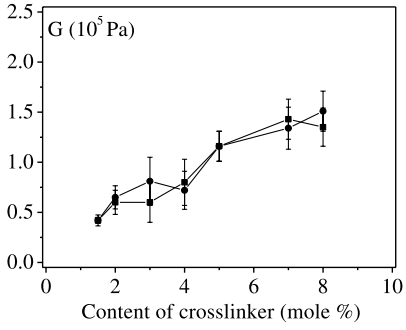

Fig. 4. Equilibrium shear modulus as a function of content of crosslinker for PAAm gels in the initial state (●) and in the equilibrium of swelling (■).

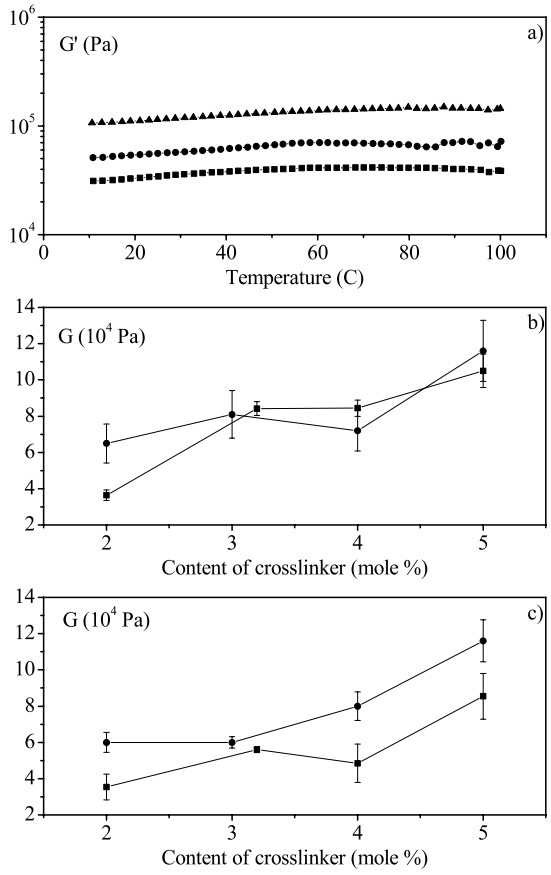

Fig. 5. (a) Storage modulus as a function of temperature for PAAm gels of content of crosslinker 2 (■), 4 (●) and 5% (▲) loaded with Gox (50 mg/mL) in the initial state; (b) Equilibrium shear modulus as a function of content of crosslinker for PAAm gels in the initial state: (●) empty gel and (■) loaded gel; (c) Equilibrium shear modulus as a function of content of crosslinker for PAAm gels in the equilibrium of swelling: (●) empty gel and (■) loaded gel.

increases with the content of crosslinker of the gel. In Fig. 5(b) we compare the evolution of the equilibrium modulus with the content of crosslinker for both: pure PAAm gels and PAAm gels with entrapped GOx in the initial state. We can observe, within the experimental error, an analogous behaviour in the evolution of the modulus with the content of crosslinker for both series. In contrast, gels with entrapped enzyme have lower modulus than pure gels when the systems are studied in conditions of swelling equilibrium, as can be observed in Fig. 5(c).

3.3. Application of the gels loaded with GOx to a glucose biosensor

Gels with immobilized GOx were used as biological component of an amperometric glucose sensor. The electrode was prepared by depositing an exact weight of freeze-dried powdered gel on the surface of a platinum electrode, which was then covered with a dialysis membrane. The resulting electrode was placed in a three-electrode cell and acted as the working electrode. The

electrode was then washed with 0.1 M phosphate buffer and a potential of +600 mV vs Ag/AgCl was applied to the sensor until the background current had decreased to a constant level.

The principle of the determination of the current response is based on the formation of hydrogen peroxide during the enzyme-catalyzed reaction [25]. The current response resulting from oxidation of hydrogen peroxide in the electrode was measured by amperometry under steady-state conditions [26]. The calibration curve of the biosensor, measured in stirred solutions at 25 °C is shown in Fig. 6. The average response for glucose of this biosensor was 135 nA/mM and the response time 600 s. Furthermore, the linearity of the electrode response can be monitored controlling the load of GOx in the gel.

4. Discussion

4.1. Molecular weight between crosslinks

It is interesting to study the elastically effective crosslink densities of the PAAm gels as a function of the content of crosslinker. For this purpose the crosslinking reactions were carry out until complete conversion and the final gels were swollen in deionized water until equilibrium was reached.

From the equilibrium degrees of swelling of the PAAm hydrogels, the number average molecular weight between adjacent crosslinks, M_c, were evaluated using the Peppas and Merrill [27] swelling equation for tetrafunctional affine networks:

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\left(\frac{\upsilon}{V_1}\right)\left[\ln(1-\upsilon_{2,s}) + \upsilon_{2,s} + \chi_1 \upsilon_{2,s}^2\right]}{\upsilon_{2,r}\left[\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)^{\frac{1}{3}} - 0.5\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)\right]} \quad (1)$$

and for tetrafunctional phantom networks:

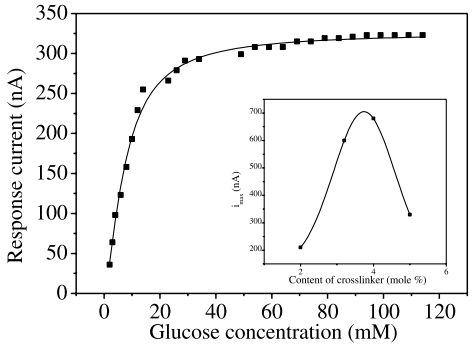

Fig. 6. Response current of the biosensor (PAAm gel of content of crosslinker 5% and GOx concentration 50 mg/mL) as a function of glucose concentration. In the inset, we show i_{max} as a function of the content of crosslinker of the PAAm hydrogels.

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\left(\frac{\upsilon}{V_1}\right)\left[\ln(1-\upsilon_{2,s}) + \upsilon_{2,s} + \chi_1 \upsilon_{2,s}^2\right]}{\frac{1}{2}\upsilon_{2,r}\left(\frac{\upsilon_{2,s}}{\upsilon_{2,r}}\right)^{\frac{1}{3}}} \quad (2)$$

where M_c is the number average molecular weight between crosslinks, M_n is the number average molecular weight of the polymer before crosslinking, ν is the specific volume of the polymer (0.741 mL/g for PAAm), V_1 is the molar volume of the swelling solvent (18 mL/mol for water), χ_1 is the Flory polymer–solvent interaction parameter (0.48 for the system PAAm-water), $\nu_{2,s}$ is the swollen polymer volume fraction (polymer volume fraction after equilibrium swelling) and $\nu_{2,r}$ is the initial polymer volume fraction (polymer volume fraction immediately after crosslinking but before swelling).

It should be noted that the factor (1-2Mc/Mn) is the correction for network imperfections resulting from chain ends; this factor reduces to one for perfect networks and it is also approximated to one for crosslinking polymerizations.

Using the experimental $\nu_{2,s}$ and $\nu_{2,r}$ and the values of parameters indicated above, we estimated the molecular weights between crosslinks using Eqs. (1) and (2). The results are represented in Fig. 7. In this figure the continuous line represents the stoichiometric molecular weight between crosslinks of the gel chains calculated from the Bis content in the reaction mixtures: M_c(theoretical) = M_r/2X, where M_r is the molecular weight of AAm and X is the ratio mole of Bis/mole of AAm.

The molecular weight between crosslinks obtained experimentally decreases with increasing crosslinking degree. As shown in Fig. 7 good agreement between the theoretical and experimental values from both affine and phantom models was obtained.

The molecular weight between crosslinks can also be estimated by means of viscoelastic measurements. The theory of polymer networks predicts the shear modulus G for polymer gels obtained by solution crosslinking copolymerization to be [28]:

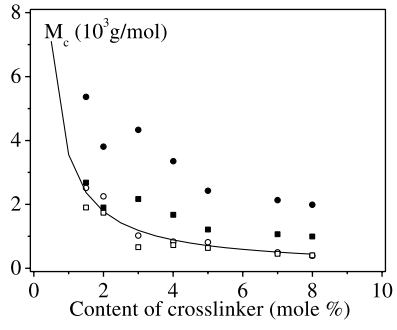

Fig. 7. Average molecular weight between crosslinks as a function of content of crosslinker for PAAm gels: (solid line) theoretical, (○) from swelling measurements (affine model), (□) from swelling measurements (phantom model), (■) from rheological measurements (phantom model) and (●) from rheological measurements (affine model).

$$G = \left(1 - \frac{2}{f}\right) \frac{\rho RT}{M_c} \upsilon_{2,s}^{1/3} \upsilon_{2,r}^{2/3} \quad (3)$$

where f is the functionality of the crosslinks ($f=4$ for tetrafunctional networks), ρ the polymer network density (1.350 g/mL for PAAm), R the gas constant and T the temperature. The use of the front factor (1-2/f) is appropriate for diluted systems such as gels (Phantom networks). For more concentrated Affine networks of functionality 4 the front factor turns into 1.

Using experimental values of G, $\nu_{2,s}$,$\nu_{2,r}$ and equation (3) M_c was determined. As can be seen in Fig. 7 no good agreement between experimental values of M_c determined by viscoelastic and swelling measurements was obtained. It must be noted that, as stated in the literature, the thermodynamic swelling experiments give generally more reliable results of M_c than the viscoelastic experiments. In the latter case entanglements (which tend to increase the modulus) and other defects of the chains, as closed loops and loose ends (which do not contribute to the network elasticity), have a stronger influence on the mechanical properties, and ultimately the calculated values of M_c than on the swelling properties. Nevertheless, the considerable difference between M_c values calculated from swelling experiments (which reasonably fit the theoretical values) and the values calculated from mechanical experiments points to a rather heterogeneous structure of the hydrogels in accordance with previous results in the literature (cyclisation of polymer chains, formation of microgels, pendant chain ends, etc.) [16,20].

4.2. Effect of GO_x on the viscoelastic and swelling properties of PAAm hydrogels

It has been shown (Section 3) that the effect of the GOx content on the viscoelastic properties of PAAm gels depends on whether the initial state or the equilibrium swelling state is considered. In the initial state gels loaded with GOx present a similar modulus value than empty gels. On the other side, in the equilibrium swelling state empty gels have modulus values higher than loaded gels. This contradictory behaviour can be understood if we consider the results presented in Fig. 8. In Fig. 8 the evolution of the volume fraction of the polymer in the hydrogels is shown as a function of crosslinking degree for both loaded gels and empty gels in the initial state and in the state corresponding to the equilibrium of swelling. As can be seen, in the initial state the volume fraction of polymer in the hydrogels is independent of: (i) the crosslinking degree and (ii) the presence of GOx.

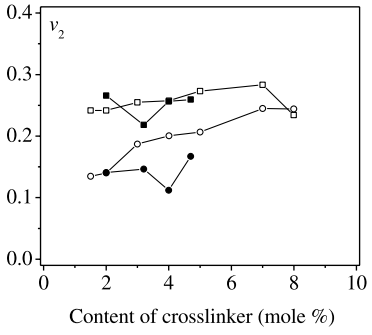

Fig. 8. Evolution of the volume fraction of the polymer as a function of content of crosslinker for PAAm gels in the initial state, v_{2r}: (□) empty gel and (■) gel loaded with GO_x; and in the equilibrium of swelling, $v_{2\,s}$: (○) empty gel and (●) gel loaded with GO_x.

Fig. 8 also shows the effect of GOx on the evolution of the polymer volume fraction with the degree of crosslinking for the gels swollen to equilibrium: For the gels loaded with GOx, the polymer volume fraction is, within the experimental error, rather independent of the degree of crosslinking. Moreover, the polymer volume fraction is lower for loaded gels than for empty gels (gels loaded with GOx swell to a higher extent than empty gels). The fact that the swelling equilibrium degree is independent of the crosslinking degree for GOx loaded gels can be attributed to osmotic effects. The presence of GOx makes the gel to absorb an excess of water to equilibrate the osmotic pressure of the enzyme. The driving force for the swelling of the loaded gel is thus of osmotic rather than elastic nature. The swelling behaviour is consistent with the elastic behaviour and can account for it: the degree of swelling at equilibrium is higher for gels loaded with GOx than for empty gels and subsequently, the equilibrium modulus is smaller (see Eq. (3)) for loaded gels than for empty gels for the same degree of crosslinking.

4.3. Detection of glucose by the biosensor

The steady-state current i_k for an amperometric enzyme electrode under kinetic control is given by the Eq. (4) [26]

$$i_k = \frac{nFAdk_c[E_0][S]}{2(K_M + [S])} = \frac{i_{max}[S]}{K_M + [S]} \quad (4)$$

where n is number of electrons in the electrodic reaction, F is the Faraday constant, A is the area of the electrode and d is the diffusion layer thickness, [S] is the concentration of substrate, k_c and K_M are the catalytic and apparent Michaelis–Menten constant, respectively and i_{max} is the maximum current response which is proportional to the concentration of GOx [E_0]. We have observed Michaelis–Menten behaviour when we work with free enzyme (dissolved in the electrochemical cell) but this is not the case for the entrapped enzyme. In the later case the fitting of the variation of i_k with the substrate concentration is better described by the Hill expression [26]:

$$i_k = \frac{i_{max}[S]^n}{K_M + [S]^n} \quad (5)$$

where $n=1.4$ is the Hill cooperativity coefficient. The result of fitting the experimental data with Eq. (5) is showed by the

continuous line in Fig. 6. Cooperative interactions may appear in enzymes with two identical subunits like GOx and it seems that immobilization in gels favours the cooperative behaviour.

In the inset of Fig. 6 we show i_{max} as a function of the crosslinking of the gel. The continuous line is a guide for the eye to illustrate the upward trend of i_{max} up to 3.5% followed by a decrease to higher crosslinking content. This behaviour of i_{max} is attributed to the decrease of the porosity of the gel with increasing the crosslinking, slowing down the diffusion of the substrate towards the catalytic site of the enzyme.

5. Conclusion

The analysis and interpretation of the viscoelastic and swelling properties of polyacrylamide hydrogels on the basis of different theories points to the existence of an inherent inhomogeneous structure in a microscopic scale.

Glucose oxidase can be immobilised in polyacrylamide gels by aqueous crosslinking copolymerisation of acrylamide and *N*,*N*′-methylene bisacrylamide in the presence of the enzyme. The effects of the entrapment of the enzyme on the properties of polyacrylamide gels can be summarized as follows: (i) in the initial state the introduction of GOx does not affect the gel modulus, (ii) gels loaded with GOx swell to a greater degree than empty gels, due to osmotic effects introduce by the presence of the enzyme and therefore (iii) in the equilibrium of swelling, gels loaded with GOx possess a smaller modulus value than empty gels. In order to elucidate the effect of GOx on the molecular structure of PAAm hydrogels, solid state NMR spectroscopy and small angle neutron scattering studies are now undertaken.

Polyacrylamide hydrogels with immobilized GOx can be used as biological component in an amperometric biosensor for glucose detection.

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