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High stability amperometric biosensor based on enzyme entrapment in microgels

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Abstract

The preparation and characterization of an amperometric glucose biosensor based on the entrapment of glucose oxidase (GOx) in a polyacrylamide microgel is described. This study proves that polyacrylamide microgels provide an excellent matrix for GOx immobilization that can be used as a biological material in amperometric biosensors. The interference produced by ascorbic and uric acid has been eliminated by including acrylic acid in the polymeric matrix. With this modification, we obtain an adequate device for glucose determination in complex samples such as blood and serum. The study of the temperature effect in the response of biosensors indicates that swelling of the microgels directly influences the enzymatic activity. Thus, the behaviour of the enzyme in the swollen microgels is similar to the enzyme in solution, but the enzyme's activation energy increases when the water content in the microgels decreases. One important property of these biosensors is their remarkable stability. After 4 months of its manufacture, there is no loss in the initial response. Furthermore, the enzymatic activity of freeze-dried microgels containing enzyme remains unaltered for at least 18 months.

Keywords: Glucose; Amperometric biosensor; Glucose oxidase; Polyacrylic microgels

1. Introduction

Among the many parameters used to characterize a biosensor some of them evaluate functional properties such as sensitivity, stability, response time, reproducibility and so on, while others like biocompatibility are related to clinical applications. Zhang et al. [1] observe that most clinical applications have been restricted to academic studies rather than to routine clinical monitoring because of the poor biocompatibility of available materials used as host matrix of biomolecules. Consequently, developments in biosensor design are focussed on the technology of new materials, especially new copolymers that promise to solve the biocompatibility of the device offering the prospect of a more widespread use of biosensors.

Immobilization of enzymes on the electrode surface is a key factor in fabricating a biosensor and various immobilization methods have been employed in preparation of reagentless amperometric enzyme electrodes. One method consists of encapsulating within polymers. In particular, the glucose oxidase has been successfully immobilized on electropolymers [2–9], hydrogel polymers [10–19], sol–gel [20,21] and more recently polymer latex [22,23].

The inherent biocompatibility of some hydrogels together with their ability to simulate natural tissues makes them potentially important electrode-coating materials. Hydrogels are described as cross-linked macromolecular networks [24] which undergo reversible volume changes in response to external stimuli, like pH or temperature, and they are known to exist swollen and shrunk depending on the surrounding environment. Hydrogels are excellent carriers of biomolecules, which can be immobilized within hydrogel matrices through physical entrapment and, in this context, two different geometries has been presented, namely, disc

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and microparticles. Microgel particles based on cross-linked polymers form an interesting group of hydrogels since they share properties with macrogels at the time that show typical features of colloidal systems.

In microencapsulation, the biocatalyst is trapped in an inert three-dimensional polymer network providing larger enzyme loading than covalent grafting or simple adsorption [23]. The enzyme stability in these matrices is usually the key factor determining the lifetime of the biosensor. Moreover, this method has several advantages like: (i) high activity due to the high surface-to-volume ratio, (ii) it is chemically simple, (iii) it forms flexible and strong membranes with no haemolysis in distilled water and (iv) the enzyme is protected against contamination and biodegradation by the polymeric matrix, which increases the biosensor lifetime.

One of the challenges of diabetes research is to find a microdevice capable not only of measuring glucose level in blood but also to release insulin as a function of the glucose level. One important criterion determining the feasibility of hidrogels in insulin delivery is their optimal performance under physiological conditions. Albin et al. [25] studied GOx immobilized in polyacrylamide and poly(dimethylaminoethyl metacrylate) gels in the form of macroporous and microporous matrices. More recently, studies conducted by Podual et al. [26] on glucose-sensitive membranes indicated that hydrogels could be used successfully to achieve feedback-controlled release of insulin because the gels present a reversible swelling in response to the glucose concentrations of the surrounding medium. The resulting changes in the mesh size modulate the release of insulin physically imbibed in the gels.

Even though, it has been proven that microencapsulation is a good immobilisation method, it is difficult to find biosensors with this host matrix. Sol–gel derived glasses have emerged as materials to microencapsulate biomolecules [20,27,28], but due to their low conductivity they have been used in optical sensors, and rarely in amperometric sensors. Microparticles prepared with methacrylate [13], alginate [18] and artificial latexes [23] have been showed as convenient host matrices to encapsulate enzymes and versatile materials to modify electrodes surfaces.

In this paper, we report a new biosensor based on polyacrylamide microgel using the polymeric matrix for enzyme entrapment. Two polymer compositions have been used to encapsulate GOx: the first one contains acrylamide as monomer and bisacrylamide as cross-linker agent (PMP1), while in the second one, (PMP2) part of acrylamide has been substituted by acrylic acid in order to provide a negative charge to the microparticles. We have also investigated the effect of the crosslinking, pH, temperature, the performance of the prepared enzyme sensor and its application to the determination of glucose in human serum. We are aware of the great number of reported glucose biosensors [29,30] but we selected GOx just as a model enzyme to essay a new immobilisation system and its application in an amperometric sensor.

2. Experimental

2.1. Chemicals

Acrylamide (AA) was purchased from Panreac, *N*,*N*′-methylenebisacrylamide (BIS) from Aldrich, ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED) and Span80 from Fluka. Glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, 5000 units/g protein was obtained from Sigma. D(+)-glucose was from Merck. Phosphate buffer solutions were prepared from stock solutions of sodium dihydrogen phosphate (Panreac) and the pH was adjusted using sodium hydroxide. The dialysis membrane (12,000–14,000 MWCO) was purchased from Spectrum Medical Industries. All reagents were used as received without further purifications. Doubly distilled water was further purified with a MilliQ water system (Millipore).

2.2. Emulsion preparation

Polyacrylamide (PAA) microparticles have been prepared using the concentrated emulsion polymerization method. The crosslinking, η, given by the expression, $\eta = \frac{\mathrm{BIS(g)}}{\mathrm{AA(g)+BIS(g)}} \times 100$, was varied between 1.6 and 5.82%, changing the crosslinker, (BIS), concentration and keeping constant the amount of monomer, (AA). The concentrated emulsion was prepared with a low fraction of the oil phase, which constitutes the continuous phase, according to procedures outlined in the literature [31–33]. In order to obtain the W/O concentrated emulsion, 5 mL of an aqueous solution consisting of AA (1.25 g), BIS (from 20 to 72.5 mg depending on η), ammonium persulfate (25 mg) and GOx (250–900 mg), was added with a syringe to the continuous oil phase (750 μL of dodecane and 250 μL of Span 80). The emulsion was homogenized by magnetic stirring and purged with nitrogen to remove residual oxygen. The polymerization was started by adding TEMED (63 μL) to the emulsion. After 1 h of reaction, the polymer was precipitated and washed with a cold phosphate buffer solution pH 7.2. Microparticles were isolated by centrifugation (10,000 rpm) for 10 min at 10 °C and the supernatant was analysed. The liquid only showed enzymatic activity for microparticles with $\eta = 1.6\%$ but not for the other crosslinking fractions. After solvent separation, microparticles were freeze-dried to remove residual solvent and water. The polymerization generates polyacrylamide microparticles with diameters between 0.9 and 15 μm. Negatively charged microparticles were achieved including acrylic acid as monomer, being 1/1 the ratio (w/w) of acrylamide/acrylic acid in the aqueous solution of the emulsion. These particles have similar size to the previous ones and they will be named PMP2 to distinguish from microparticles with only acrylamide, which are named PMP1. In a separated paper we have reported the characterisation of the microparticles by DSC, X-ray diffraction and scanning electron microscopy [33].

2.3. Apparatus and measurements

Amperometric measurements at constant potential were performed with a Metrohm Polarecord potentiostat, Model E-506. All electrochemical measurements were performed using 0.1 M phosphate buffer in a three-electrode cell with a platinum electrode as working electrode, an saturated calomel electrode, SCE as reference electrode and a platinum counter electrode. Absorbance measurements were carried out with a Cary 300-Bio UV–vis spectrophotometer. Scanning electron micrographs (SEM) of the microparticles were obtained with a JEOL JSM-6400 at an acceleration voltage of 20 kV and 5000× magnification.

2.4. Electrode preparation

Platinum electrodes were washed ultrasonically with hexane, acetone, isopropanol and distilled water. Next, they were heated at 60 °C for 30 s with a solution comprising a 1:1:5 volume ratio of aqueous ammonia (0.1 M), hydrogen peroxide (20%) and distilled water. Subsequently, the electrode surfaces were polished with alumina slurry and any residual abrasive particles were removed ultrasonically in DI water. An exactly weighed amount of microgel particles was placed and held at the platinum electrode surface by a dialysis membrane. The resulting electrode was washed with phosphate buffer and overoxidized at +0.6 V vs. SCE until the background current decreased to a constant level.

3. Results and discussion

3.1. Effect of crosslinking ratio on the immobilized enzyme behaviour

In order to select microparticles with better properties as biosensor component, GOx was entrapped in microgels with different crosslinking degrees. Fig. 1 shows the biosensor response versus glucose concentration, at 25 °C and pH 6.0 for microgels with crosslinking from 1.6 to 5.8%. Given that the use of the maximal response temperature requires an additional instrumentation and a slightly thermal denaturation appears, pH of maximal biosensor response (6.0) but room temperature (25 °C) were selected to carry out these experiences.

Considering that the amount of water entrapped in the microparticle depends on the crosslinking and the pore size, the following results would be a reflection of the microgel swelling properties. Thus, as we have proved by differential scanning calorimetry [33] and have been also reported by several authors, when crosslinking increases from 1.6 to 5.8%, a swelling decrease is observed and the entrapped water is reduced up 50%. On the other hand, at $\eta = 1.6\%$, the pore size is large enough to allow a considerable loss of enzyme. Since V_{max} is proportional to the concentration of enzyme, the lower amount of enzyme entrapped in these

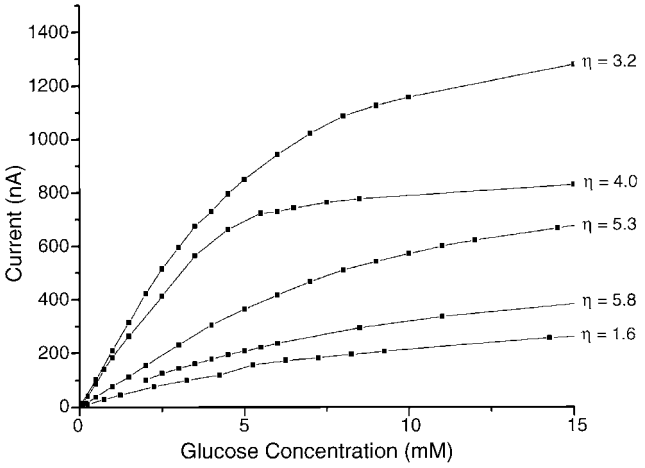

Fig. 1. Influence of crosslinking on the biosensor response studied by calibration plot for glucose in stirred 0.1 M phosphate buffer (pH 6.0), at a potential of +0.6 V vs. SCE and 25 °C.

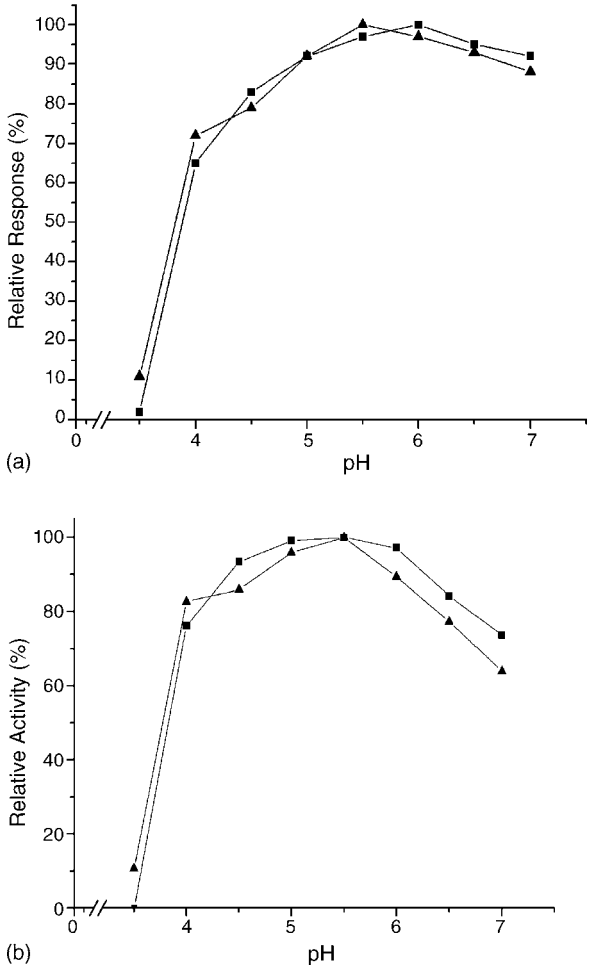

Fig. 2. The pH profiles of PMP1 (■) and PMP2 (▲) biosensors at 20 mM glucose concentration, $\eta = 3.2\%$. Experimental conditions as in Fig. 1.

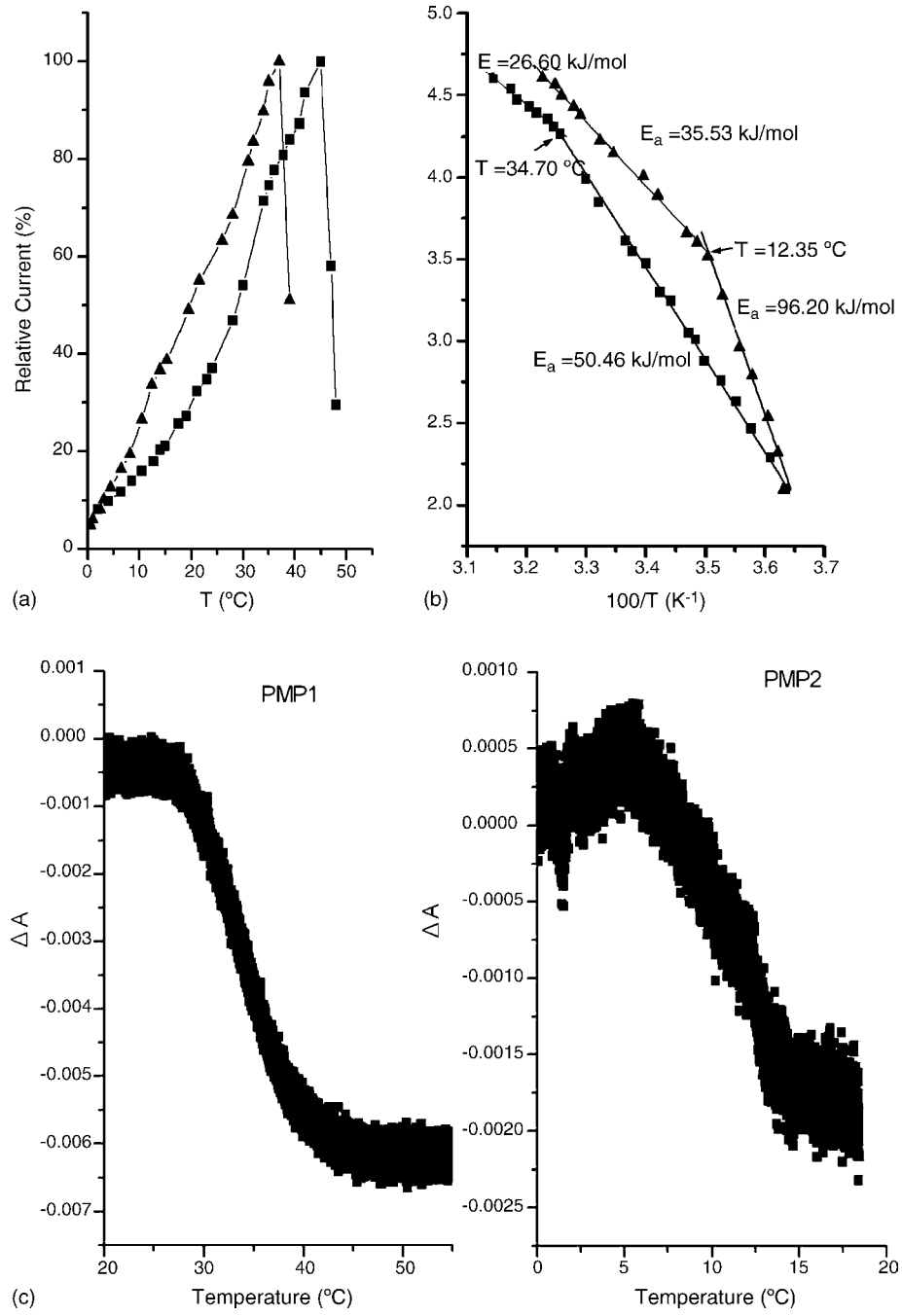

Fig. 3. (a) Temperature profiles of PMP1 (■) and PMP2 (▲) biosensors registered at 20 mM glucose concentration. (b) Arrhenius plots for immobilised enzyme in both PMP1 (■) and PMP2 (▲) electrodes. Experimental conditions as Fig. 2. (c) The variation of absorbance with temperature in an enzyme free cross-linked polyacrylamide (PMP1) and polyacrylamide/poly(acrylic acid) (PMP2) gels.

microparticles would explain the low V_{max} obtained with these microgels. Swelling becomes more difficult at higher η values because of the decreasing of pore size, consequently, more enzyme is retained inside the microgel, V_{max} increases as well as the current measured by the biosensor, which reaches a maximum at $\eta = 3.2\%$. When the crosslinking is further increased, the pore size becomes so small that hampers the substrate diffusion towards the encapsulated enzyme, explaining the decrease of the biosensor response observed for $\eta > 3.2\%$. According with these results, microgels with $\eta = 3.2\%$ were selected as enzyme immobilization system. By contrast, PMP2 microparticles prepared with $\eta = 3.2\%$ showed a lower response versus glucose than PMP1 ones with the same crosslinking and they require a higher enzymatic load to obtain a biosensor with the same response. We attribute this behaviour to the negative charge of the acrylic acid that hinders the entrapment of the also negatively charged enzyme at pH 7.0 (pI 4.3).

3.2. Characterization of the biosensor performance

3.2.1. Effect of pH

The effect of the pH on the biosensor response was examined in 0.1 M acetate/phosphate buffer solutions from pH 3.5 to 7. As the electrochemical oxidation of the H_2O_2 is a process that involves two protons, we checked the pH effects on the H_2O_2 oxidation at the bioelectrode (from 3.5 to 7.0), and this current was eliminated from the global biosensor response. Fig. 2 shows the bell-shaped pH profiles obtained without this correction, corresponding to the biosensor response (Fig. 2a), and with correction (Fig. 2b), showing the immobilized enzyme activity as a function of pH. PMP1 and PMP2 electrodes showed a similar behaviour, with an optimum pH of 5.5–6.0 also coincident with the optimum pH reported to the free enzyme [34,35]. These results indicate that the acid/base behaviour of the GOx is not affected by the immobilization process.

As Carr and Bowers [36] affirmed, when there is an excess of enzyme, a large change in pH, should have very little effect on the product concentration in the first-order kinetic regime. In contrast, at high substrate concentration, the electrode current will be very dependent on the enzyme activity, and therefore, should be strong function of pH. Therefore, we have performed the study with two different glucose concentrations, one corresponding to the first-order kinetic regime, 0.25 mM, and the second one corresponding to the saturation domain, 20 mM and, in agreement with Fig. 2, no difference in the result was observed. The following experiences were carried out in pH 6.0 phosphate buffer solutions.

3.3. Effect of temperature

The current response of PMP1 and PMP2 glucose electrodes was measured in the temperature range 0–50 °C (Fig. 3). Measurements were performed in a thermostated and stirred vessel, under O_2 saturation conditions to be sure that the oxygen concentration does not limit the rate of the reaction. Glucose concentration was 50 mM. The response increased gradually with temperature (Fig. 3a), and the PMP1 electrode reached a maximum value at 45 °C while the maximum level of PMP2 electrode was found at 37 °C. Over these values, a sharply decrease in the biosensor response was observed, probably due to the enzyme thermal denaturation, as have been also reported by others authors [37]. If comparing with the optimum temperature of free GOx which have been reported to be 30 °C, it seems that the immobilizing support stabilizes to the enzyme, becoming less susceptible to the temperature-induced conformational changes [35].

As can be see in Fig. 3b, the Arrhenius plot shows two regions. The PMP1 electrode exhibits two straight lines that intersect at 34.7 °C while in the case of PMP2 electrode the intersection temperature shifts to 12.35 °C. Discontinuous Arrhenius plots have already been reported for other systems [25,38,39] and such behaviour has been attributed

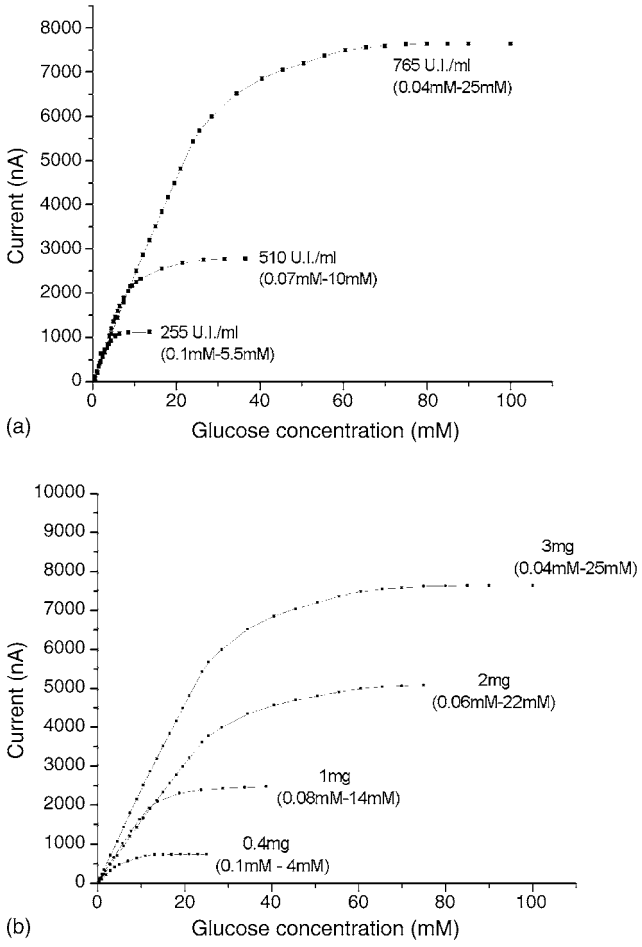

Fig. 4. Calibration curves for glucose (25 °C), as a function of (a) enzyme immobilized into the microgel (3 mg of microparticles) and (b) mass of microparticles placed on electrode surface (765 IU of enzyme/mL of dispersed phase). Experimental conditions as in Fig. 3.

to conformational changes of the immobilized enzyme with temperature. At high temperature, a lower activation energy is observed than at low temperature. The activation energies were calculated from the two slopes and were found to be 26.60 and 50.46 kJ mol^{-1} for PMP1 electrode and 35.53 and 96.20 kJ mol^{-1} for PMP2 electrode. These values are quite different from the free enzyme reported value which was found to be 14.63 kJ mol^{-1} [40], thus, supporting the idea that the entrapped enzyme undergoes some modifications in their structure during the immobilization process, specially at low temperatures. However, other authors [38] attribute the existence of two regions in Arrhenius plot as a shift in control rate by the enzyme reaction or by diffusion. We have verified the temperature-dependent swelling of hydrogels from the changes in optical density (absorbance) observed by absorption spectrometry ($\lambda = 500$ nm), see Fig. 3c. The decrease in optical density observed in the hydrogel when temperature increases could be due to the inclusion of water into the hydrogel during the swelling and, as consequence, to the reduction in the hydrogel refractive index, as Loxley and Vincent affirm [41]. As the absorbance and the slopes

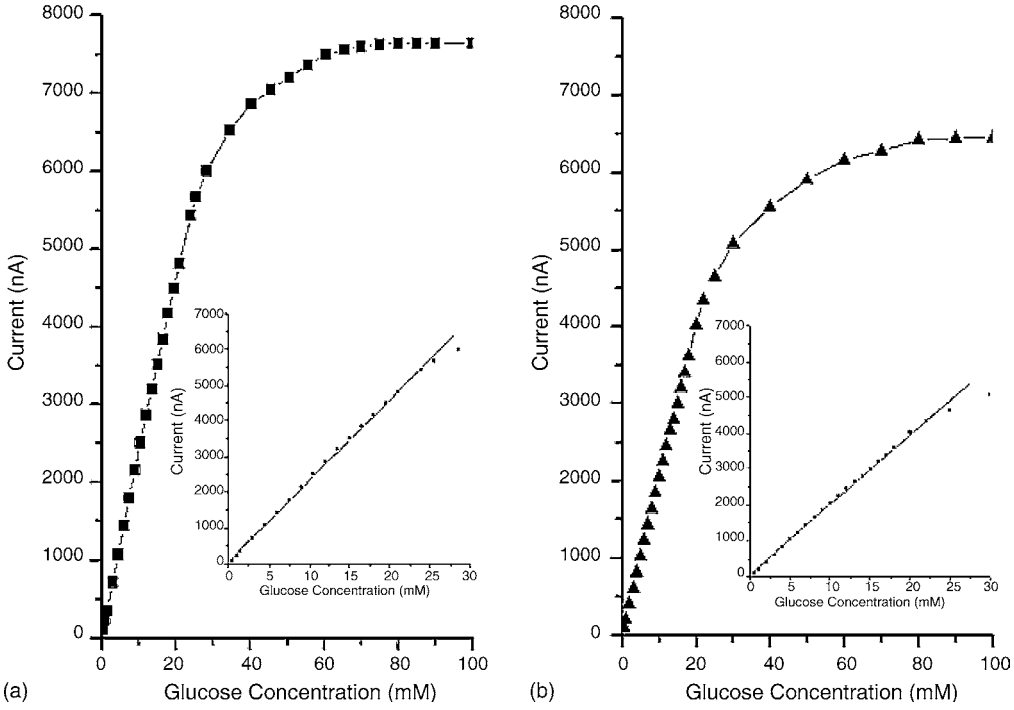

Fig. 5. PMP1 (■) and PMP2 (▲) calibration curves for glucose obtained in stirred phosphate buffer at 25 °C, pH 6, η = 3.2%, 3 mg microparticles and 765 IU enzyme/mL of dispersed phase. Insets show the linear response of both biosensors up to 25 mM glucose.

in Arrhenius plot change at a very coincident temperatures, around 35 °C (PMP1) and 10 °C (PMP2), we attribute both effects to the swelling of the hydrogel, producing significant changes in the substrate diffusion. Hence, the enzyme activity is greatly influenced by the volume phase transition of the polymer surrounding the enzyme. Above the temperature of the volume phase transition, the microgel is swollen, the enzyme is surrounded by water and its activation energy comes closer to that of the enzyme in solution. However, in the shrunk phase the amount of water retained inside the hydrogel decreases, hindering the enzyme–substrate union, and increasing the activation energy. The incorporation of acrylic acid to the network shifts the swelling of the hydrogel to lower temperature. In the case of PMP2, both regions of Arrhenius plots present activation energies higher than the corresponding PMP1 counterparts. We attribute this behaviour to the negative charge that acrylic acid confers to the polymeric matrix at this pH (5.5), increasing the interaction between the microgel and the negatively charged enzyme and obstructing the enzymatic reaction. All further experiments were conducted at 25 °C.

3.4. Effect of loading

The linear behaviour of the calibration curves showed in Fig. 1 ends at 5 mM glucose concentration, close to the blood glucose values in healthy individuals, however, glucose levels >5 mM, characteristic of diabetic patients could not be measured with this device. In order to extend the calibration range of the device, we have studied the effect of enzymatic load. The study was performed first, changing the amount of enzyme immobilized into the microgel (Fig. 4a) and then, varying the amount of microparticles placed on electrode surface (Fig. 4b). As shows Fig. 4a, the linear range reaches to 25 mM when the concentration of GOx in soluble phase of microemulsion goes from 255 to 765 IU/mL and the same effect was observed when the amount of microparticles placed on the electrode increased up to 3 mg (Fig. 4b), but in this case, the response time was also bigger. Biosensors loaded with 1 mg of microparticles required 69 s to achieve a

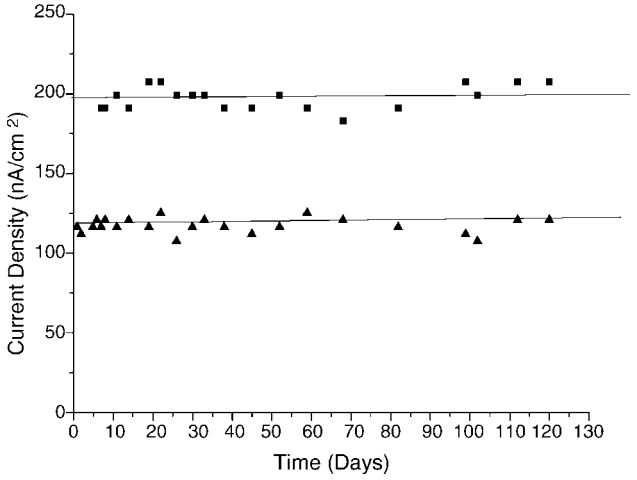

Fig. 6. Storage stability profiles for PMP1 (■) and PMP2 (▲) biosensors. The biosensors were stored frozen in phosphate buffer at −4 °C when not in use.

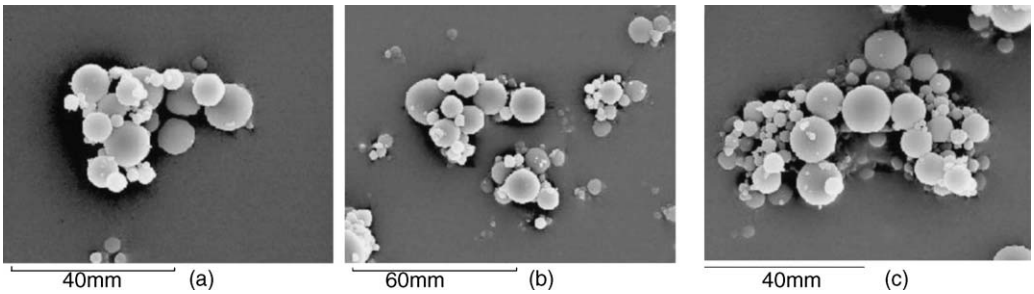

Fig. 7. Scanning electron micrograph of PMP1 and PMP2 freeze-dried microparticles. (a) PMP1, 2 months after their synthesis, (b) PMP1, 8 months after their synthesis and (c) PMP2, 2 months after their synthesis.

95% steady state current increasing up to 135 s in biosensors with 3 mg of microparticles. We have opted for a loading of 3.0 mg of microgel and, regarding to the amount of enzyme entrapped intro the microgel, it will depend on the application of the device.

3.5. Linear response

The linearity is maintained at 25 °C by both biosensors in the range 0.1–20 mM glucose as is illustrated in Fig. 5. The response of PMP1 follows the equation $y = 22.1 + 203.5x$ (with $r = 0.999$) and that of PMP2 is $y = 25.8 + 209.2x$ (with $r = 0.998$), being y the current expressed in nA and x the substrate concentration in mmol l^{-1}. A detection limit of 3×10^{-5} M was estimated for both biosensors with a signal-to-noise ratio equal 3.

3.6. Stability

The stability of the electrodes has a critical importance in pharmaceutical and industrial applications. To characterize the electrode stability, they were stored in a frozen phosphate buffer solution and, periodically, the response of the biosensor to a 0.5 mM glucose solution was measured and recorded as a function of the elapsed time. Biosensors were defrosted only to performance the stability measurements, remaining the rest of the time frozen in the buffer solution. Measurements were carried out when swelling equilibrium was attained and biosensor current was stable. The electrodes revealed a remarkable stability, as it is shown in Fig. 6. This study is still on course and we have found that biosensor exhibit a 100% of the initial signal at least after 125 days. In a further attempt to obtain information on the stability of the biosensor, the morphology and structure of the microparticles have been studied by scanning electron microscopy. Microparticles were preserved freeze-dried, and as micrographs in Fig. 7 reveal, they do not exhibit significant changes 8 months after their synthesis. Moreover, their enzymatic activity remained almost invariable after this storage time.

3.7. Interferences

Because of the large overvoltage required for the oxidation of hydrogen peroxide, the enzymatic electrode operates at a high potential of +0.6 V versus SCE, and therefore, the glucose detection may be disturbed by the presence of interfering species such as ascorbic and uric acid in the sample. However, due to the negative charge of these substances at biological pH, the incorporation of a negatively charged monomer as acrylic acid into the polymeric matrix let to a significant improvement in the performance of the resultant glucose sensors containing entrapped GOx. In order to observe the effect of this negative charge and also to investigate the amount of acrilic acid necessary to remove the interference, we begin the study substituting gradually acrylamide by acrylic acid. As it can be observed in Fig. 8, the higher the proportion of acrylic acid, the smaller the biosensor response. For a ratio 1:1 of acrylic acid:acrylamide, the ascorbic and uric acid current was totally eliminated. We have used this microgel composite for the rest of biosensors. Fig. 9 illustrates the efficiency of the acrylic acid inclusion in the microgels to remove interfering species. As can be seen in the figure, the response generated by ascorbic and uric acid were similar to glucose when acrylamide microparticles were used. However, when acrylamide was replaced by acrylic acid in a ratio of 1:1, the glucose response remained unchanged whereas for ascorbic and uric acids no response was detected.

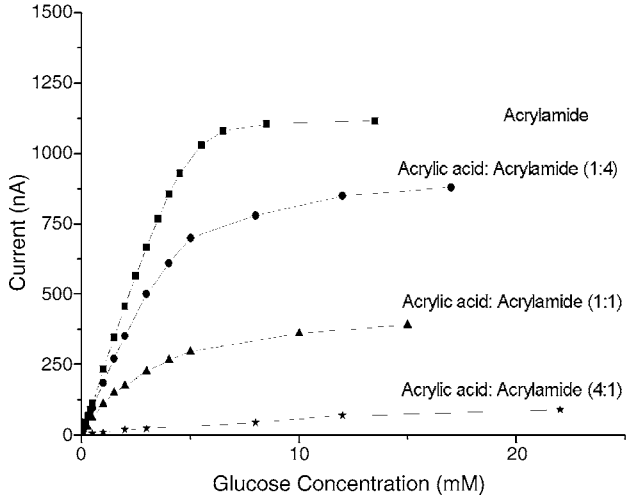

Fig. 8. Effect of the ratio acrylic acid:acrylamide on the biosensor response. Experimental conditions as in Fig. 5.

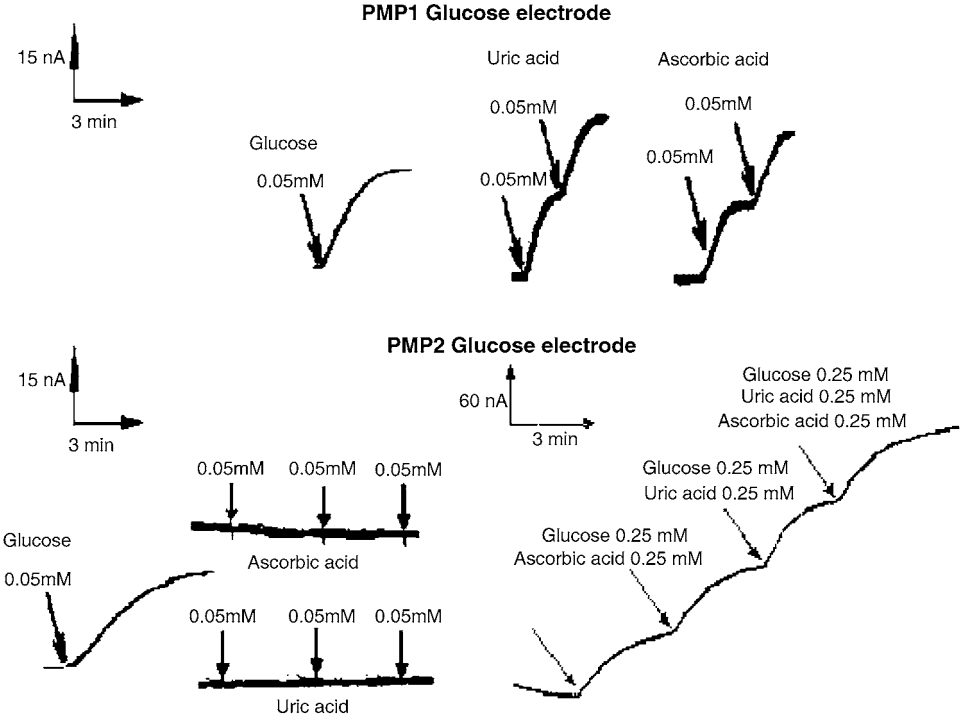

Fig. 9. PMP1 and PMP2 biosensor response to glucose, ascorbic and uric acids.

Table 1
Analysis results of the serum with PMP1 and PMP2 biosensors and spectrophotometric method[a]

Sample	Glucose (mg/dL) reference method	Glucose (mg/dL) PMP1	R.S.D. (%)	PMP1 deviation[b] (%)	Glucose (mg/dL) PMP2	R.S.D. (%)	PMP2 deviation[b] (%)
1	74	80 ± 1	1.5	+8.4	76 ± 2	2.24	+2.6
2	75	82 ± 1	1.37	+9.9	74 ± 1	1.59	−1.3
3	77	80 ± 2	2.01	+3.4	75 ± 2	2.10	+2.2
4	72	79 ± 1	1.48	+10.0	70 ± 2	2.30	−2.9
5	76	80 ± 1	0.65	+5.0	74 ± 1	0.15	−2.3

[a] Average of three measurements.
[b] Deviation: deviation between the reference method and the biosensor results.

3.8. Applications

To check the applicability of the biosensors in real samples, five serum pools were analysed with both PMP1 and PMP2 biosensors. The results are given in Tables 1 and 2. A parallel analysis, performed by the spectrophotometric method of hexokinase described by Young [42], showed a good correlation with the proposed methods, especially in the case of the PMP2 biosensor. The R.S.D. (Table 1) was never above 2.30%, revealing the good precision of both biosensors. According to these results, measurements of glucose in samples so complex as the serum could be performed by the biosensor based on PMP2, with good precision and accuracy. When comparing the glucose concentration of five serum obtained by the biosensors and the spectrophotometric method, we observe that the PMP1 biosensor gave always val-

Table 2
Recovery studies using the PMP1 and PMP2 glucose oxidase biosensors[a]

Sample	Glucose added (mg/dL)	PMP1 biosensor		PMP2 biosensor	
		Glucose found[a] (mg/dL)	Recovery (%)	Glucose found[a] (mg/dL)	Recovery (%)
1	72	71.82	99.75	67.69	94.01
2	72	75.72	105.20	68.80	95.56
3	72	74.54	103.53	73.38	101.92
4	72	74.58	103.58	72.36	100.50
5	72	70.76	98.27	74.24	103.11

[a] Average of measurements.

ues 10% higher than those obtained by the reference method while the results obtained with the PMP2 biosensor were similar to those of the reference method, and differences between them were positive and negative and never overcame 2.9%. These results confirm that the serum samples contain substances which interfere in the glucose quantification if PMP1 electrode is used. In contrast, the negative charge of acrylic acid present in PMP2 electrode seems to prevent the approach of negative species to the electrode, keeping the biosensor free of this type of interference. Table 2 shows the result of the recovery, always around 100%, that indicate the accuracy of two biosensors in the measurement of glucose. The interference observed in PMP1 biosensor, probably due to the presence of ascorbic or uric acid, is an interference no possible to detect in the recovery study.

4. Conclusions

In this study, we have verified that polyacrylic microgels provide an excellent matrix for enzyme (GOx) immobilization and its application as the biomaterial of an amperometric biosensor. We have also prepared microparticles with a ratio 1/1 of acrylamide/acrilic acid, to charge negatively the surface, and thus, get rid off interferences caused by species with a negative net charge. With this modification, the interferences produced by the ascorbic and uric acid were removed proving the suitability of the proposed device in the quantification of glucose in samples as blood and serum. The temperature dependence of biosensor response was studied and we associate the change in the enzyme activation energy to the swelling of microgel, that is, to changes in the enzyme environment. Concerning to the stability of the devices, 4 months after their preparation, the response was practically the initial one. In addition, once the microgel with entrapped enzyme was being freeze-dried, their enzymatic activity remained unaltered for several months.

Acknowledgments

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