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Synthesis and characterization of microparticles based on poly-methacrylic acid with glucose oxidase for biosensor applications

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ABSTRACT

In the line of the applicability of biocompatible monomers pH and temperature dependent, we assayed poly-methacrylic acid (p-MAA) microparticles as immobilization system in the design of enzymatic biosensors. Glucose oxidase was used as enzyme model for the study of microparticles as immobilization matrices and as biological material in the performance of glucose biosensors. The enzyme immobilization method was optimized by investigating the influence of monomer concentration and cross-linker content (N′,N′-methylenebisacrylamide), used in the preparation of the microparticles in the response of the biosensors. The kinetics of the polymerization and the effects of the temperature were studied, also the conversion of the polymerization was determinates by a weight method. The structure of the obtained p-MAA microparticles were studied through scanning electron microscopy (SEM) and differential scanning microscopy (DSC). The particle size measurements were performed with a Galai-Cis 1 particle analyzer system. Furthermore, the influence of the swelling behavior of hydrogel matrix as a function of pH and temperature were studied. Analytical properties such as sensitivity, linear range, response time and detection limit were studied for the glucose biosensors. The sensitivity for glucose detection obtained with poly-methacrylic acid (p-MAA) microparticles was 11.98 mA M^{-1} cm^{-2} and 10 μM of detection limit. A Nafion® layer was used to eliminate common interferents of the human serum such as uric and ascorbic acids. The biosensors were used to determine glucose in human serum samples with satisfactory results. When stored in a frozen phosphate buffer solution (pH 6.0) at −4 °C, the useful lifetime of all biosensors was at least 550 days.

1. Introduction

Among numerous reports about glucose biosensors, the enzyme immobilization on electrodes is the first important step in fabrication, which also plays a vital role in the biosensor performance. Different immobilization methods have been assayed such as covalent attachment [1,2], entrapment in a suitable matrix [3], adsorption onto insoluble materials [4], conjugation [5], ionic-covalent hybridization [6,7], etc. Each method has its own advantages and limitations. Among these, polymeric entrapment presents several advantages that make it highly interesting. The enzymatic reporter molecule is less affected than when other methods are employed (i.e covalent attachment), because the reporter is entrapped in the matrix.

Emulsion polymerization is a well-known method for preparation of the latex polymers with defined structures. The synthesis of the microparticles usually does not lead to ideal core–shell morphology. Depending on the compatibility of the components in the interface, these can be mixed on the molecular level with continuous concentration gradient. Organic matrixes made of cross-linked hydrogels based on methacrylates have been proven non cytotoxic, non-immunoreactive and their porosity could be controlled by the amount of cross-linker used in their synthesis [8,9].

In the search for a suitable matrix for enzyme immobilization, other biocompatible polymers, pharmacologically inert were sought since they could have different responses to external stimuli such as temperature and pH [10–12]. After studying the pH-dependent polymer and temperature-dependent polymer (poly (ethylene glycol) methyl ether methacrylate (p-PEGMEM) and poly (dimethylaminoethyl) methacrylate (p-DMAEM), respectively [13,14], this production focuses on the study of the behavior of a polymer p-(methacrylic acid) (p-MAA) that has both features.

p-MAA is an ionizable hydrophilic polymer. Cross-linked

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p-MAA is able to swell in water. Its swelling behavior is greatly pH-dependent due to the ionization/deionization of the carboxylic acid groups [15,16]. At low pH, usually less than 5.5, the –COOH groups are not ionized and keep the p-MAA network at its collapsed state [17]. At high pH values, the –COOH groups are ionized, and the charged COO– groups repel each other, leading to p-MAA swelling. p-MAA exhibits thermosensitive properties as well, however, at high polymer concentrations (> 5 wt% in water); its LCST is equal to 50 °C [18]. For pH above the pK_a of MAA, the LCST transition is suppressed [19,20]. The reported values of T_g for p-MAA are 205 °C [21].

Amperometric enzyme electrodes and especially glucose oxidase-based biosensors had been described extensively during the almost 50 past years, and there are far more than 5000 publications related to these types of biosensors. Since the first report on glucose enzyme biosensors by Clark and Lyons [22], the analysis of glucose has attracted intense research interest due to the increasing incidence of diabetes in the population of developed countries [23,24]. In this scenario, amperometric biospecific enzyme glucose biosensors have been generally considered in terms of the simple operation, sensitive determination, fast response, and low cost [25–27]. The fabrication of biosensors with biocompatible materials will allow the monitoring in vivo of glucose and other compounds. Future progress in biosensors design will certainly focus upon the technology of new materials that solve the biocompatibility problem. Another decisive factor is the immobilization of enzymes, with complete retention of their biological activity, in matrices with good diffusion properties for substrates.

In this task, our first purpose was to encapsulate glucose oxidase (GOx) inside p-MAA microgels with different amount of cross-linker followed by the concentrated emulsion polymerization method [28], as well as the study of the influence of some polymerization parameters (monomer concentration, kinetics of polymerization). The chosen enzyme from *Aspergillus niger* (EC 1.1.3.4) was taken as a redox enzyme model because of its high stability, good catalytic ability, commercial availability, and moderate cost. Moreover, GOx is a widely studied enzyme that provides a suitable model system for the development of enzyme electrodes [29–35]. Secondly, we have investigated the properties of microparticles of GOx-immobilized in hydrogel microparticles, and the modifications induced by the enzyme in the glass transition temperature as well as in the microstructure of the gels. And finally, we have investigated the performance of the amperometric biosensors, based on glucose oxidase (GOx) immobilized in biocompatible microparticles, for the determination of glucose.

2. Experimental

2.1. Chemicals and buffers

Methacrylic acid (MAA), GOx (EC 1.1.3.4) (425 U mg^{-1}) from *Aspergillus niger,* D-(+)-glucose, ascorbic acid, uric acid and Nafion® 5 wt% (Nafion® purum 5% in a mixture of lower aliphatic alcohols and water), were purchased from Sigma (St. Louis, MO, USA). N,N´-methylenebisacrylamide (BIS) from Aldrich (St. Louis; MO, USA), ammonium persulfate (PSA), N,N,N′,N′ tetramethylethylendiamine (TEMED) and the surfactant Span 80 from Fluka (Buchs, Switzerland). Phosphate and Acetate/phosphate buffer solutions were prepared from stock solutions of sodium dihydrogen phosphate and sodium acetate (Panreac). The dialysis membrane (12,000–14,000 MWCO) was purchased from Spectrum Medical Industries. All reagents did not need any modification and the water was Milli Q quality (Millipore, Milford, MA, USA). Serum samples were kindly provided by Health Analysis Center belonging to the University Complutense of Madrid.

2.2. Apparatus and measurements

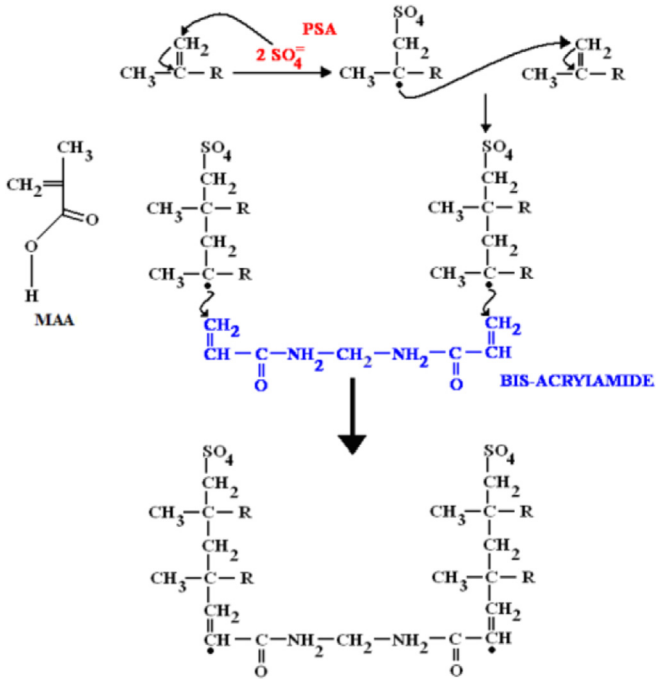
The microgel particles were examined using scanning electron micrographs (SEM) with a JEOL JSM-6400 microscope (JEOL, Japan) operating at an acceleration voltage of 20 kV and with 5000 magnification. The grid with the microparticles was dried, and replicas were produced by shadowing gold deposited with a Balzers Sputter Coater (SCD-004). Particle size measurements in the range 2–150 μm were performed with a Galai-Cis-1 particle analyzer system. The glass transition temperature and the degradation temperature of the microgels were investigated by DSC. The DSC measurements were carried out in a Mettler 820 differential scanning calorimeter equipped with a cooler operated by liquid nitrogen. The DSC cell was used for heat, treating the samples that weighted about 5.0 mg. The X-ray diffraction studies were performed with a Philips Xpert PW3050 diffractometer. The diffractograms were recorded covering an angular interval between $2\theta = 5$ and 50°, and using a step size of 0.01° with time per step of 1 s. The pH of the buffer solution was adjusted using a Metler Toledo MP-230 pH-meter. In addition, amperometric measurements at constant potential were carried out at a Metrohm Polarecord potentiostat, Model E-506. Electrochemical measurements were performed in 0.05M acetate/0.05M phosphate buffer and in 0.1 M phosphate buffer, using a three-electrode cell with a platinum working electrode, a SCE reference electrode and a platinum counter electrode. All experiences were carried out in thermostated cells with the help of a circulation thermostat Julabo (−50, 200 °C). Calibration plots were obtained by measuring the current response, after successive additions of substrate solution into a stirred electrolyte solution (10 mL), corresponding to the enzyme saturation concentration. Sensitivity was expressed as the slope of the calibration curve. Detection limit was calculated according with the criterion of ratio signal-to-noise equal 3. The response time was the time needed to reach 95% of the steady-state current after a substrate addition. In order to study the catalytic enzyme immobilization system efficiency, a comparative study of the measured absorbances of solutions generated once the enzymatic reaction with a UV–visible spectrophotometer CARY 300 Bio was performed at 353 nm.

2.3. Sample preparation

p-MAA microgels with varying amount of cross-linker N,N´-methylenebisacrylamide (BIS), have been prepared using the concentrated emulsion polymerization method [28]. The immobilization of GOx was carried out by adding the enzyme (425 U mg^{-1}) in the aqueous phase of the concentrated emulsion. The amount of cross-linker, ($\eta = n_{BIS}/(n_{BIS} + n_{monomer})$% where n_{BIS} and $n_{monomer}$ are the number of moles of BIS and monomer respectively) was varied between 0.13% and 1.48%. During the synthesis of the microgels (around 1.5 h) the temperature was controlled and kept below 25 °C to preserve the enzyme properties. The polymeric reaction of p-MAA and BIS is shown in the Scheme 1.

2.3.1. Control of the temperature of polymerization

A key factor in the immobilization of enzymes is the temperature to which the polymerization is carried out. The polymerization is exothermic and the temperature reached depends on the amount of initiator used in the synthesis, in this task it was PSA. Therefore, we have prepared different syntheses varying the amount of initiator from 9.86, 10.96 and 21.92 mM. Fig. 1 shows the range of polymerization temperature for the different concentration of initiator. In all cases the temperature of polymerization increases when increasing the concentration of initiator, keeping the temperature between 19.0 and 23.0 °C for



Scheme 1. Mechanism of polymerization reaction of p-MAA with BIS.

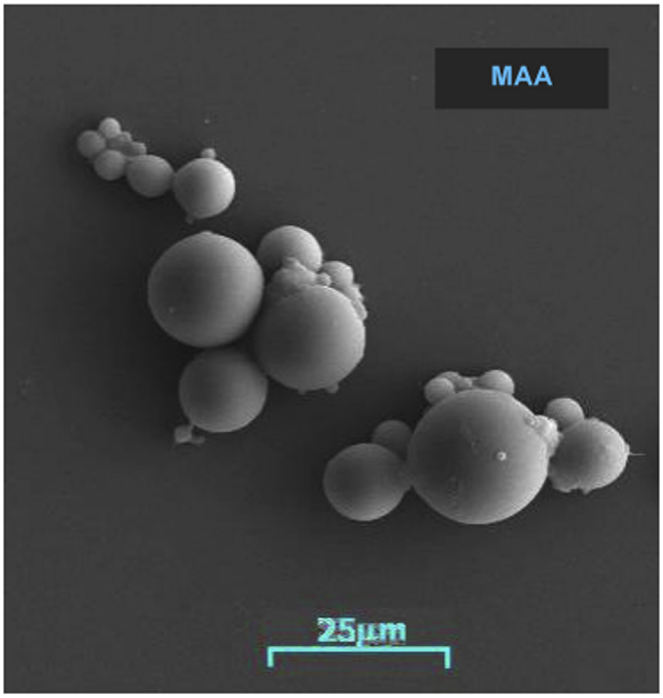


Fig. 1. SEM micrograph of freeze-dried p-MAA microparticles.

p-MMA (Fig. 1). As a consequence of these results, 10.96 mM was the concentration selected, to prevent thermal denaturation of the enzyme GOx.

2.3.2. *Kinetic of polymerization*

Aiming at maintaining the enzyme in the reaction media as little as possible, the kinetics of the polymerization reaction was studied. For this purpose, 10 vials were filled with 250 µl of concentrated emulsion. In every vial, the polymerization began at different times, stopping the reaction in all the vials simultaneously. The resulting microgels were washed with cold methanol,

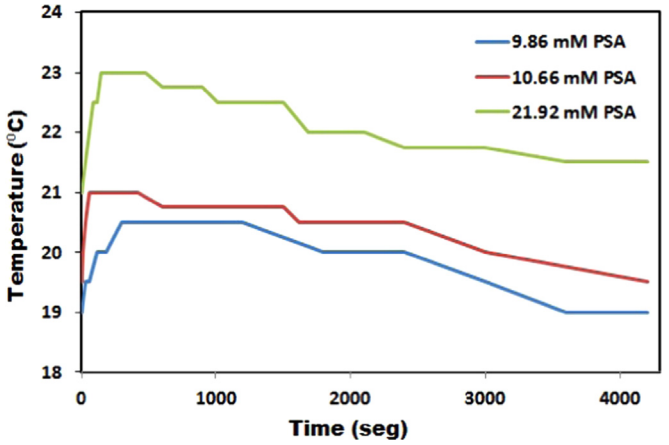


Fig. 2. Range of polymerization temperature for the different initiator's concentration.

centrifuged during 20 min at 10,000 r.p.m. and finally dried in an oven at 100 °C for 24 h. Subsequently the sample was weighed.

Fig. 2 shows the degree of conversion of monomer when a concentration of PSA of 10.96 mM is used. As it is observed in the graph, the degree of conversion of monomer reaches 99% once passed 90 min of reaction. In the following syntheses, the reaction of polymerization is considered completed after 90 min.

To verify if there were changes in the kinetics of polymerization, we have studied two factors – the quantity of opening agent (PSA) used (Fig. 3), and the temperature of the polymerization reaction (Fig. 3a). We observed that the degree of conversion of monomer in all the polymers reaches 99% passed 70 min of reaction with 21.92 mM PSA, 90 min of reaction with 10.92 mM PSA and 110 min of reaction with 9.86 mM PSA. Although the degree of conversion of monomer required less time when the concentration of PSA is 21.92 mM, as it was studied previously (Fig. 2), the temperature reached with this concentration is also higher. Therefore 10.92 mM was chosen as PSA concentration. On the other hand, it was seen that the temperature that needed less time to achieve the conversion is 40 °C (Fig. 3b), nevertheless this temperature does not support the enzymatic characteristics of the GOx, on what we decided to use 25 °C as temperature of synthesis.

2.4. *Overall reaction of glucose biosensor*

The electrode surface (diameter 3 mm) was polished with 0.05 µm alumina slurry paste. After polishing, any residual abrasive particles were removed ultrasonically in ethanol and subsequently in distilled water. An exactly weighed amount of micro gel particles were placed on the electrode surface and fixed with a dialysis membrane. The resulting electrode was washed with phosphate buffer and overoxidized at +0.6 V versus SCE until the background current decreased to a constant level.

The biosensor response is based on an indirect measurement that correlates the amount of glucose with the concentration of hydrogen peroxide. Because the redox centres in GOx are insulated within the enzyme molecule, direct electron transportation to the surface of the electrode does not occur to any measurable degree. GOx is first reduced by the substrate glucose and then reoxidized by oxygen. As it is shown in Scheme 2, oxygen behaves as electron acceptor for reduced GOx leading to the formation of hydrogen peroxide that is oxidized at the electrode and results in the current that is detected by amperometry.

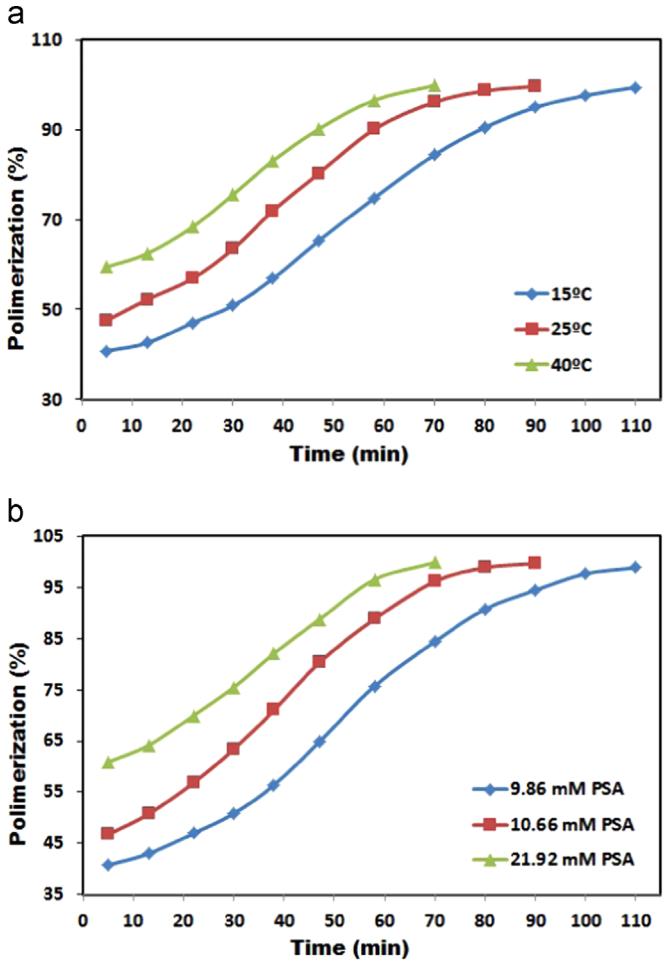
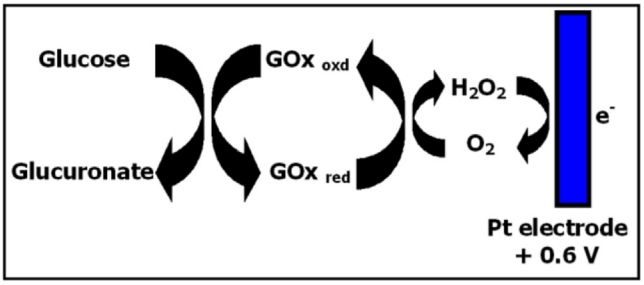


Fig. 3. Monomer conversion degree as a function of temperature (a) or as a quantity of PSA (b).



Scheme 2. Proposed working mechanisms of the p-MAA based glucose biosensors when the electrode is polarized at +0.6 V vs. SCE.

3. Results and discussion

3.1. Characterization of the microparticles

Fig. 4 shows the scanning electron micrograph (SEM) of p-MAA microparticles (in a range of 2–150 μm) with entrapped GOx with a cross-linking of η=0.37% for p-MAA. The microparticles in all cases were found to be spherical and polydisperse.

According with the results obtained with the Particle-Size Analyzer System, the microparticles present a wide distribution of size with diameters varying between 2 and 12 μm for all the microparticles, and with an average size of 5.04 μm for p-MAA, very close to the average size 5 μm of the microparticles without GOx and the same cross-linking content. As Fig. 4 illustrates, more than the 75% of the microparticles present a size comprised between

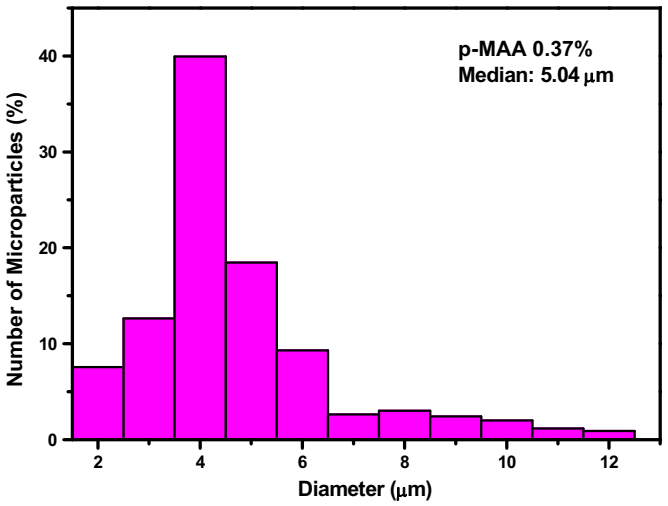


Fig. 4. Size distribution of the microgel particles dispersed in buffer η=0.37%.

3 and 5 μm. The studied microparticles were spherical and polydisperse. Similar results were found when other methacrylate microparticles were used [13,14].

3.2. Structure of the microparticles

To verify the structure of the microparticles, diffraction studies were carried out with empty microparticles and enzyme-containing microparticles to observe the differences between both. Spectra were performed at different η of cross-linking for the selected polymer. In the Fig. 5 the diffractograms for p-MAA microparticles compared with other p-PEGMEM and p-DMAEM microparticles, showed the characteristic peaks of an amorphous polymer structure, as manifested for the microparticles without enzyme, however, crystalline peaks also appeared, which matched with the enzyme alone (inlet). These results corroborate the existence of the enzyme caught in the microparticles. X-ray scattering spectrums of the polymer are also shown in Fig. 5. It was found that diffraction peaks of the polymers contain the crystalline and dispersion peaks.

This can be explained considering that the immobilization is carried out in an oily medium, in which polymer chains are dissolved, and the enzyme is dispersed as small crystals. These

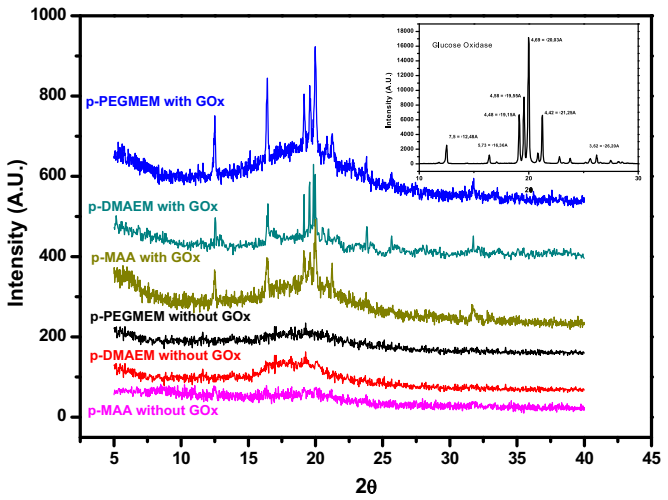


Fig. 5. X-ray diffraction pattern of methacrylate microparticles with and without GOx immobilized inside and the comparison between these microparticles and X-ray diffraction of free GOx.

crystals are part of the inner phase of this emulsion. Given that methacrylates have a small molecular weight, the synthesized matrix has a low density, allowing the beam to reach the three-dimensional structure of the enzyme.

The maintenance of enzyme activity on the supporting materials is crucial in the biosensor designs, because the secondary conformational variations of the enzyme can affect its activity markedly. Herein, X-ray diffraction is utilized to check the secondary conformation variations of the polypeptide chain of GOx on the microparticles. As shown in Fig. 5, peaks obtained for the free enzyme (inlet), was also observed when the enzyme was immobilized on the microparticles. These results reveal that the secondary structure and bioactivity of GOx molecules can be reserved when immobilized on the microparticles.

3.3. Glass transition temperature

The glass transition temperature of the methacrylates microgels was measured as a function of the cross-linking. To measure the glass transition, thermal analysis of the polymer was carried out with a heating rate 15 °C/min and over the temperature range −150 to 0 °C under nitrogen atmosphere. After this treatment the glass transition can be clearly identified in the DSC scan of all methacrylate microgels. For p-MAA Fig. 6(e) indicates that the T_g values were located between 219.18 °C and 222.18 °C, similar to the reported in literature [21,36,37]. The T_g increases as well as the % of cross-linking. Similar behavior was previously reported to p-PEGMEM and p-DMAEM microparticles [13,14].

3.4. Optimization of the synthesis method

3.4.1. Monomer concentration

A study of the monomer concentration when preparing the immobilization matrix and its behavior in the biosensor characteristics were done. p-MAA microparticles synthesized at pH 6.0 η=0.37%, containing 765 U mg^{-1} of GOx. Different concentrations of monomer in the aqueous phase were employed: 0.75, 1.19, 1.59 and 2.9 M for MAA. The results presented in Tables 1 and 2 showed the values of the steady-state response curves obtained at the platinum electrode.

The maximum current response was found for monomer concentration 1.59 M (MAA). The optimal values of response for the polymer are highlighted in bold, and were obtained from the calibration curves. As shown, the optimum concentration of monomer used in the synthesis of the microparticles varied depending on the molecular weight of the monomer used, so that, the lower the molecular weights, the monomer used concentration increases.

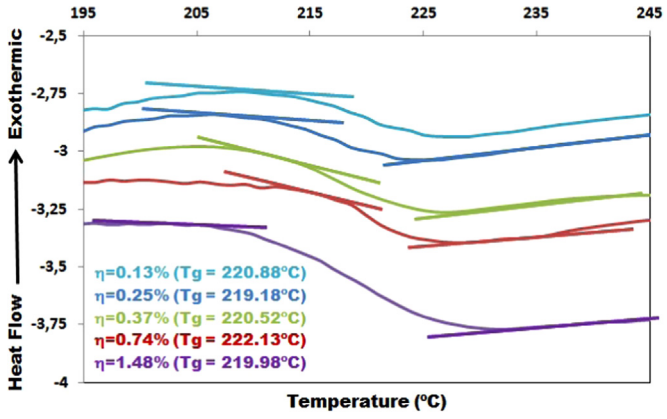


Fig. 6. Glass transition temperatures of p-MAA microparticles as a function of BA ratio.

Table 1
Relationship between monomer concentrations and current response.

p-MAA	
P_m=86,09 g/mol	
d =1015 g/ml	
Monomer concentration (mM)	**Current response (μA)**
795 mM	3.2
1193 mM	6.5
1590 mM	8.8
2903 mM	4.1

Table 2
Activation energy from the Arrhenius plot for p-MAA microparticles.

Glucose Biosensor	Activation Energy (AE)	Breakpoint temperature
p-MAA microparticles	37.41 KJ/mol 93.33 KJ/mol	14.13 °C

A further increase in the monomer concentration decreases the current response. This behavior was attributed to the decreasing of pore size with increasing monomer concentration that introduces a higher diffusional barrier hindering the motion of both, the substrate towards the enzyme catalytic site, and the production of the enzymatic reaction towards the electrode. Decreasing the monomer concentration also results in a decrease of the response due to the loss of immobilized enzyme as it was demonstrated by measuring enzymatic activity in the supernatant liquid obtained after the synthesis of the microgels.

3.4.2. Effect of the cross-linking content

GOx was entrapped in methacrylate microgels with different η to estimate the optimal pore size required for its immobilization. As η decreases, the pore size in the microgels becomes higher, so the amount of water entrapped into methacrylate microparticles also increases, leading to a greater degree of swelling [28]. This fact can affect the activity of the entrapped enzyme.

Fig. 7 shows the calibration curves of the biosensor response versus glucose concentration for microparticles with cross-linking between 0.13% and 1.48% for p-MAA. The monomer concentration selected to obtain these curves were described previously, and the amount of entrapped GOx was 765 U mg^{-1}.

The optimum response occurs for microgels with η=0.37%. All

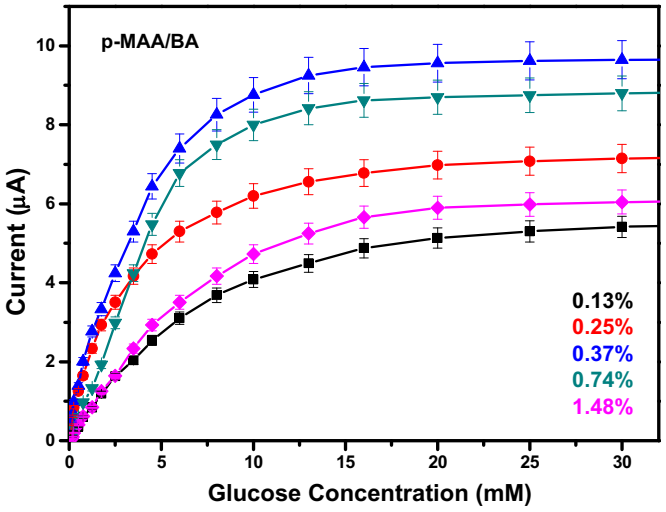


Fig. 7. Influence of cross-linking on the biosensor response studied by calibration plot for glucose in stirred 0.1 M sodium phosphate buffer, pH 6.5, at a potential of +0.6 V vs. SCE and 25 °C.

the methacrylates microparticles prepared with lower fractions of cross-linking show a pore size too large to efficiently retain the enzyme. By contrast, the decrease of the response for $\eta \geq$ optimum % is attributed to a higher substrate diffusional barrier imposed by the cross-linking. Accordingly, microparticles with $\eta = 0.37\%$ and monomer concentration 1.19 M were selected for subsequent measurements performed in this work to optimized the biosensor response with respect to the working conditions.

In order to find out how the reaction of polymerization affects to the effectiveness of the enzyme entrapment on enzymatic activity, the enzymatic activity of microparticles with the GOx enzyme and enzyme from the same amount of free enzyme activity was measured using an enzymatic Spectrophotometric method [38]. The results obtained show that the enzymatic activities of microparticles dispersions were 87.0% of the enzymatic activity of the enzyme dissolution for p-MAA. Similar results were found for p-PEGMEM and p-DMAEM microparticles (data not published). That is, the proposed method involves immobilizing a loss of 13.0% of the enzymatic activity according to the polymer used, demonstrating that these matrices were good immobilization system, preserving the enzymatic properties of GOx.

3.5. Optimization of the biosensor response

Aiming to optimize the design of the biosensor to achieve maximum response and in the shortest time, there has been an individualized study of experimental factors that can influence on the response of the biosensor, such as the applied potential, pH, temperature and enzyme loading.

3.5.1. Working potential

The dependency of the biosensor response (steady state current at the electrode) on the applied potential was investigated in 0.05 M acetate/phosphate buffer solution, pH 6.0, containing 0.25 mM glucose. The response of the enzyme electrode increased with the applied potential up to +0.60 V vs. SCE and levels off for higher values. The operating potential of +0.60 V vs. SCE was used in all subsequent measurements.

3.5.2. pH

The influence of pH on the glucose biosensor response to 30 mM glucose was checked in 0.05 M acetate/0.05 M phosphate buffer solutions from pH 4.0–8.0 at 25 °C. Considering the optimum pH of biosensors studied for each of the matrices, it is observed in all cases as the optimum pH was maintained between 6.0 and 6.5, which coincides with the maximum activity and pH stability of glucose oxidase. The optimal pH was similar with the optimum pH reported for the free enzyme which indicates that the catalytic function of GOx is not affected by the immobilization process.

To not change the nature of the anions as they may interact with the substrate and enzyme, the study of the influence of pH was performed in the matrices studied with a buffer solution composed of 0.05 M acetate/0.05 M phosphate, adjusting the pH to the desired value.

P-MAA has ionizable functional groups, a carboxyl group, which confers a different charge depending on the pH used. In the case of p-MAA, whose pKa value is 4.66 and the ionizable functional group is a carboxyl group, which means that the load present in the higher pKa values will be negative, which means swelling microgels, while below the pKa values for the load presented is positive, which means the collapse of the microgels.

To study polymer–enzyme interaction, the load of GOx should be considered according to the pH. Considering that the isoelectric point of the GOx is 4.2, its net charge was negative in the range of pH 5 to 8 studied.

At these pH values (6.0 or 6.5), the GOx enzyme had negative charge, while the present pH dependent polymers for p-MAA had positive charge. This charge difference between the parent and the enzyme is reflected in the response of the biosensor. These results were also observed for p-DMAEM microparticles [14], because an electrostatic attraction between enzyme (negatively charged), and polymer matrix (positively charge) occurred. The observed results may be due to interactions between the matrix and the enzyme, which are conditioned by the charges of both, and this leads to an improvement of the analytical properties of the proposed biosensor.

3.5.3. Temperature

When temperature was studied, it was observed that when the working temperature exceeds 30 °C, the current intensity decreases due to the beginning of the process of enzyme denaturation. This temperature of 30 °C is considered optimal for all subsequent in each of the matrices experiments. Although the matrices used are temperature dependent, the possible effect of the temperature dependence should be generated in obtained responses for the biosensors with different matrices unseen reflected, because the optimum temperature obtained, could cause swollen microgels.

Comparing the results obtained in the Arrhenius plot with glucose oxidase biosensors studied, two linear segments with slopes two activation energies (AE) are derived and observed. The AE in the region of low temperatures varies in the range from 43.11 to 93.33 KJ/mol (Table 3). AE obtained with biosensors of

Table 3
Analytical properties of the methacrylates biosensors as a function of the quantity of microparticles placed on electrode surface and the enzyme immobilized into the microparticles. Experimental conditions: 0.1 M phosphate buffer, pH 6.0 and +0.6 V vs. SCE.

Enzyme load			Sensitivity (mA M^{-1} cm^{-2})	J_{max} (μA cm^{-2})	Lineal range (M)	R^2 (N) [a]	DL (μM)
Quantity of microparticles in the biosensors (mg)		Quantity of enzyme in the microparticles (U mg^{-1})					
p-MAA	1	765	4.0	61.9	$1.0\ 10^{-5}$ – $1.0\ 10^{-2}$	0.9969 (15)	20
	2	765	6.6	80.4	$1.1\ 10^{-5}$ – $6.0\ 10^{-3}$	0.9964 (19)	10
	3	255	7.8	99.8	$1.0\ 10^{-6}$ – $6.2\ 10^{-3}$	0.9981 (16)	20
	3	425	9.6	112.0	$1.0\ 10^{-6}$ – $7.3\ 10^{-3}$	0.9977 (19)	20
	3	765	12.0	126.6	$9.0\ 10^{-6}$ – $8.3\ 10^{-3}$	0.9979 (19)	10

[a] Points of linear range in calibration curve.

polymeric microparticles in high temperature zone is higher to that obtained for GOx solution (14.6 KJ/mol) [39]. However, similar values in AE to those found when PAAm microparticles are used [40], demonstrating that the enzyme activity has not been affected by the proposed system immobilization.

The value of the breakpoint of the straight lines was 12.86 °C at 14.13 °C. Breakpoints in similar characteristics were found in other microparticles obtained with the same system of synthesis [13,14]. Elevation in the values obtained for the AE, could be due to the increase of monomer necessary in the synthesis to obtain different microparticles.

3.5.4. Loading

Glucose levels of diabetic patients are higher than 5 mM (> 90 mg/dL), therefore, with the aim of a possible application, we have extended the calibration range of the biosensor. Based on previous works [39] we have chosen as reference an enzymatic load of 765 U mg^{-1} and we tested two methods of modifying the amount of enzyme in the surface of the electrode: i) changing the amount of enzyme immobilized within the microgel, and ii) varying the amount of microparticles placed on electrode surface. The linear range reaches 6.2×10^{-3} M and 8.3×10^{-3} M when the amount of GOx used in the synthesis is 255 U mg^{-1} and 765 U mg^{-1} respectively. These values slightly increased by increasing the amount of microparticles placed on the electrode surface from 1 up to 3 mg. The increase of the linearity owes in both cases to the increase of the quantity of existing enzyme, which improves the above mentioned linearity. However in both cases the sensitivity increased and, for this reason, we have selected for later experiments microparticles prepared with 765 U mg^{-1} GOx and a load of 3 mg of microparticles on the electrode. Table 3 summarized the analytical properties of the biosensors prepared with the later conditions.

3.6. Analytical performance of the biosensors

The calibration curves for each biosensor, displays the steady state current density as a function of the glucose concentration (Fig. 8). Under all the optimal experimental conditions for the biosensor prepared with p-MAA, under optimal conditions (0.37%, potential +0.6V, pH 6.0 and 30 °C), the calibration curve has presented the following properties: sensitivity of 11.98 mA M^{-1} cm^{-2}, maximum current density 126.63 μA cm^{-2}, linear range between 9.0×10^{-6} and 8.26×10^{-3} M, and detection limit 10 μM. This information is similar to the majority of the existing biosensores that use the same system of immobilization. It is necessary to emphasize the value obtained of the linear range,

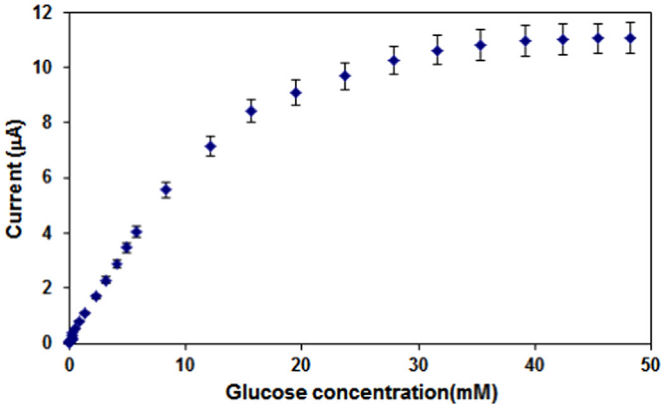


Fig. 8. Response to the glucose of the biosensor fabricated with p-MAA microparticles under all the optimal experimental conditions in stirred 0.1 M phosphate buffer, pH 6.0, potential of +0.6 V versus SCE and 25 °C.

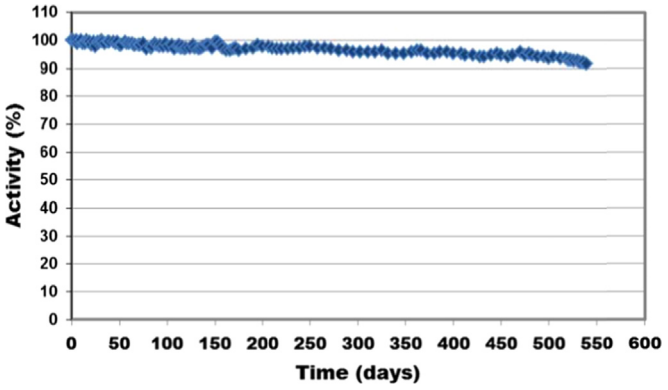


Fig. 9. Storage stability profiles p-MAA biosensor. The biosensor was stored frozen in phosphate buffer at −4 °C when not in use.

which is included for values of concentration of glucose up to 150 mg/dL.

Stability is a basis requirement of glucose biosensors. In general, the decrease of activity of enzyme-based biosensors was caused by leaching and inactivation of enzyme. The inactivation of enzyme is mainly caused by unsuitable temperature, and the leaching of enzyme depends on the absorbent structure. The stability study was carried out considering both stabilities of the biological material as the analytical device. One remarkable characteristic of the microparticles is the stability of the enzyme immobilized into the network. As it has been demonstrated, the enzyme activity of the freeze-dried microparticles was unalterable at least for 16 months after their synthesis. To study the stability of the biosensors the response to 0.25 mM glucose solution, measured at +0.6 V versus SCE and at 30 °C was periodically registered keeping the electrodes stored in a phosphate buffer solution at −20 °C between measurements. We have found that the biosensors exhibited a 90% of the initial signal 550 days for p-MAA (see Fig. 9) after its preparation. Furthermore, if we compare the stability of the biosensors prepared by enzyme entrapped in methacrylate microgels with the stability of those prepared by using covalent attachment of the enzyme [41] or electrostatic interactions [42] it is observed that the enzyme entrapped in methacrylate microgels could provide better enzymatic stability.

When comparing the results obtained with the proposed biosensors and that previously reported values using a similar immobilization system (polymeric microparticles), it was observed an improvement in the analytical properties as sensitivity, and the maximum current density. The significantly increasing of the stability, detection limit and lineal range, seem the most interesting characteristics of the proposed biosensors, compared with the biosensor previously reported by our research group [28,40,43]. However these results are slightly lower than those obtained with p-DMAEM [14]. These results may be due to interactions between the matrix and the enzyme, which are conditioned by the charges of both, and that in this case being influenced by the analytical properties of the proposed biosensor.

The detection limit was 10 times smaller than previously reported values, the linear range increased one order of magnitude compared with other devices prepared using different immobilization techniques such as layer-by-layer self-assembly films, or deposition on the electrode surface [44–48], and the stability improved from 150 days to 550 days with these biosensors [44–50].

3.7. Interference study

Glucose is an important component in biological and food samples. Some components of those samples that possibly

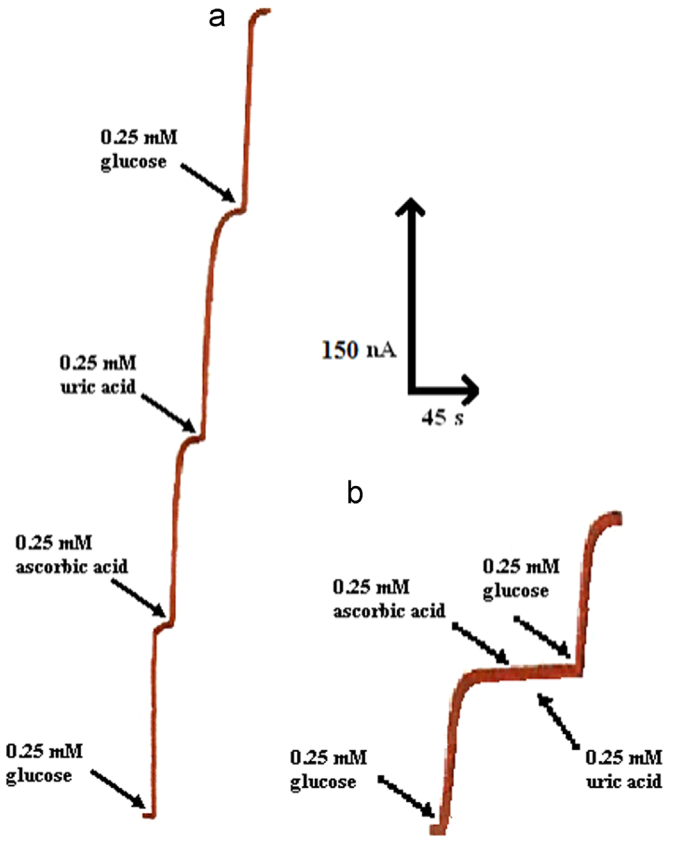


Fig. 10. GOx-biosensor response to 0.25 mM glucose, ascorbic and uric acid (a). GOx-Nafion®-biosensor response to 0.25 mM glucose, ascorbic and uric acid (b).

interfered with glucose determination were tested. The enzymatic electrode operated at a reading potential of +0.6 V vs. SCE corresponding to the oxidation of hydrogen peroxide. At this potential, the H_2O_2 detection is disturbed by the presence of interfering species such as uric acid (UA) and ascorbic acid (AA) when physiological samples are analyzed since these acids present negative charge at the working pH (6.0–7.0). The interferences caused by electroactive molecules were eliminated using a Nafion® layer covering the electrode surface, since Nafion® is a negatively charged polyelectrolyte whose effect on the negatively charged substrates is noticeable.

Thus, a layer of Nafion® (50 μL) was applied on the electrode surface. The layer was dried in air for 15 min and subsequently the electrode was kept at 80 °C for 45 min. The microparticles with GOx obtained from each studied methacrylates, were then placed over the Nafion® film and covered with the dialysis membrane. As it is shown in Fig. 10a, for the biosensor with p-MAA microparticles prepared without Nafion® (GOx-biosensor), the interfering signals of 0.25 mM UA and AA are significant. On the contrary, when the GOx-Nafion®-biosensor was calibrated, the signals arising from interferents disappeared (Fig. 10b). However, the intensity current due to 0.25 mM of glucose was slightly affected and remained nearly constant, varying from 150 nA in the biosensor without Nafion® to 145 nA in the biosensor with Nafion® for p-MAA, resulting still useful to eliminate the signs of these interfering species.

3.8. Application of biosensor for determination of glucose in human serum

In order to demonstrate the feasibility of the glucose biosensor in real sample analysis, the biosensor was employed to measure glucose in human blood sample. The glucose concentration of five serum pools were analyzed using both the GOx-biosensor and GOx-Nafion® biosensor. The glucose of these serums was also analyzed by the spectrophotometric method of hexokinase [51] which was used as reference method. The result showed a good correlation with the reference method, especially in the case of the biosensor prepared with Nafion®. The glucose concentration calculated with the biosensor without Nafion® was always slightly higher than those obtained with the reference method (differences between 1.0–5.1%), due to the contribution of the AA and UA oxidation in the obtained signals. By contrast, when Nafion® was included in the biosensor, the concentration of glucose measured with both methods was similar and the values were never higher than ±1.1% expressed as standard deviations, confirming that depositing a layer of Nafion® on the electrode surface removed the interference caused by these species present in the serum. A good precision was obtained with all the biosensors RSD <2.2% (Table 4).

Furthermore, the recoveries observed with both biosensors were always between 99.6% and 101.5% (Table 5). The results indicate the glucose biosensor is feasible for practical analysis.

4. Conclusions

The effects caused by the immobilization of GOx in p-MAA microgels can be summarized as follows: (a) The size of the microgel particles is not increased by the inclusion of GOx; (b) The effect of the entrapped enzyme in the swelling of the microgels is insignificant; (c) The T_g values observed are quite similar to the obtained by other authors. The decrease of T_g observed in microgels, is attributed to the increasing of the cross-linking amount in the microgel; (d) The diffractograms of microgels with enzyme corroborated the existence of the enzyme caught in the microparticles. The diffractograms of cross-linked microgels present different maximums similar to those of the free enzyme.

Microparticles based on methacrylates present an interesting matrix for GOx immobilization and can be used as biological element in glucose biosensors. The optimal conditions of the

Table 4
Quantitative results obtained from the analysis of the serum by the spectrophotometric method (reference), GOx-biosensor, and GOx-Nafion®-biosensor

SAMPLE		GLUCOSE (mg/dl) REFERENCE METHOD GOx-BIOSENSOR	GLUCOSE[a] (mg/dl)	RSD(%)	GOx-BIOSENSOR Deviations[b] (%)	GLUCOSE[a] (mg/dl) GOx-NAFION® BIOSENSOR	RSD (%)	GOx-NAFION® BIOSENSOR Deviations[b] (%)
p-MAA	1	99.0	104.1 ± 1.9	1.9	+5.1	100.1 ± 0.5	0.5	+1.1
	2	89.0	92.6 ± 2.0	2.3	+4.1	88.8 ± 2.0	2.2	−0.2
	3	176.0	178.9 ± 0.2	1.2	+1.8	174.0 ± 0.9	1.9	−1.0
	4	116.0	117.2 ± 1.5	1.2	+1.0	114.8 ± 1.2	1.8	−0.5
	5	93.0	95.7 ± 0.3	0.3	+2.8	93.5 ± 0.4	0.4	+0.5

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

Table 5
Recovery studies using both glucose oxidase biosensors[a].

SAMPLE		GLUCOSE ADDED (mg/dl)	GOx-BIOSENSOR GLUCOSE FOUND[a] (mg/dl)	RECOVERY (%)	GOx-NAFION®-BIOSENSOR GLUCOSE FOUND[a](mg/dl)	RECOVERY (%)
p-MAA	1	72.0	72.1 ± 0.8	100.2	72.8 ± 1.6	101.2
	2	72.0	72.1 ± 0.8	100.1	73.0 ± 1.9	101.5
	3	72.0	71.6 ± 1.1	97.5	72.0 ± 2.1	100.0
	4	72.0	71.9 ± 0.7	94.8	71.7 ± 2.2	99.6
	5	72.0	71.5 ± 1.2	99.3	72.0 ± 1.5	100.0

[a] Average of tree measurements.

synthesis were: monomer concentration 1.59 M and $\eta = 0.37\%$. Furthermore, the response of the biosensor is optimal for the following working conditions: pH 6.0; temperature 30 °C; 3 mg of particles on the electrode; and an enzyme load of 765 UI mg^{-1}. The significantly increasing of the stability seem the most interesting characteristics of the proposed biosensors, compared with the biosensor previously reported by our research group and even compared with others enzyme biosensors. The analytical properties obtained from the proposed biosensor for the determination of glucose presented the following properties: Under optimal conditions ($\eta = 0.37\%$, potential +0.6 V, pH 6.0 and 30 °C), we realized a calibration curve in which the glucose biosensor presented the following properties: sensitivity of 11.98 mA M^{-1} cm^{-2}, maximum current density 126.63 μA cm^{-2}, linear range between 9.0×10^{-6} and 8.3×10^{-3} M, and detection limit 10 μM.

The interferences caused by species with negative net charge were eliminated by a Nafion layer covering the electrode surface. The biosensor has been successfully applied to the determination of glucose in serum samples. The stability studies for all the methacrylate microparticles confirmed that the immobilization process enhanced significantly the enzymatic stability and allowed its reuse.

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