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An amperometric sensor employing glucose oxidase immobilized on nylon membranes with different pore diameter and grafted with different monomers

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Abstract

Glucose oxidase (GOD) was immobilized on nylon membranes having three different pore diameters and chemically grafted with glycidyl methacrylate (GMA) or butyl methacrylate (BMA). Hexamethylenediamine (HMDA) and glutaraldehyde (GA) were used as spacer and coupling agent, respectively.

The biochemical and electrochemical behaviour of the membranes has been studied as a function of pH, temperature and glucose concentration with reference to the grafted monomer and the membrane pore diameter. The behaviour of the soluble GOD has also been studied in order to see the modification induced by the immobilization process on the enzyme activity.

It was found that the values of the biosensor sensitivity, maximum saturation current and electrochemical affinity increase with the membrane pore diameter, indipendently of the nature of the graft monomer. Opposite behaviour was found relatively to the extension of the linear response ranges and the average response times.

With reference to the parameters increasing with the pore diameter it was found that membranes grafted with GMA had higher values than those of the membranes grafted with BMA. The contrary occurred to the values of the parameters decreasing with the increase of the pore diameter.

Biochemical and electrochemical results have been discussed in terms of the different limitations to the diffusion of substrate and reaction products across the catalytic membrane introduced by the different pore diameters and by the different hydrophobicity of the graft monomers.

Keywords: Glucose Oxidase; Catalytic Membranes; Biosensors; Grafting technique; Glucose determination

1. Introduction

Amperometric enzyme electrodes combine the specificity of the enzyme in recognizing molecular species with the direct transduction of the reaction rate into an electrical signal, i.e. a current. This

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class of sensors is used in an increasing number of biotechnological applications, particularly clinical, environmental and in food industry [1–7]. Enzymes in biosensors are employed immobilized on supports. For this reason the immobilization techniques became an important field of research addressed to prepare catalytic membranes usefully employed in bioreactors or biosensors [8–11].

Grafting technique assumed a relevant role in this context. By means of grafting, chemical or by γ-radiation, inert membranes, unable to bind enzymes in the absence of specific treatments, become good supports to be loaded with catalysts. Recently, our research was focused on the preparation of catalytic nylon or teflon membranes, chemically or by γ-radiations grafted, and on the study of their behaviour in isothermal and non-isothermal bioreactors [12–22], as well as in non-isothermal biosensors [23–25]. Grafting technique offers the advantage of modulate some physical properties of the membranes, such as diffusive and thermo-osmotic permeabilities, according to the nature of the grafted monomer and to the amount of grafting, measured as grafting percentage. Nylon membranes are particularly useful owing to their biocompatibility and resistance to microbial attack. Another advantage is the possibility of obtaining these membranes with different pore diameters. By using grafting copolymerization of different monomers on the same membrane or by grafting the same monomer on membranes with different pore diameters, different extension of the linear response range of a biosensor can be obtained.

In this paper, we will discuss the results relative to the electrical response of a biosensor when different catalytic membranes are separately employed. The membranes were obtained by immobilizing glucose oxidase (GOD) on nylon membranes grafted with glycidyl methacrylate (GMA) or butyl methacrylate (BMA). The untreated membranes had three different pore diameters and were grafted up to the same value of the grafting percentage. Catalytic membranes have also been characterized on the biochemical point of view. When possible, comparison with the behaviour of the soluble GOD was carried out to know the modifications induced by the immobilization process on enzyme activity.

2. Apparatus, material and methods

2.1. The biosensor

The biosensor used, represented in Fig. 1a, was constituted by a cylindrical cavity in which the

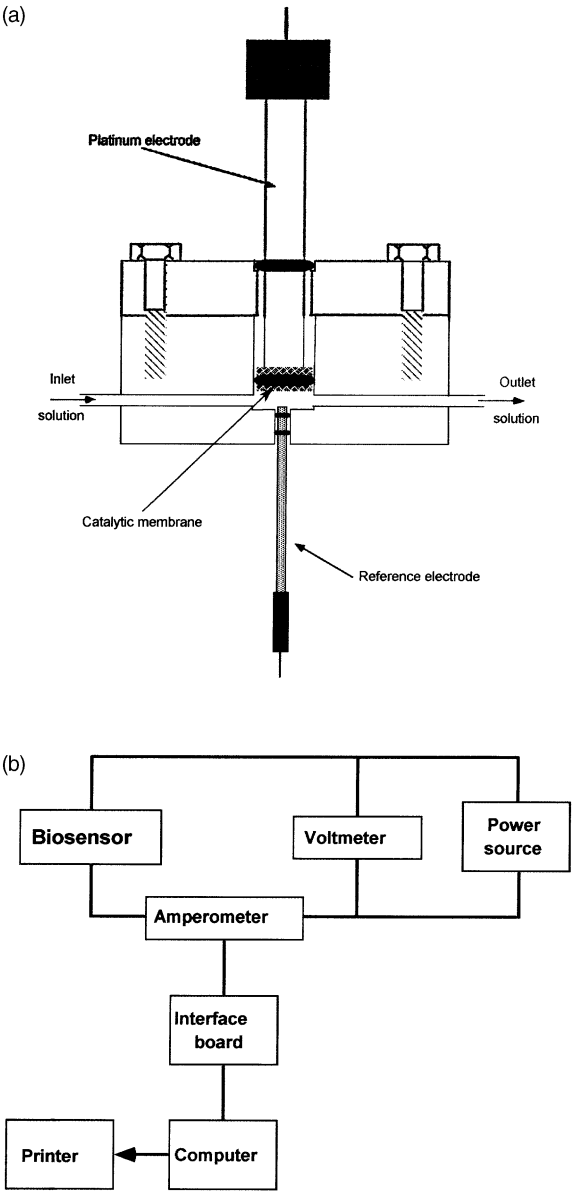


Fig. 1. (a) Schematic representation of the biosensor; (b) block diagram of the apparatuses employed.

glucose solution flows at a rate of 3 ml/min through an hydraulic circuit, thermostatted at the required temperature and driven by a peristaltic pump. The cell volume was 1 ml. The catalytic membrane was positioned by means of an O-ring to the surface of the anode, constituted by a platinum disk, 6 mm in diameter, while the reference electrode was a Ag/AgCl electrode (Flexref model WPI, USA). A 700 mV potential difference between the two electrodes, the anode being positive, was ensured by means of an external power source Goodwill GPS 3030D (Good will Instrument Co., Poipei, Taiwan) and controlled by a digital multichannel recorder Philips KS 3460 (Philips, Almedo, The Netherlands). Under the electric potential difference used, the hydrogen peroxide produced by the catalytic reaction was oxidized according to the reaction: $H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$.

The current measured at the output of the biosensor, was proportional to glucose concentration.

In Fig. 1b, a block diagram of all the apparatuses employed is represented.

2.2. Materials

As solid support to be grafted, nylon Hydrolon membranes, a precious gift from Pall (Pall Italia, Milano, Italy), were used. These hydrophobic membranes, 150 μm thick, had a nominal pore size of 0.2, 1.2 and 3 μm, respectively. Pore size is defined as the size of the diameter of the smallest particles retained, since in the membrane there are no "classical" pores but irregular cavities, constituted by the interstices between the nylon fibers.

GMA or BMA were used as monomers to be grafted. HMDA and GA were used as spacer and coupling agent, respectively.

GOD(EC 1.1.3.4) was used as a catalyst. The enzyme catalyzes the reaction:

β-D-glucose + H_2O → D-gluconic acid + H_2O_2.

Horseradish peroxidase (POD, EC 1.11.1.7) was used to measure the H_2O_2 concentration produced by the enzyme reaction.

All chemical products, with the exception of the GOD, were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA) and used without further purification. GOD was purchased from Fluka (Fluka Chemic AG, Buchs, Switzerland).

2.3. Methods

2.3.1. Preparation of the catalytic membranes

Two different types of catalytic membranes have been prepared. The first one, identified henceforward as GMA_x, was a nylon-poly(GMA)-HMDA-GA-GOD; while the second one, identified as BMA_x, was a nylon-poly(BMA)-HMDA-GA-GOD. The subscript x is related to the membrane pore diameter: 0.2, 1.2 or 3 μm. In this a way, e.g. the symbol $BMA_{1.2}$ indicates the nylon membrane with pore diameter of 1.2 μm and grafted with BMA.

The preparation of catalytic membranes was carried out by means of two steps: grafting copolymerization and enzyme immobilization.

(a) *Grafting copolymerization*: Grafting copolymerization for both membrane types was carried out by dissolving as initiating system $K_2S_2O_8$ and $Na_2S_2O_3$ in a 1/1 (v/v) water/ethanol mixture. Initiator concentration, reaction time and reaction temperatures were different in the preparation of the two membrane types.

To obtain the nylon-poly(GMA) membranes the untreated nylon membranes were immersed, for 60 min at 40 °C, in a reaction vessel filled with the water/ethanol solution containing 0.6 M GMA, 0.008 M $K_2S_2O_8$, 0.008 M $Na_2S_2O_3$ and 0.03 mM $CuCl_2$.

To obtain the nylon-poly(BMA) membranes the untreated nylon membranes were immersed, for 30 min at 55 °C, in a reaction vessel filled with the water/ethanol solution containing 0.425 M BMA, 0.006 M $K_2S_2O_8$ and 0.012 M $Na_2S_2O_3$.

At the end of the grafting process, both membranes types were treated with dimethyl ketone to remove the produced homopolymer, and dried until a constant weight was reached. Grafting percentage (G, %) was determined by the difference between the membrane masses before (M_b) and after (M_a) the grafting process through the expression:

$$G = \frac{M_a - M_b}{M_b} \times 100 \quad (1)$$

(b) *Enzyme immobilization*: Enzyme immobilization was carried out by using three successive treatments. With the first one, the spacer HMDA was

attached to the grafted support; with the second one, the membrane was activated by the interaction which the GA; with the third one, the enzyme was immobilized on the activated support through covalent attachment to the GA.

Nylon-poly(GMA)-HMDA membranes were obtained by immersing the grafted membranes in a 2% (v/v) HMDA aqueous solution for 30 min at room temperature, while for the preparation of the nylon-poly(BMA)-HMDA membranes a 50% (v/v) HMDA aqueous solution was used for 30 min at 60 °C. After this step, both membrane types were washed with water to remove the unreacted amines, then treated for 1 h at room temperature with a 2.5% (v/v) GA aqueous solution. After further washing with double-distilled water and 0.1 M acetate buffer solution, pH 5.0, both membrane types were treated for 16 h at 4 °C with the same buffer solution containing GOD at a concentration of 1 mg/ml. At the end of this step, the membranes were washed with the buffer solution in order to remove the unbound enzymes.

The overall process of grafting, membrane activation and enzyme immobilization is illustrated in Figs. 2 and 3 for membrane GMA_x and BMA_x, respectively.

The amount of immobilized GOD was calculated by subtracting the amount of the proteins recovered in the solution at the end of immobilization process and into the solutions used to wash the enzyme derivatives from the amount of the proteins initially used for the immobilization. Protein determination was carried out by the method of Lowry et al. [26].

2.3.2. Biochemical measurements of GOD activity

GOD activity was measured by means of an enzymatic method for the determination of the hydrogen peroxide. It is based on the enzyme horseradish peroxidase which in the presence of 2,2′-azino-bis(3-ethylbenzothyazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide gives a coloured complex. The intensity of the solution colour, spectrophotometrically measured at 405 nm, is proportional to the hydrogen peroxide concentration. The procedure was the following: 0.1 ml of the sample to be analyzed plus 0.1 ml of POD (50 μg/ml) were added to 2 ml of 0.1 M acetate buffer, pH 5.0, containing ABTS at a concentration of 10 mM. The concentration of ABTS was in excess in respect to that of H_2O_2 so that the reaction was limited by the concentration of the latter product. The reaction was allowed for 10 min at 25 °C, till the amount of H_2O_2 was consumed. A calibration curve of the H_2O_2 concentration as a function of the absorbance gave an angular coefficient equal to 2 m/M.

(a) *Activity measurements of soluble enzyme*: Soluble GOD activity has been measured according to the following procedure: 0.1 ml of GOD solution (1 mg/ml) were added to 1.9 ml of the β-D-glucose solution at the appropriate concentration, temperature and pH, so that the enzyme concentration was 50 μg/ml. Every 2 min, 0.1 ml of the reaction solution was taken out and added to 0.4 ml of a 0.1 M HCl aqueous solution to stop the enzyme reaction. The H_2O_2 concentration was then measured according to the procedure described earlier. In this way, the H_2O_2 production as a function of time was obtained. The angular coefficient of the straight line gives the enzyme activity measured as μmol/min.

(b) *Activity measurements of insoluble enzyme*: To measure the activity of the catalytic membranes, having a surface of $16 \pm 0.5\ cm^2$, they were put in a reaction vessel filled with 20 ml of the glucose solution at the required concentration, temperature and pH. Samples of 0.1 ml were extracted at regular time intervals and processed in the same way used for the free enzyme. The angular coefficient of the H_2O_2 production as a function of time gave the activity of the catalytic membranes, expressed as mol/min.

2.3.3. Current measurements

The current, constituting the output signal from the biosensor, was measured by means of a digital multimeter Philips PM 2525, interfaced to a personal computer by means of a National Instruments IEEE-488 interface board, model GPIB PCII (National Instruments, Austin, TX, USA). The response time of the instrument was 0.4 s, with a minimal range in dc of 1 μA and a resolution of 100 pA.

The software for data acquisition, written by us in the Quick Basic programming language, accounted for the value of the background current, which was

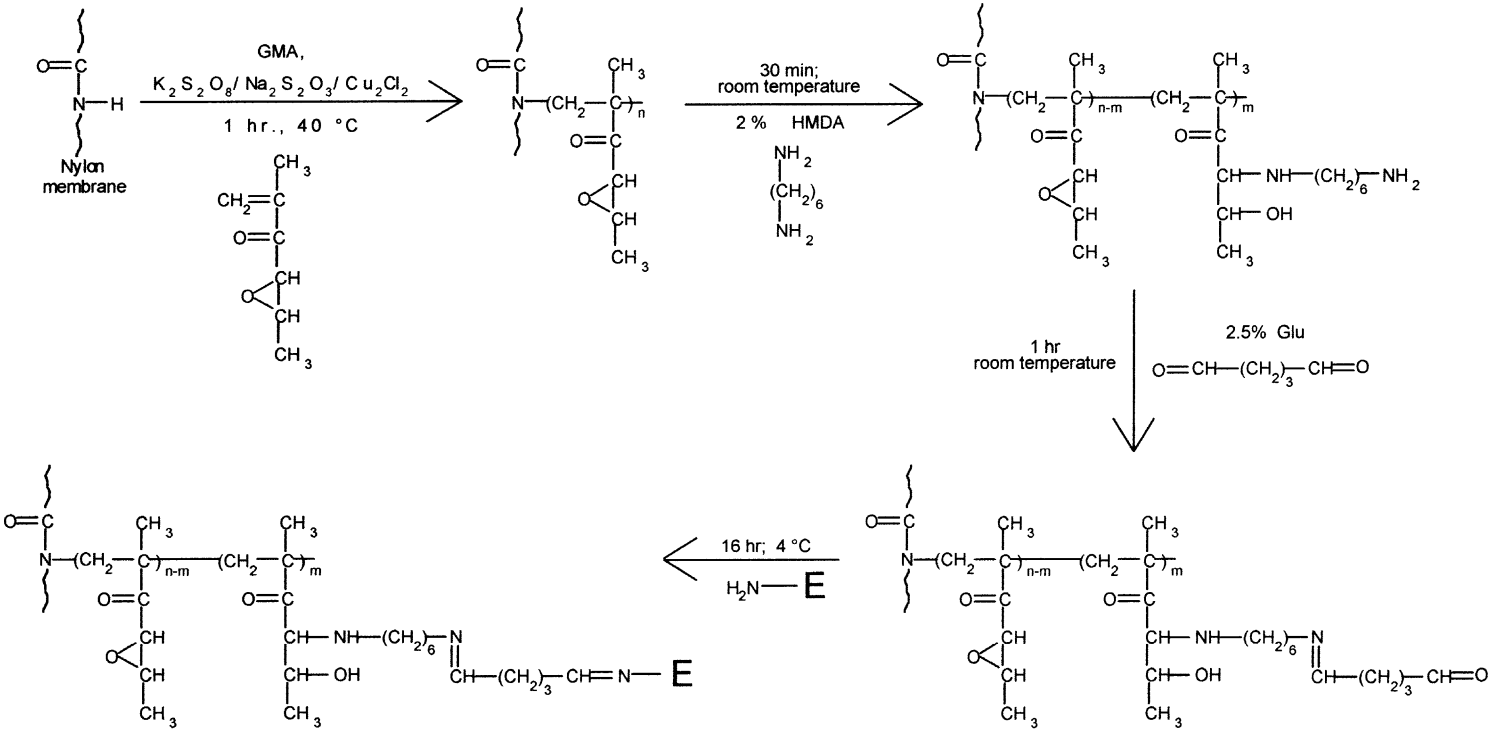


Fig. 2. Representation of the processes of grafting, membrane activation and enzyme immobilization to obtain membrane GMA_x.

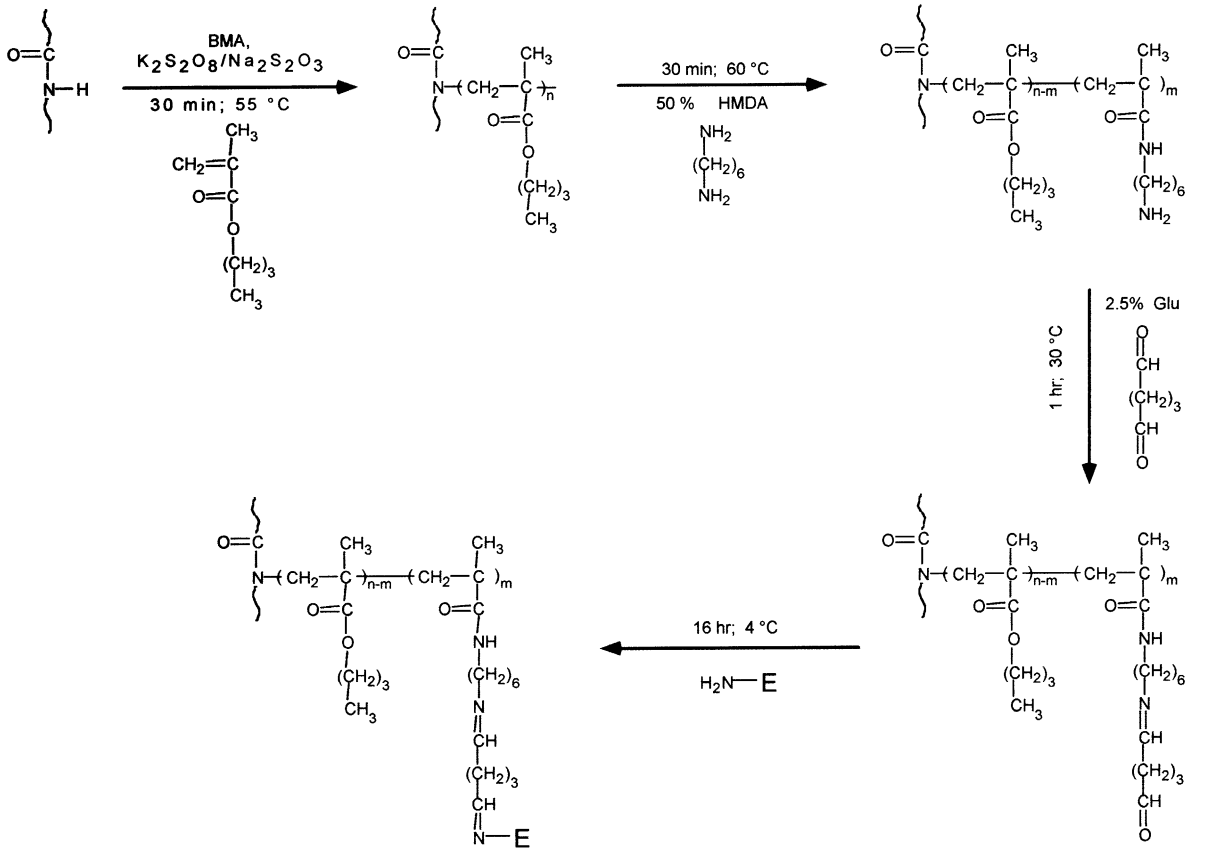


Fig. 3. Representation of the processes of grafting, membrane activation and enzyme immobilization to obtain membrane BMA_x.

continuously subtracted from the subsequent values of the measure.

Each experiment was carried out according to the following procedure. A cleaning 0.1 M acetate buffer solution, pH 5.0, was circulated through the experimental apparatus to remove possible traces of glucose or hydrogen peroxide coming from previous experiments and until the current decreased to a constant value smaller than 100 nA, considered as background current. Once this value was reached, the data acquisition system was started and the cleaning solution replaced with that containing the glucose concentration to be measured. In Fig. 4, a typical current/time curve is reported. The first part of the curve, where the anodic current is still equal to the background current, is related to the substitution of the cleaning solution with the glucose one. During this phase the glucose concentration inside the catalytic membrane starts to increase. When the analyte oxidation is high enough to give an appreciable current, the latter starts to increase, too. Later on the glucose concentration inside the catalytic membrane reaches a steady-state value where the enzyme reaction rate is equal to the rate of substrate mass transport by diffusion [27]. This steady-state corresponds to the saturation value of the current, I_{sat}. We have calculated that the minimum solution volume required to reach the steady-state current was of 20 ± 2 ml. When the experiment is ended, at t_f, the current value decreases owing to the substitution of the glucose solution with the cleaning one.

According to this phenomenological description, it is possible to define two physical parameters: the steady-state response of the biosensor, i.e. the saturation current I_{sat}, and the response time τ_r, which is the measure of the time needed for the current to rise from 10 to 90% of its maximum value.

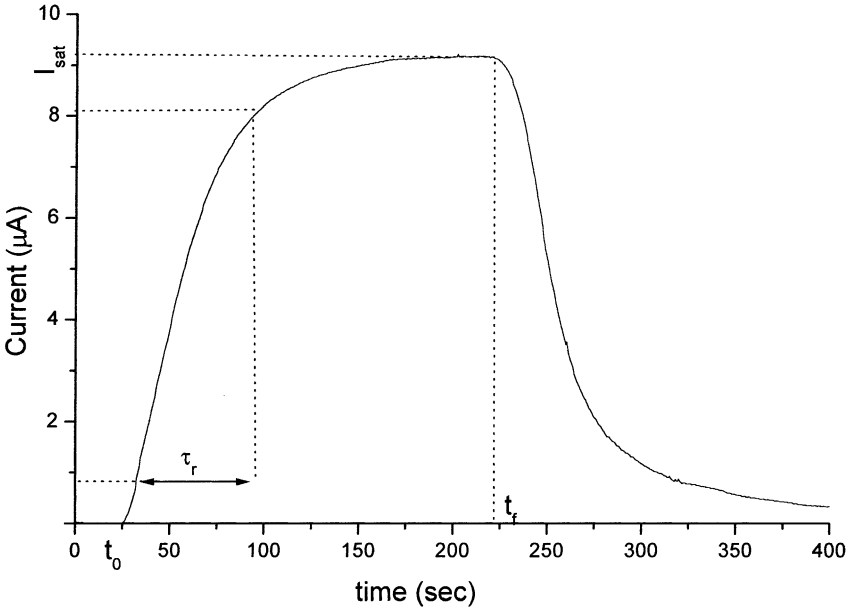


Fig. 4. Typical current/time response of the biosensor.

2.3.4. Time stability of the catalytic membranes

Time stability of catalytic membranes was assessed by measuring every day the electrical response under the same experimental conditions, i.e. 20 mM glucose in 0.1 M acetate buffer solution, at pH 5.0 and 25 °C. After about two initial days, during which the membranes gave unreproducible fluctuating values, a stable condition was reached, which remained constant for more than 1 month.

When not used, the membranes were stored at 4 °C in 0.1 M acetate buffer solution, pH 5.0.

2.3.5. Treatment of the experimental data

All points reported in the figures are the average of five independent runs carried out under the same experimental conditions. The experimental errors never exceeded 4.0%.

3. Results and discussion

Before discussing the catalytic behaviour of the two membrane types, we report in Table 1 a summary of

Table 1
Characteristics of catalytic supports

Catalytic membrane composition	Membrane pore diameters (μm)	Grafting percentage (%)	Amount of immobilized GOD (mg cm^{-3})	Coupling yield (%)	Absolute activity (μmo1/(min mg^3))	Specific activity (μmo1/(min mg))	Activity retention (%)[a]
Nylon-poly(GMA)	0.2	21.6 ± 0.6	8.1 ± 0.2	45.0	1.54 ± 0.05	0.19 ± 0.01	7.60
	1.2	21.4 ± 0.5	8.0 ± 0.2	45.0	1.59 ± 0.04	0.20 ± 0.01	7.83
	3.0	21.7 ± 0.7	7.9 ± 0.3	44.9	1.75 ± 0.06	0.22 ± 0.01	8.62
Nylon-poly(BMA)	0.2	21.2 ± 0.6	8.3 ± 0.2	45.0	1.17 ± 0.03	0.14 ± 0.01	5.77
	1.2	21.5 ± 0.7	8.4 ± 0.3	45.1	1.27 ± 0.04	0.15 ± 0.01	6.26
	3.0	21.4 ± 0.5	8.1 ± 0.2	45.0	1.38 ± 0.03	0.17 ± 0.01	6.80

[a] The activity retention has been calculated considering 2.5 μmol/(min mg) the specific activity of the free GOD.

the properties of each of the membranes. Inspection of the values listed in Table 1 shows that the grafting percentage is equal for the six membranes. This result was not fortuitous and due to serendipidity, in so much as the experimental conditions to obtain the same grafting percentage were previously searched. As a consequence of the same values of grafting percentage, similar amounts of immobilized GOD and "coupling yields" were obtained. The latter parameter is defined as the ratio of the amount of immobilized enzyme to the amount of soluble enzyme used for the immobilization process. On the contrary, the values of the absolute and specific activities are affected by the nature of the grafted monomer. Indeed, the average value of the absolute activities of membranes belonging to GMA_x group is 1.63 μmol/(min cm^3), a value about 1.3 times higher than the corresponding value of membranes belonging to BMA_x group. The same ratio is present when the specific activities are considered. This means that when GOD is immobilized through GMA, it retains more activity in respect to the soluble counterpart, i.e. its affinity for the substrate appears to be better. Absolute activity is referred to the activity of 1 cm^3 of the catalytic membrane, while the specific activity is referred to 1 mg of immobilized enzyme. The activities reported in Table 1 were measured under the experimental conditions: $T = 250\,^{\circ}C$, 0.1 M acetate buffer, pH 5.0, and 200 mM, a concentration value at saturation for both free and immobilized form of GOD. The volume of all membranes employed in this experiment and in the other experiments relative to the enzyme membrane activity was 0.24 ± 0.01 cm^3. Magnetic stirring was ensured during the activity measurement of both the enzyme forms, soluble and insoluble.

The experimental results are separately reported in two groups, the first one relative to the dependence of the biochemical and electrical responses on the nature of the graft monomer, taking constant the membrane pore diameters; the second one to the dependence on membrane pore diameters and on the two graft monomers. In this way, in the first group the comparison is done between the behaviour of $GMA_{0.2}$, $BMA_{0.2}$ and the soluble GOD; in the second group the comparison is carried out between the membranes obtained with the two monomers separately grafted on the membranes having pore diameter 0.2, 1.2 or 3.0 μm.

3.1. Dependence on the nature of the graft monomer

3.1.1. pH dependence

In Fig. 5a, the relative enzyme activity as a function of pH has been reported. The experimental conditions were: $C_0 = 20$ mM glucose concentration and $T = 25\,^{\circ}C$. Acetate buffer was used for the pH range between 3 and 5.5; citrate buffer for the range between 5.5 and 6.5, and Tris–HCl buffer between 6.5 and 10. All buffer concentrations were 0.1 M. At the point of pH overlapping the enzyme activity was found to be independent of the buffer nature. The relative activity of the free enzyme has also been reported to allow comparison between the behaviour of the soluble and insoluble GOD. The experimental conditions in the measurements relative to the soluble GOD were the same that for the immobilized ones and have been carried out in presence of magnetic stirring. From Fig. 5a, a shift of the optimum pH towards more alkaline values is observed for both forms of immobilized GOD in respect to the value of the soluble enzyme. The optimum pH value occurs at 5.1 for the soluble enzyme, at 6.0 for the enzyme immobilized on membrane $BMA_{0.2}$ and at 6.8 for membrane $GMA_{0.2}$. The position of the optimum pH for the soluble enzyme was similar to that reported by other authors [28–30] which found for the free GOD a maximum of activity around to pH 5.5. Analogous pH shifts towards more alkaline values upon immobilization were found by other authors [28,30–32]. This behaviour is due to the partitioning effect by which the H^+ and OH^- concentrations in the micro environment where the enzyme is operating are changed in respect to their values in the bulk solution owing to the interaction with the carrier (and the grafted monomer). The absolute values of the enzyme activities at the position of the pH optimum were 0.25 μmol/min for the free enzyme, 1.25 and 0.94 μmol/(min cm^3) for membrane $GMA_{0.2}$ and $BMA_{0.2}$, respectively.

By defining "optimum pH range" the range in which the relative enzyme activity is comprised between 90 and 100%, it is possible to observe that this range occurs between 4.1 and 6.0 for the free enzyme; between 5.5 and 8.0 for membrane $GMA_{0.2}$ between 4.5 and 75 for membrane $BMA_{0.2}$.

Employing membrane $GMA_{0.2}$ or $BMA_{0.2}$, we have studied the electrical response of our biosensor as a

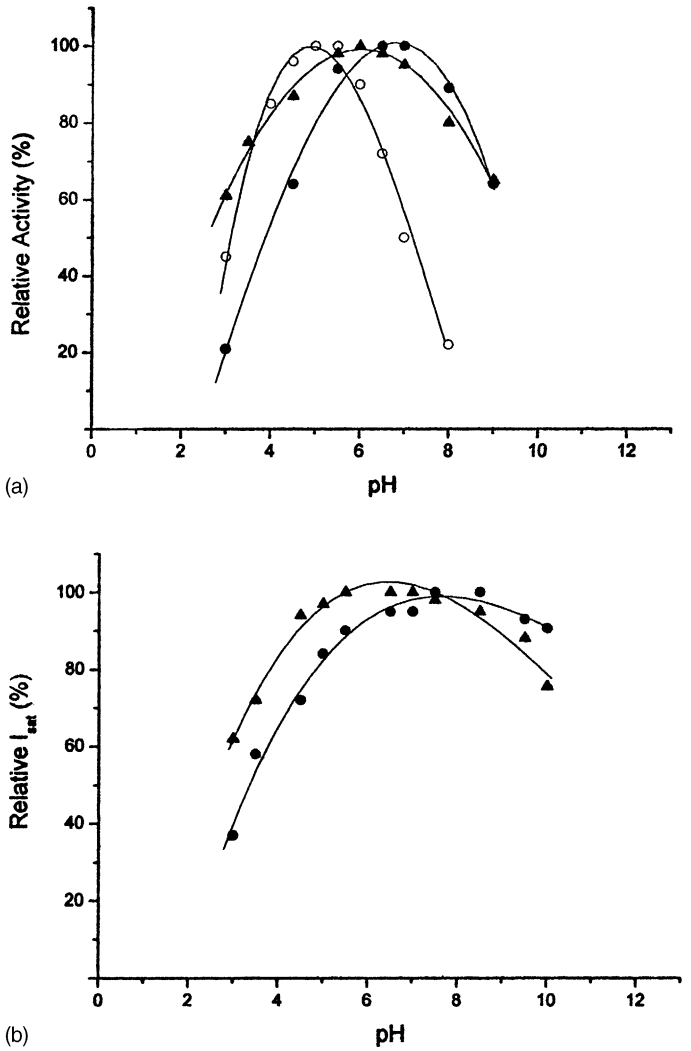


Fig. 5. Relative enzyme activity (a) and relative saturation currents (b), as a function of pH. Symbols: (○) free enzyme; (●) membrane $GMA_{0.2}$; (▲) membrane $BMA_{0.2}$.

function of pH under the conditions of the experiments reported in Fig. 5a. The results of this investigation are reported in Fig. 5b. It is possible to observe that both membranes show a shift of the position of the optimum saturation current of more than one unit towards higher pH values in respect to the optimum enzyme activity. The position of the maximum value of the saturation current, indeed, occurs at pH 7.0 for membrane $BMA_{0.2}$ and at pH 8.0 for membrane $GMA_{0.2}$. The absolute values of the saturation currents at the maximum value are 14.4 and 5.1 μA for membrane $GMA_{0.2}$ and $BMA_{0.2}$, respectively. The pH profiles of the saturation currents appear broader than the corresponding profiles of the relative enzyme activity, while the pH difference between the positions of the two electrical maxima remain practically unchanged in respect to the same difference in the biochemical behaviour. In addition, the "optimum pH range" of the electrical response for both membranes is larger than that found in the biochemical response. In the case of membrane

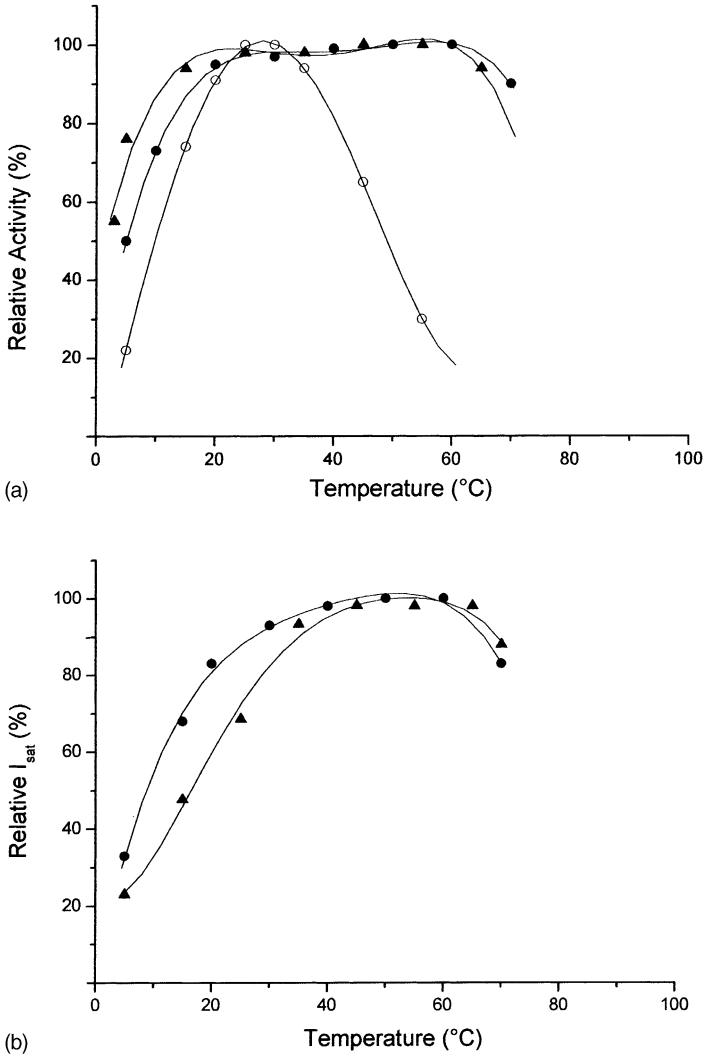


Fig. 6. Relative enzyme activity (a) and relative saturation currents (b), as a function of temperature. Symbols: (○) free enzyme; (●) membrane $GMA_{0.2}$; (▲) membrane $BMA_{0.2}$.

$GMA_{0.2}$, the optimum pH range is between 6 and 10, while between 4.6 and 9 for membrane $BMA_{0.2}$.

3.1.2. *Temperature dependence*

In Fig. 6a, the relative catalytic activity of both membrane types $GMA_{0.2}$ and $BMA_{0.2}$ has been reported as a function of temperature. The experimental conditions were: $C_0 = 20$ mM glucose concentration in 0.1 M acetate buffer, pH 5.0. The relative activity of the free enzyme has also been reported to allow comparison between the behaviour of the soluble and insoluble GOD. The experimental conditions in the measurements relative to the soluble GOD were the same that for the immobilized ones and have been carried out in presence of magnetic stirring. Inspection of the results reported in Fig. 6a shows that it is impossible to define an optimum value for the activity of membrane $GMA_{0.2}$ and $BMA_{0.2}$, since both

membranes exhibit a large range in which the activity is constant at the maximum value. We have repeated the experiments in this range at least six times, but we always obtained the same experimental average values with errors less than 4%. From Fig. 6a, a shift of at least 15 °C of the optimum temperature for the enzyme reaction appears upon immobilization. Indeed the optimum temperature occurs around 30 °C for the soluble GOD, and at 45 ± 20 °C for both insoluble forms of the enzyme. The absolute values of the enzyme activity at the position of the optimum temperature were 0.25 μmol/min for the free enzyme, 1.25 and 0.92 μmol/(min cm^3) for membrane $GMA_{0.2}$ and $BMA_{0.2}$, respectively. More interesting is the effect of the immobilization on the "optimum temperature range", defined as the temperature range in which the enzyme exhibits a relative activity between 90 and 100%. The "optimum temperature range" occurs between 22 and 35 °C for the free GOD, between 15 and 66 °C for membrane $BMA_{0.2}$; between 19 and 69 °C for membrane $GMA_{0.2}$. Increases of the thermal stability range are generally observed as a consequence of the immobilization [33]. Analogous increases have been observed by us with urease immobilized on nylon-poly(BMA) membranes [21] or with β-galactosidase immobilized on nylon-poly(BMA) membranes [20] with different percentage of grafted BMA. What appears interesting in our present results is the extent of the range in which the activity-temperature profile takes values constant and equal to 100% upon the immobilization process. We have no explanation for this behaviour.

In Fig. 6b, the relative saturation currents measured with the two catalytic membranes are reported as a function of temperature. The experimental conditions were the same as in Fig. 6a. Inspection of the results in Fig. 6b shows that the relative saturation currents of membrane $GMA_{0.2}$ and $BMA_{0.2}$ unlike the biochemical behaviour, exhibit a well defined position of the optimum temperature and a clear bell-shape profile. The optimum temperature occurs at about 57 °C for membrane $BMA_{0.2}$, and at about 50 °C for membrane $GMA_{0.2}$. The absolute values of the saturation currents at the maximum value are 13.3 and 7.14 μA for membrane $GMA_{0.2}$ and $BMA_{0.2}$, respectively. In view of the application of this type of biosensor in the clinical field, it is interesting to observe that the body temperature lies within "the optimum temperature range" of the electrical response, which goes from 30 to 70 °C for membrane $GMA_{0.2}$, and from 33 to 60 °C for membrane $BMA_{0.2}$. The "optimum temperature range" of the electrical response is the range in which the relative saturation currents are comprised between 90 and 100%. It was impossible to perform electrical measurement at temperatures higher than 70 °C, since at these temperatures we observed air bubbles disturbing the measurements.

3.1.3. Concentration dependence

In Fig. 7a, the catalytic activity of membranes $GMA_{0.2}$ and $BMA_{0.2}$ are reported as a function of the initial glucose concentration. The experimental conditions were: $T = 25$ °C and 0.1 M acetate buffer, pH 5.0. As expected, a Michaelis–Menten behaviour is observed for both membrane types. Moreover, the activity of membrane $GMA_{0.2}$ is higher than that of membrane $BMA_{0.2}$. From the data of Fig 7a, it is possible to derive the kinetic constants for GOD immobilized on the two membranes, when the experimental points there reported are replotted in form of Hanes plots. The K_m^{app} for membrane $GMA_{0.2}$ results equal to 3 ± 0.1 and 5 ± 0.2 mM for $BMA_{0.2}$.

Even if the values of the K_m^{app} look similar, an accurate examination of these values suggests that the immobilization procedure and the nature of the monomer grafted affect very much the enzyme reaction. So, when BMA is used the affinity of immobilized GOD for the substrate is halved in respect to that of the free enzyme which we found to be 2 ± 0.1 mM. In addition, the different hydrophobicity degree of BMA and GMA creates, particularly in the membrane pores, different limitations to diffusion of substrate (or reaction products) toward (or away from) the catalytic site. These limitations are responsible for the differences in the apparent K_m^{app} values between membrane $GMA_{0.2}$ and $BMA_{0.2}$. It must be remembered that the hydrophobicity of BMA is higher than that of GMA.

In Fig. 7b, the saturation currents are reported as a function of glucose concentration. The experimental conditions were the same of Fig. 7a. From the results in Fig. 7b, it clearly appears that the saturation currents exhibit a Michaelis–Menten behaviour, the values of membrane $GMA_{0.2}$ being higher than those of membrane $BMA_{0.2}$, at each of the glucose concentrations explored. Both curves of Fig. 7b can be expressed in

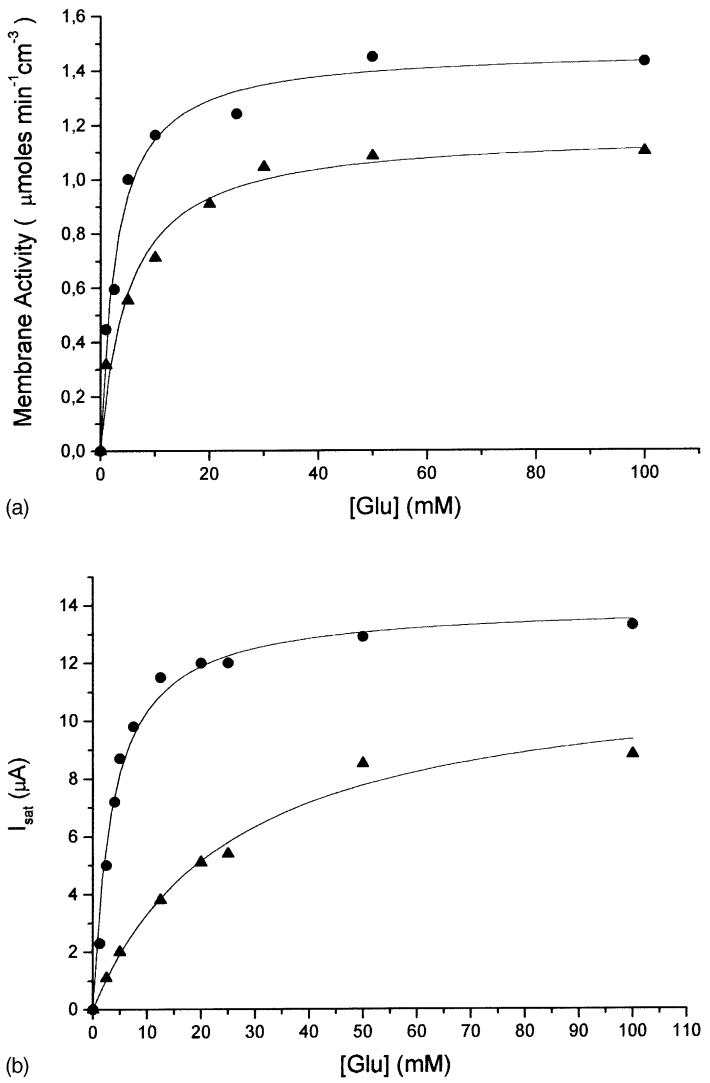


Fig. 7. Membrane activity (a) and saturation currents (b), as a function of the initial glucose concentration. Symbols: (●) membrane $GMA_{0.2}$; (▲) membrane $BMA_{0.2}$.

the electrochemical Hanes form:

$$\frac{C}{I} = \frac{C + K_m^{elect}}{I_{max}} \quad (2)$$

where C is the glucose concentration (mM), I the steady-state current (μA) at the glucose concentration C, I_{max} the maximum steady-state current (μA) and K_m^{elect} is the apparent electrochemical Michaelis–Menten constant (mM). The values electrochemical kinetic constants calculated by means of Eq. (2), are reported in Table 2. These values indicate that GOD immobilized on membrane $GMA_{0.2}$ exhibits an apparent K_m^{elect} value lower than that measured for membrane $BMA_{0.2}$. These results agree with those relative to the biochemical K_m^{app}. The higher K_m^{elect} and K_m^{app} values of membrane $BMA_{0.2}$ in respect to that of membrane $GMA_{0.2}$ can be attributed to the greater hydrophobicity of BMA molecules in respect to that of GMA. In this way the diffusion of substrate and reaction products across the catalytic

Table 2
Electrochemical parameters

Catalytic membrane composition	Membrane pore diameter (μM)	K_m^{elect} (mM)	I_{max} (μA)
Nylon-poly(GMA)	0.2	4.8 ± 0.1	14.5 ± 0.4
	1.2	3.0 ± 0.1	15.0 ± 0.3
	3.0	1.8 ± 0.1	16.5 ± 0.5
Nylon-poly(BMA)	0.2	25.0 ± 0.7	11.0 ± 0.3
	1.2	8.0 ± 0.2	12.0 ± 0.3
	3.0	5.3 ± 0.2	13.0 ± 0.4

membrane results more restricted in the membrane $BMA_{0.2}$ than in membrane $GMA_{0.2}$.

To verify this hypothesis, we have calculated the diffusion coefficient D_m (cm^2/s) across the two catalytic membranes through the expression [34]:

$$I_{sat} = -\frac{2FAD_m}{\delta}\left(\frac{1}{\cosh\phi} - 1\right)C \quad (3)$$

correlating the saturation current I_{sat} (μA) to the glucose concentration C (mM). In this expression, F is the Faraday constant (C/mol), A the electrode surface (cm^2), δ the membrane thickness (cm) and ϕ is the Thiele modulus given by:

$$\phi = \delta\sqrt{\frac{V_m^*}{K_m^{app}D_m}} \quad (4)$$

where V_m^* is the maximum reaction rate for unit membrane volume (μmol/(s cm^3)) and K_m^{app} the apparent Michaelis–Menten constant (mM) for immobilized GOD.

Eq. (3) is a transcendent equation in the variable D_m and for this reason it cannot be solved by means of analytic methods. By using numerical methods and a computer program, we have obtained the following numerical solutions: $D_m = 9.0\times10^{-7}$ cm^2/s for membrane $GMA_{0.2}$ and $D_m = 7.8\times10^{-8}$ cm^2/s for membrane BMA_{02}. From these values, it is evident that the diffusion across membrane $GMA_{0.2}$ is higher than that across membrane $BMA_{0.2}$, confirming the role of the hydrophobicity of the grafted monomers in modulating the transmembrane matter transport. The relevance of the diffusive over the kinetic process in our case is also confirmed by the values of the Thiele modulus for the two membranes, these values being 1.5 and 3 for $GMA_{0.2}$ and $BMA_{0.2}$, respectively. These values were obtained by means of Eq. (4) and by putting in it the D_m values previously calculated.

3.2. Dependence on membrane pore diameter

In Fig. 8a, the saturation currents measured with the biosensor operating with the three nylon-poly(GMA) membranes are reported as a function of the initial glucose concentration. Each curve is referred to a nylon membrane with a determined pore diameter. The experimental conditions were $T = 25\,^{\circ}C$ and 0.1 M acetate buffer, pH 5.0. From Fig. 8a, it clearly appears that at each substrate concentration the saturation currents are function of the membrane pore diameter, increasing with the increase of the latter parameter. This behaviour is explained by considering that in the presence of larger pores the diffusion of substrate (or reaction products) towards (or away from) the catalytic site is facilitated. As a consequence the biochemical and electrochemical reactions occur at higher rates that in the presence of smaller pores. This observation is confirmed by the values of the apparent electrochemical K_m^{elect} obtained when the experimental data of Fig. 8a are plotted in form of Hanes plots according to the Eq. (2).

The values of the apparent K_m^{elect} are listed in Table 2 together with the values of I_{max}.

In Fig. 8b, the saturation currents measured with the biosensor operating with the membranes belonging to the BMA_x group are reported as a function of the initial glucose concentration. Each of the three curves is referred to a nylon membrane with a determined pore diameter. Again the saturation currents for each membrane type increase with the increase of the glucose concentrations exhibiting a Michaelis–Menten behaviour. In addition, it is possible to observe that

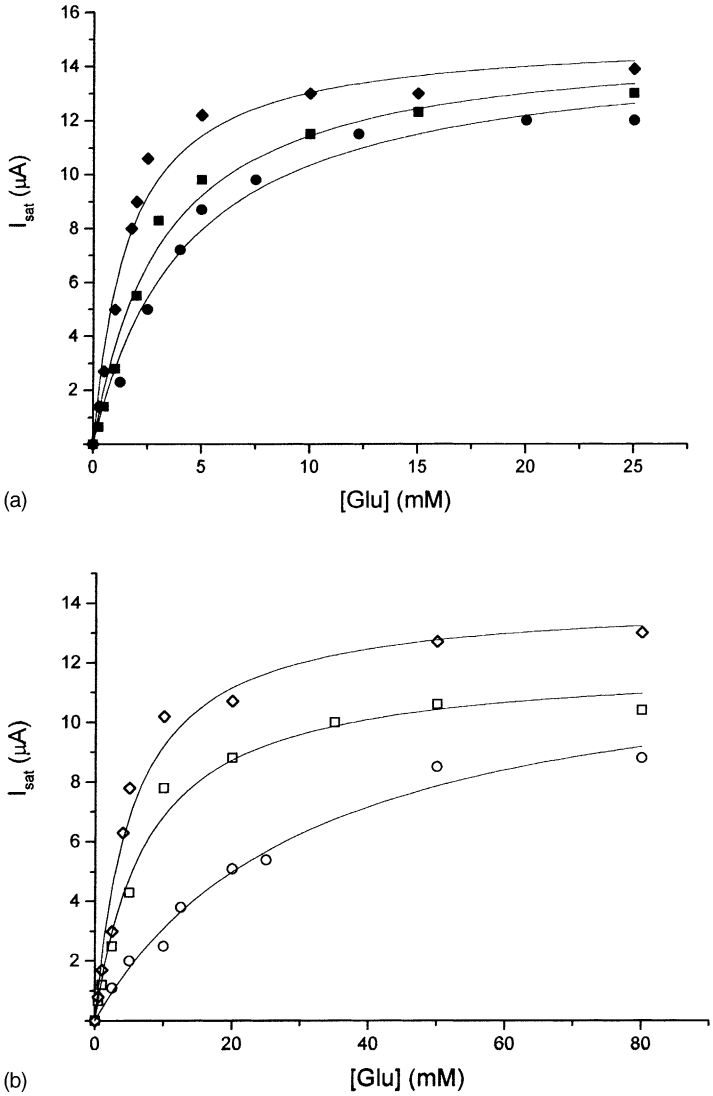
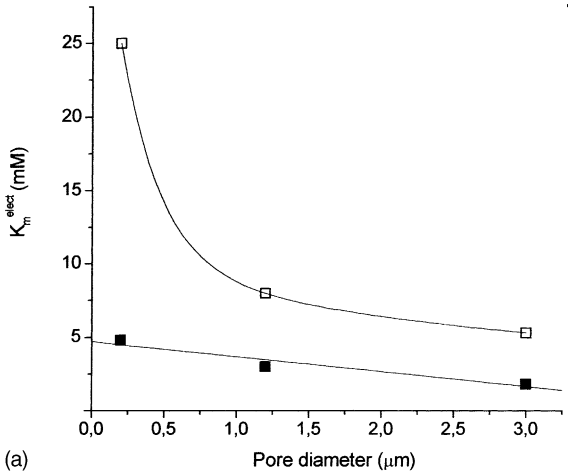


Fig. 8. Saturation currents as a function of the initial glucose concentration. (a) GMA_x membranes; (b) BMA_x membranes. Symbols: (●) membrane $GMA_{0.2}$; (■) membrane $GMA_{1.2}$; (◆)membrane $GMA_{3.0}$; (○) membrane $BMA_{0.2}$; (□) membrane $BMA_{1.2}$; (◇)membrane $BMA_{3.0}$.

at each glucose concentration the saturation currents increase with the increase of the membrane pore diameter.

When the electrochemical parameters of each of the six membranes reported in Table 2 are put in the graphical form as in Fig. 9a and b, interesting observations can be drawn. The electrochemical affinities of both membranes types Fig. 9a) increase with the increase of the pore diameter. In addition, at each pore diameter, membranes grafted with GMA show higher electrochemical affinity than membranes grafted with BMA. In Fig. 9b, the relative values of the K_m^{elect} are reported as a function of the pore diameter, by assigning 100% to the value of the membrane with pores of 0.2 μm. An exponential decrease of the K_m^{elect} is found for both membrane types, the percentage



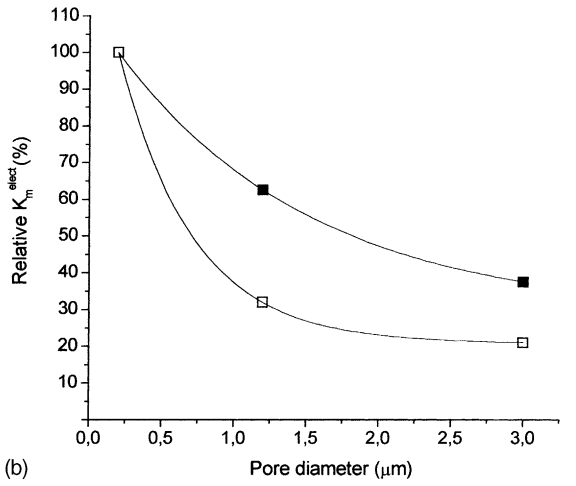
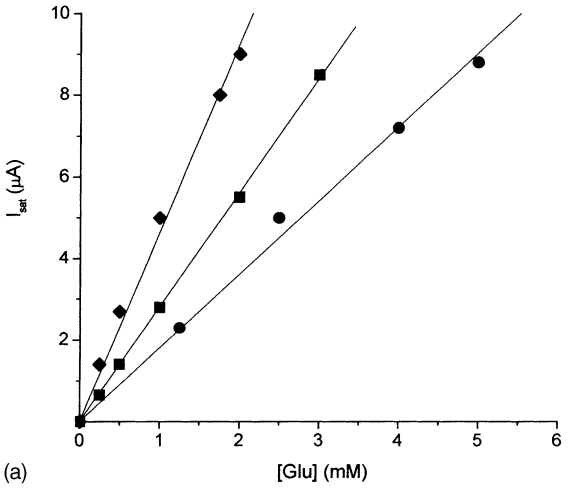


Fig. 9. (a) Apparent electrochemical constants as a function of pore diameter; (b) relative apparent electrochemical constants as a function of pore diameter. Symbols: (■) membranes GMA_x; (□) membranes BMA_x.



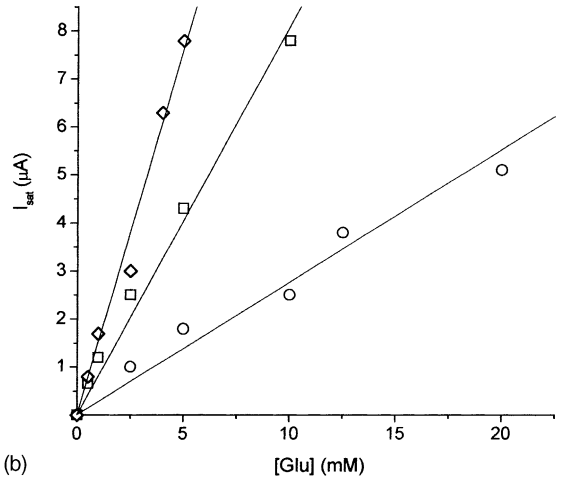


Fig. 10. Calibration curves in the linear range: saturation currents vs. glucose concentrations. Symbols: (●) membrane $GMA_{0.2}$; (■) membrane $GMA_{1.2}$; (◆) membrane $GMA_{3.0}$; (○) membrane $BMA_{0.2}$; (□) membrane $BMA_{1.2}$; (◇) membrane $BMA_{3.0}$.

decrease of the nylon-poly(BMA) membranes being higher than that found for the nylon-poly(GMA) membranes. Also the results in Fig. 9a and b can be explained by considering the more hydrophobic nature of BMA in respect to GMA. Analogous conclusions about the hydrophobic degree of BMA_x and GMA_x membranes can be deduced considering the values of the I_{max} The saturation currents increase with the increase of the electrochemical affinities, so that the I_{max} corresponding to the nylon-poly(GMA) membranes are higher than those of the nylon-poly(BMA) membranes.

With reference to the employment of our membranes in measurements of glucose concentrations in samples of different origin, it is interesting to focalize the attention to the calibration curves in which our biosensor gives linear responses. In Fig. 10a and b, the calibration curves relative to the nylon-poly(GMA) and to the nylon-poly(BMA) membranes are reported, respectively. Data in both figures show that: (i) the

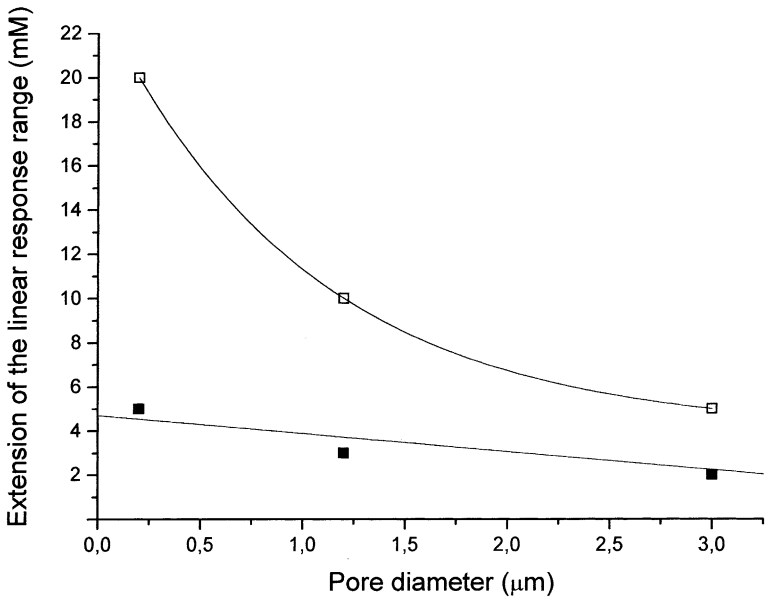


Fig. 11. Extension of the linear response ranges as a function of membrane pore diameter. Symbols: (■) membranes GMA_x; (□) membranes BMA_x.

ranges in which the biosensor exhibits linear responses for each membrane type decrease with the increase of their pore diameter; (ii) the membranes grafted with BMA exhibit linear ranges higher than those grafted with GMA when the same pore diameter is considered. In Fig. 11, the ranges of linear response for each of the two types of catalytic membranes are reported as a function of their pore diameter. As for the results relative to the apparent K_m^{elect}, the ranges of linear response of the catalytic membranes grafted with BMA exponentially decrease with the pore diameter, while those relative to the catalytic membranes grafted with GMA show an apparent linearly decrease. The similarity of the results reported in Figs. 9a and 11 confirm the working hypothesis that a common physical cause is responsible for the two behaviours, this cause being the greater hydrophobic of the BMA monomer in respect to that of GMA.

Coming back to Fig. 10a and b, the calibration curves of both membranes are interpolated by linear regression equations of the type $y = ax$, where y is the saturation current, measured in μA, and x is the glucose concentration, measured in mM. The "a" value, measured in μA/mM, represents the sensitivity of the biosensor. The "a" values for each of the six catalytic membranes are listed in Table 3. In Table 3, the average response times of the biosensor in the linear range are also reported for each of the six membranes used

Table 3
Biosensor electrical characteristics

Catalytic membrane composition	Membrane pore diameter (μm)	Sensitivity (μA/mM)	Average response time (s)
Nylon-poly(GMA)	0.2	1.85	38
	1.2	2.80	32
	3.0	4.50	30
Nylon-poly(BMA)	0.2	0.26	95
	1.2	0.75	65
	3.0	1.60	45

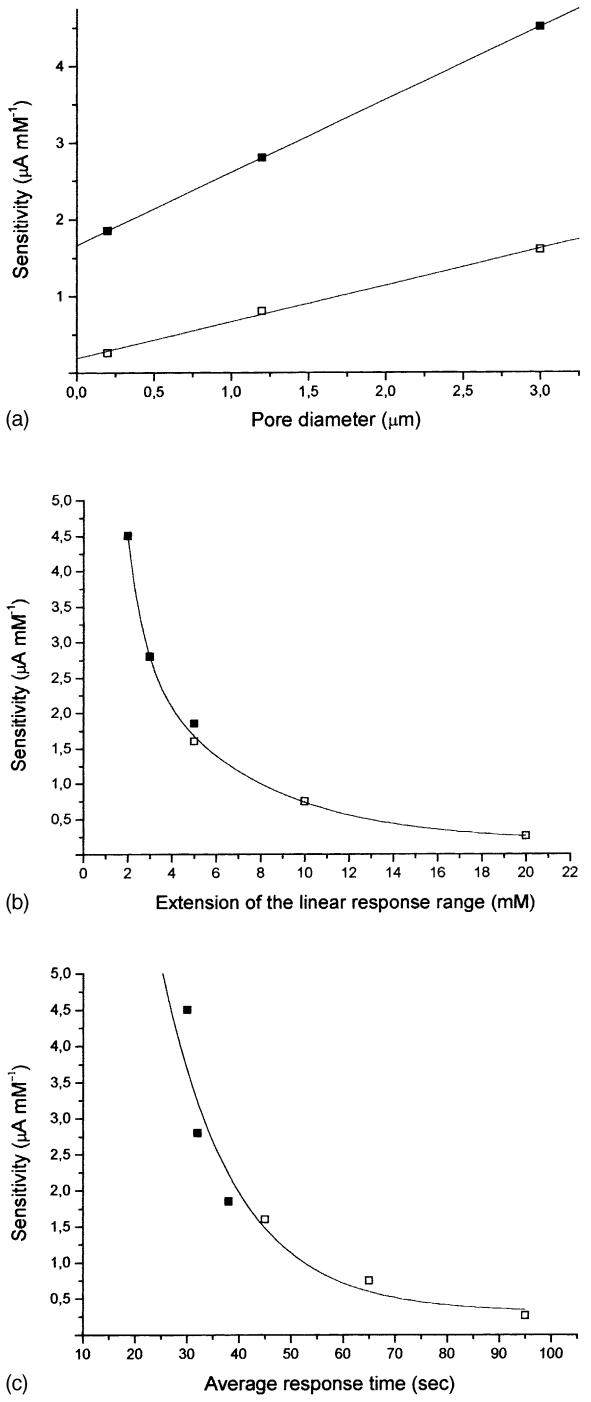


Fig. 12. Biosensor sensitivity as a function of: (a) membrane pore diameter; (b) linear response range; (c) the average response time. Symbols: (■) membranes GMA_x; (□) membranes BMA_x.

in this research. Average response times have been obtained by averaging the response times obtained for each of the concentrations explored in the linear range.

In Fig. 12a, the values of the biosensor sensitivity are reported as a function of the pore diameter of each of the two catalytic membrane types. Results in Fig. 12a show the linear increase of the biosensor sensitivity with the increase of the membrane pore diameter. Of course this linearity is the result of the circumstance that we have prepared membranes endowed with the same grafting percentage. We have demonstrated [20], indeed, that some physical properties of the grafted membranes, such as hydraulic or thermo-osmotic permeabilities, are strongly affected by the grafting degree.

4. Conclusions

Form all the results reported above it is possible to derive some interesting conclusions on the characteristics of our membranes and their behaviour in our biosensor.

Each membrane type exhibits as a function of the pore diameter: (a) a decrease of the linear response range; (b) an increase of the sensitivity; (c) a reduction of the average response time. In addition, the biosensor sensitivity decreases with the increase of the linear range (Fig. 12b) and with the increase of the average response time (Fig. 12c), while the latter parameter increases with the increase of the linear response range (Fig. 13). In each of the last three figures there is a good experimental link between the results obtained with the catalytic membranes grafted with GMA and those grafted with BMA. These results indicate that it is possible to modulate the diffusive transport of substrate and reaction products across a catalytic membrane either by changing the diameter of its pores or by grafting monomers with different hydrophobic degree, when a membrane with a determined pore diameter is used. In our case a membrane with large pore diameter (3 μm) and grafted with a monomer endowed with high hydrophobicity (BMA) is equivalent, in respect to the electrical response (and hence in respect to the diffusive behaviour), to a membrane with small pore diameter (0.2 μm) and grafted with a monomer endowed with small hydrophobicity degree (GMA).

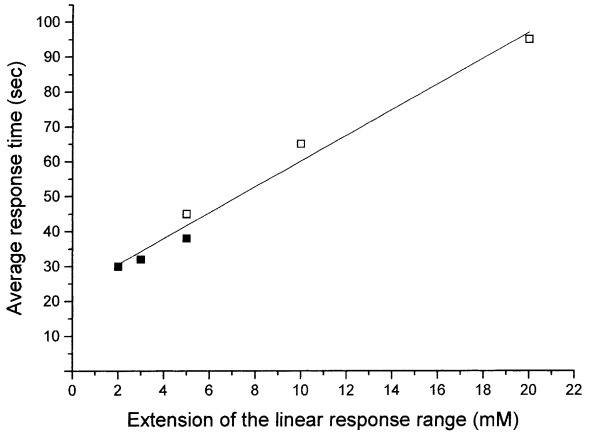


Fig. 13. Average response time as a function of the extension of linear response range. Symbols: (■) membranes GMA_x, (□) membranes BMA_x.

Membranes grafted with BMA are useful when the biosensor is employed in a large glucose concentration range (up to 20 μM), even if the sensitivity results low and the average response time consequently great. On the contrary, membranes grafted with GMA can be usefully employed in a small glucose concentration range (up to 5 μM), but they offer high sensitivity and small average response time.

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