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Glucose Sensors Based on Enzyme Immobilization onto Biocompatible Membranes Obtained by Radiation-Induced Polymerization

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ABSTRACT

Amperometric glucose biosensors based on glucose oxidase immobilized onto poly(2-hydroxyethylmethacrylate) membranes obtained by γ radiation-induced polymerization were constructed. In a three-electrode configuration, smooth or platinized platinum electrodes with different shapes were used, in order to detect the amount of hydrogen peroxide produced in the glucose oxidation. A saturated calomel electrode and a platinum foil were used as a reference and counterelectrode, respectively. The biocompatible obtained sensors were characterized as regards the temperature effect, the response, and lifetime. The determination of glucose in standard solutions was carried out, and linear calibration curves were obtained. Depending on the electrode configuration, the sensor had a response time of 1–4 min, and the measuring range extended from 5×10^{-5} to $4 \times 10^{-3} M$.

Index Entries: Glucose sensor; radiation-induced polymerization; enzyme electrode; poly(2-hydroxyethylmethacrylate); glucose oxidase.

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INTRODUCTION

The development of amperometric glucose sensors continues to receive considerable attention. It is well known that glucose oxidase (GOD) catalyzes the oxidation of glucose to gluconolactone, and this property is utilized in the construction of immobilized enzyme electrodes. The immobilization is an important key in determining the characteristics of the glucose sensors.

Recently (*1*) we developed a new method of physically immobilizing GOD in poly(2-hydroxyethylmethacrylate) (HEMA) membranes, and we prepared a glucose sensor based on an oxygen Clark electrode. The immobilization procedure used was based on low-temperature g radiation-induced polymerization of an aqueous solution of the enzyme mixed with glass-forming acrylic or methacrylic esters (*2–5*). The main characteristic of the polymer matrix obtained by this method is its porous structure, which arises owing to the ice dispersed in the cooled monomer. The enzyme appears to be physically entrapped on the walls of the pores. With this immobilization technique, there is no need for polymerization initiators or organic solvents.

On account of the possibility of obtaining membranes of different shapes and with good mechanical characteristics, the purpose of the present work was to construct some kinds of glucose sensors having small dimensions, fast responsiveness, accuracy, long lifetime, and biocompatibility.

The electrode response was obtained from the amperometric detection of hydrogen peroxide, which is a product of glucose oxidation by the enzyme in the presence of oxygen. A considerable effort has been expended in the study of this type of detection, including the use of smooth platinum (*5*), platinized platinum (*7–9*), and platinized carbon electrode (*10–12*).

The use of g irradiation to obtain suitable polymers for the purpose of modifying electrodes has been reported by Galiatsatos et al. (*13*) and Hajizadeh et al. (*14*). These authors have shown that glucose oxidase and lactate oxidase can be immobilized in a poly(vinylalcohol) matrix on a platinized graphite electrode, obtaining an enzyme layer sandwiched between two polymer layers.

Here we describe the fabrication of three types of electrodes:

1. Flat smooth platinum;
2. Smooth platinum wire; and
3. Platinized platinum wire.

The response characteristics of the resulting biosensors are discussed.

EXPERIMENTAL

Chemicals

Glucose oxidase (EC 1.1.3.4, type II-S from *Aspergillus niger*, 18,000 U/g solid), peroxidase (POD, EC 1.11.1.7, type VI from horseradish, 250

purpurogallin units/mg solid), *o*-anisidina dihydrochloride, and β-D-glucose were obtained from Sigma Chemical (St. Louis, MO). Stock solutions were prepared in bidistilled water or buffer solution, and stored in the dark at 4°C. Glucose solutions were allowed to mutarotate overnight at room temperature before use.

2-Hydroxyethylmethacrylate, obtained from Aldrich Chemie (Stenheim, Germany) was purified in the following way. An $NaHCO_3$-saturated solution was added to HEMA in the 1:1 ratio (v/v), then continuously stirred for 20 h, and subsequently salted out with NaCl. Methacrylic acid-free HEMA was thus separated from the aqueous phase, dried with anhydrous Na_2SO_4, and fractionally distilled at reduced pressure under N_2 flushing.

Trimethylol-propane trimethacrylate (TMPTM), as crosslinking coagent, from Janssen (Beerse, Belgium) was used as received. Nafion perfluorinated ion-exchange resin (5% w/v in a mixture of lower aliphatic alcohols and 10% water), from Aldrich Chemie, was used as supplied. All other analytical-grade chemicals were purchased from Carlo Erba (Milano, Italy) or Merck (Darmstadt, Germany).

GOD Immobilization

GOD was immobilized according to the procedure described elsewhere (*1*). A deareate solution of HEMA (polymer/water ratio 1:1, w/w) and GOD (4 mg/g polymerization mixture) in 0.1*M* phosphate buffer at pH 6.5 was irradiated at −78°C with a dose of 5.4 kgy by exposure to a ^{60}Co γ-ray source (dose rate: 1.8 kgy/h). TMPTM had been added to the mixture in the proportion of 4% of amount of monomer. With this procedure, a sponge-like material is obtained with the enzyme entrapped in the small cavities produced by the ice crystals during freezing of the solution.

For the preparation of the membranes to be applied to flat electrode, the matrix was then sliced into disks of about 200-μm thickness with a microtome; the disks were carefully washed five times with a phosphate buffer (25 mL, pH 7) for 24 h.

In order to prepare the wire electrode, the mixture of HEMA and GOD was dropped into a cavity of the electrode (*see* Preparation of Electrodes section) and then polymerized directly by exposure to the γ-ray source at −78°C.

Measurements of Enzyme Activity

GOD activity was determined according to the procedure reported in the Worthington Manual (*15*). The assay mixture consisted of 2.5 mL *o*-dianisidine-buffer solution (0.26 m*M* in 0.1*M* phosphate buffer, pH 6), 0.3 mL β-D-glucose (18% w/v), and 0.1 mL peroxidase (50 purpurogallin U/mL). The whole mixture was incubated at 25°C for 5 min in a spectrophotometer, and the reaction was initiated by the addition of 10 μL GOD solution (0.1 mg/mL).

The absorbance at 460 nm was determined spectrophotometrically and converted into enzyme activity by means of reaction kinetics. In order to determine the activity of the immobilized enzyme, a membrane was placed in a glucose solution (0.3 mL of 1*M* glucose + 2.5 mL of phosphate buffer at pH 6) and incubated at 25°C with shaking for 2 min. The piece was then removed, and this solution was combined with the activity assay mixture containing *o*-dianisidine and POD. The activity was determined from the absorbance at 460 nm through the use of a calibration curve.

Apparatus

Batch experiments were performed with an electrochemical cell that had a working volume of 20 mL in a three-electrode configuration. The three electrodes were connected to an AMEL (Milano, Italy) model 559 potentiostat. A saturated calomel electrode and a platinum foil with large area were used as the reference and counter electrode, respectively. A smooth or platinized platinum wire was used as a working electrode. All experiments were carried out in a temperature-controlled cell by using a Haake F3-C thermostatic bath.

Spectrophotometric measurements were carried out with a Hitachi U-3200 spectrophotometer equipped with a 1-cm quartz thermostated cell. The oxygen concentration in the solution was measured with an Orion (Boston, USA) model 97-08 oxygen electrode.

Preparation of Electrodes

As shown in Fig. 1, three types of electrodes were prepared:

1. Flat platinum electrode;
2. Smooth platinum; and
3. Platinized platinum wire covered with silicone tube.

Electrode A

Platinum wire (0.5-mm diameter) was sealed in a glass tube and then polished with alumina powder to prepare its flat surface. Two microliters of Nafion solution were dropped on the surface and then dried at room temperature for 30 min so as to cover the platinum. Then an HEMA membrane disk (5-mm diameter and about 200-μm thickness) containing the immobilized GOD was placed on the flat electrode surface and fixed with a Teflon™ cap having a 3-mm hole. The fixing system and the resistance of the membrane allowed its easy replacement, and also the possibility of using it again.

Electrode B

A piece of platinum wire (1-mm diameter and 3 cm long) soldered to a copper wire was polished with a fine emery paper and then insulated with silicone tubing. The tubing was circularly cut 2 mm from the tip and

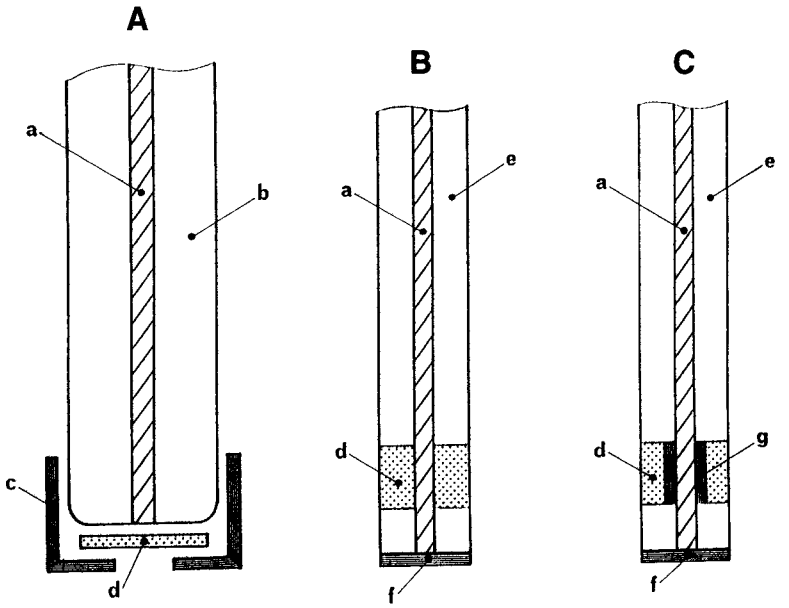

Fig. 1. Schematic design of sensors. A, Flat platinum; B, smooth platinum wire; C, platinized platinum wire. **a**, Platinum; **b**, glass; **c**, Teflon cap; **d**, membrane; **e**, silicone tubing; **f**, araldite; **g**, platinum black.

then stripped to form a cavity 1 mm wide. Two microliters of Nafion were dropped into the cavity and dried at room temperature. Successively a mixture of HEMA and GOD was dropped into the same cavity over the Nafion film. The electrode was exposed to the γ irradiation at $-78°C$ in order to induce the polymerization and cover the platinum surface. The electrode was washed three times with a phosphate buffer (5 ml, pH 7) for 24 h.

Electrode C

This was prepared in the same way as electrode B, but the exposed platinum surface was preliminarly platinized by reducing hexachloroplatinic acid (3% w/v) in the presence of lead acetate with an applied current of 3 mA for 3 min at room temperature.

The membrane thicknesses of electrodes B and C were estimated as being about 300 μm. When not in use, the membranes and the electrodes were stored at 4°C in 0.1*M* phosphate buffer at pH 7.

Determination of Glucose

The determination of glucose was performed with three-electrode system by applying a polarization voltage of +600 mV to the smooth platinum electrode and +330 mV to the platinized platinum electrode against a saturated calomel electrode. The resulting current was measured with an amperometer. All measurements were carried out in a 0.1*M*

phosphate buffer at pH 7 in a thermostated cell at 25°C. Calibration of the electrodes was carried out following standard additions of glucose solutions to 20 mL buffer solution.

RESULTS AND DISCUSSION

The membranes obtained as described in the Experimental section show good mechanical properties and, in the case of the type A electrode, can be assembled or detached many times without any decrease in the enzyme performance.

In order to determine the amount of activity truly fixed in the matrix, several pieces of the polymer were rinsed with phosphate buffer (25 mL, pH 6) for 24 h, and then the washing solution was assayed for the enzyme activity. This process was repeated five times, using the same sample. After the fifth washing, the matrix also was assayed for enzyme activity. The retained activity was found to be about 70% of the total introduced, but the greater part of it passed into solution with the first two washings. Only 5–6% was really fixed permanently. A reduction of about 30% of the original activity was attributed to the damages caused by the highly energetic radiation. In fact, aqueous solutions of the enzyme exposed to γ radiation under the same immobilization conditions showed a decrease in enzyme activity of about 35–40%.

The activity of immobilized GOD in the membranes measured spectrophotometrically was found to be 15 nmol/min corresponding to about 70 nmol/min/cm^2. The activity fixed in the membrane attached to the wire electrode was found to be about 4–6 nmol/min.

The specific responses of the examined electrodes expressed in current per surface area were: A = 25 μA/cm^2, B = 15 μA/cm^2, C = 30 μA/cm^2, for a glucose concentration of 2 mmol/L.

These values are comparable to those for glucose electrodes described by Cronenberg et al. (*9*) and Galiatsatos et al. (*13*), but lower than those obtained by Ikariyama et al. (*7*).

It is very interesting to note that, with the platinized platinum electrode, it was possible to work at an applied potential lower than with smooth platinum, because the platinum black catalyzes the oxidation of hydrogen peroxide. Such catalytic property, chiefly because of its very porous surface, has been observed by other authors with platinized graphite (*13,14*) and also with rhodium electrodeposited onto carbon fiber (*16*). The lowering of the operating potential (+330 mV) in comparison to unplatinized platinum electrodes (+600 mV) is advantageous because the sensor response is less susceptible to electrochemical interferences.

In fact, ascorbic and uric acid, two common interferents in biological samples, interfere with hydrogen peroxide detection on platinum electrode at an applied potential of +600 mV. The oxidation potential of these compounds shows a little shift toward negative values on the

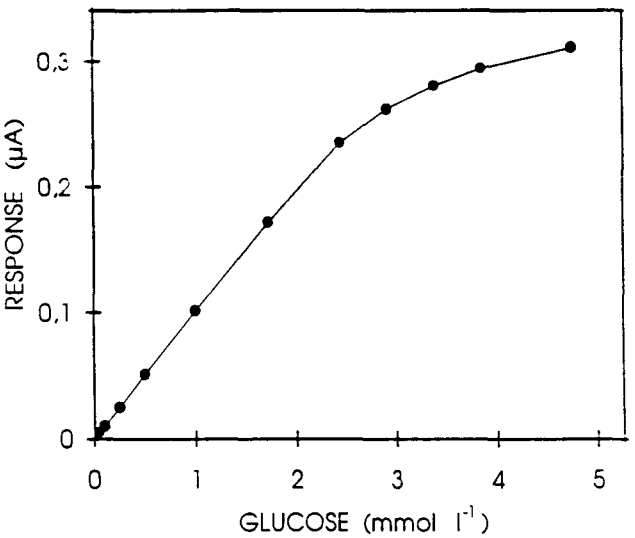

Fig. 2. Calibration graph for sensor A, obtained at pH 7 and 25°C.

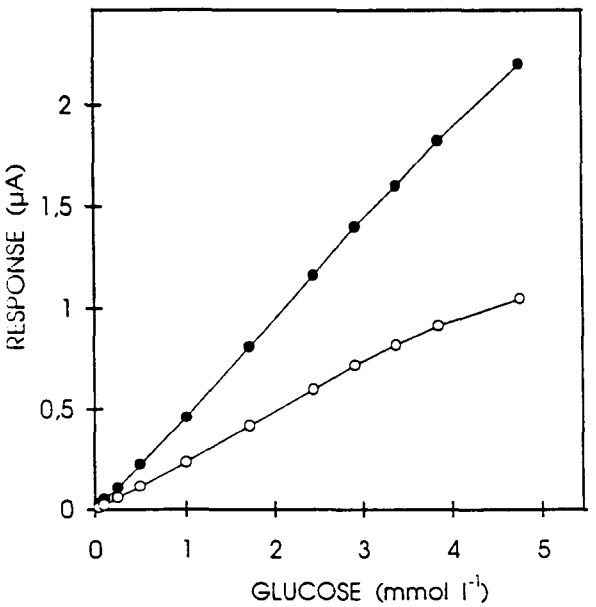

Fig. 3. Calibration graph for sensors (○) B and (●) C, obtained at pH 7 and 25°C.

platinized surface, but at +330 mV, uric acid does not interfere, whereas ascorbic acid can interfere because it is oxidized at potentials lower than +330 mV on both the platinized and unplatinized platinum electrodes.

After the electrodes were covered with the Nafion film, the responses to ascorbate (0.28 m*M*) and urate (0.15 m*M*) were reduced to zero.

Typical calibration curves for the sensors obtained at 25°C and pH 7 are presented in Figs 2 and 3. The optimum pH value had been previously found at 6.5 (*1*).

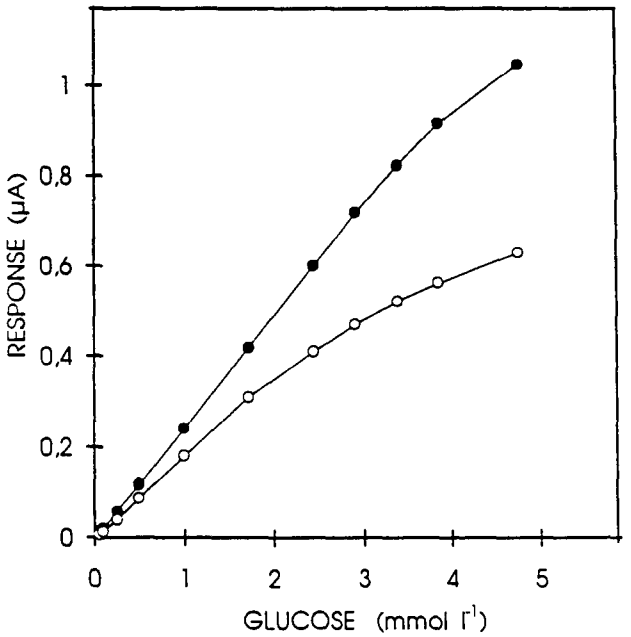

Fig. 4. Response of sensor B at different oxygen concentrations at pH 7 and 25°C. (●) 0.25 m*M*; (○) 0.021 m*M*.

Figure 2 shows the response of the A electrode (flat platinum). The linear range was verified between 50 μM and 2.5 m*M*. In this range, the linear regression equation is $y = 10.31\,x - 0.015$, $R^2 = 0.9994$, where y is the electrode response in μA and x is the glucose concentration in mmol/L.

Figure 3 shows the response of the B and C wire electrodes. The linearity range was verified between 50 μM and 3.5 m*M* for the B sensor ($y = 4.06\,x + 0.015$, $R^2 = 0.9998$) and between 50 μM and 3.85 m*M* for the C sensor ($y = 2.10\,x + 0.010$, $R^2 = 0.9998$). The average detection limit was 25 μM. Measurements repeated five times with the same sample containing 0.5 mmol/L of the substrate showed a relative SD < 3% for each sensor.

The loss of linearity at higher concentrations was probably the result of oxygen limitation and also perhaps the inhibiting effect of the high hydrogen peroxide level on the enzyme activity. This was a temporary effect, because the electrodes did not show a permanent loss of sensitivity when operated for some time at the considered concentration of glucose.

The concentration of dissolved oxygen in the solution strongly influenced the calibration as can be seen from Fig. 4. The linear range and sensitivity were decreased considerably after bubbling pure nitrogen through the solution.

The response curves of the sensors at several temperatures are shown in Figs. 5, 6, and 7. The response increased gradually until 50°C, whereas the optimum temperature for the free enzyme is 35°C. This result suggests that the immobilization process increased the thermostability of glucose oxidase.

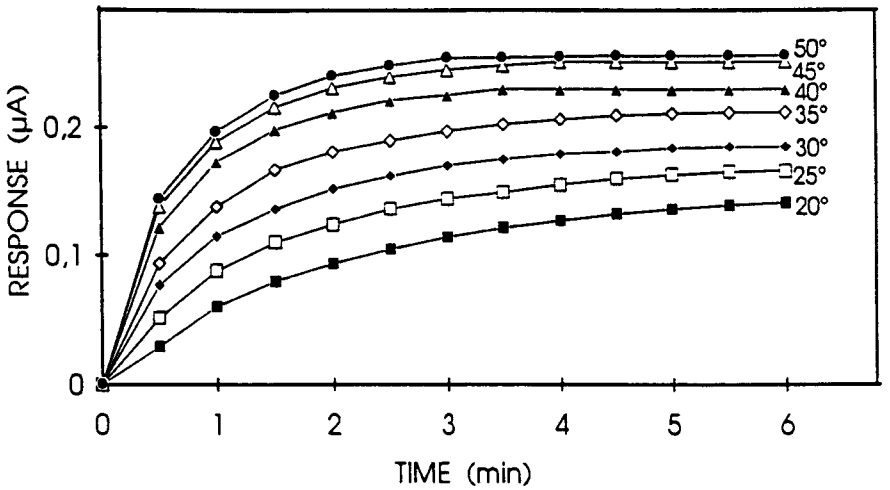

Fig. 5. Response time of sensor A under various temperatures at pH 7. Glucose concentration: 1.7 m*M*.

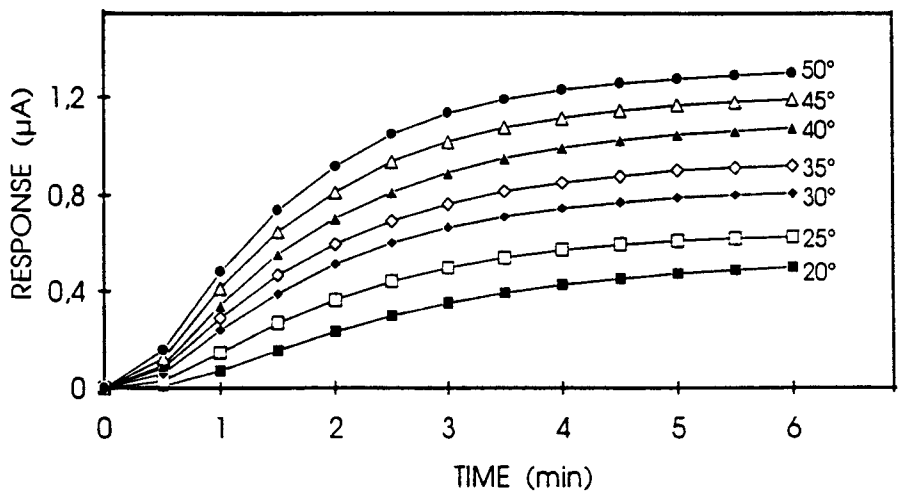

Fig. 6. Response time of sensor B under various temperatures at pH 7. Glucose concentration: 2.4 m*M*.

The same figures also show the response time of the sensors. For the A electrode the time required to reach a 95% response at 40°C was <2 min, but at 20°C was about 4 min. The response time of the B sensor was about 5 min and was greatly influenced by the temperature. The response time of the platinized platinum electrode was <1 min and was not significantly temperature-dependent.

The difference in the response times between the A and B electrodes was ascribed to the different thicknesses of the membranes, whereas the fast response of the C electrode was the result of the catalytic effect of platinum black. The value obtained for this last electrode was comparable with that reported by Galiatsatos et al. (*13*).

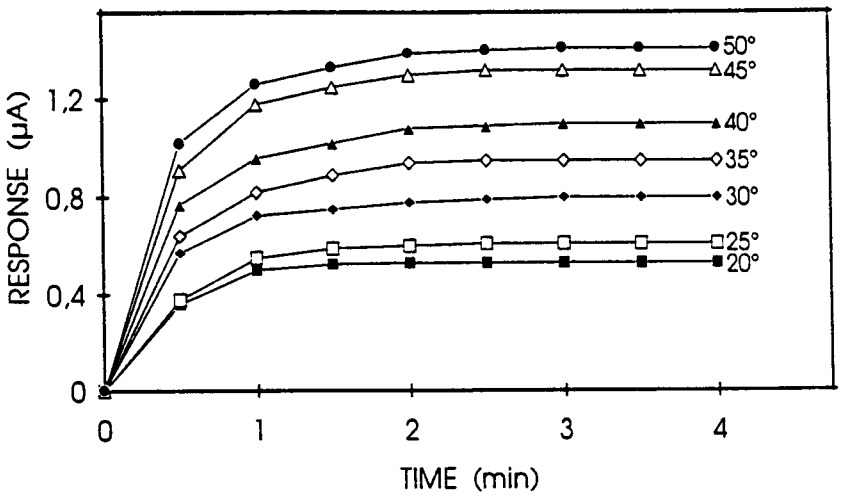

Fig. 7. Response time of sensor C under various temperatures at pH 7. Glucose concentration: 0.12 m*M*.

The greater thickness of the membrane increases the upper limit of the linear range because the diffusion of substrate is restricted to the underlying enzyme layer, but causes a slower response time. Electrodes obtained with membranes prepared by exposure to different levels of γ irradiation in the 5.4–20 kgy range did not show significant changes in the response to glucose.

Generally, enzymes can undergo rapid deactivation under the same irradiation conditions in aqueous solutions and at room temperature. In this case, GOD was stabilized by immobilization in HEMA matrix. This effect can be ascribed to the free radical scavenging property of the polymer (*14*) and to a higher radioresistance of the enzyme at low temperature (*5*).

Figure 8 reports the response of the sensors with respect to the time after immobilization step. It can be observed that about 1 mo after the immobilization, the obtained response is decreased by about 25% for the A and about 30% for the B and C electrodes compared with the initial value. Under normal operating conditions, mechanical limitations of the membrane were not observed, and the sensors could be used repeatedly for long time without any decrease in the enzyme performance.

CONCLUSIONS

This work suggests that HEMA membranes, obtained by γ radiation-induced polymerization, are a good method for enzyme immobilization on electrode surfaces. This method offers several advantages, including the immobilization achieved without the use of organic solvents and chemical initiators, and the good mechanical properties of the membranes.

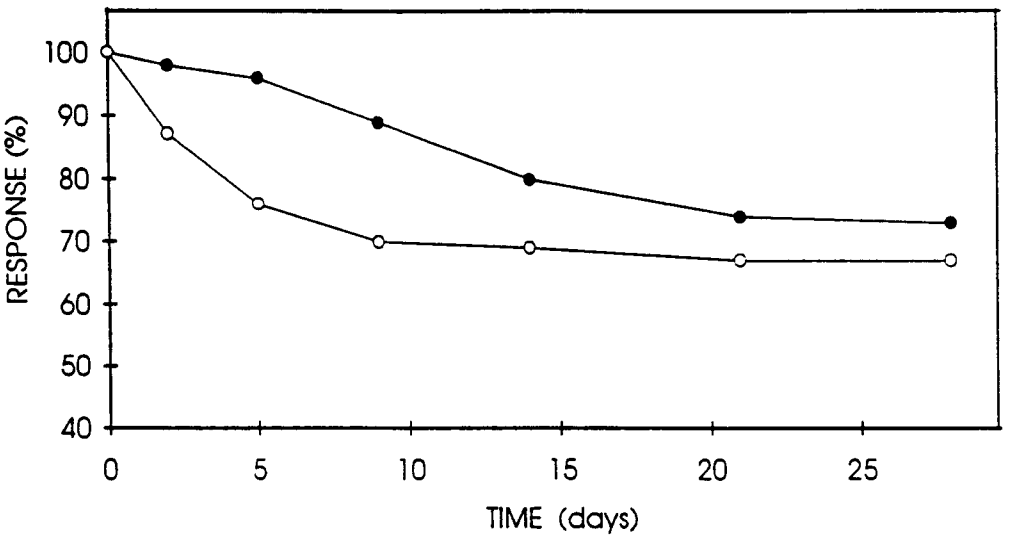

Fig. 8. Lifetime of (●) sensor A and (○) sensors B and C under normal operating conditions; enzymatic sensors stored in phosphate buffer at pH 7 and 4°C after each series of measurements.

The response and lifetime of the constructed biosensors are comparable with the values obtained for other enzymatic amperometric sensors.

This technique also permits sensors of different shapes to be obtained that are biocompatible and resistant to higher temperatures. In particular, owing to the polymerization method used, it seems possible to construct enzyme electrodes with small size and rapid response time.

Linear range, correlation coefficient and accuracy are very good. Moreover, the addition of a Nafion film and the low applied potential at the platinized platinum electrode reduce the interferences owing to the negatively charged compounds, such as ascorbate and urate.

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