A Glucose Oxidase Immobilized Electrode Based on Modified Graphite

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Z. Naturforsch. **57c,** 705–711 (2002); received March 25/May 6, 2002

Glucose Oxidase, Enzyme Electrode, Hydrogen Peroxide Electroreduction

Glucose oxidase (E. C. 1.1.3.4) was immobilized on electrochemically modified graphite to obtain an enzyme electrode. The working surface of the electrode was coated with gelatine to prevent desorption of the enzyme. In substrate (glucose) solutions the amperometric signal of the enzyme electrode was due to the electroreduction of H_2O_2 generated in the enzyme layer. The linearity of the electrode response was found up to a substrate concentration of 300 μM at a working potential of 0 mV (vs. Ag/AgCl). It was shown that the electrode did not respond to L-ascorbic and uric acid at that working potential. The response time was about 2 min. The enzyme electrode keeps about 50% of its initial activity after a one-week storage at 4 °C.

Introduction

The interest towards glucose oxidase (GOD) – a flavoenzyme, is determined by its use in development of electrochemical biosensors for blood or urine glucose monitoring. The enzyme shows high substrate specificity for β-D-glucose oxidation with molecular oxygen:

$$\beta\text{D-glucose} + \text{GOD–FAD} \rightleftharpoons \text{GOD-FAD}\cdot H_2 + \beta\text{D-gluconolactone}$$

$$\text{GOD-FAD}\cdot H_2 \rightarrow \text{GOD-FAD} + H_2O_2$$

Various methods for development of glucose biosensors have been described, with a common detection principle based on the registration of electrical signal generated by:

i) direct electron transfer (DET) from the enzyme active site to the electrode surface in presence of glucose. Although a DET process was observed in presence of glucose (Alvarez-Icaza and Schmid, 1994) the authors did not report application of this phenomenon for glucose biosensor development;
ii) electron exchange between the electrode surface and the enzyme mediated by shuttles of electrons (mediators). This approach is based on replacement of oxygen, the natural acceptor, by artificial electron acceptors such as ferrocenes (Liu *et al*., 1998; Vaillancourt *et al*., 1999; Pandey and Upadhyay, 2001), heterocyclic dihydropolyazines (Kulys *et al*., 1998), potassium ferricyanide (Ge *et al*., 1998), osmium complexes (Reiter *et al*., 2001), conducting organic salts (Lowry and O'Neill, 1992; Centonze *et al*., 1997) or polymers (Cosnier *et al*., 1999).
iii) electrochemical transformation of hydrogen peroxide generated by the enzyme-catalyzed oxidation of glucose. Hydrogen peroxide formed can be either oxidized on the electrode surface at anodic potentials (Koshy *et al*., 1993; Furbee *et al*., 1994; Yang *et al*., 1998; Celej and Rivas, 1998) or reduced electrochemically (Jonsson-Pettersson, 1991; Celej and Rivas, 1998; Cosnier *et al*., 1999; Cosnier *et al*., 2000).

The main drawback of the glucose biosensors based on H_2O_2 electrooxidation is the high potential applied at the working electrode, which makes such sensors responsive to interfering substances. To avoid this disadvantages either elimination of electrochemically-active interferents by pre-electrolysis of the sample solutions (Koshy *et al*., 1993) were used or lowering of the potential applied by electrode modification (Furbee *et al*., 1994; Yang *et al*., 1998; Celej and Rivas, 1998).

The most selective biosensing of glucose was achieved using enzyme electrodes based on mediators or H_2O_2 electroreduction. In both cases the electrochemical process takes place at low potentials applied (at potentials near 0 V for the hydrogen peroxide reduction), where a large variety of substances and typical components of biological fluids (such as L-ascorbic acid, uric acid, glutathi-

one, physiological levels of neurotransmitters and their metabolites, etc.) exhibit no electrochemical activity whatsoever. Therefore, all described glucose biosensors are characterized with continuous labor-consuming procedures for electrode preparation.

An efficient and relatively simple procedure was developed by Horozova and co-workers for graphite electrochemical modification with microquantities of platinum-group metals (Horozova *et al.*, 1997). The modified graphite electrodes exhibit catalytic activity on H_2O_2 electroreduction and show long-time stability (over one year). The present study deals with the development and characterization of a GOD-immobilized enzyme electrode based on a modified graphite electrode using its catalytic activity on hydrogen peroxide electroreduction.

Experimental

Materials

Glucose oxidase (GOD) (E. C. 1.1.3.4) – from *Aspergillus niger* (ZMP, Peshtera, Bulgaria), with activity of $50\ U \times mg^{-1}$ (1 U corresponds to the amount of the enzyme which oxidizes 1 μmol glucose per min at pH = 7.0 and 25° C); β-D-glucose (Valerus, Sofia, Bulgaria). Hydrogen peroxide and chemicals used for preparing buffer solutions: $Na_2HPO_4 \times 12H_2O$, citric acid, KOH, H_3PO_4, were purchased from Fluka. All solutions were prepared with double distilled water. The β-D-glucose solution (5×10^{-3} M in phosphate-citrate buffer, pH = 7.0) was allowed to mutarotate for 24 h before use.

Inert pads of graphite "GMZ"™ with geometric surface S = 1.6–1.8 cm^2 ($0.7 \times 0.7 \times 0.3$ cm) were used. The structural characteristics of graphite are as follows: specific surface $0.8\ cm^2 \times g^{-1}$, density $1.56–1.70\ g \times cm^3$ and porosity 20–25%. The graphite pads were kindly provided by Prof. Bogdanovskiy, State University of Moscow, Russia.

Preparation of the electrodes

The enzyme electrode was prepared on the basis of a modified graphite electrode which catalyses hydrogen peroxide electroreduction. The graphite pads were modified with microquantities of (Pt + Pd). The catalytically active components were deposited in a potentiostatic regime ($E_r^{deposit}$ = +0.05 V vs. reversible hydrogen electrode) by a brief electrolysis ($t_{deposit}$ = 10 s) from the following electrolyte: 2% $PtCl_6 \times 6H_2O$ + 2% $PdCl_2$ + 0.1 M HCl in the ratio (Pt+Pd) (10:90%) (Horozova *et al.*, 1997).

Enzyme immobilization

Three different procedures for GOD immobilization on modified graphite electrode were investigated:

A. a 50 µl drop of GOD solution ($c = 50\ mg \times ml^{-1}$) was deposited onto the electrode surface and was allowed to dry at room temperature. Then the working surface was covered with two layers of gelatine (50 mg gelatine dissolved in 1 ml phosphate-citrate buffer, pH = 7.0 to obtain a 5% solution);
B. two layers of glucose oxidase suspended in gelatine (50 mg GOD in 1 ml 5%-gelatine solution at 37° C) were applied onto electrode surface and dried in argon; and
C. GOD adsorbed on an electrochemically activated modified graphite electrode and then covered with two layers of GOD suspension in gelatine (50 mg GOD in 1 ml 5%-gelatine solution at 37° C).

The electrochemical pretreatment of the modified graphite electrode was a cathode-anode cyclization (30 min) within the potential range of – 0.58 – +0.35 V (vs. Ag/AgCl). Just before immobilization, the graphite electrode was polarized for 2 min at E = 1.5 V. The adsorption of GOD was carried out under static conditions by immersing the graphite electrode in the enzyme solution with a concentration of $50\ mg \times ml^{-1}$, in phosphate-citrate buffer (pH = 7.0) for 24 h at 4 °C. After adsorption the electrode was dried in the air, at room temperature, for about 45 min. Then the working surface of the prepared electrode was coated with 2 layers of 5% gelatine solution containing GOD (using a capillary glass tube). After applying each layer, the electrode surface was dried with argon.

After completing the measurements the enzyme electrodes were carefully washed with bidistilled water, dried in the air at room temperature for about 30 min and then stored in a refrigerator at 4° C until measurement. When necessary the immobilized enzyme could be removed from the

electrode surface by treating of the electrode for ~20 min in hot double distilled water (50–60° C) regenerating the bare modified graphite electrodes. The processed electrode material can be stored for more than one year in bidistilled water (at room temperature) and used repeatedly.

Apparatus and measurements

All electrochemical measurements were performed in a three-electrode cell with separated anode and cathode compartments. An Ag/AgCl electrode was used as a reference electrode, and platinum wire as a counter electrode. The electrochemical setup also involved a bipotentiostat, type BiPAD (TACUSSEL, Villeurbanne, France); a generator, type EG-20 (Elpan, Lubawa, Poland); a digital voltmeter, type 1AB105 (ZPU, Pravets, Bulgaria). The solutions were bubbled with argon during the measurements. The biosensor was characterized by the polarization curves' method in potentiostatic regime (phosphate-citrate buffer pH = 7.0).

The experimental data were obtained by consecutive addition of portions of 5×10^{-3} M glucose solution to the phosphate buffer in the cell with simultaneous registration of the current. The time to reach a steady-state value of the current did not exceed 2 min.

For maintaining constant temperature a thermostat UH (VEB MLW Prüfgeräte-Werk, Medingen, Germany) was used. A pH-meter OP-208 (Radelkis, Budapest, Hungary) was used for the buffer solutions preparation.

Results and Discussion

Modified graphite electrode as a catalyst of hydrogen peroxide electroreduction

The polarization curves in presence of H_2O_2 and interfering substrates such as ascorbic acid, uric acid and glutathione within the range from –140 to 400 mV (vs. Ag/AgCl) are presented by Fig. 1. Cathode current of hydrogen peroxide electroreduction (curve 1) is observed up to 310 mV and reached a constant value between 0 to 200 mV. Within the potential range of hydrogen peroxide electroreduction (from –140 to 310 mV) the interfering agents produce minimal anode currents (up to 16 μA; curves 2–4).

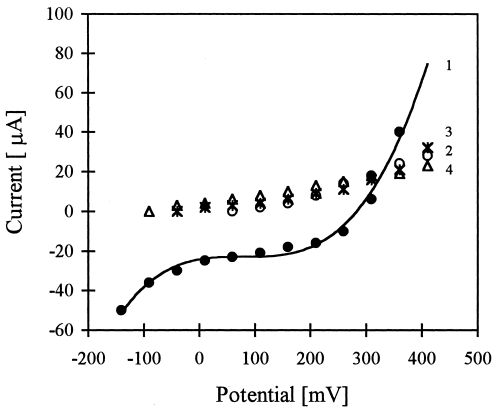

Fig. 1. Polarization curves of graphite electrode modified with microquantities of Pt + Pd (10 + 90%) in presence of: 1) 1×10^{-4} M hydrogen peroxide; 2) 1×10^{-5} M uric acid; 3) 1×10^{-5} M glutathione; 4) 1×10^{-5} M L-ascorbic acid. Background electrolyte: phosphate-citrate buffer (pH = 7.0).

The stability of graphite electrode was studied after a one-year storage in bidistilled water. The polarization curves in the presence of H_2O_2 recorded with freshly prepared and one-year stored modified graphite indicated the same cathodic current values were registered even after a year, which show a very good electrode stability as well as reproducibility of the results. One possible explanation of this finding is that after electrochemical modification of the graphite they are not studied immediately, as freshly prepared, but after about 2–3 weeks. This period is probably long enough to complete the natural aging of the electrode surface.

The electrode response as a function of H_2O_2 concentration was studied within the potential range from 0 to 150 mV where limited current of hydrogen peroxide electroreduction was observed. As the background as the steady-state cathodic currents generated on hydrogen peroxide electroreduction increased proportionally to the potential applied. The sensitivity of modified graphite electrode determined as the slope of the linear portion dI/dC is 0.45 ± 0.02 $\mu A \times \mu M^{-1}$. The electrode response depends linearly on the H_2O_2 concentration up to about 300 μM within the potential range investigated (E = 0 to 150 mV).

The current-potential dependencies of modified graphite electrode recorded in background electrolyte (circles) and in glucose solution (triangles)

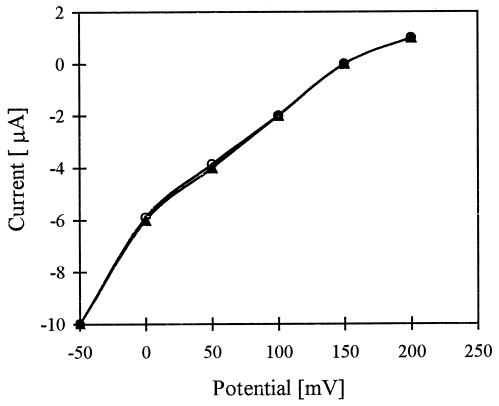

Fig. 2. Polarization curves of modified graphite electrode in background electrolyte (circles) and in 5.5×10^{-5} M glucose solution (triangles). Background electrolyte: phosphate-citrate buffer (pH = 7.0).

are compared on Fig. 2. Within the potential range from −50 to 200 mV the polarization curves in both cases are practically identical which proves that no electrochemical process is observed on modified graphite in glucose solution.

Enzyme electrode based on modified graphite. Optimization of enzyme immobilization

As a rule, the enzyme loading affects the enzyme electrode sensitivity. Increasing the amount of immobilized GOD (using consequently the immobilization procedures A, B and C – described in Experimental section) cause an improved electrode sensitivity and an expanded linear range of the electrode response as a function of substrate concentration was detected. Electrode sensitivities and the corresponding linear range of the electrode response as a function of glucose concentration were determined at a working potential of 0 mV as follows:

– Sensitivity 0.022 ± 0.005 $\mu A \times \mu M^{-1}$ and linearity up to 210 μM were obtained using the immobilization procedure A;
– Sensitivity 0.033 ± 0.005 $\mu A \times \mu M^{-1}$ and linearity up to 250 μM were obtained for the immobilization procedure B;
– Sensitivity 0.043 ± 0.005 $\mu A \times \mu M^{-1}$ and linearity up to 300 μM were obtained for the immobilization procedure C;

Dependencies of enzyme electrode response on glucose concentration at E = 0 mV established that higher sensitivity (determined as the slope of the linear portion, dI/dC) was registered using the third procedure for GOD immobilization. All further results reported were realized with enzyme electrode obtained by procedure C of GOD immobilization.

Operational characteristics of the enzyme electrode

To determine the working potential of the enzyme electrode the dependency of steady-state current on substrate concentration was studied within the potential range from E = −50 mV to E = 150 mV (Fig. 3). The higher electrode sensitivity was detected at E = −50 mV (curve 1), E = 0 mV (curve 2), and E = 50 mV (curve 3). Therefore, at E = −50 mV the linear portion of relationship determined is shorter than at potentials 0 and 50 mV (curves 2 and 3, respectively). The last two were selected as optimal working potentials because of higher sensitivity and extended linear dependence of the electrode response on substrate concentration (up to approx. 300 μM). Detection limit of 10 μM at the optimal working potential was determined at a signal to noise ratio 3:1.

The calibration graph of the electrode for glucose (background subtracted current versus concentration) at working potential E = 0 mV, and the corresponding Eadie-Hofstee plot are given at Fig. 4. It is seen from Fig. 4-a that the electrode

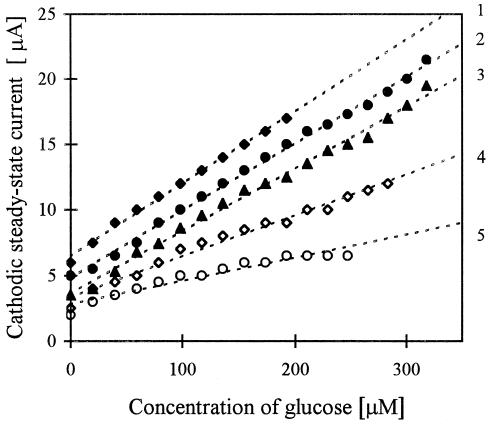

Fig. 3. Linear portions of the enzyme electrode response as a function of glucose concentration and the corresponding trend lines (dotted) at potentials: 1) −50 mV; 2) 0 mV; 3) 50 mV; 4) 100 mV; 5) 150 mV. Background electrolyte: phosphate-citrate buffer (pH = 7.0).

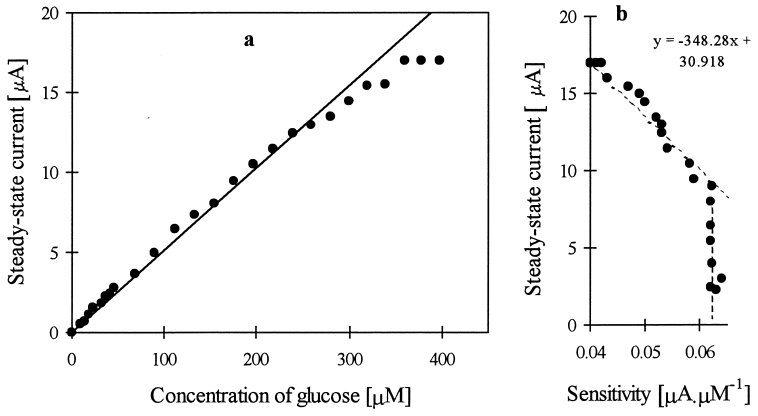

Fig. 4. Steady-state current (background subtracted) of the biosensor (at 0 mV vs. Ag/AgCl): (a) as a function of glucose concentration; (b) as a function of the electrode sensitivity (background subtracted steady-state current divided by glucose concentration at which it is measured); working temperature: 23° C.

response increases linearly as a function of glucose concentration up to 300 μM, and reaches a constant value (saturation) at substrate concentrations exceeding 360 μM. The values of the steady-state current from Fig. 4-a are presented in Fig. 4-b (the electrochemical Eadie-Hofstee plot) as a function of electrode sensitivity (determined as the ratio between the steady-state current and the substrate concentration at which it is measured). The electrode sensitivity remained practically constant at glucose concentrations up to ~ 150 μM. This vertical region imply a probably diffusion control over the electrochemical process. The inclining region indicates that within the concentration range from 150 to approximately 300 μM the reaction is controlled by enzyme kinetics. The value of the apparent Michaelis-Menten constant K_M^{app} = 320 ± 30 μM was calculated using regression analysis of experimental data for this region. The horizontal region at Fig. 4-b observed at high glucose concentrations is probably connected with a substrate saturation of the immobilized enzyme.

Electrode response on interfering substances present

The influence of interfering substances such as *L*-ascorbic acid and uric acid normally presented in blood samples on electrode performance was studied at working potentials (E = 0 and 50 mV). The values of the steady-state current (background subtracted) obtained in *L*-ascorbic acid, uric acid and glucose solutions are compared in Table I. It is seen that no current increase is observed increasing L-ascorbic and uric acid concentrations at these working potentials. At E = 100 mV in L-ascorbic acid solutions anodic currents were found that increased as a function of its concentration. This finding proves above suggested conclusion that E = 100 mV is not a suitable working potential.

Dependence of the electrode response on temperature

The temperature effect on the electrode response was studied at working potentials E = 0 mV and E = 50 mV within the temperature

Table I. Steady-state current, (background subtracted) of the enzyme electrode at different concentrations of glucose, L-ascorbic and uric acid, temperature 23° C.

Substrate	$I_S - I_0$ [μA] at E = 0 mV			$I_S - I_0$ [μA] at E = 50 mV		
	C = 20 μM	C = 40 μM	C = 80 μM	C = 20 μM	C = 40 μM	C = 80 μM
β-D-glucose	1.5	2.5	5.5	1.8	2.7	5.0
L-ascorbic acid	0.0	0.1	0.2	0.0	0.2	0.3
Uric acid	0.0	0.0	0.2	0.0	0.0	0.2

Table II. Electrode sensitivity and linear range of the electrode response at working potentials $E = 0$ mV and $E = 50$ mV within the temperature interval from 15 to 30° C.

Potential [mV]	t [°C]	dI/dC [μA × μм$^{-1}$]	Linear range [μм]
0	15	0.029	300
	20	0.032	300
	25	0.044	300
	30	0.049	300
50	15	0.028	300
	20	0.030	300
	25	0.041	300
	30	0.048	300

range from 15 to 30° C (Table II). The dependence of the steady-state current on substrate concentration remains linear within the temperature range investigated, and the electrode sensitivity (dI/dC) increases as a function of temperature. At potentials $E = 0$ mV and $E = 50$ mV within the temperature range investigated the enzyme electrode sensitivities and the linear parts of the response as a functions of glucose concentration were found practically identical.

Stability of the enzyme electrode

The stability of the discussed enzyme electrode as a function of storage time (days) was studied. The activity of enzyme electrode was estimated as the maximum current value measured at high glucose concentrations (where the electrode response does not depend on substrate concentration). It was found that after 7 days (approximately 10 working hours) storage at 4 °C the maximum current value is about 50% of the initially registered electrode response i.e. the enzyme electrode saves approximately 50% of its initial activity.

Acknowledgment

Authors express their gratitude to the University of Plovdiv Research Fund, for the financial support of this research.

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