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BIOSENSOR FOR XANTHINE WITH IMPROVED SENSITIVITY AND DETECTION LIMIT

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ABSTRACT

An interference-free amperometric biosensor for the quantitative xanthine analysis is described. Xanthine was monitored through the electrochemical reduction of hydrogen peroxide (produced upon its enzyme-catalyzed oxidation) at a constant applied potential of -50 mV (vs. Ag/AgCl). The biosensor was designed on the basis of a graphite electrode modified with microquantities of platinum and palladium (mixed in the ratio 30% Pt : 70% Pd). The analytical performance of the produced biosensor was compared with previously reported results obtained with a different basic transducer (graphite modified with 10% Pt : 90% Pd). The here presented biosensor exhibits operational characteristics, such as electrode sensitivity, linearity range and detection limit much better than the earlier reported.

Keywords: Modified graphite electrodes, Hydrogen peroxide electroreduction, Xanthine oxidase, Xanthine, Biosensor.

INTRODUCTION

Determination of xanthine (uremic toxin) in human blood and urine is important in clinical assays – it is one of the most widely used markers to assess renal function.

The levels of xanthine in plasma and urine samples are routinely measured by such analytical techniques as high-performance liquid chromatography (HPLC) [1,2,3], chemiluminescence (CL) [4], or UV-spectroscopy, whose common drawback is the painstaking procedures for sample preparation. In contrast, an electrochemical biosensor, which combines the high substrate specificity of a chosen biological catalyst (e.g. an enzyme) with the simplicity of operation with electrochemical

equipment, would offer the advantages of a rapid, highly selective, compact, and convenient for handling method for the determination of the target analyte. In addition, it is cost-effective since a small amount of the immobilized enzyme can be repeatedly used for up to several hundreds of assays.

The detection of xanthine with amperometric biosensors, reported so far in the literature is achieved through:

i) electrochemical oxidation of the reaction products such as uric acid and hydrogen peroxide (both produced upon enzymatic oxidation of the analyte) [5, 6];

ii) the redox conversion of organic compounds capable of effectively shuttling electrons between the enzyme active site and the electrode (so-called mediators) [7-11]. As efficient mediators for xanthine oxidase electrochemical transformation as low-molecular weight species, freely diffusing between the electrode surface and the enzyme, such as cobalt phtalocyanine [7] and Prussian blue [8], as organic polymers in which the enzyme was incorporated [9-11]. The later afford the opportunity for developing reagentless enzyme electrodes, since the mediator is assembled together with the biocatalyst on the transducer surface. The applicability of all these biosensors to detecting xanthine in blood serum and urine samples was proven.

A substantial problem in biosensors design is to find the way to eliminate the unwanted electrochemical transformation of interfering substances, present in real samples. The use of a Nafion membrane or other polyelectrolyte multilayer films [12] to block the access of electroactive species to the electrode surface has been reported.

Our research is focused on the development of a simple, sensitive enzyme electrode with interference-free response, which can be used for quantitative determination of xanthine in mammalian bio-liquids. In this paper an amperometric xanthine biosensor with improved sensitivity and detection limit is described. A modified graphite matrix, effective in the elimination of the interferences from uric acid, ascorbic acid and glutathione is used as the basic electrode.

EXPERIMENTAL

Materials

Xanthine oxidase (XOD) (E.C. 1.1.3.2) from buttermilk (Fluka) with an homogeneous activity 8 U mg^{-1} (1 U corresponds to the amount of enzyme which oxidizes 1 μM xanthine per minute at pH 7.5 and 25^0C); xanthine , H_2O_2, uric acid, L-ascorbic acid, glutathione and the chemicals used to prepare buffer solutions: $Na_2HPO_4.12H_2O$, KOH and H_3PO_4, were purchased from Fluka. Gelatine – analytical grade (Chimtek, Bulgaria) was employed as a 5% suspension in phosphate buffer (pH=8.4) for electrode coating formation. All solutions were prepared with bidistilled water.

Preparation of the electrode

Inert pads of graphite (type GMZ with geometric surface area S=1.6 – 1.8 cm^2; 0.7x0.7x0.3 cm) were used. The structural characteristics of this graphite are as

follous: specific surface 0.8 $cm^2 g^{-1}$, density 1.56 – 1.7 g cm^3, porosity 20 – 25%. The graphite pads were modified with microqantities of platinum and palladium mixed in the ratio Pt:Pd 30%:70%. The catalytically active components were deposited in a potentiostatic regime ($Er^{deposit}$= + 0.05V vs. reversible hydrogen electrode) via a brief electrolysis ($t_{deposit}$=10 s) from the following electrolyte: 2%$PtCl_6.6H_2O$ + 2% $PdCl_2$ + 0.1M HCl in the ratio Pt:Pd (30:70%) [6].

Enzyme immobilization

The enzyme immobilization was carried out on an electrochemically activated electrode surface. The electrochemical pretreatment of the modified graphite electrode started with a cathode-anode cycling (30 min) within the potential range from -0.60 to +0.35 V (vs. Ag|AgCl). Just before immobilization, the electrode was polarized for 2 min at E = 1.5 V. The adsorption of XOD was carried out under static conditions by immersing the graphite electrode in the enzyme solution with a 10^{-5} M concentration, in phosphate buffer (pH = 8.4) for 24 h at 4^0C. 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 a layer of 5% gelatine solution containing XOD (5 mg XOD in 1 ml 5%-gelatine solution at 37 °C) using a capillary glass tube. After applying the layer, the electrode surface was dried with argon.

After completing the measurements the enzyme electrode was 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 doubly distilled water (50 – 60 °C) thus regenerating the bare modified graphite electrode. The processed electrode material can be stored for more than one year in bi-distilled water (at room temperature) and used repeatedly.

The analytical performance of the enzyme electrodes based on Pt:Pd(30:70)/Cgmz and Pt:Pd (10:90)/Cgmz (which will be further denoted in the text as EE type B and EE type A, respectively), were compared. The later was fabricated according to a previously reported protocol [6].

Apparatus and measurements

All electrochemical measurements were performed in a three-electrode cell with separated compartments (working volume 11-15 mL). 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 amperometric data were obtained by successive addition of aliquots of $8.6.10^{-4}$ M xanthine solution to the phosphate buffer in the cell with simultaneous registration of the current at constant potential. 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. The pH of the buffer solutions was adjusted with a pH-meter OP - 208 (Radelkis, Budapest, Hungary).

RESULTS AND DISCUSSION

A series of modified graphites, based on the commercially available graphite (type GMZ) whose surface was modified with micro- and nano-deposits of catalytically active components (consisting of Pt and Pd mixed in various ratios) was tested as electrode materials at the electrochemical reduction of hydrogen peroxide. The graphite modified with 30% Pt : 70% Pd was chosen as the basic transducer for designing the xanthine biosensor since it showed an excellent electrochemical performance at the process of interest (electroreduction of H_2O_2). This electrode showed both the lowest background current and the highest sensitivity (dI/dC=0,82 ± 0,02 $\mu A.\mu M^{-1}$) in combination with wide range strict linear dependence of the electrode response (up to 600 μM) at an applied potential of –50 mV (Ag/AgCl) [13].

High selectivity of the assay is one of the factors of crucial importance to consider when developing amperometric biosensors, because a waste variety of electrochemically active species are normally present in the real samples, which can potentially contribute to the output response thus compromising the analytical results. In this connection, it should be pointed out that the modified graphite demonstrated full inertness towards a variety of potentially interfering compounds. Under the selected experimental conditions no electrochemical response was achieved to uric acid, ascorbic acid or glutathione, present in the concentration of 10^{-5} M, which exceeds the possible physiological levels.

The polarization curves of modified electrode over the potential range from -300 to +300 mV (Ag/AgCl) in the presence of 10^{-4} M H_2O_2 were examined at both pH=7 and pH=8.4, as depicted at Fig.1. The potential ranges where the reduction current reaches limited values (plateau region) were found nearly the same at both pH investigated. A slightly extended plateau region (from –150 mV to +150 mV) was observed at pH 8.4 as compared to pH 7.0, where the plateau lies between –150 mV and +50 mV.

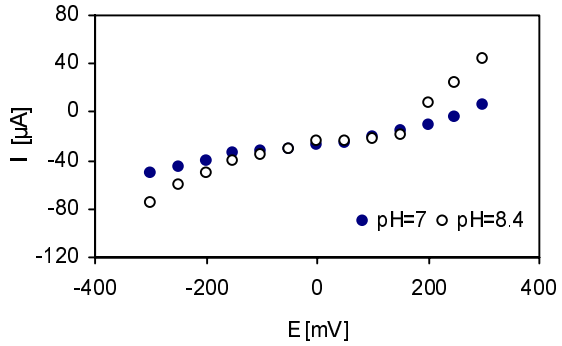

Figure 1. *Polarization curves of graphite modified electrode Pt:Pd(30:70)/Cgmz in 0.1mM H_2O_2, background electrolyte: phosphate buffer; reference electrode Ag/AgCl; temperature 20^0C*

Modified graphite electrode Pt:Pd(30:70)/Cgmz was used as the basic transducer for developing an amperometric xanthine biosensor for highly selective quantitative analysis of xanthine through immobilization of XOD according to an already reported protocol [13] (EE type B).

Operational parameters determined for the EE type B at an applied potential of -50 mV (vs. Ag/AgCl) at 25^0C are presented in Table 1. The linearity range for this enzyme electrode was found twice as large as for the EE type A, while the sensitivity of the first one was estimated to be almost one order of magnitude higher than the sensitivity of the later. The calibration graph (Fig.2-A) shows strict linear dependence of the steady-state electrode response (for the EE type B) on xanthine concentrations up to 70 μM; the electrode sensitivity was determined as the slope of the linear portion of the calibration graph as 0.39 $\mu A.\mu M^{-1}$. At substrate concentrations exceeding 80 μM XOD, the calibration graph turnes to a horizontal line, which most probably is due to the enzyme saturation with xanthine. The biosensor produced demonstrated considerably lower detection limit to xanthine – 1.5 μM (determined at signal to noise ratio 3:1) as compared to 4.5 μM for EE type A. The better analytical performance of the EE type B as compared to the EE type A could be ascribed to several factors acting synergistically. From the one hand, the basic transducer for designing the EE type B possesses enhanced catalytic activity and sensitivity towards hydrogen peroxide electrochemical reduction. From the other, the immobilized on its surface xanthine oxidase was chosen with higher homogeneous activity than this ane used to develop the EE type A.

The apparent Michaelis constant Km^{app} , which is a basic kinetic parameter, was calculated both from the Lineweaver-Burke plot (1/C vs 1/I) (Fig 2-B) and Eadie-Hofstee plot (not shown). The obtained values were found practically identical: Km^{app}=58.4 μM (r^2=0.996) determined from the LB-plot; and Km^{app}=58.6 μM (r^2=0.995) estimated using the EH-method. Higher Km^{app} value than this reported for EE type A results in wider linearity range for the xanthine biosensor type B.

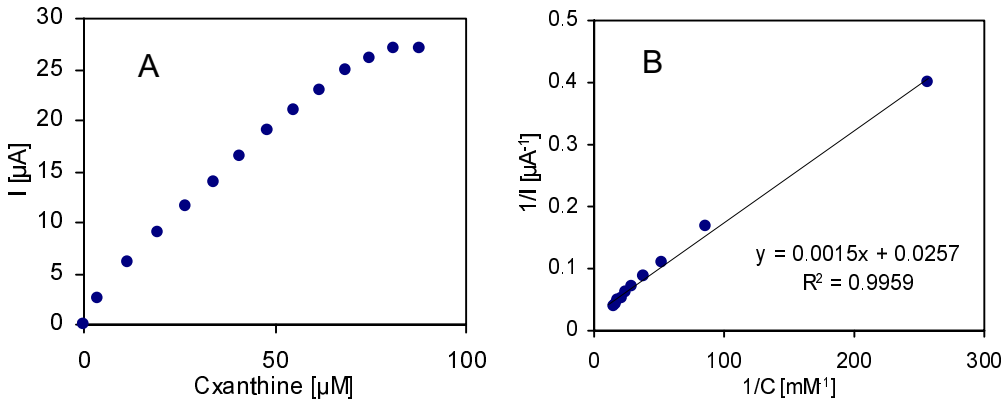

Figure 2. *A - Steady-state response of the enzyme electrode type B as a function of xanthine concentration; B – Lineweaver – Burke plot. Applied potential -50mV(vs. Ag/AgCl); background electrolyte: 0.1 M phosphate buffer, pH=8.4; temperature 25 ^{0}C*

CONCLUSIONS

The enzyme electrode with considerably improved operational characteristics was prepared using graphite electrode modified with Pt and Pd, mixed in the ratio 30%:70% and XOD with higher homogeneous activity, than previously used [6]. The enzyme electrode produced, tested under the selected experimental conditions (ambient temperature, 25^0C; working potential -50mV; pH 8.4) showed the following operational characteristics:

- a detection limit of 1.5 μM;
- a linear range up to 70 μM;
- a sensitivity of 0.39 $\mu A.\mu M^{-1}$;
- no response to glutathione, ascorbic acid and uric acid present in higher than the physiological levels.

A brief comparison of the xanthine biosensor presented here with previously published results [6] (accomplished with an enzyme electrode based on graphite modified with 10%Pt+90%Pd) showed that the new biosensor displayed better electrode sensitivity with larger linear range, lower detection limit and higher selectivity (practically inert towards interference substances as ascorbic acid, uric acid and glutathione).

Table 1. *Operational parameters of xanthine oxidase-based enzyme electrodes; working potential -50mV (vs. Ag/AgCl); background electrolyte phosphate buffer pH=8.4; temperature 25^0C*

Enzyme electrode type	Sensitivity, $\mu A.\ \mu M^{-1}$	r^2	Linearity, μM	Detection limit, μM	I_{max}, μA	Km^{app}, μM
EE type A	0.3	0.98_4	40	4.5	10	30
EE type B	0.39	0.98_3	70	1.5	39	58.5

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