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In memoriam prof. dr. Liviu Oniciu

AMPEROMETRIC BIOSENSOR FOR ETHANOL BASED ON A PHENOTHIAZINE DERIVATIVE MODIFIED CARBON PASTE ELECTRODE

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ABSTRACT. A new amperometric biosensor for ethanol, based on carbon paste electrode modified with alcohol dehydrogenase (ADH), polyethylenimine (PEI) and using a phenothiazine derivative (DDDP; 16*H*,18*H*-dibenzo[c,1]-7,9-dithia-16,18-diazapentacene) as redox mediator for NADH recycling, was developed. The biosensor response is the result of mediated oxidation of NADH, generated in the enzymatic reaction between ADH and ethanol (in the presence of NAD^+). The biosensor sensitivity (calculated as the ratio I_{max}/K_M^{app}) was 0.035 mA M^{-1} and the detection limit was 0.26 mM, while the linear response range was from 0.1 to 20 mM ethanol.

Keywords: *amperometric biosensors, alcohol dehydrogenase, ethanol, nicotinamide adenine dinucleotide, phenothiazine derivative, polyethyleneimine, modified carbon paste electrodes.*

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+/NADH) dependent dehydrogenases catalyze the oxidation of compounds of great interest in analysis, such as carbohydrates, alcohols and aldehydes. Selective, sensitive and simple devices for the monitoring of ethanol are required from different fields such as biotechnology, food and clinical analysis [1] and, consequently, a lot of biosensors for ethanol detection were proposed [1-15].

Because the direct electro-oxidation of NADH, required for its recycling in biosensor functioning, involves high overpotentials on conventional electrodes [16-18], many efforts were directed towards discovering and characterizing new efficient electrocatalysts [19,20]. Among the most frequently investigated mediating schemes are those based on the direct adsorption of electron mediators onto electrode surface to obtain modified electrodes with electrocatalytic activity for NADH oxidation [21].

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Taking advantage of their remarkable strong adsorption on graphite surface associated with high electrocatalytic efficiency, organic dyes, i.e. phenazines, phenoxazines and phenothiazines derivatives were extensively used as electrocatalysts for NADH oxidation [22-24]. Continuing our preoccupation in this domain [13,25] and taking advantage of a new phenothiazine derivative, 16*H*,18*H*-dibenzo[c,1]-7,9-dithia-16,18-diazapentacene (DDDP), which was successfully used to design efficient electrocatalytic schemes for NADH recycling [26-28], the possibility to develop a simple and inexpensive biosensor for ethanol determination, by immobilization of alcohol dehydrogenase and polyethylenimine (PEI) on carbon paste modified with DDDP, was investigated.

RESULTS AND DISCUSSIONS

1. Bioelectrocatalysis at DDDP modified carbon paste electrodes

In alcohol dehydrogenase (ADH) based biosensor, the enzyme catalyzes the oxidation of ethanol to acetaldehyde, in the presence of nicotinamide adenine dinucleotide (NAD^+) and the reduced NADH can be detected amperometrically, according to the following reactions:

$$CH_3\text{-}CH_2\text{-}OH + NAD^+ \xleftrightarrow{ADH} CH_3\text{-}CHO + NADH + H^+ \quad (1)$$

$$NADH + M_{ox} \longrightarrow NAD^+ + M_{red} + H^+ \quad (2)$$

$$M_{red} \longrightarrow M_{ox} + 2e^- + H^+ \quad (3)$$

This approach has some important characteristics for ethanol monitoring in real samples, because it is not oxygen dependent and is more selective for ethanol [11].

In the present case, ADH and the oxidized form of DDDP (as electrocatalyst) are both present in the carbon paste, whereas NAD^+ is dissolved into the electrolyte solution. When ethanol is added to the stirred solution contacting the biosensor, the enzymatic reaction 1 occurs and NADH diffuses to the DDDP-modified carbon paste electrode, where it is catalytically oxidized back to NAD^+ (reaction 2). The electrochemical re-oxidation of the mediator (reaction 3) yields an analytical signal proportional to the rate of ethanol oxidation, which itself is proportional to the ethanol concentration if the concentrations of the other reactants are kept constant and ADH is unsaturated. A steady state current will be achieved if the enzyme and mediator are efficiently retained in the carbon paste electrode and the reaction rates of reactions 2-3 are high enough, allowing a continuous and fast recycling of NAD^+.

Electrical communication of the redox-active center of enzymes with an electrode surface is a fundamental element for the development of amperometric biosensor devices [30]. For this reason carbon paste was chosen as electrode material for ADH immobilization. In a previous work [28] it

was demonstrated that the DDDP-modified carbon paste electrode (DDDP-CPEs) can efficiently catalyze the oxidation of NADH. The present results show that the DDDP-CPEs can also catalyze the oxidation of enzymatically generated NADH from the reaction of NAD^+ and ethanol catalyzed by ADH (reactions 1-3).

Since NAD^+ and mediator concentrations are constant, the increase in the electrocatalytic current depends only on the ethanol concentration (NADH formation), and this characteristic was used as the basis of the development of a biosensor for ethanol determination.

The cyclic voltammograms recorded for DDDP-modified carbon paste electrode, incorporating ADH, in the presence of NAD^+ and ethanol (results not shown) proved that the electrode is able to sustain the catalytic cycle described by reactions 1-3. After addition of 10 mM NAD^+ and 50 mM ethanol, a good electrocatalytic effect of DDDP for the enzymatically produced NADH was clearly observed (the anodic current is enhanced and the cathodic one is diminished). Obviously, no catalytic current can be observed in the absence of NAD^+ and/or ethanol (results not shown).

2. Influence of NAD^+ concentration

The NAD^+ coenzyme also plays a major role in the biosensor mechanism (see reactions 1-3). Thus, the effect on the biosensor response was evaluated for 50 mM ethanol, at different NAD^+ concentrations in the electrolyte solution (results not shown). It was observed that the response increases with increasing NAD^+. Based on these results, 10 mM of NAD^+ was employed in the development of further biosensors.

3. Response to ethanol of ADH-PEI-DDDP-CPE

Fig. 1A presents the ADH-PEI-DDDP-CPE amperometric response to successive injections of ethanol, and gives qualitative information on the response rate, as well as on the signal stability. In order to diminish the mass transport effect on the biosensor response the ADH-PEI-DDDP-CPE was rotated with 500 rpm.

The biosensor response time was very short, reaching of its $t_{95\%}$ in 1 minute, as observed in fig. 1A. This response time is good considering that it is a carbon paste electrode.

Fig. 1B shows a calibration curve obtained from 1 to 100 mM of ethanol, in 0.1 M phosphate buffer at pH 7. The values of the kinetic parameters (I_{max} and K_M^{app}) were calculated by fitting the experimental data to the Michaelis-Menten equation (fig. 1B). A linear response is observed up to 20 mM ethanol.

The sensitivity for ethanol (estimated as the I_{max}/K_M^{app} ratio) of ADH-PEI modified carbon paste based biosensor was 0.035 mA/M.

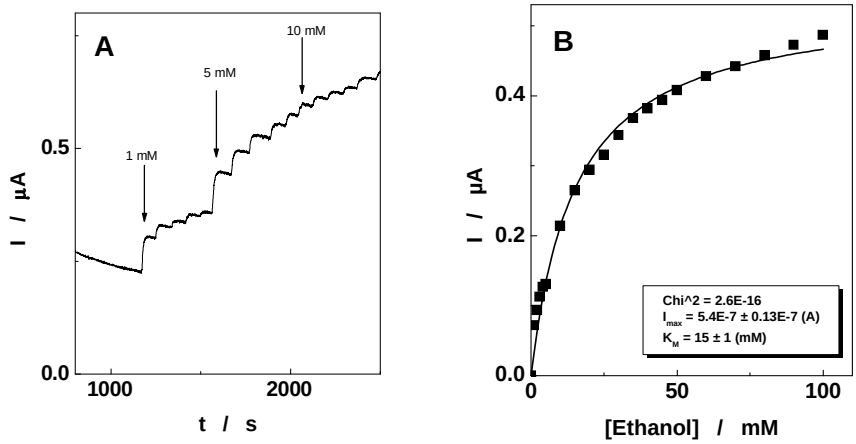

Figure 1. (A) Amperometric response to successive additions of ethanol and (B) calibration plots for ADH-PEI-DDDP-CPE. Experimental conditions: applied potential, +430 mV *vs.* Ag|AgCl/KCl$_{sat}$; supporting electrolyte, 0.1 M phosphate buffer pH 7 containing 10 mM NAD^+; rotation speed, 500 rpm.

Kinetic parameters were also estimated using the Lineweaver–Burk, Hanes–Woolf and Eadie–Hoffstee linearizations of Michaelis-Menten equation (table 1). The values obtained for K_M^{app}, I_{max} and sensitivity are in good accordance with those obtained by Michaelis-Menten fitting (fig. 1B). This behavior was attributed to the good reproducibility of the ADH-PEI-DDDP-CPE response, reflected by small fluctuations of the experimental data involved in the calibration curve (fig. 1B).

The value of K_M^{app} is higher than those observed for the free enzyme in solution (3.2 mM for dissolved ADH; Pt rotated disk electrode; using hexacyanoferrate(III) as mediator; at pH 8.8 [31]) and for immobilized enzyme in carbon paste electrode (10 mM for ADH immobilized using glutaraldehyde/bovine serum albumin cross-linking procedure, in Meldola Blue adsorbed on silica gel modified niobium oxide [11]). As expected, the ADH immobilization lead to an increase of K_M^{app} value in comparison with the corresponding values obtained when ADH was dissolved in solution. Additionally, a small increase (of 1.5) was observed between the values of K_M^{app} for the present study and that obtained in the above example [11]. These results showed that the immobilization procedure did not promote a significant change in the enzyme selectivity/activity [11].

Detection limits around 0.26 mM ethanol could be estimated considering a signal/noise ratio of 3.

Table 1.

Kinetic parameters of DDDP-ADH-PEI-CPE biosensors.
Experimental conditions: as in figure 2.

K_M^{app} (mM)	I_{max} (μA)	Sensitivity (μA M^{-1})	R / no. of exp. points
Lineweaver – Burk linearization			
17.1 ± 1.8	0.55 ± 0.02	32.2 ± 2.3	0.9942 / 13
Hanes- Woolf linearization			
17.8 ± 1.7	0.56 ± 0.01	30.9 ± 2.4	0.9990 / 15
Eadie – Hoffstee linearization			
18.2 ± 1.8	0.57 ± 0.02	31.3 ± 4.2	0.9886 / 13

The biosensor showed a good operational stability, as verified by data from repetitive analyses recorded over 6 h periods of continuously operating.

Also, the proposed biosensor presented good storage stability, which allowed measurements with the same response, for 1-2 days, when the biosensor was stored in a refrigerator, at 4^0C. Decreasing of response towards ethanol with 88 %, after three days of storing is due to enzyme deactivation, because DDDP-CPE presents a good stability for NADH oxidation and DDDP remains immobilized in carbon paste more than a month [28].

CONCLUSIONS

The phenothiazine derivative, 16*H*,18*H*-dibenzo[c,1]-7,9-dithia-16,18-diazapentacene, adsorbed on carbon paste electrode was very useful for a simple and effective way to develop biosensors for ethanol determination.

The analytical signal is due to the electrocatalytic oxidation of enzymatically generated NADH at ADH-PEI-DDDP-carbon paste electrodes. The proposed ADH-PEI-DDDP-CPE biosensor exhibited a good sensitivity (0.035 mA/M), a fast response ($t_{95\%}$ < 1 min.) and a linear domain of concentration up to 20 mM, as well as a good operational and storage stability.

EXPERIMENTAL SECTION

Materials

Alcohol dehydrogenase (ADH), EC 1.1.1.1. from yeast, was obtained from Sigma (St. Louis, MO, USA). The phenothiazine derivative, 16*H*,18*H*-dibenzo[c,1]-7,9-dithia-16,18-diazapentacene (DDDP) was synthesized according to a previously published procedure [29]. The supporting electrolyte used in the electrochemical cell was a solution of 0.1 M sodium phosphate, pH 7.0 (Merck, Darmstadt, Germany).

Potassium chloride was purchased from Merck (Darmstadt, Germany) and absolute ethanol (99.7 %) from Kemetyl (Stockholm, Sweden). Polyethylenimine (PEI) and NAD^+ were purchased from Sigma (St. Louis, MO, USA).

Preparation of the DDDP-modified carbon paste electrodes

100 µl of a 0.001% (w/v) DDDP solution prepared in tetrahydrofuran (Labscan Limited, Dublin, Ireland) were added to 100 mg of carbon powder and adsorption of the mediator was allowed to proceed in vacuum until total evaporation of the solvent. DDDP-modified carbon paste electrodes (DDDP-CPEs) were obtained by thoroughly mixing the obtained DDDP-modified carbon paste with 25 µl of paraffin oil.

Preparation of the ADH-PEI-modified carbon paste electrodes

To 10 mg of DDDP-modified carbon paste, 200 µl solution formed by 5 mg ADH (400 U / mg) and 1 ml of 0.2 % (w/v) PEI was added, and adsorption of the enzyme was allowed to proceed in vacuum until a dried carbon powder was obtained.

The modified carbon paste was put into a cavity of an in-house made Teflon holder using pyrolytic graphite in the bottom for electric contact and then screwed onto a rotating disk electrode device (RDE; EG&G Model 636, Princeton, Applied Research, Princeton, NJ, USA). The final geometrical area of the modified carbon paste electrodes was equal to 0.071 cm^2.

Electrochemical measurements

Cyclic voltammetry and rotating disk electrode experiments were carried out using a conventional three-electrode electrochemical cell. The modified carbon paste was used as working electrode, a platinum ring as counter electrode and an $Ag|AgCl/KCl_{sat}$ as reference electrode. An electrochemical analyzer (BAS 100W, Bioanalytical Systems, West Lafayette, IN, USA) was connected to a PC microcomputer for potential control and data acquisition. For rotating disk electrode experiments an EG&G rotator (Princeton Applied Research, Princeton, NJ, USA) was used.

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