STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, XLIX, 1, 2004

THIRD GENERATION AMPEROMETRIC BIOSENSOR BASED ON MEDIATED ELECTRON TRANSFER BETWEEN ALCOHOL DEHYDROGENASE AND 1,1,2,2,3,3,4,6,7,8,9-UNDECACHLORO-1,2-DIHYDRO-PHENOTHIAZINE

DELIA M. GLIGOR, GRAZIELLA L. TURDEAN, LIANA M. MURESAN and IONEL CATALIN POPESCU

Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University of Cluj, 11 Arany Janos Str. Cluj-Napoca 400028 Romania. E-mail:ddicu@chem.ubbcluj.ro

ABSTRACT. A new amperometric biosensor for ethanol was obtained using a graphite electrode modified with 1,1,2,2,3,3,4,6,7,8,9-undecachloro-1,2-dihydro-phenothiazine as transducer, and alcohol dehydrogenase (ADH) cross-linked with glutaraldehyde, as NAD^+/NADH dependent detecting enzyme. The ethanol amperometric detection is based on the oxidation of NADH generated in the enzymatic reaction between ethanol and NAD^+ in the presence of ADH. The biosensor response increases linearly with ethanol concentration up to 2 M and the detection limit was 1.5 mM. The biosensor activity remains relatively stable for about 2 days.

Keywords: amperometric biosensors, alcohol dehydrogenase, nicotinamide adenine dinucleotide, phenothiazine derivative, modified electrodes.

INTRODUCTION

The majority of redox enzymes widely used in amperometric biosensor research are NAD^+/NADH-dependent dehydrogenases [1,2]. Their electrical connection with electrodes reveals several fundamental limitations that prevent their broad use for biosensor applications. Thus, the NAD^+/NADH redox couple: (i) operates by a diffusion controlled mechanism; (ii) exhibits poor electrochemical properties at bare electrodes; (iii) requires a large overvoltage because its direct electrochemical reduction/oxidation is kinetically unfavorable [3-5]. So, an effective way to avoid these problems is the mediated electrocatalytic oxidation of NADH.

The phenothiazine derivatives are one of the compounds more frequently used as mediators to design efficient electrocatalytic schemes for NADH recycling. Thus, to develop biosensors based on NAD^+-dependent enzymes it is important to integrate the enzyme-NAD^+ cofactor and the catalyst for NADH oxidation as an assembly that electrically communicates with the amperometric transducer [4,6,7].

Because ethanol is an important compound in medicine, biotechnology and food industry, amperometric methods based on using of either alcohol oxidase (AOX) [7] or alcohol dehydrogenase (ADH) [1,8-12] to an appropriate electrode are employed. The interest in ADH instead of AOX for ethanol detection is due to the fact that in the first enzymatic reaction oxygen is not involved [13].

The alcohol dehydrogenase (ADH) has been immobilized onto the electrode surface by physical entrapment using Nafion membrane [14] or a dialysis membrane [9], into electropolymerised polypyrrole film or *o*-phenylenediamine [13] as well as by cross-linking using glutaraldehyde/albumin [15,16].

Recently [17], it was demonstrated that a graphite electrode modified with 1,1,2,2,3,3,4,6,7,8,9-undecachloro-1,2-dihydro-phenothiazine (G/UDP) shows a significant reduction of the NADH oxidation overpotential and a remarkably stable voltammetric response. In this context, the aim of the present study was to use this new electrochemical interface for NADH detection in order to develop and characterize a G/UDP/ADH amperometric biosensor for the ethanol detection.

EXPERIMENTAL SECTION

Chemicals

β-nicotinamide adenine dinucleotide, NAD^+ was purchased from Merck (Darmstadt, Germany), alcohol dehydrogenase (ADH, alcohol:NAD^+ oxidoreductase; EC 1.1.1.1., from bakers yeast, activity of 340 U mg^{-1}) was obtained from Sigma (St. Louis, MO, USA) and both used as received. Glutaraldehyde (25 % w/v), obtained from Aldrich (Steinheim, Germany), was diluted to 2.5 % (v/v) with distilled water. Dimethylformamide was purchased from Merck (Darmstadt, Germany) and absolute ethanol (96 %) was obtained from Prodvinalco, Cluj-Napoca.

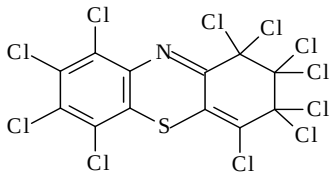
The phenothiazine derivative, 1,1,2,2,3,3,4,6,7,8,9-undecachloro-1,2-dihydro-phenothiazine (UDP, see Scheme 1) was kindly offered by Prof. Silberg (Department of Organic Chemistry, "Babes-Bolyai" University), who synthesized it as described previously [18]. The supporting electrolyte was a 0.1 M phosphate buffer solution (pH 7.0), prepared using appropriate amounts of Na_2HPO_4 and NaH_2PO_4 (Merck, Darmstadt, Germany). All other reagents were of analytical grade and used as received.

All solutions were prepared with distilled water. The ethanol aqueous solution and the ADH stock solution were prepared in phosphate buffer solution (pH 7) and stored at 4^0 C.

Enzymatic activity determination of alcohol dehydrogenase

Before use, the enzymatic activity of ADH was spectrophotometrically tested (Jasco V530, PC connected spectrophotometer). The UV-VIS absorption spectrum of a phosphate buffer solution (pH 7), containing 10^{-3} M ethanol and 10^{-3} M NAD^+, was compared with that of the same solution containing in addition 476 units ADH/ml. In the presence of ADH, a maximum of absorption located at 330 nm proves the enzymatic formation of β-NADH, according to reaction 1:

$$\text{Ethanol} + \beta - \text{NAD}^+ \xleftrightarrow{\text{ADH}} \text{Acetaldehyde} + \beta - \text{NADH} + \text{H}^+ \quad (1)$$



Scheme 1. *Structure of 1,1,2,2,3,3,4,6,7,8,9-undecachloro-1,2-dihydro-phenothiazine (UDP).*

Preparation of the G/UDP-modified electrodes

A spectrographic graphite rod (Ringsdorff-Werke, GmbH, Bonn-Bad Godesberg, Germany), of ~ 3 mm diameter, was wet polished on fine (grit 400 and 600) emery paper (Buehler, Lake Bluff, In., USA). Then, a graphite piece of suitable length was carefully washed with distilled water, dried, and finally press-fitted into a PTFE folder in order to obtain a graphite electrode, having in contact with the solution, a flat circular surface of ~ 0.071 cm^2.

Spreading onto the graphite surface 5 μl of 10 mM UDP dissolved in dimethylformamide, and leaving the graphite for 20 minutes at room temperature to evaporate the solvent the G/UDP-modified electrodes were obtained. Before immersion in a test solution, the G/UDP-modified electrodes were carefully washed with doubly distilled water.

Preparation of the G/UDP/ADH bioelectrodes

In order to immobilize the ADH on the G/UDP-modified electrodes a cross-linking technique with glutaraldehyde (GA) was used. Thus, 3 mg of ADH were dissolved in 100 μl of 0.1 M phosphate buffer solution (pH 7) and was stirred thoroughly with 50 μl glutaraldehyde (2.5 % v/v). Spreading onto the electrode surface 5 μl ADH-GA mixture the G/UDP/ADH bioelectrodes were obtained. This procedure was repeated three times. Before use the resulting bioelectrodes were incubated at 4^0 C, for least 1 h in a phosphate buffer solution (pH 7).

Electrochemical measurements

Electrochemical experiments were carried out using a typical three-electrode electrochemical cell. The modified graphite electrode was used as working electrode, a platinum ring as counter electrode and a saturated calomel electrode (SCE) or Ag|AgCl (KCl_{sat}) as reference electrodes.

Cyclic voltammetry experiments were performed on an electrochemical analyzer (Autolab-PGSTAT 10, EcoChemie, Utrecht, The Netherlands) connected to a PC for potential control and data acquisition.

Amperometric measurements were carried out with the G/UDP/ADH biosensor at an applied potential of +150 mV *vs.* Ag|AgCl (KCl_{sat}) and under constant stirring using a modulated speed rotator (model AMSFRX, Pine, Grove City, Pa., USA). The current-time data were collected using a computer-controlled BAS CV-50W voltammetric analyzer (Bioanalytical Systems, West Lafayette, In., USA). The experiments were performed in phosphate buffer solution (pH 7) containing 1 mM NAD^+. The background current was first allowed to decay to a constant value, and

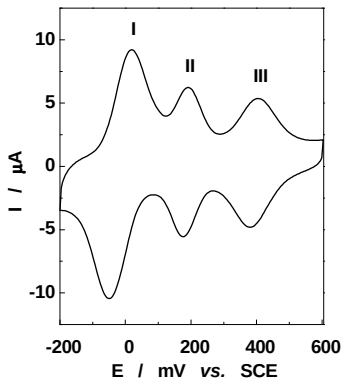


***Fig. 1**. Voltammetric response of UDP adsorbed on spectrographic graphite. Experimental conditions: starting electrode potential, -200 mV vs. SCE; scan rate, 50 mV s^{-1}; supporting electrolyte, 0.1 M phosphate buffer (pH 7.0).*

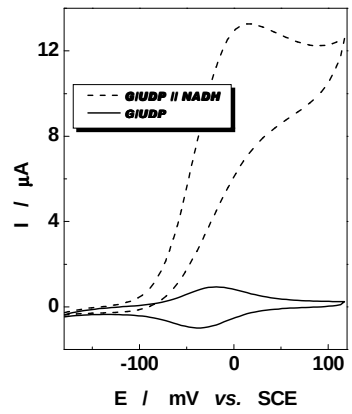


Fig. 2. *NADH electrocatalytic oxidation at G/UDP-modified electrode: voltammetric response in 0.1 M phosphate buffer solution (pH 7.0) (_____) and in the presence of 5 mM NADH (.....). Experimental conditions: scan rate, 5 mV s^{-1}; surface coverage, ~3.0 nmol cm^{-2}; starting potential, -180 mV vs. SCE.*

then the ethanol was added. The steady-state current was measured under constant solution stirring.

Results and discussion

1. Electrochemical behavior of the G/UDP modified electrode

The voltammetric response recorded for G/UDP electrodes (Fig. 1), exhibited three peak pairs placed at following formal standard potentials: $(E^{0'})_I$ = -15; $(E^{0'})_{II}$ = 185; $(E^{0'})_{III}$ = 396 mV *vs.* SCE (pH 7.0). The first peak pair of UDP was attributed to the formation of the 1,2,3,4,6,7,8,9-octachlorophenothiazinyl cation radical [19]. The second and the third peak pairs were supposed to correspond to two different dimers formed during the electrode potential cycling, namely 3,10'-bis(1,1,2,2,3,3,4,6,7,8, 9-undecachloro-1,2-dihydro-phenothiazine) and 10,10'- bis(1,1,2,2,3,3,4,6,7,8,9-undecachloro-1,2-dihydro-phenothiazine) [17]. All further results will refer exclusively to the first peak pair, because its redox formal potential is well placed in the for amperometric detection [20]. The electrochemical analysis of the voltammetric response, corresponding to the formation of the UDP cation radical involves a quasi-reversible, $1e^-/1H^+$ transfer. The UDP formal redox potential was found pH-dependent with a slope of 52 mV/ΔpH [17].

2. Electrocatalytic activity of the G/UDP modified electrode

Starting from the favorable electrochemical behavior of the UDP adsorbed on graphite, its electrocatalytic activity towards NADH oxidation was tested by cyclic voltammetry (Fig. 2).

In the presence of NADH, a remarkable enhancement of the anodic peak current, associated with the progressive diminishing of the cathodic one, proved the electrocatalytic effect of UDP. The electrocatalytic efficiency, estimated as the

$(I_{cat})_{NADH}/(I_{cat})$ ratio, at an applied potential of +15 mV *vs.* SCE, was found equal to 5, when the NADH concentration was 5 mM.

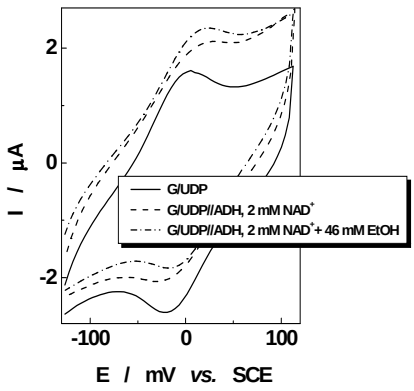
Taking as reference the potential for NADH electro-oxidation at bare graphite electrodes [21,22], an overpotential decrease higher than 400 mV was observed. The calibration curve of G/UDP-modified electrode for NADH (results not shown), recorded at an applied potential of +15 mV *vs.* SCE, exhibited a linear range up to ~1 mM NADH and a sensitivity of 1.0 mA/(M.cm^2).

3. Electrochemical behavior of G/UDP/ADH bioelectrode

3.1. Detection of NADH generated by enzymatic reaction

The above-presented results showed that the G/UDP-modified electrode exhibits a significant catalytic activity toward NADH oxidation. However, it was necessary to check if its sensitivity is enough high to allow the detection of the enzymatically generated NADH resulting from reaction (1). In order to verify this supposition, the G/UDP voltammetric response in a phosphate buffer solution was compared to that corresponding to the same electrode in contact with a buffer solution containing simultaneously dissolved ADH and NAD^+ as well as with that recorded at G/UDP-modified electrode immersed in a buffer solution containing dissolved ADH, NAD^+ and ethanol (Fig. 3). It can be seen that the presence of ethanol in the solution containing NAD^+ and ADH lead to a significant increase of the anodic peak current, proving the ability of the G/UDP-modified electrode to detect the enzymatically generated NADH.

3.2. Analytical performances of G/UDP/ADH bioelectrode

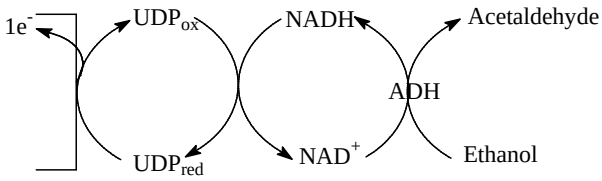


ADH catalyzes the oxidation of ethanol with a simultaneous reduction of an equivalent amount of NAD^+ to NADH (reaction 1) and, subsequently, the generated NADH reduces UDP_{ox} (Scheme 2).

Fig. 3. *Cyclic voltammograms at G/UDP-modified electrode in phosphate buffer solution (pH 7.0) (———), in the presence of 2 mM NAD^+ and 100 U ml^{-1} ADH (— —) and after addition of 46 mM ethanol (-.-.-.). Experimental conditions: starting electrode potential, -130 mV vs. SCE; scan rate, 5 mV s^{-1}.*

During the anodic scan the reduced mediator (UDP_{red}) will be reoxidized at the electrode and, consequently, the anodic peak current will increase significantly. A catalytic current will be not observed if any component shown in Scheme 2 is absent.

By fixing the NAD^+ concentration in solution, the increase of the peak current should depend only on the ethanol concentration. This is the basis for the ethanol determination via NADH amperometric detection, using the G/UDP/ADH bioelectrode.



Scheme 2. *Scheme of the electrochemical coupled enzymatic oxidation of ethanol*.

The batch amperometric response of G/UDP/ADH bioelectrode to successive additions of ethanol, observed at an applied potential of +150 mV *vs.* Ag/AgCl (KCl_{sat}) and a fixed (1 mM) NAD^+ concentration [9], corresponds to a typical Michaelis–Menten kinetics (Fig. 4).

A linear response was observed up to 0.6 mM ethanol for G/UDP-modified electrode in the presence of dissolved ADH and up to 2 M ethanol for the G/UDP/ADH bioelectrode. A detection limit of 0.2 mM ethanol was calculated for the G/UDP-modified electrode and 1.5 mM ethanol for the G/UDP/ADH bioelectrode (signal-to-noise ratio of three). The response time necessary to reach 95% of the steady state signal was almost independent on the substrate concentration and, in both cases, its average value was less than 50 s.

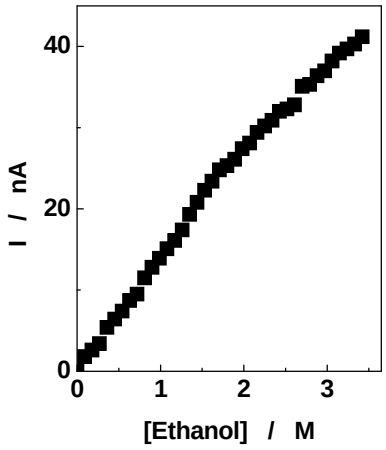


Fig. 4. *Calibration curve for ethanol at G/UDP/ADH modified electrode. Experimental conditions: applied electrode potential, +150 mV vs. Ag|AgCl; electrode rotation speed, 300 rpm; supporting electrolyte, 0.1 M phosphate buffer (pH 7.0).*

The stability of the G/UDP**/**ADH bioelectrode was evaluated by measuring periodically its response towards ethanol. The response remained at 90% of the initial value for 2 days and decayed to half of its initial value after one week, indicating the G/UDP**/**ADH bioelectrode displays a relatively stable response for ethanol. *Experimental*

The decrease in the response towards ethanol might be due mainly to the loss of ADH enzyme activity, because it was noticed that the G/UDP electrode exhibited a higher stability (more than a week) for NADH detection [17].

For immobilized enzymes used to obtain amperometric biosensors, the observed electrochemical response may be controlled either kinetically or by sub-

strate transport. For an immobilized enzyme reaction that is kinetically controlled, the steady-state current, I_{SS}, is proportional to the initial rate of the enzymatic process. In order to obtain the kinetic parameters of the biosensor, three different linearization of Michaelis-Menten equation (Lineweaver–Burk, Hanes–Woolf and Eadie–Hoffstee) were used. The kinetic parameters obtained for G/UDP/ADH bioelectrode are presented in Table 1. As expected, the ADH immobilization lead to an increase of K_M^{app} value and a decrease of sensitivity in comparison with the corresponding values obtained when ADH was dissolved in solution (K_M = 3 M and S = 45 nA/M).

A survey of the literature shows that the value of apparent constant Michaelis–Menten, K_M^{app}, for ADH varies between 3.2 mM (for dissolved ADH; Pt rotated disk electrode; using hexacyanoferrate(III) as mediator; at pH 8.8) [23] and 41.7 mM ethanol (for ADH immobilized on a nickel hexacyanoferrate modified microband gold electrode, using glutaraldehyde/bovine serum albumin cross-linking procedure; at pH 7.5) [9]. The difference between the results obtained in this study and those presented above could be due to the restricted substrate access at the electrode surface, as a result of diffusion constraints created by the enzyme layer. This finding confirms the fact that the value of K_M^{app} is not an intrinsic property of a given enzyme, but it is strongly dependent of the microenvironment around the immobilized enzyme and the transductor geometry.

Table 1.

Analytical parameters for G/UDP/ADH bioelectrode.

K_M^{app} (M)	I_{max} (μA)	Sensitivity (nA/M)	R/ no. of exp. points
Lineweaver – Burk linearization			
7.2 ± 0.8	0.12 ± 0.01	17.7 ± 0.5	0.9984 / 22
Hanes – Woolf linearization			
7.5 ± 0.9	0.13 ± 0.01	17.5 ± 0.5	0.9833 / 22
Eadie – Hoffstee linearization			
7.2 ± 1.0	0.12 ± 0.01	17.6 ± 4.2	0.9617 / 20

Conclusions

The results indicate that the new bioelectrode, obtained by ADH cross-linking with glutaraldehyde on the surface of a G/UDP-modified electrode, is a promising detector for ethanol. Its analytical signal is based on the electrocatalytic oxidation of NADH enzymatically generated in the reaction between ethanol and NAD^+, in the presence of ADH. G/UDP/ADH bioelectrode responds rapidly to ethanol with a detection limit of 1.5 mM. The response current increases linearly with ethanol concentration up to 2 M. The bioelectrode maintains its activity for about 2 days.

Acknowledgements

Financial support from CNCSIS (Grant CNCSIS A_T 26/43-2003) and Romanian Academy (GAR 43/2003) is gratefully acknowledged. Authors acknowledge Professor I. A. Silberg, Department of Organic Chemistry, “Babes-Bolyai” University Cluj-Napoca (Romania) for kindly providing UDP.

REFERENCES

[1] L. Gorton, A. Lindgren, T. Larsson, F. D Munteanu, T. Ruzgas, I. Gazaryan, *Anal. Chim. Acta*, **1999**, 91, 400.
[2] L. Gorton, E. Dominguez, Rev. *in Mol. Biotechnol.*, **2002**, 82, 371.
[3] Z. Samec, P. J. Elving, *J. Electroanal. Chem.*, **1983**, 144, 217.
[4] M. Zayats, A. B. Kharitonov, E. Katz, A. F. Buckmann, I. Wilner, *Biosens. & Bioelectron.*, **2000**, 15, 671.
[5] H.-L. Schmidt, W. Schuhmann, *Biosens. & Bioelectron.*, **1996**, 11, 127.
[6] J. Katrlik, J. Svorc, M. Stred'ansky, S. Miertus, *Biosens. & Bioelectron.*, **1998**, 13, 181.
[7] M. Boujtita, J. P. Hart, R. Pittson, *Biosens, & Bioelectron.*, **2000**, 15, 257.
[8] L. T. Kubota, L. Gorton, *Electroanalysis*, **1999**, 11, 719.
[9] C.-X. Cai, K.-H. Xue, Y.-M. Zhou, H. Yang, *Talanta*, **1997**, 44, 339.
[10] W. J. Blaedel, R. C. Engstrom, *Anal. Chem.*, **1980**, 52, 1691.
[11] F. Tobalina, F. Pariente, L. Hernandez, H. D. Abruna, E. Lorenzo, *Anal. Chim. Acta*, **1999**, 395, 17.
[12] A. Karyakin, E. Karyakina, W. Schuhmann, H.-L. Schmidt, *Electroanalysis*, **1994**, 6, 821.
[13] M. J. Lobo Castanon, A. J. Miranda Ordieres, P. Tunon-Blanco, *Biosens. & Bioelectron.*, **1997**, 12, 511.
[14] A. Karyakin, O. A. Bobrova, E. A. Karyakina, *J. Electroanal. Chem.*, **1995**, 399, 179.
[15] M. Somasundrum, J. V. Bannister, J. Chem. Soc., *Chem. Commun.*, **1993**, 1629.
[16] F. Y. Bernadette, R. L. Christopher, *Anal. Chem.*, **1987**, 59, 2111.
[17] D. Gligor, L. Muresan, I. C. Popescu, I. A. Silberg, *Rev. Roum. Chim.*, **2002**, 47(10-11), 953.
[18] C. Bodea, I. Silberg, *Rev. Roumaine Chim.*, **1964**, 9, 425.
[19] G. Cauquis, A. Deronzier, J. -L. Lepage, D. Serve, *Bull. Soc. Chim. France*, **1977**, (3-4), 295.
[20] L. Gorton, *Electroanalysis*, **1995**, 7, 23.
[21] H. Jaegfeldt, T. Kuwana, G. Johansson, *J. Am. Chem. Soc.*, **1983**, 105, 1805.
[22] A. Torstensson, L. Gorton, *J. Electroanal. Chem.*, **1981**, 130, 199.
[23] M. K. Ciolkosz, J. Jordan, *Anal. Chem.*, **1993**, 65, 164.