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FORMATE DEHYDROGENASE-MODIFIED CARBON PASTE ELECTRODES FOR AMPEROMETRIC DETECTION OF FORMATE

Florentina-Daniela MUNTEANU,[a] Delia GLIGOR,[b] Ionel Cătălin POPESCU[b] and Lo GORTON[*a]

[a] Department of Analytical Chemistry, Lund University, P.O. Box 124, SE-22100 Lund, Sweden
[b] Department of Physical Chemistry, Faculty of Chemistry and Chemical Engineering, "Babeş-Bolyai" University, Arany Janos 11, 400028 Cluj-Napoca, Roumania

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A simple and inexpensive amperometric biosensor for formate detection was obtained by immobilization of formate dehydrogenase (FDH) on graphite powder, modified with a new phenothiazine derivative (DDDP; 16*H*, 18*H*-dibenzo[c,1]-7,9-dithia-16,18-diazapentacene). Two different enzyme environments were used: FDH was adsorbed on DDDP-modified graphite powder in the presence of polyethylenimine (PEI) as well as in its absence. In both cases, when 10 mM NAD^+ were added in the supporting electrolyte (phosphate buffer, pH 7.0), the biosensor developed a response to formate, obeying Michaelis-Menten kinetics with a linear domain up to 3 mM formate. The biosensor sensitivity, calculated as the ratio I_{max}/K_M^{app}, was better in the second case (0.962 mA/M), but the best detection limit (3 μM) was observed in the first case.

INTRODUCTION

Redox enzymes have been widely used in amperometric biosensor research.[1-7] The largest group of redox enzymes known today is the nicotinamide dinucleotide (NAD^+/NADH) dependent dehydrogenases.[8-11] Coupling the electrocatalytic oxidation of NADH by an appropriate mediator to the reaction catalyzed by the NAD^+ dependent dehydrogenase enzymes enables the construction of amperometric biosensors for a large variety of biologically important species in biotechnology and analysis.[12-14]

NAD^+-dependent formate dehydrogenase (FDH; formate:NAD^+ oxidoreductase, EC 1.2.1.2) from methyloprotic bacterium *Pseudonomas* sp. is one of the most extensively characterised NAD^+-dependent dehydrogenases.[15,16] FDH is composed of two identical subunits each comprising two domains: a coenzyme binding domain and a substrate-binding domain.[17] FDH was purified from several sources including eukaryotes, archaea and bacteria. In general, FDHs from aerobic organisms reduce NAD^+, have a high K_M for formate and are insensitive to O_2.[18] The optimum pH range for substrate binding is 5.5–10.5.[19]

FDH catalyzes the oxidation of formate to carbon dioxide. Formate reacts with FDH in the presence of NAD^+, which is reduced in the process to NADH. In turn NADH should be reoxidized at the electrode surface in a biosensor format.

Formate as substrate for FDH is one of the cheapest hydrogen sources and the oxidation product CO_2 can be easily removed from the reaction mixture.[18,20] Formate is a common substance found in the atmosphere, museum cabinets, exhaust emissions, natural and seawaters, sediments[21,22] and is a product of degradation of metal-cyano compounds by fungi.[22]

A limited number of devices for the real-time determination of formate, based on a biosensor using FDH and a chemically modified electrode, have been recently introduced.[14] These biosensors are mainly based on electrocatalytic oxidation of NADH with modified electrodes such as polypyrrole/ferrocyanide[23,24] and 3,4-dihydroxibenzaldehyde.[25] The major drawbacks of these biosensors are the low sensitivity, poor stability and slow response. A gaseous biosensor for formic acid has been developed using Meldola's blue as mediator, the biosensor suffering from very short lifetime due to its constructional limitation.[21]

[*]Corresponding author: lo.gorton@analykem.lu.se

In this context, 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 formate determination, by immobilization of formate dehydrogenase on graphite powder modified with DDDP, was investigated.

EXPERIMENTAL SECTION

Chemicals

Recombinant NAD^+-dependent formate dehydrogenase (FDH) from *Pseudonomas* sp. 101 expressed in *E. coli* with an enzymatic activity of 180 U/mL (30°C) and purity higher than 95% was provided by Prof. V. Tishkov (Moscow University).

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 at pH 7.0 (Merck, Darmstadt, Germany).

Potassium chloride and formic acid were purchased from Merck. 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 graphite powder with 25 μL of paraffin oil.

Preparation of the FDH-modified carbon paste electrodes

- No PEI addition: To 14 mg of DDDP-modified graphite powder 0.5 mL of FDH solution was added, and adsorption of the enzyme was allowed to proceed in vacuum until a dried carbon powder was obtained.
- With PEI addition: In a first step, adsorption of FDH on DDDP-modified graphite powder was realized as mentioned above. Then, 23 μL of 0.2% (w/v) PEI were added to the resulting paste and the final drying was allowed to proceed in vacuum.

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 the working electrode, a platinum ring as the counter electrode and an Ag|AgCl (KCl_{sat}) as the 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.

RESULTS AND DISCUSSION

The response of the amperometric biosensor presented in the text below is based on the following sequence of reactions:

$$\text{Formate} + NAD^+ \xleftrightarrow{\text{FDH}} CO_2 + NADH + H^+ \quad (1)$$

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

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

It should be remembered that FDH and the oxidized form of DDDP (as electrocatalyst) are both present in the carbon paste, whereas NAD^+ is dissolved into the electrolyte solution. When formate 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 formate oxidation, which itself is proportional to the formate concentration if the concentrations of the other reactants are kept constant and FDH 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^+.

Previously, it was shown that at DDDP-modified carbon paste electrode the oxidation of NADH occurs efficiently.[28] The cyclic voltammograms presented in Fig. 1 clearly show that in the presence of NAD^+ and formate the DDDP-modified carbon paste electrode, incorporating FDH, is able to sustain the catalytic cycle described by reactions (1-3). Thus, the enhancement of the anodic peak current associated with the diminishing of the cathodic one proves the strong electrocatalytic effect of DDDP for the enzymatically produced NADH. Obviously, no catalytic current can be observed in the absence of NAD^+ and/or formate (Fig. 1).

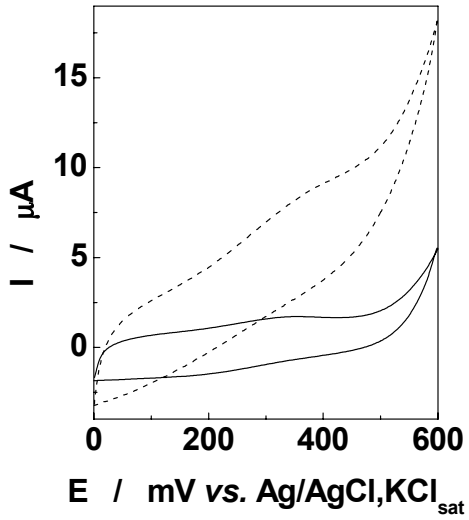

Fig. 1 – Cyclic voltammograms of FDH-DDDP-CPE recorded in 0.1 M phosphate buffer solution, pH 7 (—) and in 0.1 M phosphate buffer solution, pH 7, containing 10 mM NAD^+ and 30 mM formate (----). Experimental conditions: starting potential, 0 mV *vs.* Ag|AgCl/KCl_{sat}; scan rate, 5 mV s^{-1}.

Influence of NAD^+ concentration

From kinetic and thermodynamic considerations[30] it can be presumed that a higher level of NAD^+ leads to a shift of the equilibrium described by reaction (1) towards product formation. For this reason, the effect of NAD^+ concentration on the biosensor response was investigated at a constant concentration of formate (50 mM). This concentration was chosen to be high enough in order to ensure saturation of FDH. A plot of steady-state current as a function of NAD^+ concentration is presented in Fig. 2. It is obvious that the steady-state current increases with an increase in the concentration of NAD^+. Based on these results and in order to ensure that the response of the sensor was less dependent of the concentration of NAD^+, a value of 10 mM NAD^+ was employed for all further measurements.

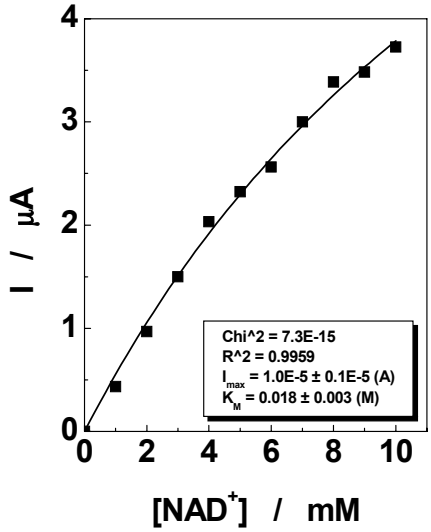

Fig. 2 – Dependence of the current intensity for 50 mM formate observed at FDH-DDDP-CPE as a function of the concentration of NAD^+ in the buffer. Experimental conditions: see Fig. 1. All current values were measured at +430 mV *vs.* Ag|AgCl/KCl_{sat} at FDH-DDDP-CPE.

Response to formate of FDH-DDDP-CPE

Fig. 3 depicts the FDH-DDDP-CPE amperometric response to successive injections of 1 mM formate, and gives qualitative information on the response rate ($t_{95\%}$ ~ 70 s) as well as on the signal stability. In order to diminish the mass transport effect on the biosensor response the FDH-DDDP-CPE was rotated with 500 rpm.

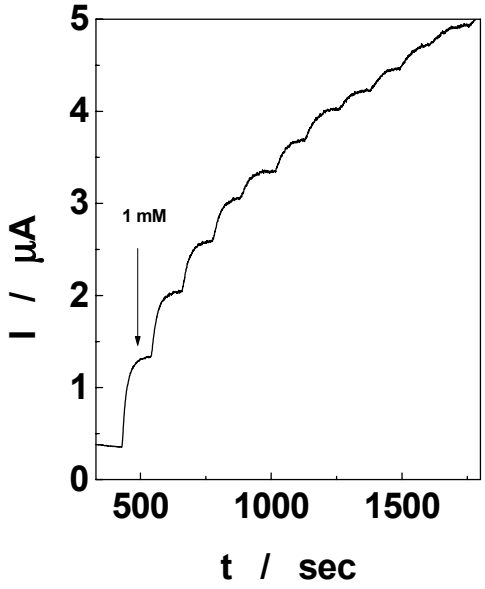

Fig. 3 – Amperometric response of the FDH-DDDP-CPE to successive increments of 1 mM formate. Experimental conditions: applied potential, +430 mV *vs.* Ag|AgCl/KCl_{sat}; supporting electrolyte, 0.1 M phosphate buffer (pH 7.0) containing 10 mM NAD^+; electrode rotation speed 500 rpm.

The dependence of the FDH-DDDP-CPE and FDH-PEI-DDDP-CPE response *vs.* formate concentration was shown to obey Michaelis-Menten kinetics (Figs. 4A and 4B). The values of the kinetic parameters (I_{max} and K_M^{app}) were calculated by fitting the experimental data to the Michaelis-Menten equation (Table 1). Undoubtedly, such an assumption is a simplification of the real process. However, it can be used to calculate the electroanalytical parameters of the biosensor. A linear response is observed up to 3 mM in both cases.

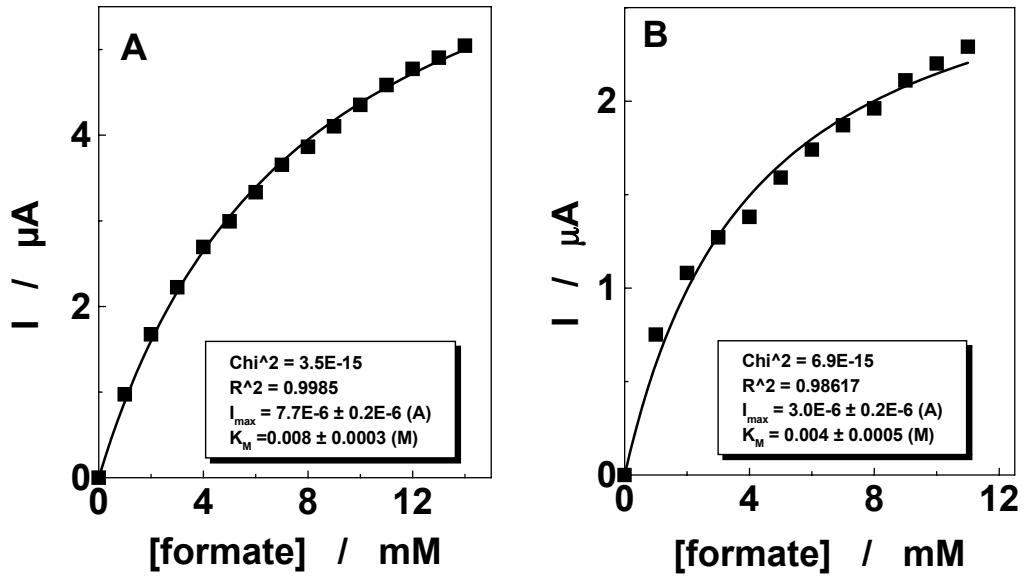

Fig. 4 – Calibration curves to formate at (A) FDH-DDDP-CPE and (B) FDH-PEI-DDDP-CPE. Experimental conditions: see Fig. 3.

The biosensor sensitivity (estimated as the I_{max}/K_M^{app} ratio) revealed that the FDH-DDDP-CPE was significantly more sensitive (0.962 mA/M) than FDH-PEI-DDDP-CPE (0.750 mA/M). When PEI was added

in the carbon paste composition the K_M^{app} decreased from 8 mM to 4 mM. This observation suggests that addition of PEI induces an increase in the affinity of the mediator to bind NADH, due to the presence of a positively charged polyelectrolyte in the carbon paste composition.

Table 1

Kinetic parameters for the response to formate of the amperometric biosensor, estimated by fitting the experimental data to the Michaelis-Menten equation (M-M) and by Hanes-Woolf linearization of the calibration curve (H-W)

Parameters	FDH-DDDP-CPE		FDH-PEI-DDDP-CPE	
	M-M[a]	H-W[b]	M-M[c]	H-W[d]
I_{max} (μA)	7.7 ± 0.2	7.6 ± 0.3	3.0 ± 0.2	3.6 ± 0.2
K_M (mM)	8.0 ± 0.3	7.3 ± 0.7	4.0 ± 0.5	6.5 ± 0.7
S (mA/M)	0.96 ± 0.05	1.04 ± 0.05	0.75 ± 0.07	0.55 ± 0.03

[a]see Fig. 4A; [b]R/N = 0.9976 / 14; [c]see Fig. 4B; [d]R/N = 0.9986 / 8.

On the other hand, for FDH-PEI-DDDP-CPE the K_M^{app}, I_{max} and sensitivity (S, mA/M) values, calculated from the Hanes-Woolf linearization, were found to be different from those obtained from the experimental data fitting to the Michaelis-Menten formalism (see Table 1). This discrepancy was attributed to the lower reproducibility of the FDH-PEI-DDDP-CPE response, reflected by a higher fluctuation of the experimental data involved in the calibration curve (Fig. 4B).

Unexpectedly, the K_M values estimated for immobilized FDH were close to that reported for the free enzyme (K_M = 7 mM), obtained from spectrofluorimetric measurements, performed in phosphate buffer (pH 7) at 37°C.[19] This finding suggests that the microenvironment existing in carbon paste around the immobilized enzyme is quite mild, affecting to a very low extent its activity.

The detection limit (calculated for a signal/noise ratio of 3) was found to be 10 μM for FDH-DDDP-CPE and 3 μM for FDH-PEI-DDDP-CPE. As a concluding remark, it can be stated that the FDH-DDDP-CPEs are good amperometric sensors for formate, having a wide linear range and a relatively low detection limit. For four independent determinations, performed for a formate concentration of 1 mM, an average response current of 0.73 μA, with a relative standard deviation of 0.02 μA, was obtained.

The stability of the FDH-DDDP-carbon paste electrode was evaluated by measuring periodically the response to formate, when the biosensor was stored at 4°C. The response retained 88% of its initial value for three days, indicating that FDH-DDDP-carbon paste electrodes display a quite stable response to formate. The decrease in the response to formate was attributed to a loss of the enzyme activity, because the DDDP carbon paste electrodes exhibit excellent stability for NADH oxidation.[28]

Future work will include studies for improvement of the sensitivity and detection limit using other redox mediators and at the same time higher attention will be paid to the possibility of making an amperometric biosensor for formate determination, which can be used at a lower working potential. It is worthy to note that the system described here can be easily adapted to other substrates using their corresponding dehydrogenases.

CONCLUSIONS

The investigations described in this paper aimed at obtaining an amperometric biosensor for formate determination. The analytical signal is due to the electrocatalytic oxidation of enzymatically generated NADH at FDH-DDDP-carbon paste electrodes. The biosensor FDH-DDDP-CPE exhibits a good sensitivity (0.962 mA/M) and a low limit of detection (9.7 μM), as well as a fast response and a linear domain of concentration up to 3 mM.

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