J Solid State Electrochem (2008) 12:175–180
DOI 10.1007/s10008-007-0378-1

ORIGINAL PAPER

Electrocatalytic detection of NADH and ethanol at glassy carbon electrode modified with electropolymerized films from methylene green

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Received: 26 April 2007 / Revised: 8 May 2007 / Accepted: 14 June 2007 / Published online: 13 July 2007

Abstract The oxidation of NADH on electropolymerizing methylene green (MG)-modified glassy carbon electrode (GCE) is described. The modified electrode shows an excellent electrocatalytic activity toward NADH oxidation, reducing its overpotential by about 650 mV and exhibits a wide linear range of 5.6–420 μM NADH with the detection limit of 3.8 μM. The electrode displays a good reproducibility and stability and the coexisting species does not affect the determination of NADH. The application in the amperometric biosensing of ethanol using alcohol dehydrogenase enzyme (ADH) also has been demonstrated with this electrode. MG-modified GCE can not only be used to detect NADH in biochemical reaction, but also can be used as the potential matrix of the construction of dehydrogenases biosensor.

Keywords NADH · Methylene green · Detection · Alcohol dehydrogenase · Ethanol · Electropolymerization

Introduction

β-Nicotinamide adenine dinucleotide (NADH) is involved as a cofactor in several hundred enzymatic reactions of NAD^+/NADH-dependent dehydrogenases. The dehydrogenase enzymes catalyze the oxidation of a variety of families, such as alcohol, lactate, glucose, aldehyde, and carbohydrate, which are of immense interest from the analytical point of view [1–3]. The studies of electrochemical oxidation of NADH related to the development of amperometric biosensors are of considerable interest [4–8]. Owing to the large overvoltage encountered for NADH oxidation at ordinary electrodes [9] and surface fouling associated with the accumulation of reaction products [10], considerable effort has been devoted to provide new electrode materials or new methods that will reduce the overpotential for NADH oxidation and increase the stability of the electrode.

During the last three decades, several mediators such as ortho- and paraquinones, phenylenediamines, phenoxazines, alkylphenazines, and phenothiazines [11–13] for NAD^+/NADH regeneration have been proposed. Methylene green (MG) has also been applied to sense NADH, but it has always been immobilized on the surface of the electrode by adsorption [14–16], which resulted in the instability and tended to desorption [17].

Among all the strategies for incorporating the mediator in the electrode configuration, the electropolymerization has proved to be most reliable [18]. The permselective electropolymerized film covers the whole electrode surface, improving the selectivity and the stability of the biosensor [19, 20], in some cases, improving even the sensitivity [21] and avoiding fouling of its surface and having the better reproducibility. Recently, some mediators such as azure B [22] and Meldola's blue [23] have been reported to electropolymerize on the surface of the electrode to sense NADH. This paper reports the electropolymerization of MG on the surface of the glassy carbon electrode (GCE) for the oxidation of NADH and the construction of a highly sensitive and stable NADH biosensor. Chen reported electropolymerization of MG and its electrocatalysis for the oxidation of NADH [24]. But the linear range is narrow and the detection limit is not low.

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Furthermore, the quantification of ethanol is very important in many different areas like clinical, forensic, agricultural and environmental analysis, food, beverage, and pulp industries [25, 26]. Various methods including enzymatic and nonenzymatic methods have been developed [27, 28]. The enzymatic methods are based on the use of either alcohol dehydrogenase (ADH) or alcohol oxidase enzymes. The methods based on ADH have received much interest, requiring the coenzyme NAD^+; ADH catalyzes the oxidation of primary alcohols (other than methanol) in the presence of NAD^+. The voltammetric sensing of ethanol using ADH is based on the electrochemical detection of enzymatically generated NADH during the enzymatic reaction. Although Chen et al. reported electropolymerization of MG [24], it was not applied to sense ethanol. In this paper, the MG modified electrode is used to sense ethanol using ADH and NAD^+.

Experimental section

NADH, NAD^+, and ADH were purchased from Sigma and used as received. MG was purchased from Beijing Chemical Co. Phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of K_2HPO_4 and KH_2PO_4 and adjusting the pH with H_3PO_4 or NaOH. All other chemicals were of analytical grade and were used without further purification. All solutions were made up with doubly distilled water.

The GCE (3 mm in diameter) was polished to a mirror-like finish with 1.0, 0.3, and 0.05 μm alumina slurry (Bühler) followed by rinsing thoroughly with doubly distilled water. The electrodes were successively sonicated in 1:1 nitric acid, acetone, and doubly distilled water, and then allowed to dry at room temperature. Electropolymerization of MB on GCE was carried out using cyclic voltammograms in 0.1 M pH 6.2 PBS containing 0.5 mM MB in a potential range from −600 to 1,300 mV at a scan rate of 100 $mV \cdot s^{-1}$. After successively cycling for 60 cycles, the electrodes were taken out and washed with doubly distilled water thoroughly.

Cyclic voltammetric measurements were performed on CHI 660 electrochemical workstation (CH Instruments, USA). All electrochemical experiments were carried out in a cell containing 5.0 mL 0.1 M pH 6.2 PBS at room temperature (25±2 °C) and using a platinum wire as auxiliary, a saturated calomel electrode as reference, and the MG modified electrode as working electrodes. In amperometric experiments, some ADH and NAD^+ were dissolved in 0.1 M pH 6.2 PBS, before the addition of the ethanol. After the background current reached to a steady state value, aliquots of a stock solution of ethanol in buffer were added. It was carried out by applying a potential of 50 mV on a stirred cell at (25±2 °C). The sensor response was measured as the difference between total and residual currents.

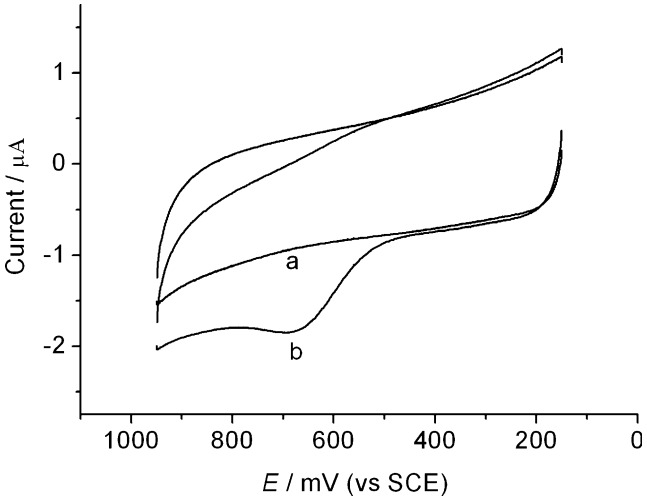

Fig. 1 Cyclic voltammograms of a bare GCE in the absence (*a*) and presence (*b*) of NADH at 100 $mV \cdot s^{-1}$ in 0.1 M pH 6.2 PBS

Results and discussion

Oxidation of NADH

Figure 1 showed the cyclic voltammogram of a bare GCE for the oxidation of NADH. It was found that no peak was observed at a bare GCE (curve a) and upon addition of NADH, the oxidation resulted in an oxidized peak with the anodic potential of 700 mV vs SCE (curve b).

The main objective of the present investigation is to utilize MG-modified electrode for the electrocatalytic sensing of NADH. MG is a two-electron mediator and the structure is shown in Fig. 2. MG reacts with NADH, followed by the regeneration of both MG and biologically active NAD^+. The electrochemical response depends on the electrochemical oxidation of MG used in a sensor design, as given by

$$NADH + MG_{(OX)} \rightarrow NAD^+ + MG_{(red)} + H^+ \quad (1)$$

$$MG_{(red)} \rightarrow MG_{(OX)} + e^- \quad (2)$$

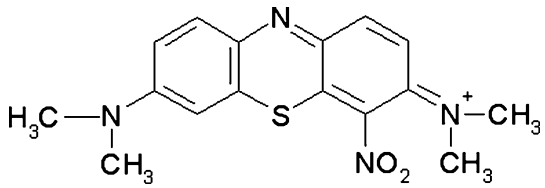

Fig. 2 The structure of methylene green (MG)

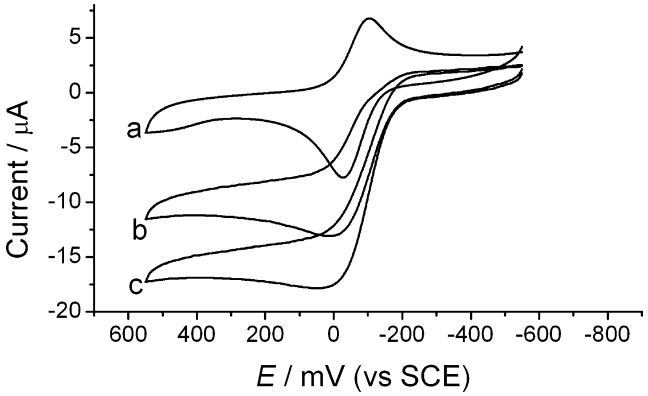

Fig. 3 Cyclic voltammograms of MG-modified electrode in 0.1 M pH 6.2 PBS containing 0 (*a*), 100 μM (*b*), and 200 μM (*c*) NADH at 100 mV·s^{-1}

Figure 3 depicted the typical cyclic voltammogram demonstrating the electrocatalytic activity of MG-modified electrode towards the oxidation of NADH. A couple of stable redox peaks with the formal potential of −71 mV vs SCE (curve a in Fig. 3) was observed at MG-modified GCE without the addition of NADH which was the same formal potential as that obtained for MG in aqueous solution (−70 mV vs SCE) [29]. Upon the addition of NADH, a dramatic enhancement in the anodic peak current associated with a decrease in the cathodic peak current was observed which came from the mediated oxidation of NADH to NAD^+ (curve b in Fig. 3). NADH oxidation at different concentrations of NADH at the MG-modified electrode was shown as curves b and c in Fig. 3 which covered the values of a great relevance in biosensor design and application.

The amperometric responses of the MG-modified electrode upon successive additions of 1.0 mM NADH to 0.1 M

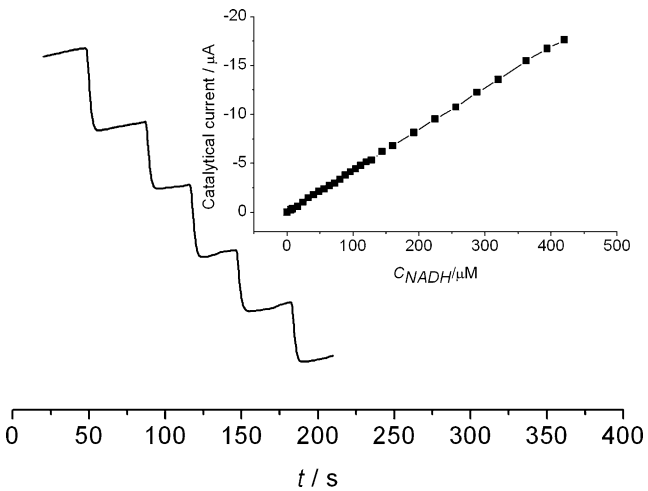

Fig. 4 Amperometric responses of MG modified GCE at the potential of 50 mV upon successive additions of 5.0 μL 5.6 mM NADH to 5.0 mL 0.1 M pH 6.2 PBS. *Inset* Plot of electrocatalytic currents vs NADH concentration

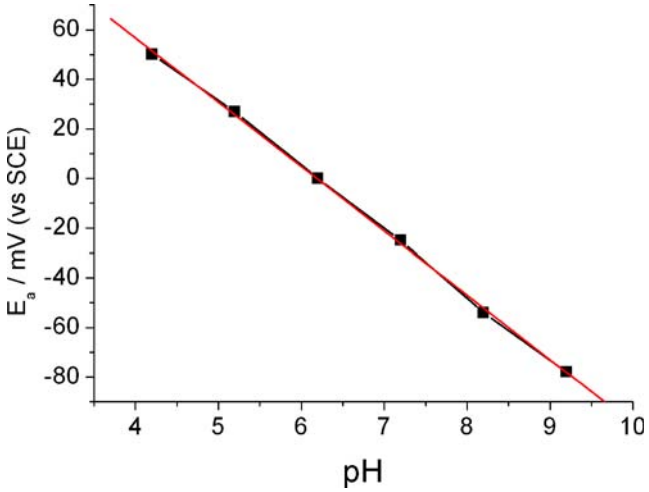

Fig. 5 Plot of anodic potential for the oxidation of 0.10 mM NADH on the solution pH (0.1M PBS) at the modified GCE. Scan rate: 100 mV·s^{-1}

pH 6.2 PBS at an applied potential of 50 mV were shown in Fig. 4. Upon addition of an aliquot of NADH to the buffer solution, the oxidation current increased steeply to reach a stable value. The enzyme electrode achieved 95% of the steady-state current in less than 10 s. The results demonstrated clearly that the electrocatalytic response was very fast, which could be used as an efficient sensor for NADH detection.

The magnitude of the catalytic current was proportional to the solution concentration of NADH over the range of 5.6–420 μM (inset in Fig. 4). The linear range was wider than that of 3–50 μM from GCEs modified with transition metal complexes containing 1,10-phenanthroline-5,6-dione ligands [30] and 10^{-4}–10^{-2} M from enzyme modified by electropolymerization of aminobenzene isomer and pyrroloquinolinequinone (PPQ) on electrode [31]. The detection limit was 3.8 μM at a signal to noise ratio of 3 which was lower than 5×10^{-4} M from electrode modified by PPQ [31] and 8.2 μM from MG adsorbed on the electrode [14] which indicated that the polymer-modified electrode exhibited a better catalytic activity for NADH.

The experimental parameters of scan rate, buffer solution, pH, and applied potential were investigated. The

Table 1 Dependence of the catalytic current as a function of the applied potential, obtained for MG-modified GCE in the presence of 0.1 mM NADH in 0.1 M pH 6.2 PBS

Applied potential (mV)	Δi (μA)
−150	0.1
−100	1.4
−50	4
0	4.6
50	5
100	5

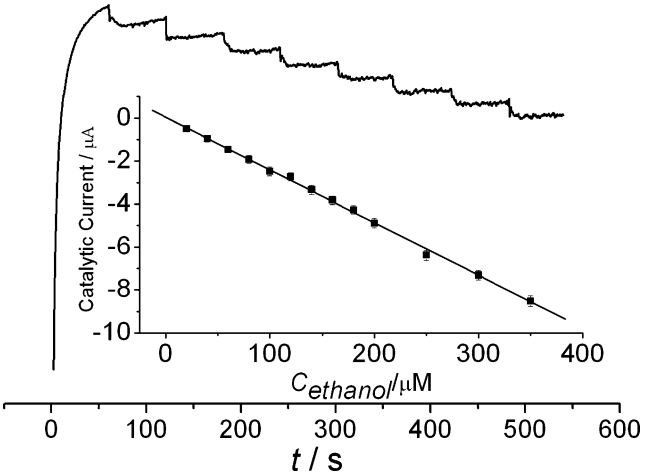

Fig. 6 Amperometric responses of MG-modified GCE upon successive additions of 5 μL 20 mM ethanol to 5.0 mL 0.1 M pH 6.2 PBS at the potential of 50 mV. *Inset* Linear curve of ethanol sensor

effect of scan rate on the oxidation of the NADH at MG-modified electrode was evaluated. With an increasing scan rate from 20 to 200 mV s^{-1}, the anodic peak potential of MG shifted to a more positive value. The peak currents were proportional to the square root of scan rate, indicating a diffusion-controlled behavior.

The effect of the buffer solution on the oxidation of NADH was investigated. Three buffer solutions, i.e., PBS, Britton-Robinson (B-R), and tris–HCl were used and it was found that the oxidation of NADH was not affected by the buffer solutions. So, we chose PBS which was closer to the life system.

The effect of pH on NADH sensor lay in two main aspects: the peak potentials and the peak currents. The optimal pH for enzymes varied with immobilization method and microenvironment around them. The maximum current response occurred at pH 6.2. An increase in solution pH caused a negative shift in anodic peak potentials. Figure 5 showed plot of the anodic potential vs pH (from 4.2 to 9.2) produced a line with the slope of 26.1 mV·pH^{-1}, indicating one proton and two electrons attending the electron transfer process.

The effect of the applied potential on the oxidation of NADH was investigated and the results were presented in Table 1. As could be observed the NADH electrooxidation began at −100 mV vs SCE and it reached a maximum at 50 mV vs SCE. The increase in amperometric response was because of the increased driving force for the fast oxidation of MG. Thus, this potential was applied to obtain the analytical curve for NADH.

Stability, reproducibility, and interference of NADH detection

An extremely attractive feature of the prepared sensor is whether it is stable. It was found the electrode could keep 93% of its initial current response when successively swept for 60 cycles from −550 to 550 mV, which suggested it could circumvent NADH surface fouling effects.

The storage stability of NADH biosensor stored in 0.1 M pH 6.2 PBS was examined by checking periodically its relative response currents in PBS containing 0.1 mM NADH. After a storage period of 1 month in 0.1 M pH 6.2 PBS the biosensor showed a 10% loss of activity.

The fabrication reproducibility of five electrodes, made independently, showed an acceptable reproducibility with a RSD of 6.2% for the current determined at 0.1 mM NADH. The recoveries for the assays of 0.1–0.3 mM NADH were between 96–102% for ten measurements.

One of the main difficulties in the development of the sensor for the electrocatalytic sensing of NADH is the interference due to ascorbic acid, uric acid, etc. The influences of foreign species were investigated at the applied potential of 50 mV by analyzing a standard solution of 0.1 mM NADH to which interfering species were added. An amount of 0.1 mM uric acid and 0.1 mM p-acetaminophenol did not cause any observable interference to the sensor response to NADH, and only ascorbic acid at the concentration of 0.1 mM produced the relative response of about 4.0%, indicating these species

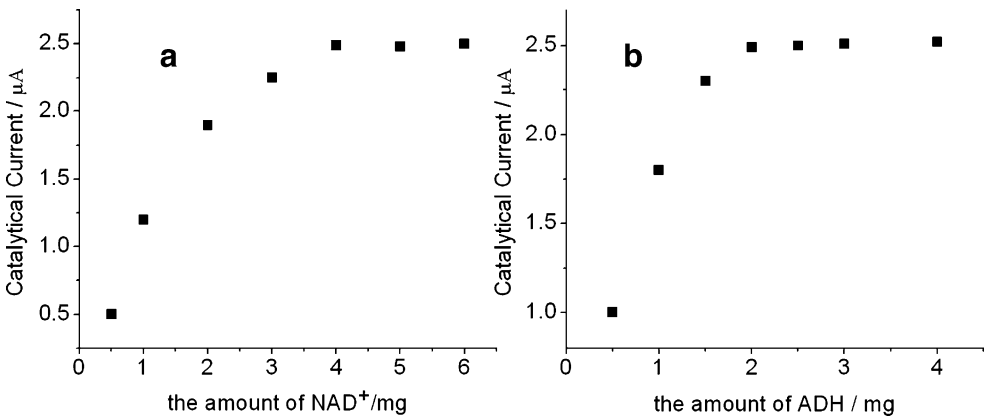

Fig. 7 Effect of the amounts of NAD^+ (keeping the amount of ADH constant) (**a**) and ADH (keeping the amount of NAD^+ constant) (**b**) for the oxidation of 0.10 mM NADH on the performance of the ethanol sensor, respectively

coexisting in the sample matrix did not affect the determination of NADH.

Amperometric biosensing of ethanol

ADH catalyzes the oxidation of ethanol and simultaneously the cofactor NAD^+ gets reduced to NADH. The reaction that occurs in the reaction is as follows [32]:

$$\text{ethanol} + NAD^+ + \text{ADH} \rightarrow \text{acetaldehyde} + \text{NADH} + H^+$$

According to the reaction above, the signal from NADH increases with increasing concentration of ethanol. Figure 6 shows the steady-state response for different additions of ethanol at a potential of 50 mV. Upon addition of ethanol to the buffer solution, the oxidation current increases steeply to reach a stable value and achieves 95% of the steady-state current in less than 20 s. The results demonstrate clearly that the electrocatalytic response is very fast. The linear curve of the sensor was shown as inset in Fig. 6. The calibration range of ethanol was done from 20 to 500 μM and the linear response range was from 20 to 350 μM with a correlation coefficient of 0.9994. The detection limit was 12 μM at a signal to noise ratio of 3 which was even lower than 0.1 mM with the SIRE biosensor P100 [33], 49 μM on Au nanoparticles [34] and 0.5 mM on mercaptopyrimidine and thiocytosine monolayer-modified electrodes [35].

The effects of the amounts of NAD^+ and ADH on the performance of the ethanol sensor were also evaluated. Figure 7a indicated that keeping the amount of ADH constant, the electrocatalytic currents increased with the increase of the amount of $NAD^+$and reached a maximum value when the amount of NAD^+ was 4.0 mg and stayed practically constant afterward. Figure 7b indicated that keeping the amount of NAD^+ constant, the electrocatalytic currents increased with the increase of the amount of ADH and reached maximum value when the amount of ADH was 2.0 mg and stayed practically constant afterward. So we chose the amounts of 4.0 mg of NAD^+ and 2.0 mg of ADH in the experiment, respectively.

MG-modified electrode imparted ethanol sensor a good long-term stability. The stability was quite good because it was not related to the activity of enzyme since ADH was added in solution and it could be applied in situ determination.

The concentration of ethanol in Budweiser beer was measured with both MG-modified electrode and the spectrophotometry. An amount of 1.0 μL sample was mixed with 10 mL 0.1 M pH 6.2 PBS. Five parallel determinations were carried out. The results from the biosensor correlated well with the results from spectrophotometry. The ethanol level was determined to be 0.09 mM close to the 0.10 mM determined by spectrophotometry, showing a good accuracy.

Conclusions

In summary, we present NADH and ethanol detection at GCE by electropolymerizing MG, which exhibits an excellent electrocatalytic activity towards the oxidation of NADH. NADH displays a good reproducibility and stability and the coexisting species in the sample matrix does not affect the determination of NADH. The new application described to biosensor development has been demonstrated by the construction of a very simple ethanol biosensor which exhibits a good performance to ethanol. MG-modified electrode provides an efficient matrix for development of NADH biosensors and a potential matrix of the construction of dehydrogenases biosensor.

Acknowledgements We gratefully acknowledge the financial support of the National Natural Science Foundation of China (nos. 20505010 and 20473038).

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