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ORIGINAL PAPER

Amplification of electrocatalytic oxidation of NADH based on cysteine nanolayers

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Abstract A biosensor for the determination of alcohol based on cysteine (Cys) nanolayers has been constructed. The utilization of the redox-inactive, self-assembled monolayer (SAM) modified gold ultra micro-electrode (UME) for NADH electroanalysis is introduced as a new modified gold Cys-UME. This Cys-UME prevents fouling and was found to be stable and ill-defined under subsequent potential sweeps, and different NADH concentrations. NADH oxidation gave a linear correlation between the peak current and NADH concentration (1–5 mM). Also, excellent electro-catalytic activity (enhancing the oxidation peak current) was observed towards NADH oxidation, with activation overpotential of about 250 mV lower than that of the bare electrode. The use of different alcohol concentrations in the presence of alcohol dehydrogenase (ADH) gave an increase in the NADH oxidation peak current, indicating that the electrochemical NADH oxidation at mild potentials could lead to an enzymatically active NAD^+.

Keywords Nanostructured monolayers · Cyclic voltammetry · Biosensors · NADH · Cysteine · Alcohol dehydrogenase

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1 Introduction

Electrochemical NADH detection is of great interest, because NAD pyridine nucleotides are ubiquitous in all living systems, and are required for the reactions of more than 450 enzymes (oxidoreductase) [1]. Also, the usage of NAD-dependent biocatalysts is very important in the chemical industry as well as for biofuel cells [2]. The electrochemical NADH oxidation has been the subject of numerous studies related to the development of amperometric biosensors [3]. There is a continued interest in the design of new sensors and biosensors with: higher sensitivity, selectivity and stability [4]. The direct electrochemical conversion of both nicotinamide adenine dinucleotides (NAD^+ and NADH) is severely affected by high overvoltage and side reactions [5]. Although the electrochemical NADH oxidation at neutral pH values is estimated to be −560 mV vs. SCE [2, 6, 7], a significant overpotential, as large as 1.0 V, is often required for direct NADH oxidation at bare electrodes [7–9]. The problems inherent to such anodic detections are; the high overvoltage encountered for NADH oxidation at ordinary electrodes, and surface fouling associated with the accumulation of reaction products [10]. Consequently, considerable efforts have been devoted towards identifying new electrode materials that will reduce the overpotential for irreversible NADH oxidation and minimize surface passivation effects. Generally, electrochemical measurements at solid electrodes are not appropriate for processes which are kinetically controlled or irreversible, since the electrode surfaces are easily poisoned by the products of red/ox reactions of the species or other impurities present in the test solution. Most of the problems in such systems, however, can be overcome by the use of ultra-microelectrodes (UMEs) [11]. UMEs have been applied as sensors in

various techniques such as: flow injection analysis [12, 13], cardiovascular monitoring and organic compound analysis [14, 15].

On the other hand, the self-assembly method is considered to be efficient for the modification of the electrode surface. The formation of self-assembled monolayers (SAMs) of: alkanethiol, dialkyl disulfide, dialkylsulfide and sulfur functional compounds on the gold electrodes is currently used for different electrochemical studies [16, 17], such as the high overpotential decrease for NADH oxidation [4, 8, 9]. Recently, the linkage of thiols SAM to enzymes has been employed in an electrochemical biosensor [18, 19]. Cysteine is a commonly used self-assembly reagent in the preparation of modified electrodes and biosensors. It is anchored onto the gold surface through a sulfur–gold interaction [20]. It is more stable and cheaper in comparison with other commonly used self-assembly reagents, including cystamine and some mercaptan compounds [20]. Recently, cysteine SAM has been used for the determination of unsaturated fatty acids [16] and also the construction of an electrochemical immunosensor [21].

Here we introduce an alcohol biosensor based on nanolayer formation with chemisorbed cysteine on a gold UME surface, presenting greater sensitivity and stability towards the oxidation of the irreversible NADH reaction and its linkage with the alcohol dehydrogenase (ADH) enzyme.

2 Material and methods

2.1 Chemicals

NADH, NAD^+ and ADH from bakers yeast (141 KDa, E.C. 1.1.1.1.) in the form of lyophilized powder, $\sim$300 units mg^{-1}, were obtained from Sigma. ADH Enzyme concentration was determined spectrophotometrically from absorbance at 280 nm using an absorption coefficient $\varepsilon_{280} = 1.89 \times 10^5\ M^{-1}\ cm^{-1}$ [22]. Cysteine was obtained from Merck. The rest of the chemicals were of analytical grade and were used without further purification. A phosphate sodium buffer solution 50 mM of pH value 7.5 was used as a supporting electrolyte. All the solutions were prepared using deionized water.

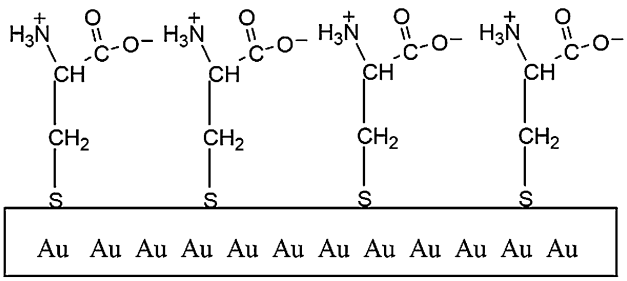
2.2 Instrumentation

All the electrochemical experiments were conducted with a setup comprising a PC PIV Pentium 900 MHz microcomputer equipped with a data acquisition board (PCL-818HG, Advantech. Co), and a custom made potentiostat. For the data acquisition and the data processing, a Delphi 6® program environment was developed in a conventional three-electrode electrochemical cell. In all measurements a reference electrode of Ag(s) | AgCl (s) | KCl (aq, 3 M) was employed. The auxiliary electrode was a Pt wire (length 1 cm and diameter 0.5 mm).

Silver epoxy (Johnson Matthey Ltd., UK) was used for the electrical contacts. The metal micro-wires (Goodfellow Metals Ltd., UK) were sealed into a soft glass capillary for the gold UMEs (radius of 12.5 μm) preparation. The modified (SAM) UME gold disc was used as the working electrode.

2.3 Surface-modified gold disc

The UME gold disc surface including 490.5 μm^2 was modified based on the affinity of the sulfur functionality group to adsorb onto the surface of Au molecules [20, 21]. Briefly, the gold disc was repeatedly polished with a fine emery paper and alumina powder. It was then sonicated in distilled water for 20 min. The polished electrodes were then electrochemically cleaned by cycling the potential between −500 and 1,600 mV in 0.5 M H_2SO_4 at a scan rate of 1 V s^{-1} for 5 min, or until the characteristic cyclic voltammogram for an Au electrode was obtained. Then, the gold electrode was cleaned with a piranha solution (a 3:7 mixture of 30% hydrogen peroxide and 98% sulfuric acid at 65 °C) for 20 min. The cysteine monolayer was formed by the spontaneous chemisorption of a cysteine solution on the cleaned gold electrode, as shown in Scheme 1. It was formed by soaking the cleaned electrodes in 0.02 M cysteine solution in deionized water for 2 h. Prior to its modification, the cysteine solution was purged with nitrogen for 5–10 min. The SAM modified Au electrodes were washed with distilled water and phosphate buffer before the initiation of the electrochemical experiments. The electrochemical sensing of NADH was carried out in a 50 mM phosphate buffer (pH 7.5) solution. All experiments were performed at least three times and reproducible results were obtained.



Scheme 1 Schematic diagram of the L-cysteine modified gold electrode

3 Results and discussion

3.1 Bare and modified UME for NADH oxidation

The electrode signal with a large background current (noise) of the large electrode surface can be resolved by UMEs [11, 23]. For this reason, the use of voltammetric techniques has been further stimulated by the advent of UMEs, because of their steady state currents, higher sensitivity owing to the increased mass transport and their ability to be used in solutions with very high resistance [24]. Figure 1 depicts the gradual decrease of the NADH oxidation peak current during successive potential cycles at bare Au UME, revealing that the electrode surface undergoes fouling during the oxidation process. It displays an ill-defined oxidation peak at around 0.9 V. This oxidation peak is decreased and shifted to a positive potential in the subsequent sweeps. The effects of the large overpotential and the surface fouling for the NADH oxidation at the ordinary bare electrode are obstacles to anodic detection. It was already reported that the bare Au electrode was unable to oxidize NADH, because of surface fouling by the intermediate complex in the following potential sweeps [8, 9, 25]. In this case, depending on the history of the electrode surface, the NADH oxidation occurred at a more positive potential (~0.9 V) and the corresponding voltammogram was ill-defined. Nonetheless, it is believed UMEs adversely affect the detection limit of the potential sweeping methods such as cyclic voltammetry [11, 26, 27]. In this study, Fig. 1 shows that the Au surface in UME undergoes fouling during the oxidation process similar to the ordinary electrodes. The fouling of the electrode surface by side reactions of the radical intermediates, formed during the oxidation process, is one of the reasons for the poor NADH electrochemical response at the bare electrode, leading to the positive shift in the oxidation potential [28].

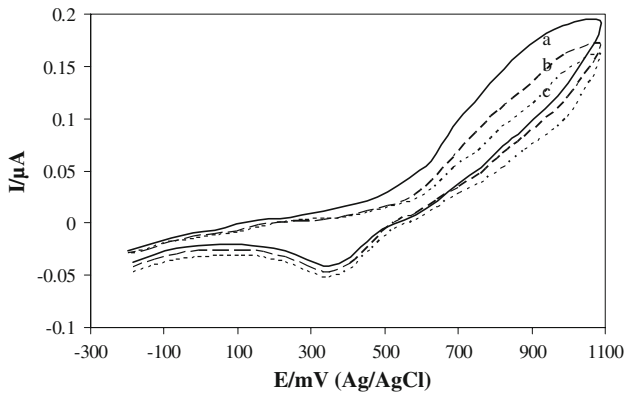


Fig. 1 Cyclic voltammograms for NADH oxidation (5 mM) at bare electrodes in a 50 mM phosphate buffer solution. The first and subsequent sweeps are represented as a, b and c. Scan rate: 60 mV s^{-1}

To overcome this problem, we modified the Au surface in the UME by SAM chemisorption of Cys as illustrated in Scheme 1. Cys molecules construct monolayers and coat gold surfaces by their 0.5 nm diameter. Figure 2 shows that the Ox/Red peak currents in the modified electrode (bold line) decreased in comparison with those of the bare one (thin line). When the gold surface was covered with the cysteine monolayer, both the anodic and the cathodic peaks diminished, showing that the formation of gold oxide was inhibited. Therefore, the Cys molecules were successfully modified on the Au surface of the UME. In Fig. 3, an approximate 250 mV decrease in the overpotential for the NADH oxidation is observed at the monolayer modified electrode, indicating that the Cys-UME monolayer facilitates NADH oxidation. The monolayer-modified electrodes gave a well-defined voltammetric signal for NADH oxidation and the peak currents were reproducible. Nevertheless, as presented in Scheme 1, the bound Cys molecules do not contain any redox mediator and are known to be electro-inactive. Figure 3 shows that modification of the UME surface with Cys shifted the oxidation peak to a less positive potential. Also the oxidation peak current was enhanced. In contrast to the bare electrode, the peak potential was not shifted to more positive potentials and the peak current did not change in the following three sweeps at the cys-Au UME (Fig. 3). It was previously reported that the electro-inactive self-assembled terminal group of the monolayer would affect the facilitation of electron transfer and NADH oxidation [25]. In the case of the cysteine modified electrode, electron tunneling was essentially unhampered for such short-chain SAMs molecules. The introduction of the cysteine layer over the gold electrode, on the one hand, may improve the hydrophilicity of the gold surface, but on the other hand, the charged cysteine molecules may facilitate catalytic effects to the NADH redox system, because of the Cys–NADH electrostatic interaction. Since the monolayer assembly covers the

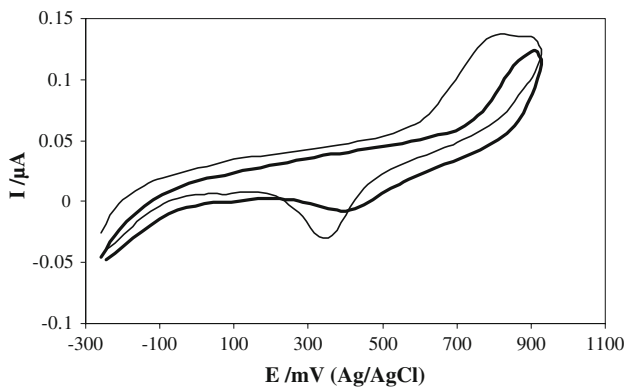


Fig. 2 Cyclic voltammograms for the bare (thin line) and the modified (bold line) electrodes in a 50 mM phosphate buffer solution. Scan rate: 60 mV s^{-1}

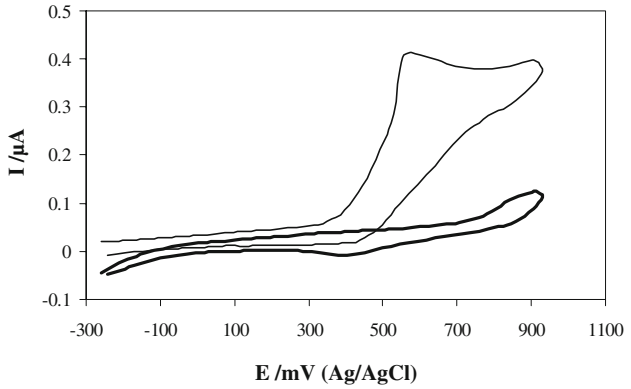


Fig. 3 Cyclic voltammograms of self-assembled monolayer gold electrode in the absence (bold line) and presence (thin line) of 5 mM NADH in 50 mM phosphate buffer solution, pH 7.5 (the first and the two subsequent sweeps during the successive potential cycles were the same). Scan rate: 60 mV s^{-1}

electrode surface, fouling of the electrode surface could not be expected as in the case of the bare electrode.

3.2 Diffusion dependency of NADH oxidation by Cys-Au UME

Peak currents vs. scan rate can give useful information about both phenomena including: diffusion controlled or adsorption controlled oxidation processes. To acquire a deeper understanding we have subtracted back current and depicted residual current for each scan rate, as in Fig. 4. The residual peak current for NADH oxidation at the Cys-UME increases linearly with the square root of scan rate (20–140 mV s^{-1}). This confirms the observations mentioned above and means that the oxidation process is diffusion controlled. In other words, the oxidation peak current increases linearly with increase of the square root of scan rate, suggesting that, in the oxidation of NADH, the intermediate complex products cannot be adsorbed on the

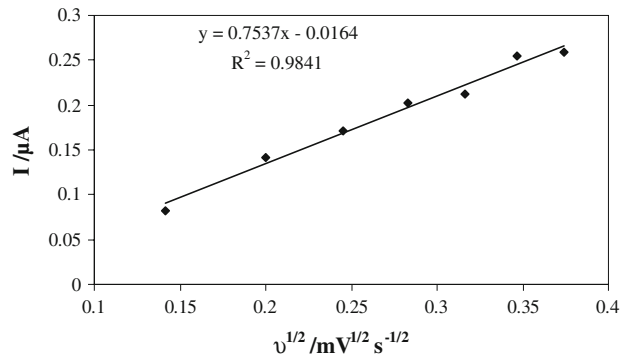


Fig. 4 Non-linear relationship between scan rate and cathodic peak current at the cysteine modified gold electrode of 5 mM NADH in a 50 mM phosphate buffer solution, pH 7.5. The back current has been subtracted for each scan rate in the current values

Au UME. These redox inactive monolayers effectively prevent fouling of the electrode and may be the reason for the facilitated oxidation at these monolayer-modified electrodes. Moreover, the facilitation of NADH oxidation can be explained by the electrostatic interaction between the cationic segment of the Cys monolayer and the anionic NADH.

3.3 Stability and NADH concentrations dependency of Cys-Au UME

According to Fig. 5, the peak intensity increased with increase in NADH concentration. The cysteine monolayer-modified UME can detect NADH as low as 1 mM. The plot of the background-subtracted currents against the NADH concentration, shows the linear cathodic peak current increase for the NADH concentration range of 1–5 mM (see inset of Fig. 5). Here it is worthwhile mentioning that the bare UME did not demonstrate any characteristic peaks for NADH oxidation in the above concentration range. To acquire information about the stability of the modified electrode, fresh Cys-UME was assessed and then kept for 48 h in phosphate buffer solution. From Fig. 6, it can be inferred that Cys-UME is stable and is able to oxidize NADH at the highest concentration, which can easily lead to surface fouling, after remaining for 48 h in the phosphate buffer solution.

3.4 Coupling Cys-UME with ADH enzyme as an alcohol biosensor

Direct electrochemical NADH oxidation at a bare metal surface did not allow regeneration of the enzymatically

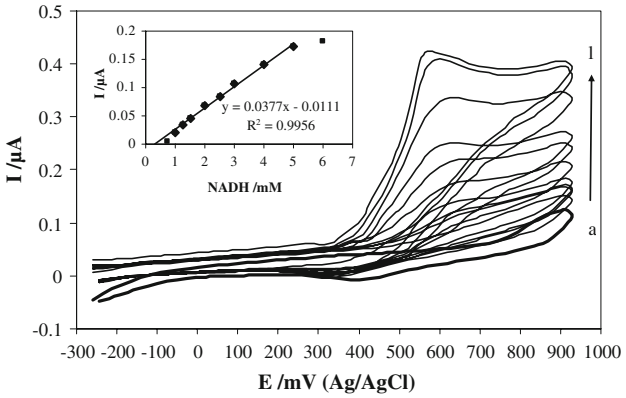


Fig. 5 Cyclic voltammograms for NADH oxidation at the self-assembled monolayer gold electrode at different NADH concentrations in a 50 mM phosphate buffer solution, pH 7.5. [NADH]: (a) 0, (b) 0.75, (c) 1, (d) 1.25, (e) 1.5, (f) 2, (g) 2.5, (i) 3, (j) 4, (k) 5, (l) 6 mM. The inset shows the linear dependency of the cathodic peak current with the NADH concentration (1–5 mM). Scan rate: 60 mV s^{-1})

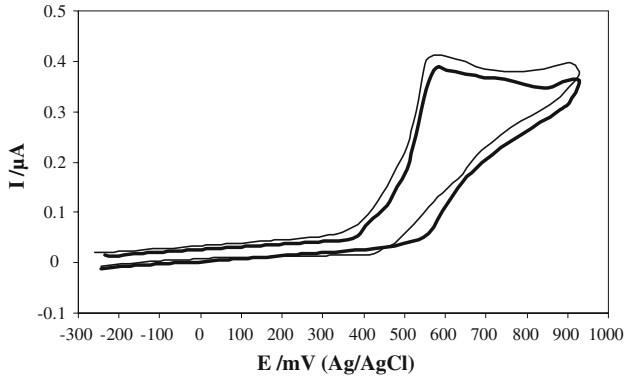


Fig. 6 Cyclic voltammograms of 5 mM NADH in 50 mM phosphate buffer solution (pH 7.5) of the fresh self-assembled monolayer gold electrode (thin line) and after remaining for 48 h (bold line) in phosphate buffer solution. Scan rate: 60 mV s^{-1}

active NAD^+, which is especially useful as a continuously supplied co-factor in enzyme-catalyzed organic synthesis or, in the electrochemical detection of dehydrogenase-based catalyzed reactions [29]. Electrochemical NADH oxidation at mild potentials can lead to enzymatically active NAD^+ and the design of alcohol biosensors [30]. The determination of ethanol is very important in: medicine, biological samples and the fermentation industry. Greatly improved NADH detection is a promising tool for the design of amperometric biosensors in combination with suitable dehydrogenase enzymes. ADH catalyzes the ethanol oxidation to acetaldehyde in the presence of NAD^+ and the reduced NADH can be oxidized electrochemically according to the following reactions:

$$NAD^+ + R\text{-}CH2OH \xrightarrow{ADH} NADH + R\text{-}CHO + H^+ \quad (1)$$

Electrochemical Oxidation

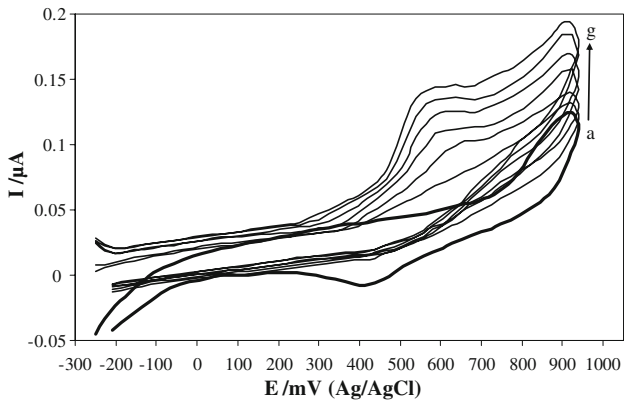


Fig. 7 Cyclic voltammograms for NADH oxidation at the self-assembled monolayer gold electrode at different ethanol concentrations in the presence of 10 mM NAD^+ and 0.1 μM ADH in a 50 mM phosphate buffer solution, pH 7.5. [Et-OH]: (a) 0, (b) 5, (c)10, (d) 15, (e) 20, (f) 25, (g) 30 mM. Scan rate: 60 mV s^{-1}

The cyclic voltammograms in the presence of different ethanol concentrations, and containing 10 mM NAD^+ are depicted in Fig. 7. It is evident that after use of the cysteine monolayer-modified UME with the ADH enzyme, the increase in the alcohol concentration results in increased intensity of the peak currents. Additionally, there is an increase in the cathodic current with addition of ethanol, illustrating electrochemical NADH oxidation as a function of the ADH-catalyzed enzymatic reaction to produce NADH again. On the other hand, NADH is oxidized to enzymatically active NAD^+ in the cathodic Cys-Au UME. As a consequence, the electrochemical oxidation of NADH at the Cys-Au UME surface was enzymatically active. This phenomenon forms the basis of an alcohol biosensor based on SAM of UME.

4 Conclusion

UMEs demonstrate steady state currents and higher sensitivity. This is a result of the increased mass transport and their application to highly resistant solutions. It is shown that bare Au UME was unable to oxidize NADH in subsequent sweeps (ill-defined oxidation peak at around 0.9 V), as ordinary Au electrodes. However, in successive potential cycles Au Cys-UME (unlike the bare one that required a large overpotential) were not fouled by oxidation products or intermediate complexes during the NADH oxidation process. The voltammetric response remained stable for 48 h. The facilitated electro-catalytic activity for NADH oxidation and reproduction of the enzymatically active NAD^+, obtained at the modified Cys-Au UME, suggests the possibility of deployment as enzymatic ethanol biosensors.

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