# ELECTROCATALYTIC OXIDATION OF NADH BY POLY-TOLUIDINE BLUE MULTIWALL CARBON NANOTUBES MODIFIED GLASSY CARBON ELECTRODE

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*This study investigates a new approach to the amperometric determination of NADH using a poly-toluidine blue multiwall carbon nanotubes modified glassy carbon electrode (PTB-MWCNTs/GCE). The currents obtained from the amperometric measurements at optimum conditions (phosphate buffer solution (pH 7.0) containing 0.1 M KCl and constant applied potential: +150 mV vs. Ag/AgCl/3.0 M KCl) were linearly correlated with the NADH concentrations. The calibration curve was obtained for NADH concentrations in a range of 0.1-100×10$^{-6}$ mol l$^{-1}$. The precision of the method was investigated by repeated measurements (n = 10) of the 10 µmol NADH and the relative standard deviation (RSD) was found to be 1.5 %. The limit of detection was found to be 0.04×10$^{-6}$ mol l$^{-1}$ for amperometric method.*

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## Introduction

NADH detection is of a great importance because the pyridine nucleotides NAD and NADP are ubiquitous in all living systems and are required for the reactions of more than 450 oxidoreductases [1]. Moreover, it can be used as the transduction reaction for designing amperometric biosensors based on dehydrogenases [2,3]. However, it is well-known that direct oxidation of NADH at a conventional solid electrode is highly irreversible, requires large activation energy, and proceeds with coupled side reactions, poisoning the electrode surface [4,5]. Different approaches have been proposed for the modification of the electrode surface of conventional electrode materials, in order to obtain modified electrodes exhibiting good electrocatalytic effect for NADH oxidation [6-20]. Among these, the strategies based on the electropolymerization of redox active monomers to fabricate the electroactive thin film on the surface of the electrode materials offer some advantages such as the sensitivity and efficiency and stability of modified electrodes for amperometric detection of NADH. Moreover, the electropolymerization approach offers a direct and easy control of surface coverage of the modified electrode [21]. The proper use of such mediators, the proper electrochemical tools for their investigation, the rationale for the selection of the proper mediator as well as the kinetic and thermodynamic constraints resulting by their use for NADH sensing have been extensively reviewed [22].

Toluidine blue (TB) is one of the popular phenothiazine derivatives, which is used commonly for preparation of chemically modified electrodes. It is a useful mediator in transporting the electrons from the reduced form of coenzyme NADH to electrode.

When immobilized at the electrode surface, TB is suitable for dehydrogenase-based bioelectrocatalytic systems for a broad variety of biosensors [23-28]. Besides, various TB based modified electrodes were used for determination of NADH [29,30], glucose [31] or chondroitin sulfate [32]. Electrochemical [33] and Raman spetroelectrochemical studies [34,35] on this mediator have been reported recently.

In this study, a thin film of poly TB was fabricated on the surface of multi-wall carbon nanotubes (MWCNTs) modified GCE because of the unique properties of MWCNTs such as large active surface area, high electronic conductivity, high mechanical resistance properties, anti-fouling capability and their ability to reduce over potential for NADH [36-39], which have promoted their extensive applications in NADH sensors.

**Material and Methods**

Reagents and Equipment

All chemicals including NADH and TB used in this study were of analytical reagent-grade (Merck) and used without further purification. The solutions throughout this work were always prepared using deionized water from a Milli-Q (Millipore, Bedford, USA) device. The stock solution of NADH (50 mmol l$^{-1}$) was freshly prepared in phosphate buffer (pH 7.0). The stock solution of TB (0.01 mol l$^{-1}$) was freshly prepared in redistilled water. The electropolymerization procedure of TB was always carried out in Britton-Robinson (BR) buffer containing 0.1 M NaNO$_3$ as supporting electrolyte. Phosphate buffer solution at pH 7.0 including 0.1 M KCl was used as supporting electrolyte for amperometric detection of NADH.

The three-electrode system consisting of GCE (working), Ag|AgCl|3.0 M KCl (reference) and platinum wire (counter electrode) connected to PalmSens (Ivium Technologies, Netherland) was used for electrochemical measurement.

Electrochemical Procedure

Before modification, GCE was polished with alumina (Al$_2$O$_3$, grain size 1 μm, 0.3 μm, 0.05 μm) on the polishing cloth, then GCE was sonicated in ethanol and deionized water to remove any trace impurities. The amount of 100 mg of the untreated multi-wall carbon nanotubes (MWCNTs) was added to 50 ml of nitric acid: sulfuric acid (3:1). The mixture was sonicated for about 1 hour at 25 °C and then was heated in 100 °C water bath for 4 hours to obtain a relatively stable suspension; then it washed with distilled water by centrifugation (4000 rpm, 10 min) to neutrality and dried for 4 hours using infrared lamp. The treated MWCNTs were dissolved in *N,N*-dimethylformamide (DMF) and were sonicated for one hour before casting 10 μl on the surface of clean GCE. The MWCNTs modified GCE was dried under an infrared lamp for DMF evaporation. Then the polymer film electrode was prepared by scanning successive cyclic voltammograms of TB on GCE in the potential range between –0.5 and +1.0 V *vs.* Ag/AgCl. In order to obtain the best electrocatalytic response of modified electrodes towards the NADH oxidation, the influence of the supporting electrolyte, monomer concentration, cycle number and anodic upper potential limit during the electropolymerization of TB were optimized.

**Results and Discussion**

Electropolymerization of TB and Its Electrochemical Behaviour

The catalytic effect of nitrate anions on the electropolymerization process of phenazines or phenoxazines has been reported in previous studies [40,41]. For that reason, BR buffer solution containing 0.1 M sodium nitrate was used as supporting electrolyte during the successive cyclic voltammetry sweep for the fabrication of poly TB on the surface of MWCNTs modified GCE.
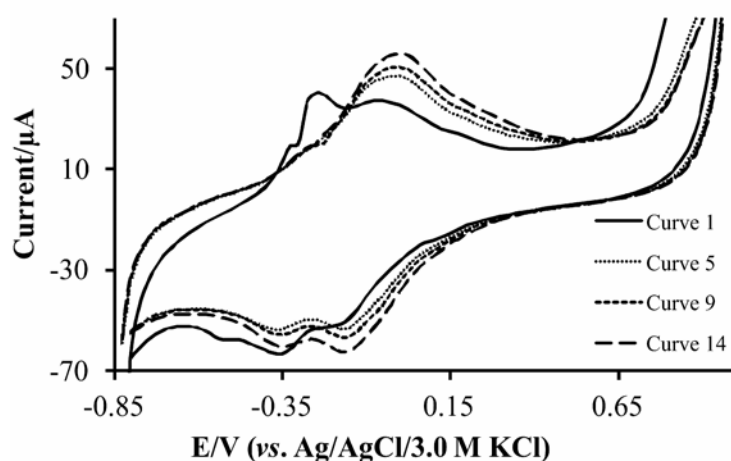


Fig. 1  Cyclic voltammograms (14 cycles) of PTB/MWCNT/GCE in BR buffer solution (pH 9.0) containing 0.1 M NaNO3, scan rate: 100 mV s$^{-1}$, potential range −0.8 and +1.0 V vs. Ag/AgCl/3.0 M KCl

Figure 1 shows the typical cyclic voltammograms of the polymer film growth during the electrolysis of TB solution in BR buffer pH 9.0. At the first scan, an anodic peak current and cathodic peak current were observed at −250 and −360 mV. This pair of redox peaks is caused by the oxidation and reduction of the monomeric form. After the first cycle, a new pair of redox peaks appeared at −100 and −160 mV. Moreover, the redox peak currents increased continuously with the increase of the number of scan. This phenomenon indicated that the electropolymerization process successfully occurred at the surface of the electrode.

The effect of supporting electrolyte, cycle number, monomer concentration and anodic upper potential used during TB electropolymerization process on electrocatalytic oxidation of NADH were investigated. Figure 2 shows the effect of these parameters on the electropolymerization of TB on the surface of the MWCNTs modified GCE. Figure 2A shows the effect of pH in the range of 3-10. At the acidic pH values, the polymerization process was not successful. For that reason, the oxidation currents of NADH obtained from the electrodes modified at
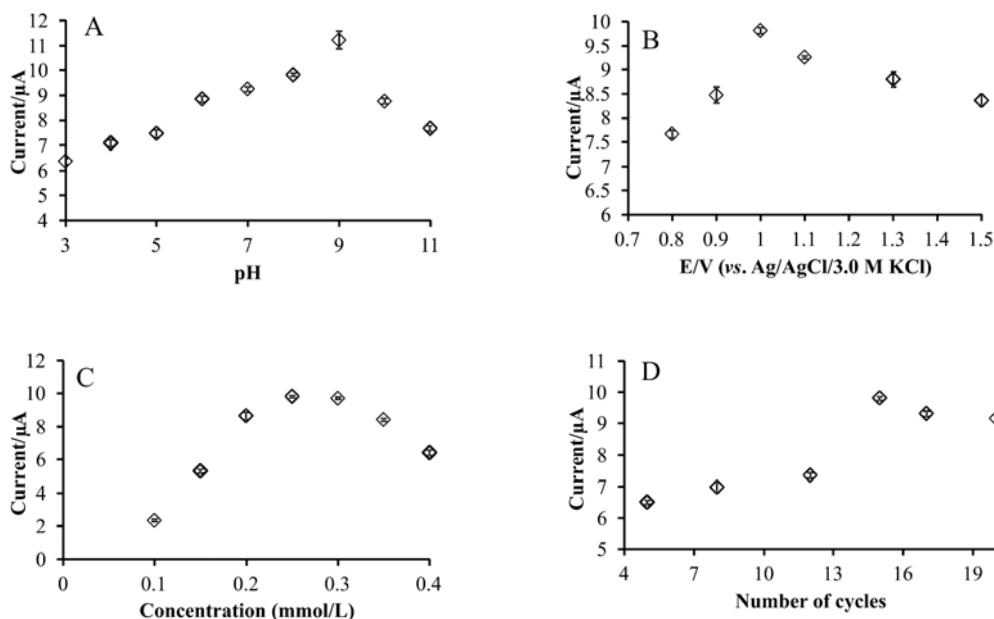
Fig. 2  The effect of A) pH, B) anodic upper potential limit, C) monomer concentration and D) cycle number to the electrocatalytic peak current of 0.2 mmol l$^{-1}$ NADH with PTB/MWCNT/GCE. Error bar represents the standard deviation of five measurements

these pH values were lower than those obtained at the basic pH values. At pH 9.0, the oxidation current of NADH was highest, which indicated that hydroxide ions showed a catalytic effect on the electropolymerization of TB.

This result was in good agreement with those reported before [30]. The potential sweep range, especially the upper potential limit, is a very important factor in the electropolymerization process. As can be seen in Fig. 2B, the optimum upper potential limit was 1.0 V. According to the theory, the electropolymerization process involves the formation of cation radicals upon electrooxidation. The primary amine groups in the chemical structure of TB can be the explanation for the formation of cation radicals at this upper potential limit. According to some previous studies, the formation of cation-radical species was possible at about 0.8 V for the electropolymerization of phenothiazines and phenoxazines if the parent monomer had primary or secondary amino groups as ring substituents [42,43]. The monomer concentration was optimized at 0.5 mM TB (Fig. 2C) and the number of cycles was chosen as 15 cycles (Fig. 2D).

Electrocatalytic Activity of PTB/MWCNT/GCE Towards NADH Oxidation

Cyclic voltammograms of PTB-MWCNTs/GCE and bare GCE were recorded in both the presence and absence of NADH in PBS solution (pH 7.0) containing 0.1 M KCl at the scan rate 50mVs$^{-1}$ (Fig. 3). In the absence of NADH, anodic and

cathodic peaks were observed at +50 mV and –75 mV, respectively, for PTB/MWCNT/GCE. The background current obtained from PTB/MWCNT/GCE was higher than the background current from bare GCE. In the presence of NADH, the anodic peak potential for the electrochemical oxidation of NADH at PTB/MWCNT/GCE and bare GCE were 100 and 475 mV, respectively. The peak potential of NADH oxidation at PTB/MWCNT/GCE shifted about 375 mV toward in negative direction compared with bare GCE. The oxidation current of NADH obtained from PTB/MWCNT/GCE (23.8 µA) was significantly higher than those obtained from bare GCE (10.9 µA).
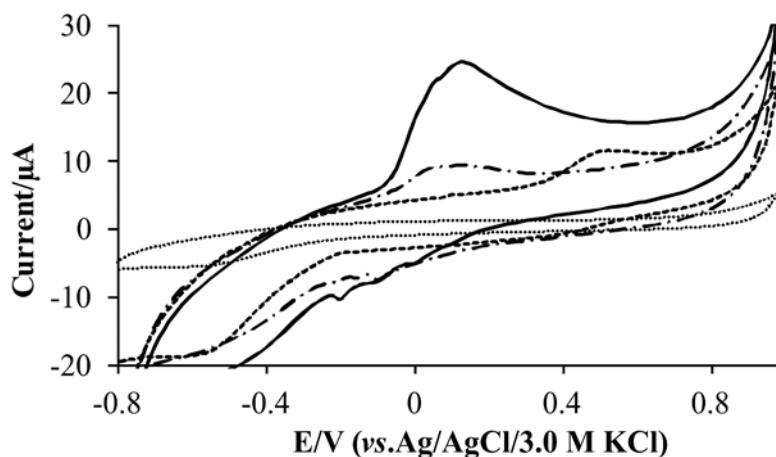


Fig. 3   Cyclic voltammograms of PTB/MWCNT/GCE in the absence of NADH (− • −), in the presence of 0.2 mmol l$^{-1}$ NADH (——); bare GCE in the absence of NADH (••••), in the presence of 0.2 mmol l$^{-1}$ NADH (− − − −). Experimental conditions: phosphate buffer solution (pH 7.0), scan rate 50 mV s$^{-1}$, potential range from –0.8 to +1.0 V vs. Ag/AgCl/3.0 M KCl

Amperometric Detection of NADH

Various values of stirring speed from 100 to 500 rpm at the fix applied potential +150 mV were employed for amperometric measurement of NADH. The concentration step of NADH was $8.0 \times 10^{-5}$ mol l$^{-1}$ for each addition to PBS (pH 7.0) containing 0.1 M KCl. The highest measured current was obtained at applied speed of stirring 400 rpm. At the stirring speeds higher than 400 rpm, the responses of amperometric detection are not stable due to the significant influence of noise. For that reason, the stirring speed 400 rpm was chosen for the next experiments.

Various values of applied potential from –100 to +500 mV at the stirring speed 400 rpm were applied for amperometric detection of NADH. The concentration step of NADH was also $8.0 \times 10^{-5}$ mol l$^{-1}$ for each addition to PBS at pH 7.0 containing 0.1 M KCl. As can be seen in Fig. 4, the increase of potential

in positive range gave the increase of the oxidation current of NADH. While oxidation current of NADH gradually increased at GCE and PTB/GCE from +150 to +300 mV, MWCNTs/GCE and PTB-MWCNTs/GCE allowed increasing the oxidation current of NADH steeply at +150 mV followed by the same values of the current ($p > 0.05$) up to +300 mV.
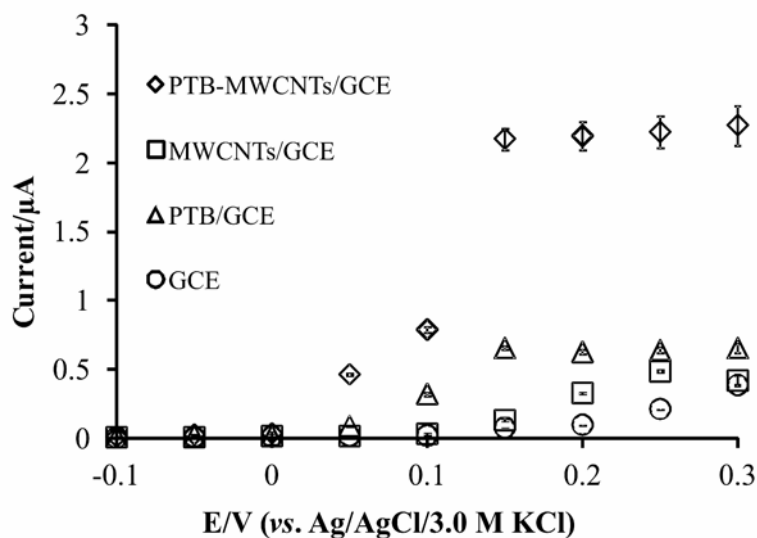


Fig. 4 The effect of applied potential to oxidation current of NADH ($10^{-4}$ mol $l^{-1}$) in phosphate buffer solution (pH 7.0) containing 0.1 M KCl. Amperometric detection, stirring speed 400 rpm. Error bar represents the standard deviation of five measurements
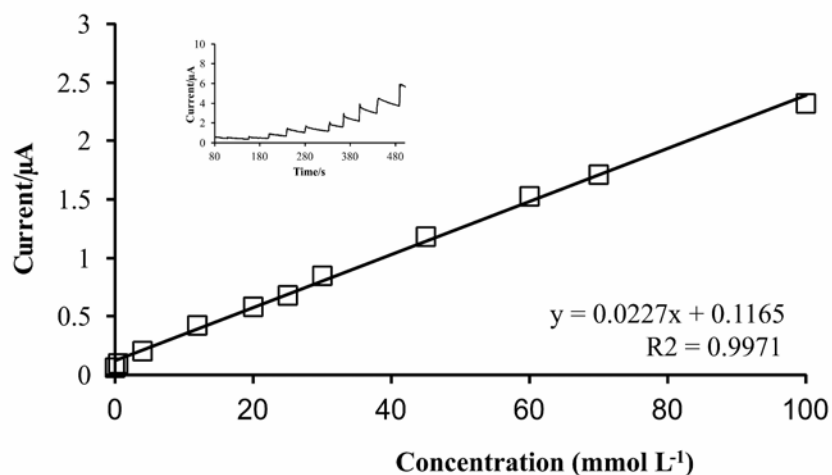


Fig. 5 Plot of oxidation current vs. concentration of NADH. Inlet: Amperometric current-time curves of NADH with various concentrations ($10^{-4}$ mol $l^{-1}$) using PTB/MWCNT/GCE. Experimental conditions: phosphate buffer solution pH 7.0 containing 0.1M KCl; applied potential, +150 mV; speed of stirring, 400 rpm

Therefore, the potential +150 mV was chosen for PTB-MWCNTs/GCE to avoid the oxidation from some interference compounds. The currents obtained at

this applied potential from PTB-MWCNTs/GCE were significantly higher than those obtained from other electrodes ($p < 0.05$)

As a result, the optimal applied potential and stirring speed for the amperometric detection of NADH were chosen as +150 mV and 400 rpm, respectively.Using these optimal conditions, amperometric currents versus various concentrations of NADH were determined using PTB-MWCNTs/GCE (Fig. 5).

As can be seen, the electrocatalytic current increased linearly up to 100 μmol l$^{-1}$, and then the linearity diminished and the response was constant up to concentration 700 μmol l$^{-1}$. In addition, degradation of the PTB-MWCNTs/GCE surface may have occurred because the response considerably decreased in repeated measurements. A linear relationship between the NADH concentration and the peak current was obtained over the concentration range $1.0\times10^{-7}$ -$2.0\times10^{-4}$ mol l$^{-1}$. The repeatability of the method was investigated by amperometric measurements of 5 μmol l$^{-1}$ and 10 μmol l$^{-1}$ NADH ($n = 20$) and the relative standard deviation (*RSD*) was found to be 7.4 % and 7.9 %, respectively. The LOD and LOQ (signal/noise = 3) was found to be 0.04 μmol l$^{-1}$ and 0.14 μmol l$^{-1}$, respectively.

## Conclusion

This study demonstrated that PTB/MWCNT/GCE showed significant electrocatalytic activity towards NADH oxidation. It was observed that the oxidation peak potential of NADH shifted from +200 mV at MWCNT/GCE to +50 mV at PTB/MWCNT/GCE. Besides, an enhancement in the oxidation peak current of NADH was observed. The amperometric detection of NADH with PTB/MWCNT/GCE was successfully applied. The detection limit of NADH was estimated at 0.04 μM in this study. It can be concluded that this paper shows the first study on the amperometric determination of NADH using a PTB/MWCNT/GCE.

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