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Poly(brilliant cresyl blue)-carbonnanotube modified electrodes for determination of NADH and fabrication of ethanol dehydrogenase-based biosensor

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ABSTRACT

A single walled-carbon nanotube (SWNT) modified with poly brilliant cresyl blue (PBCB) glassy carbon electrode has been fabricated by a simple, method in order to facilitate electrocatalytic detection of NADH. At this chemically modified electrode, NADH was determined in neutral phosphate buffer solution at 0 V (vs. SCE). The amperometric detection provided a wide linear current vs. concentration range (3.0–104.2 μM), a fast response time (within 5 s), high sensitivity [9.89 nA $(\mu M)^{-1}$] and a low detection limit (1.0 μM, $S/N = 3$). No interference was observed with a 100-fold excess of dopamine or uric acid. An ethanol biosensor also was developed using the nanocomposite modified electrode, by immobilizing ethanol dehydrogenase with carrageenan. In this case a linear ethanol concentration response was achieved in the range from 0.4 to 2.4 mM and the detection limit was estimated to be 0.1 mM ($S/N = 3$). The analytical performance achieved with the of the PBCB/SWNT nanocomposite electrode is expected to the development of novel biosensors, biofuel cells, and other bioelectrochemical devices.

1. Introduction

Nicotinamide adenine dinucleotide (NADH) is a vital cofactor in over 300 enzymatic reactions associated with NAD^+/NADH-dependent dehydrogenases. These enzymes catalyze the oxidation of the metabolites and hence can be used to develop biosensors for numerous analytes and biofuel cells with cheap fuels and use of the redox turnover of NAD^+/NADH coupled to a redox reaction (Bullen et al., 2006; Chakraborty and Raj, 2007; Heller, 2004; Zhao et al., 2007).

Although the formal potential of the NAD^+/NADH couple is −560 mV (vs. SCE) (pH 7)(Clark, 1960), typically NADH oxidation on bare solid electrodes occurs irreversibly at much high potentials, overpotential of about 1 V (Moiroux and Elving, 1978), making it unattractive to use dehydrogenases for electroanalytical purposes. The optimal potential suggested for electrochemical biosensors is −50 to 100 mV (vs. SCE) (Aizawa et al., 1976). Moreover, direct oxidation of NADH often leads to electrode fouling (Blaedel and Jenkins, 1975; Wang et al., 1992), due to radical intermediates produced in side reactions which decreases the number of turnovers (Karyakin et al., 1999a).

In order to reduce the high overvoltage and suppress radical intermediate formation, many attempts have been made to employ a simultaneous two-electron transfer mechanism using mediators such as redox-active azine dyes and their derivatives (Gorton, 1986; Malinauskas et al., 2000; Mano and Kuhn, 2001; Ni et al., 1990), quinones and their derivatives (Stoica et al., 2004), as well as metal complexes and metal nanoparticles (Cai et al., 1995; Jena and Raj, 2006; Pandey et al., 1998). Phenoxazine and phenothiazine are often considered to be highly suitable for use in chemically modified electrodes, and have been widely used (Malinauskas et al., 2000). In addition, for promotion of long-term stability and catalytic efficiency, redox dye derivatives of electropolymers have been developed (Dilgin et al., 2007; Karyakin et al., 1999a,b, 2003).

Recently carbon nanotubes (CNTs) have been used as novel electrode materials. They consist of seamlessly rolled-up graphene sheets of carbon, and can impart strong electrocatalytic effects and promote the electrochemical properties of bioanalytes and redox enzymes, due to their unique electrical, mechanical and chemical properties (Banks and Compton, 2005, 2006; Dai et al., 1996; Rivas et al., 2007; Shin et al., 2008; Treacy et al., 1996; Wong et al., 1997).

Since Wang and co-workers first explored electroanalytical application of CNTs for NADH detection at low-potential (Musameh et al., 2002; Wang et al., 2003), many efforts have been made to obtain CNTs-based complexes for the electrodetermination of NADH (Rubianes and Rivas, 2007). For example, CNTs functionalized with the azine dyes through π–π electronic and hydrophobic interactions (Li et al., 2004) have been used in biosensors and biofuel cell applications (Huang et al., 2007; Lawrence and Wang, 2006; Tsai et al., 2007; Yan et al., 2006).

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Although brilliant cresyl blue (BCB), one of the phenoxazine dyes has been applied in a modified carbon paste electrode configuration for amperometric ethanol determination (Lobo et al., 1996) and for the oxidation of NADH to fabricate anodic of biofuel cell (Gao et al., 2007), to the best of our knowledge, the synergy of BCB with carbon nanotubes has not been investigated for amperometric ethanol determination.

In the present work, a novel type of nanocomposite of single walled-carbon nanotubes (SWNTs) functionalized with poly(brilliant cresyl blue) (PBCB) is explored by electropolymerization. The PBCB/SWNT modified glassy carbon electrode (GCE) is then applied to the determination of NADH at low-potential and used to develop an ethanol dehydrogenase-based biosensor.

2. Materials and methods

2.1. Chemicals

SWCNTs (prepared by chemical vapour deposition (CVD), <2 nm in external diameter with a purity of ≥95%) were purchased from Shenzhen Nanoport, China. SWNTs were chemically purified and shortened prior to use by ultrasonicating in concentrated nitric and sulfuric acid (V/V = 1:3) for 6 h at 40 °C. A stable and homogeneous suspension was obtained by dispersing 2 mg of these acid-treated SWNTs into 4 mL water and ultrasonicating for 15 min. Brilliant cresyl blue, β-nicotinamide adenine dinucleotide in reduced form (NADH), carrageenan and ethanol dehydrogenase (ADH, EC 1.1.1.1, 337 units/mg) were purchased from Sigma (USA). All other chemicals were of at least analytical grade purity. Supporting electrolytes used for electrochemical experiments were 0.1 M PBS (0.05 M NaH_2PO_4 + 0.05 M K_2HPO_4). High purity water (18.23 MΩ cm) was produced by an Aquapro-System (Aquapro Co., China) purification system.

2.2. Electrode modification

A GCE was polished sequentially with metallographic abrasive paper (10,000) using slurries of 1.0, 0.3 and 0.05 μm alumina to give a mirror like finish. After thorough rinsing with water, the electrode was successively ultrasonicated with ethanol, 1:1 nitric acid, and then water for about 1 min. Next, the GCE was transferred to the electrochemical cell for further cleaning, using cyclic voltammetry at potentials between −1.0 and +1.0 V at a scan rate of 100 mV s^{-1} in 0.2 H_2SO_4 until a stable cyclic voltammetric profile was obtained. The treated electrode was then dried under a nitrogen stream, thoroughly rinsed with water and then used immediately for modification by dropping a 3 μL aliquot of SWNT suspension onto the surface and drying in air to give a SWNT/GCE.

To prepare the finally needed chemically modified electrode, the SWNT/GCE was dipped in 0.5 mM BCB solution (in 0.1 M KNO_3 and 0.1 M pH 8.0 PBS (Karyakin et al., 1999b) for 90 min followed by 90 s polarization at +0.9 V to obtain a composite of SWNTs functionalized with PBCB (denoted as PBCB/SWNT/GCE). Before use, the PBCB/SWNT/GCE was rinsed thoroughly with water and then dipped in 0.1 M pH 7.5 PBS for 15 min to remove monoBCB residues.

For comparison purposes, the PBCB modified glassy carbon electrode (denoted as PBCB/GCE) was fabricated by the same electropolymerizing method. A SWNT functionalized with monoBCB modified glassy carbon electrode (denoted as BCB/SWNT/GCE) also was fabricated by dipping the SWNT/GCE in 0.5 mM BCB solution (in pH 8.0 PBS) for 4 h. Finally, it was rinsed thoroughly with pure water and then dipped in 0.1 M pH 7.5 PBS for 15 min to remove the loosely adsorbed BCB.

2.3. Preparation of an ethanol biosensor

An aliquot of 10 μL 30 mg mL^{-1} ADH in 0.4% (W/W) carageenan hydrogel mixed with DMF in a 3:1 (V:V) ratio was casted onto the PBCB/SWNT/GCE to obtain the biocomposite modified electrode (denoted as ADH/PBCB/SWNT/GCE) and dried at 4 °C for 4 h. The ADH/PBCB/SWNT/GCE was stored in PBS before performing the amperometric experiments.

2.4. Apparatus and methods

Electrochemical measurements were performed using a CHI 812B Electrochemical Analyzer (CHInstruments, Chenhua, Shanghai, China) and a standard three-electrode cell consisting of a chemically modified or unmodified GC working electrode, a Pt wire auxiliary electrode and saturated calomel reference electrode.

The electrochemical behaviors of the electrodes were investigated in 0.1 M pH 7.5 PBS by cyclic voltammetry. The NADH experiments were performed in 0.1 M pH 7.5 PBS at 0 V using amperometry with injection of aliquots of NADH at regular intervals. For determining ethanol, the same amperometric procedure was used except that 3.0 mM of NAD^+ was added to the solution. All the experiments were repeated at least for three times.

3. Results and discussion

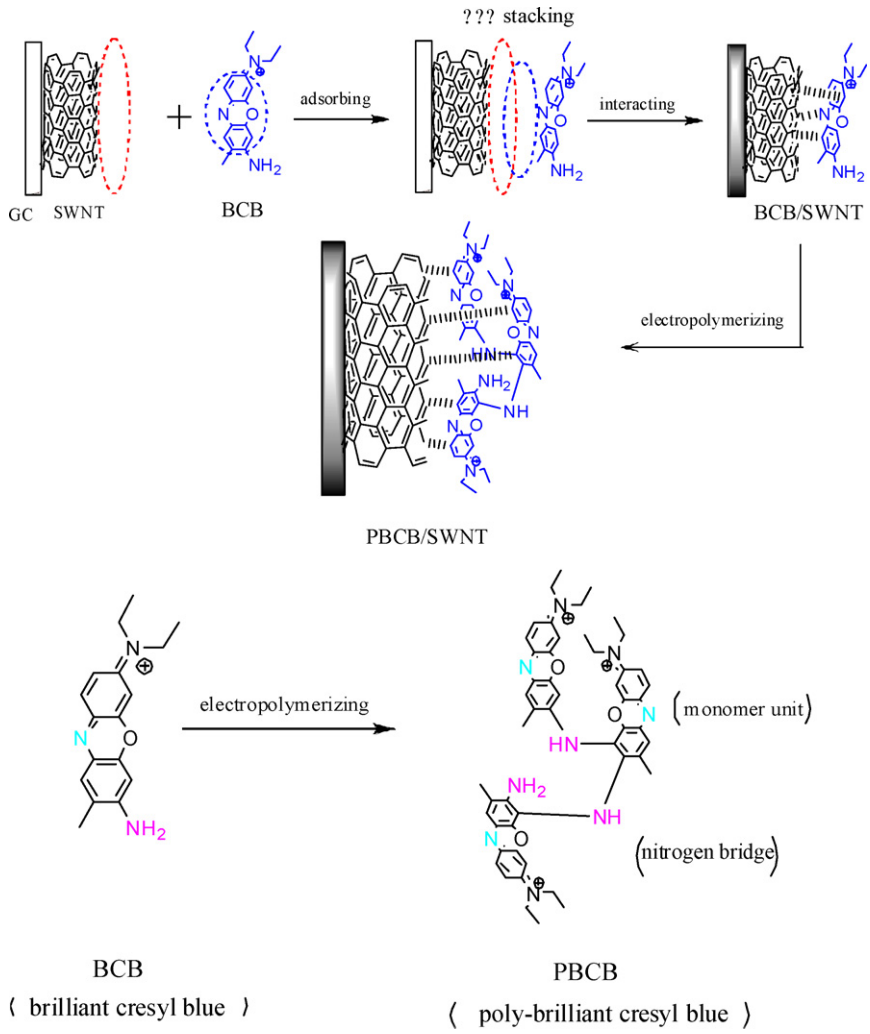
3.1. Electrochemistry of the nanocomposite of PBCB/SWNT

The fabrication of the nanocomposite of PBCB/SWNT is described in Scheme 1. Briefly, SWNT, exhibiting sidewall curvature and possessing a π–π conjugative structure with a highly hydrophobic surface, interacted with monoBCB by π–π stacking to form a 3D structure composite. The monoBCBs were crossly linked by the electropolymerization to form the stable polyBCB.

Cyclic voltammograms recorded over the potential range of −0.4 to 0.8 V at a scan rate of 20 mV s^{-1} in PBS solution exhibited two reversible redox couples, with formal potentials of −0.13 V and −0.30 V respectively, when using either the PBCB/GCE (Fig. 1c) and the PBCB/SWNT/GCE (Fig. 1d). However there was no new faradaic response obtained when using the GCE (Fig. 1a). The anodic peak at −0.1 V and cathodic peak at −0.3 V using the SWNT/GCE (Fig. 1b) are attributed to the carboxyl groups on the surface of SWNT (Shin et al., 2008). Thus, the redox couples detected originate from the presence of PBCB on the electrode surface. The peaks obtained in the PBCB/SWNT/GCE case are well resolved and the currents are larger than those obtained from the PBCB/GCE.

Differences in cyclic voltammograms obtained with monoBCB and PBCB electrodes are obvious as shown in Fig. 2. MonoBCB, exhibits its characteristic couple at −0.3 V and a shoulder located at −0.44 V (Liu and Wang, 1985) (Fig. 2a), which is not observed with PBCB (Fig. 2b)(Gao et al., 2007). There were two couples observed using PBCB modified electrodes. The one located at −0.3 V belongs to residual monoBCB which diminished with more extensive polymerization, and the other located at −0.13 V was only found with PBCB (Fig. 2b). Results are similar to those for azine-dye polymers (Rubianes and Rivas, 2007; Yang et al., 2006) where it is known that the more positive couple (−0.13 V) is associated with the bridged-nitrogens in PBCB, while the negative ones (−0.3 V) belongs to the residual monomer units in the PBCB structure. The monoBCBs were polymerized through cross-linking to PBCB, as proposed in Scheme 1.

Importantly, the PBCB/SWNT/GCE is more stable than the BCB/SWNT/GCE one. The two couples detailed at the PBCB/SWNT/GCE remained at over 90% of their initial value



Scheme 1.

after 100 cycles of potential for nearly 3 h at a scan rate of 20 mV s^{-1}. In contrast, the peak currents at the BCB/SWNT/GCE, under the same conditions, decreased by 53% after only 50 cycles. The additional covalent cross-linking of the bridged nitrogen atoms makes PBCB more stable than monoBCB, which was only absorbed by π–π stacking on the surface of SWNT.

The dependence of the formal potential on pH is linear and obeys the relationship $E^{\circ\prime}$ (V) = 0.131 − 0.0359 pH (R = 0.9991). The slope of 35.9 mV/pH unit over the pH range from 6.5 to 10.5, is near to the theoretical value (29 mV/pH) predicted for an electrochemical reaction involving the transferral of two electrons, coupled with one proton (Liu and Wang, 1985) (see S1).

Both anodic and cathodic peak currents of the positive couple increased linearly with the scan rate over the range from 20 to 200 mV s^{-1}. The linear regression equations and correlation coefficients (N = 19) are Ipa (μA) = 1.467 + 0.0727υ (mV s^{-1}), R = 0.9995

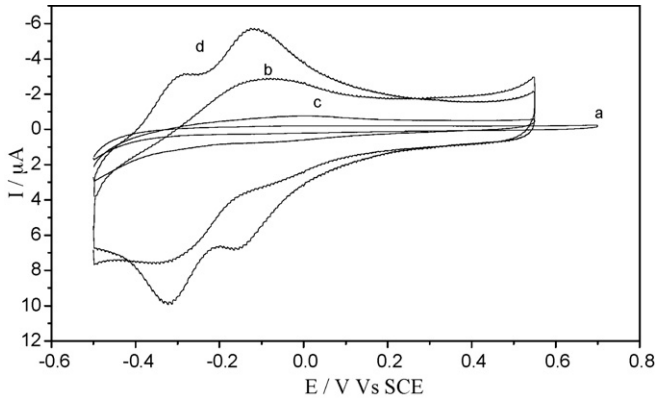


Fig. 1. Cyclic voltammograms obtained in 0.1 M pH 7.5 PBS at the PBCB/GCE (a), SWNT/GCE (b), PBCB/SWNT/GCE (c), bare GCE (d). Scan rate = 20 mV s^{-1}.

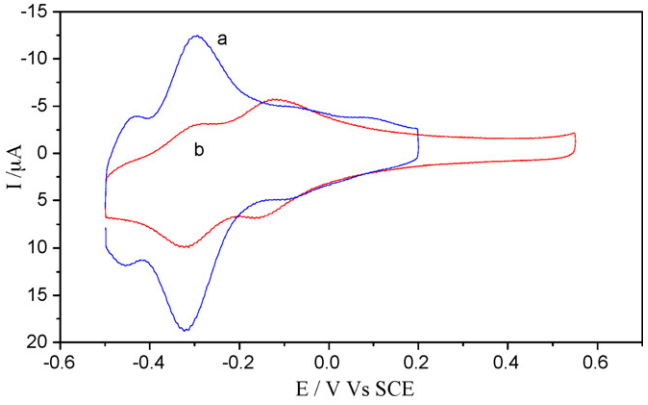


Fig. 2. Cyclic voltammograms of the PBCB/SWNT/GCE in 0.1 M pH 7.5 PBS (a) and the first cycle of CVs of the SWNT/GC electrode in 0.1 M pH 7.5 PBS containing 0.01 mM BCB (b), the scan rate of 20 mV s^{-1}.

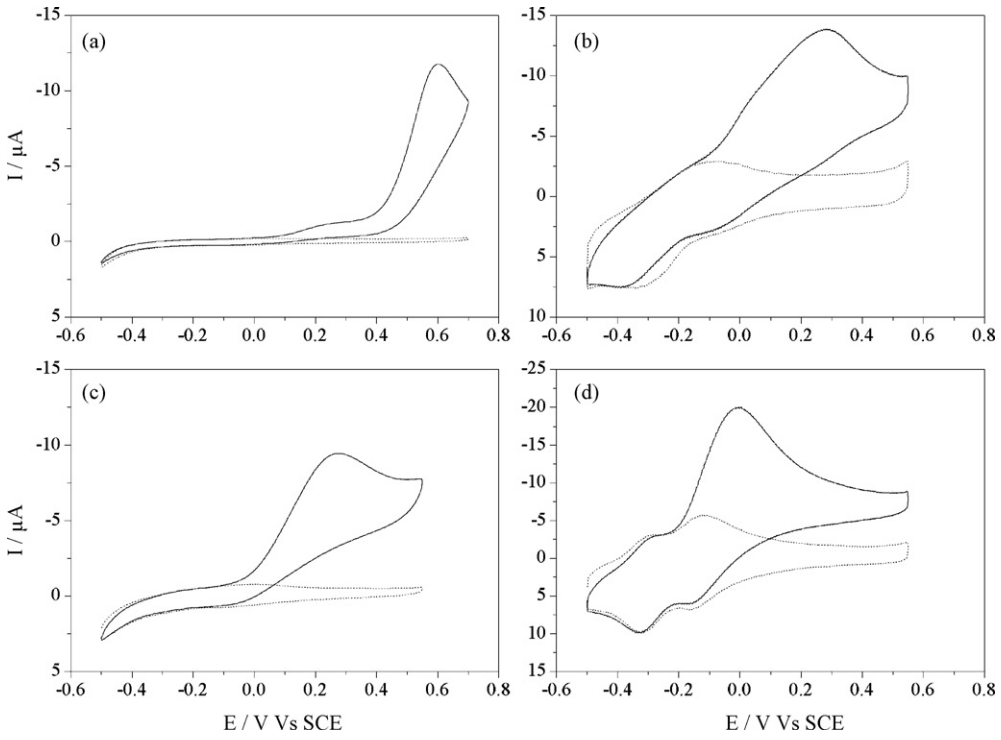


Fig. 3. Cyclic voltammograms obtained in the presence (solid) and absence (dot) of 2 mM NADH in 0.1 M pH 7.5 PBS at the bare GCE (a), SWNT/GCE (b), PBCB/GCE (c), PBCB/SWNT/GCE (d). Scan rate = 20 mV s^{-1}.

and Ipc (μA) = 1.305 + 0.0828υ (mV s^{-1}), R = 0.9987 respectively. The linear dependence of Ip on V is as expected for a surface-control redox reaction in which PBCB is covalently bonded to the surface of SWNT modified electrode.

3.2. Electrochemical response of PBCB/SWNT to NADH

NADH can be oxidized at the four different types of electrodes in the same PBS containing 2 mM NADH (Fig. 3).

The NADH oxidation process emerges at a high positive potential of 0.605 V at the bare GCE (Fig. 3a). The potential of NADH oxidation is lowered to 0.283–0.274 V using SWNT/GCE (Fig. 3b) and PBCB/GCE (Fig. 3c) respectively, which illustrates the catalytic effect of SWNT and PBCB alone towards NADH oxidation. The overpotential of NADH oxidation can be further lowered by use of a PBCB/SWNT/GCE (Fig. 3d). In this case, the catalyzed reaction occurs near 0 V, indicating the synergistic oxidation of NADH by the three-dimensional and redox-active PBCB-SWNT nanocomposite which was formed through the interaction with π-stacking between the extended π-networks of SWNTs and π-conjugative structures of PBCB (See Scheme 1).

The reaction mechanism for the electrocatalytic oxidation of NADH at the PBCB/SWNT/GCE requires that NADH is oxidized by the mediator PBCB(ox) to NAD^+ while PBCB(ox) can be reduced to PBCB(red). PBCB(red) is then in turn electrochemically oxidized to PBCB(ox) on the electrode surface. The recycling processes which leads to a substantial increase in the NADH oxidation current present below.

$$\mathrm{NADH} + \mathrm{PBCB(ox)} \rightarrow \mathrm{PBCB(red)} + \mathrm{NAD^+} \quad (1)$$

$$\mathrm{PBCB(red)} - 2e - \mathrm{H^+} \rightarrow \mathrm{PBCB(ox)} \quad (2)$$

The voltammetric response of NADH is affected by the thickness of PBCB, which can be controlled by the electropolymerization time (Karyakin et al., 1999b). The largest voltammetric response to NADH was obtained using a PBCB/SWNT/GCE prepared with a polymerizing time of 90 s. Longer electropolymerization times led a thicker film that decreases the oxidation current of NADH (see S2).

The PBCB/SWNT electrodes respond to NADH addition in the pH 7.5 PBS solution, yielding a steady-state current within 5 s. A linear relationship with a correlation coefficient R = 0.99983 for the regression equation I (nA) = 12.15 + 9.884C (μM) was obtained over the NADH concentration range of 3.0–104 μM (Fig. 4). The limit of detection was 1.0 μM (based on S/N = 3). A sensitivity of 9.88 nA (μ M^{-1}) was obtained.

The PBCB/SWNT electrode is stable with respect to its response to NADH. The peak current for 2.0 mM NADH at PBCB/SWNT electrodes remains at 95% of the initial value after being dipped in 0.1 M pH 7.5 PBS for an hour. 90% maintenance of the initial current was still observed when the PBCB/SWNT electrode was in a continuously stirred PBS solution containing 7.0×10^{-4} M NADH

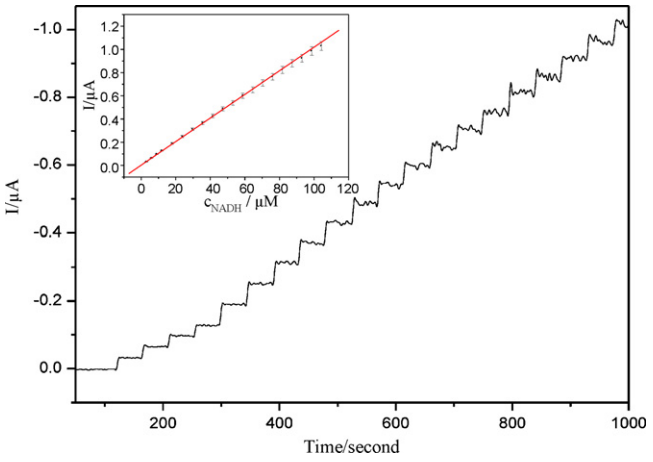


Fig. 4. Amperometric i–t curve for NADH oxidation at the PBCB/SWNT electrode (Inset: the corresponding calibration plot). Each addition was of 10 μL 3.0 mM NADH in 5 mL PBS.

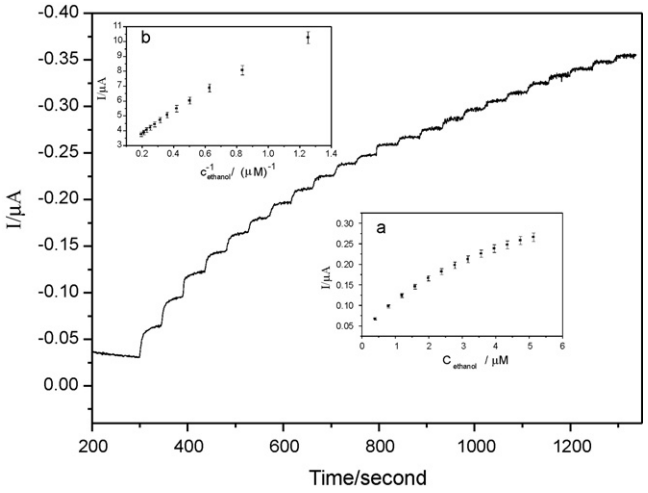


Fig. 5. Amperometric *i*–*t* curve for the sensing of ethanol by the ADH/PBCB/SWNT/GCE, in 5 mL pH 7.5 PBS containing 3.0 mM NAD^+. Each addition was of 5 μL 0.4 M ethanol. Inset: (a) corresponding calibration plot; (b) the double reciprocal plot.

for 1200 s, indicating that the electrodes are sufficiently stable for continuous measurement of NADH. Apparently, the PBCB/SWNT nanocomposites facilitate the electron transfer for the oxidation of NADH and avoid the generation of intermediates that foul the electrode surface (see S3).

The PBCB/SWNT/GCEs exhibit good reproducibility for the determination of NADH. Five measurements in pH 7.5 PBS containing 2.0 μM NADH using refreshed PBCB/SWNT/GCES, gave a relative standard derivation (RSD) of less than 3.7%.

NADH can be determined without interference from a 100-fold excess of dopamine (DA) and uric acid (UA). Thus, no change in the initial amperometric current of NADH was observed when two successive aliquots of 9.0×10^{-3} M DA and two successive aliquots of 9.0×10^{-3} M UA were sequentially injected into stirred PBS solution when a polarizing potential of 0 V was used after 9.0×10^{-5} M NADH had been injected (Fig. 5). However, the presence of a 100-fold excess of ascorbic acid (AA) caused an obvious amperometric signal increase. It has been reported that SWNT functionalized with carboxylic acid groups causes the catalytic oxidation current of AA at 0 V (Luo et al., 2001) (see S4).

Commonly, oxygen is one of the main interferences encountered in determining biological materials with biosensors. However, no measurable difference was observed when solutions of PBS was degassed with nitrogen or saturated with air under the conditions as used in our experiments. Thus, the oxygen interference can be eliminated by using this electron transfer mediator.

3.3. Amperometric ethanol dehydrogenase-based biosensor

An ethanol biosensor may be developed using the PBCB/SWNT/GCE and ADH. Glutaraldehyde cross-linking is commonly used to immobilize ADH on the surface of electrodes (Du et al., 2007; Yan et al., 2007). However, this method leads to a change of the enzymatic conformation, which causes the enzyme molecules to become inactive. ADH immobilized by carageenan hydrogel can overcome this problem (Liu et al., 2004), because its matrix exhibits moderate elasticity, high turbidity and bioaffinity with aqueous microenvironment when interacting with additives such as organic molecules, making it an ideal biopolymer for immobilizing enzymes on solid substrates.

The current increases with the concentration of ADH on the electrode surface up to 30 mg mL^{-1}. However, the current decreases at higher concentrations, because ADH, as an insulator, inhibits electron transfer and diffusion for the enzymatically produced NADH when present in large concentrations [Lee and Tsai, 2009]. Thus, an ADH concentration of 30 mg mL^{-1} was used in fabrication of the biosensor (see S5).

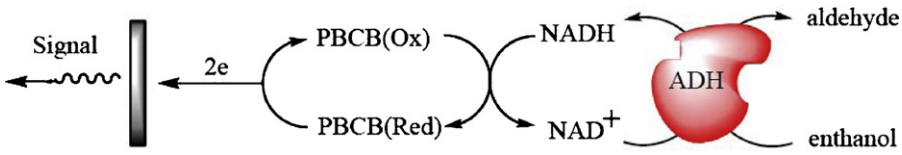
The mechanism proposed for the determination of ethanol is as follows:

The current increases with NAD^+ concentration in solution up to 3.0 mM, and then becomes relatively constant upon addition of higher concentrations (see S6).

The pH-dependence was investigated over the range of 6.5–8.5 in 4 mM ethanol solution. The amperometric response increased from pH 6.5–7.5 and then decreased above pH 7.5 (see S7).

Consequently, the determination of ethanol was performed in pH 7.5 PBS containing 3.0 mM NAD^+. A fast and stable amperometric response towards ethanol was obtained in this medium (Fig. 6). The electrode responds linearly to ethanol concentration over the range of 0.4–2.4 mM. The detection limit was estimated to be 0.1 mM with a signal-to-noise (*S*/*N*) ratio of 1:3 which was near the 90 μM on the electrodes modified by poly(dimethyldiallylammonium chloride)-MWNTs [Liu and Cai, 2007], however was higher than 50 μM on poly(nile blue A)-MWNTs [Du et al., 2007], 13 μM on poly(vinyl alcohol)-MWNTs [Tsai et al., 2007], and 5.0 μM on Meldola's Blue-MWNTs [Santos et al., 2006].

The apparent Michaelis–Menten constant K^M_{app}, which gives an indication of the enzyme-substrate kinetics, was obtained from the electrochemical version of the Lineweaver–Burk equation (Kamin and Wilson, 1980). The K^M_{app} calculated in this manner was 2.3 mM which is smaller than values of 6.30 mM (Du et al., 2007) and 5.0 mM (Liu and Cai, 2007). The smaller K^M_{app} value means that the immobilized ADH has higher enzymatic activity, and that the biosensor has a greater affinity and enhanced catalytic performance towards ethanol.



(1) ethanol + NAD^+ $\xrightarrow{ADH}$ aldehyde + NADH

(2) NADH + PBCB(Ox) ⟶ PBCB(Red) + NAD^+

(3) PBCB(Red) - 2e - H^+ ⟶ PBCB(Ox)

Fig. 6.

The biosensor retained more than 80% of its original response to ethanol after 30 days. To establish the reproducibility, five sensors were fabricated and their amperometric response towards 1.0 mM ethanol was examined. The RSD of the five determinations was 3.9%. These results indicate that this biosensor has good stability and repeatability, as required for the determination of ethanol.

4. Conclusions

A simple method of preparing a PBCB/SWNT nanocomposite electrode by electropolymerization has been developed. The modified electrode is highly sensitive towards NADH and provides a rapid response, high sensitivity, a low detection limit, a broad linear range, long-term stability and no interference from DA or UA. The excellent performance is due to the synergistic effect of PBCB with SWNTs in the catalyzation of NADH. The ethanol biosensors based on PBCB/SWNT matrix also exhibit a good analytical performance. The excellence performances of the PBCB/SWNT nanocomposites can be expected to have a wide range of potential applications in the development of novel biosensors based on dehydrogenases, biofuel cells used as the anodic biocatalysts, and other bioelectrochemical devices to establish three-dimensional bioelectrocatalytic systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.08.016.

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