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Electrochemical determination of NADH and ethanol based on ionic liquid-functionalized graphene

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ABSTRACT

It is firstly reported that low-potential NADH detection and biosensing for ethanol are achieved at an ionic liquid-functionalized graphene (IL-graphene) modified electrode. A substantial decrease (440 mV) in the overvoltage of the NADH oxidation was observed using IL-graphene/chitosan coating, with oxidation starting at ca. 0 V (vs. Ag|AgCl). And the NADH amperometric response at such a modified electrode is more stable (95.4% and 90% of the initial activity remaining after 10 min and 30 min at 1 mM NADH solution) than that at bare electrode (68% and 46%). Furthermore, the IL-graphene/chitosan-modified electrode exhibited a good linearity from 0.25 to 2 mM and high sensitivity of 37.43 $\mu A\,mM^{-1}\,cm^{-2}$. The ability of IL-graphene to promote the electron transfer between NADH and the electrode exhibited a novel and promising biocompatible platform for development of dehydrogenase-based amperometric biosensors. With alcohol dehydrogenase (ADH) as a model, the ADH/IL-graphene/chitosan-modified electrode was constructed through a simple casting method. The resulting biosensor showed rapid and highly sensitive amperometric response to ethanol with a low detection limit (5 μM). Moreover, the proposed biosensor has been used to determine ethanol in real samples and the results were in good agreement with those certified by the supplier.

1. Introduction

β-Nicotinamide adenine dinucleotide (NADH) is involved as a cofactor in several hundred enzymatic reactions of NAD^+/NADH-dependent dehydrogenases (Bergel et al., 1989). The electrochemical oxidation of NADH has attracted considerable attention due to its significance both as a cofactor for dehydrogenase enzymes and its role in the electron-transfer chain in biological system, and also due to the need to develop amperometric biosensors for substrates of NAD^+-dependent dehydrogenases (Gortona and Domínguezb, 2002; Lobo et al., 1997; Wu et al., 2007). Problems inherent to such anodic detection are the large overvoltage encountered for NADH oxidation at commonly used electrodes (Blaedel and Jenkins, 1975) and surface fouling associated with the accumulation of reaction products (Wang et al., 1992). Consequently, considerable effort has been devoted toward the goal of identifying new electrode materials and new methods that will reduce the overpotential for NADH oxidation and minimize surface passivation effects. In recent years, with the great progress made in nanoscience and nanotechnology, many nanomaterials, such as polymers (Manesh et al., 2008), carbon nanotubes (Musameh et al., 2002; Tsai et al., 2007), carbon fiber (Wu et al., 2007) and titanium containing MCM-41 (Dai et al., 2007), have been used successfully to decrease the high overpotential for NADH oxidation and minimizing surface fouling. For example, single-wall carbon nanotubes and multi-wall carbon nanotubes also eliminated surface fouling effects and exhibited good electrochemical oxidation for NADH at low potential of ca. 0.33 and 0.36 V, respectively (Musameh et al., 2002).

Graphene, considered as a "rising star" nanostructured carbon material, is a flat monolayer of carbon atoms tightly packed into a two-dimensional honeycomb lattice, and a basic building block for graphitic materials of all other dimensionalities, such as carbon nanotubes and fullerenes (Geim and Novoselov, 2007). Because of their novel properties (Li et al., 2008b; Zhang et al., 2005), such as exceptional thermal and mechanical properties, high electrical conductivity, graphene sheets have received considerable interest for potential applications in many technological fields, such as nanocomposites (Stankovich et al., 2006; Williarris et al., 2008), nanoelectronics (Gilje et al., 2007) and electromechanical resonators (Bunch et al., 2007). The biological applications of graphene, such as DNA-hybridization device and delivery of

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drugs, have also started to be concerned (Chen et al., 2008; Liu et al., 2008; Mohanty and Berry, 2008; Shang et al., 2008; Shan et al., 2009). For example, Dai et al. synthesized nanoscale graphene oxide sheets by branched polyethylene glycol (PEG) and exhibited a unique ability of graphene in the attachment and delivery of aromatic, water insoluble drugs (Liu et al., 2008). Berry et al. fabricated a novel graphene-based live-bacterial-hybrid device and a DNA-hybridization device with excellent sensitivity (Mohanty and Berry, 2008). It is noted that graphene sheets, which have a high specific surface area, tend to form irreversible agglomerates through strong π–π stacking and *van der Waals* interaction (Li et al., 2008a). Hence the prevention of aggregation is a key challenge in the synthesis and processing of bulk-quantity graphene sheets. Ionic liquids (ILs) can meet this challenge well. Due to their wide solubility and introducing a surface charge, ILs functionalized graphene sheets with good dispersibility and long-term stability in various solvents have been synthesized by our group (Yang et al., 2009). In addition, ILs-based electrochemical sensors and biosensors have also been extensively reported for direct electron transfer of various redox enzymes and detection of different types of compounds such as ascorbic acid, dopamine, hydrogen peroxide, and glucose (Lu et al., 2006; Maleki et al., 2006; Sun et al., 2007; Wei and Ivaska, 2008). These results suggested that the use of ILs could increase the sensitivity of response and facilitate efficient direct electron transfer of various redox biomolecules.

So we use the IL-functionalized graphene (Yang et al., 2009) and chitosan to construct an electrochemical biosensor for detection of NADH and ethanol. Chitosan with abundant amino groups was chosen to immobilize the IL-graphene and enzymes due to its good biocompatibility (Liu et al., 2005) and excellent film-forming ability for the solubility in slightly acidic solution due to its protonation and insolubility in solution with pH above pK_a (6.3) (Sorlier et al., 2001). The IL-graphene/chitosan-modified electrode show an obvious decrease in the overvoltage of NADH oxidation. Using alcohol dehydrogenase (ADH) as a model enzyme, a sensitive amperometric biosensor for ethanol with a low limit of detection is constructed by immobilizing ADH on an electrode surface in the IL-graphene/chitosan coating process. Such the IL-graphene provided a new, biocompatible platform for sensitive biosensors and biomolecular diagnostics.

2. Experimental

2.1. Materials

Graphite powder (320 mesh, spectroscopically pure reagent) and chitosan were purchased from Sinopharm Chemical Reagent Co. Ltd. 1-methylimidazole (≥98%, Linhai Kaile Chemicals, China) was distilled before use. 3-Bromopropylamine hydrobromide (98%) was obtained from Aldrich. ADH from saccharomyces cerevisiae (≥300 unit mg^{-1}), β-NAD and β-NADH were purchased from Sigma. Unless otherwise stated, other reagents were of analytical grade and were used as received. All aqueous solutions were prepared with ultra-pure water (≥18 MΩ cm) from a Milli-Q Plus system (Millipore). Phosphate buffer solution (PBS, 0.05 M, pH 7.4) was used in all electrochemical studies. In detection of real samples, wine (38%, V/V) was diluted with water in appropriate concentration (3%, V/V) and beer (3.6%, V/V) was used without pretreatment.

2.2. Instruments

The UV–vis absorption spectra of IL-graphene aqueous solution were collected using a CARY 500 Scan UV/Vis/NIR spectrophotometer. Fourier transform infrared spectroscopy (FTIR) was recorded on a CaF_2 substrate with a Bruker Tensor 27 Spectrometer. Transmission electron microscopy (TEM) micrographs were obtained using a JEOL 2000 transmission electron microscopy operating at 200 kV. Cyclic voltammetric measurements were performed using a conventional three-electrode cell with a platinum wire as auxiliary electrode and an Ag|gCl (saturated KCl) as reference in a CHI 660 Electrochemical Workstation (CHI, USA). Working electrodes were modified glassy carbon (GC) electrodes (d = 3 mm). Before use, GC electrodes were carefully polished to a mirror finish with 1.0-, 0.3-, and 0.05-μm alumina slurries, successively.

2.3. Preparation of IL-functionalized graphene

Graphene oxide (GO) was prepared by a modified Hummers method as originally presented by Kovtyukhova and colleagues (Kovtyukhova et al., 1999; Hummers and Offeman, 1958). IL-graphene was synthesized by an epoxide ring-opening reaction between graphene oxide (GO) and the 1-(3-aminopropyl)-3-methylimidazolium bromide (IL-NH_2) according to our previous report (Yang et al., 2009). Briefly, a solution of GO (5 mg), IL-NH_2 (10 mg), and KOH (10 mg) in ultra-pure water (10 mL) was subjected to ultrasonication for 30 min and then vigorously stirring at 80 °C for 24 h. The resulting IL-graphene was subsequently centrifuged and washed with ethanol and water. The IL-graphene was dispersed in 20 mL water and 1 mL, 2 M of $NaBH_4$ aqueous solution was added. And the reaction was stirred at 80 °C for 2 h. The IL-graphene was washed with water for three times. And the graphene without any protection was prepared by the reduction of $NaBH_4$ at 80 °C for 2 h.

2.4. Preparation of modified electrodes

Chitosan solution (pH = 5, 1 mg mL^{-1}) was prepared according to previous report (Zhang et al., 2004). 1 mg IL-graphene was added to 1 mL of 1 mg mL^{-1} chitosan aqueous solution to form homogenous dispersion with ultrasonication. 4 μL of the IL-graphene-chitosan solution was dropped onto a polished GC electrode and allowed dried in ambient air for 2 h to obtain IL-graphene/chitosan-modified electrode. The IL-graphene/chitosan/ADH modified electrode was prepared by the same procedure except for dropping 4 μL of 1 mg mL^{-1} chitosan solution containing 15 mg mL^{-1} ADH and 1 mg mL^{-1} IL-graphene and drying at 4 °C for 2 h in a desiccator. The chitosan and graphene without any protection modified electrodes were also prepared by dropping 4 μL of 1 mg mL^{-1} chitosan solution and 4 μL of chitosan solution containing 1 mg mL^{-1} graphene without any protection, respectively.

3. Results and discussion

3.1. Characterization of IL-functionalized graphene

IL-graphene was synthesized according to our previous report (schematic structure was shown in Fig. 1A) (Yang et al., 2009). The formation of IL-graphene is firstly confirmed by FTIR (as shown in Figure S1 in supporting information), which is consistent with our previous report (Yang et al., 2009). The morphology of the graphene was observed by TEM. Fig. 1B shows the TEM image of IL-graphene nanosheets, illustrating the flake-like shapes of graphene. The graphene oxides and the IL-graphene before and after the reduction of $NaBH_4$ were characterized by UV–vis spectroscopy. The UV–vis spectrum of graphene oxides (curve a in Fig. 1C) in water shows absorption peaks at 230 nm. And the absorption of water-soluble IL-graphene before the reduction of $NaBH_4$ is shifted to 260 nm, suggesting that the electronic conjugation within graphene sheets is restored after the reduction (Li et al., 2008a). After the reduction of $NaBH_4$, the absorption of IL-graphene red-shifts to 270 nm, suggesting that the electronic conjugation within

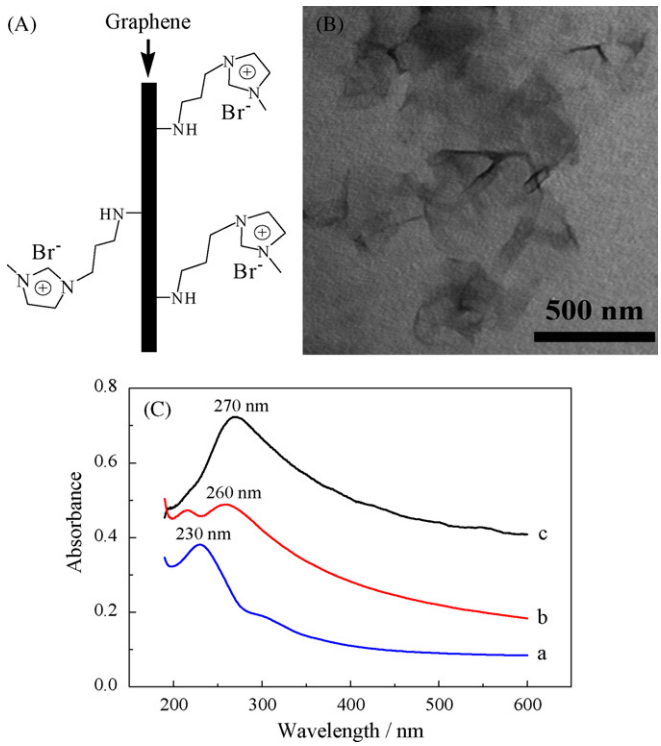


Fig. 1. (A) Schematic structure of IL-graphene. (B) TEM image of IL-graphene. (C) UV–vis spectra of graphene oxides (a), IL-graphene before the reduction of $NaBH_4$ (b) and IL-graphene after $NaBH_4$ reduction (c).

graphene sheets is restored further. The recovery of the electronic conjugation within graphene sheets is extremely important for decreasing the high overpotential for NADH oxidation, which is proved by cyclic voltammetric measurements as follow.

3.2. Electrochemical response of IL-graphene/chitosan-modified electrode to NADH

Fig. 2 shows cyclic voltammograms for the oxidation of NADH at those bare and modified GC electrodes. With bare GC electrode, the oxidation of NADH results in a broad peak with peak potential of 0.77 V (Fig. 2A). Fig. 2B shows a similar oxidation potential of 0.74 V at a chitosan-modified electrode. The IL-graphene (after $NaBH_4$ reduction)/chitosan-modified electrode shows a quite low peak potential at 0.33 V and high current signal compared to chitosan-modified electrode (Fig. 2C). The substantial negative shift (ca. 440 mV) with an onset potential at ca. 0 V and 2-fold larger current signal demonstrate that the IL-graphene can facilitate the oxidation of NADH greatly. Compared to some other nanomaterials on the NADH oxidation, such as nanoporous gold (Qiu et al., 2009), ionic liquid-protected gold nanoparticles (Shan et al., 2008) and PANI-Au composite nanotubes (Huang et al., 2008), IL-graphene/chitosan-modified electrode had lower oxidation potential. Compared to single-wall carbon nanotubes (0.33 V) and multi-wall carbon nanotubes (0.36 V) (Musameh et al., 2002), the IL-graphene/chitosan-modified electrode has similar peak potential of the NADH oxidation. In addition, the IL-graphene can be obtained more easily with low cost. So carbon nanotubes could be substituted with the IL-graphene for the oxidation of NADH.

The roles of graphene, ILs and $NaBH_4$ reduction process in the electrochemical oxidation of NADH were also investigated. Firstly, compared to chitosan-modified electrode (0.74 V, Fig. 2B), the graphene/chitosan-modified electrode showed much lower oxidation potential toward NADH (0.48 V, Fig. 2D). This indicated

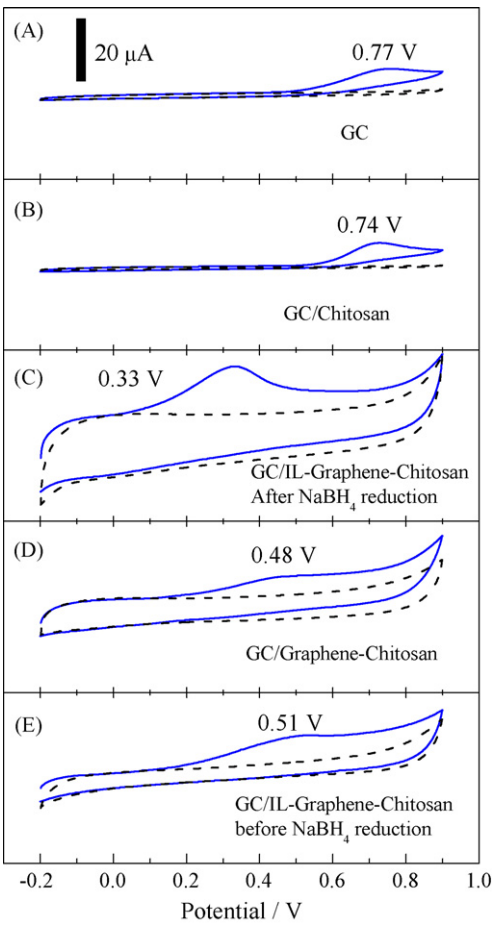


Fig. 2. Cyclic voltammograms of (A) bare GC electrode, (B) chitosan, (C) chitosan/IL-graphene (after $NaBH_4$ reduction), (D) chitosan/graphene (without any protection), and (E) chitosan/IL-graphene (before $NaBH_4$ reduction) modified GC electrodes in PBS (0.05 M, pH 7.4) containing 1 mM NADH. Scan rate: 0.05 V s^{-1}.

that the graphene could decrease the overpotential of NADH oxidation at the surface of electrode and facilitate electrochemical oxidation of NADH. That might be related to the excellent property of graphene, such as high specific surface area and electrical conductivity. Secondly, the role of ILs was also important for promoting the electrochemical oxidation of NADH. Compared to the graphene/chitosan-modified electrode without ILs protection (Fig. 2D, the peak potential at 0.48 V), the IL-graphene/chitosan-modified electrode (0.33 V, Fig. 2C) has better electrocatalytic oxidation toward NADH. These results indicate that ILs is helpful for electrochemical oxidation of NADH at a low potential, which may be due to the unique properties of ILs, such as high ionic conductivity and solubility toward various substrates. Thirdly, the electronic conjugation of the graphene had important role on the oxidation of NADH. As shown in Fig. 2E, the peak potential at IL-graphene (without the $NaBH_4$ reduction) modified electrode is 0.51 V, which is higher than 0.33 V at IL-graphene (after the reduction of $NaBH_4$) modified electrode. This result is related to the electronic conjugation of graphene. After the reduction of $NaBH_4$, the IL-graphene has better electronic conjugation than that before the $NaBH_4$ reduction (proved by UV–vis spectroscopy). And the better electronic conjugation of IL-graphene could promote electron transfer of NADH oxidation at IL-graphene and decrease the overpotential for NADH oxidation.

An extremely attractive feature of the IL-graphene/chitosan-modified electrode is its highly stable amperometric response toward NADH. Fig. 3 compares the amperometric response to

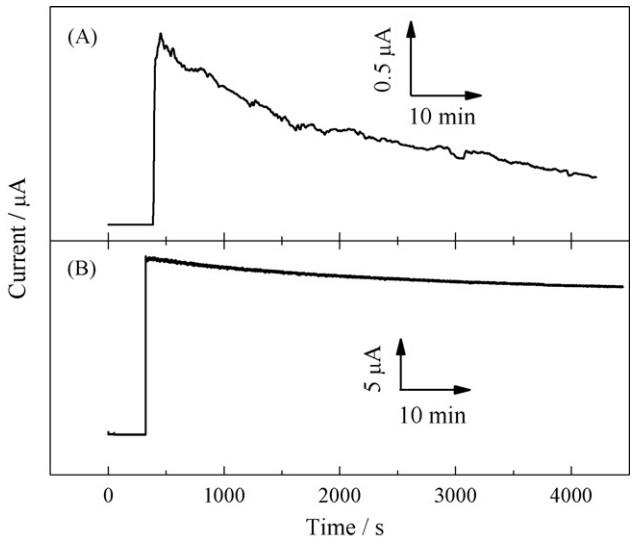


Fig. 3. Stability of the response to 1 mM NADH using (A) bare GC electrode and (B) IL-graphene/chitosan-modified GC electrode at 0.6 V in PBS (0.05 M, pH 7.4).

1 mM NADH, as recorded over a continuous 60 min period, at the bare (Fig. 3A) and IL-graphene/chitosan-modified (Fig. 3B) GC electrodes held at 0.6 V. The bare GC electrode displays a rapid decay of the signal (with up to 32%, 54% and 76% current depressions after 10, 30 and 60 min, respectively), indicating a serious inhibition of the oxidation process. In contrast, the response of the IL-graphene/chitosan-modified GC electrode remains stable throughout the entire experiment, with only 4.6%, 10% and 14% current diminutions at 10, 30 and 60 min, respectively. The stability of response at IL-graphene/chitosan-modified electrode is comparable with that at carbon nanotube modified electrode in previous report (Musameh et al., 2002).

The selective determination of NADH in the presence of AA by using the IL-graphene/chitosan-modified electrode was also investigated. Fig. 4A shows the cyclic voltammogram obtained for NADH and AA coexisting at the IL-graphene/chitosan-modified electrode. It is clear that well-defined and resolved voltammetric peaks at 0.05 and 0.36 V are observed for the electrochemical oxidation of AA and NADH, respectively. The peak separation is ca. 300 mV. Therefore, the selective determination of NADH in the presence of AA is feasible at the IL-graphene/chitosan-modified electrode.

Fig. 4B shows the amperometric response of the IL-graphene/chitosan-modified electrode at 0.45 V to the successive addition of 0.25 mM NADH in PBS. Immediately after the addition of NADH, the anodic current increased and reached a steady state within 10 s. The response displayed a good linear range from 0.25 to 2 mM with a correlation coefficient of 0.999 and good sensitivity of 37.43 $\mu A\,mM^{-1}\,cm^{-2}$.

3.3. Amperometric biosensing of ethanol

The good electrochemical oxidation performance of IL-graphene toward NADH can be used to develop amperometric biosensors for substrates of NAD^+-dependent dehydrogenases. As an example, the ethanol biosensor based on ADH and IL-grapheme was constructed. The enzyme ADH encapsulated into the IL-graphene/chitosan nanocomposite film efficiently catalyzes the oxidation of ethanol in the presence of cofactor NAD^+ (reaction mechanism shown in Scheme 1). Fig. 5 shows the steady-state response at an applied potential of +0.45 V on injecting the concentration of ethanol in 25 μM steps in 0.05 M pH 7.4 PBS. The response of the sensor was fast, and the response time was ca. 20 s. The anodic current increased linearly with ethanol concentration over the range from

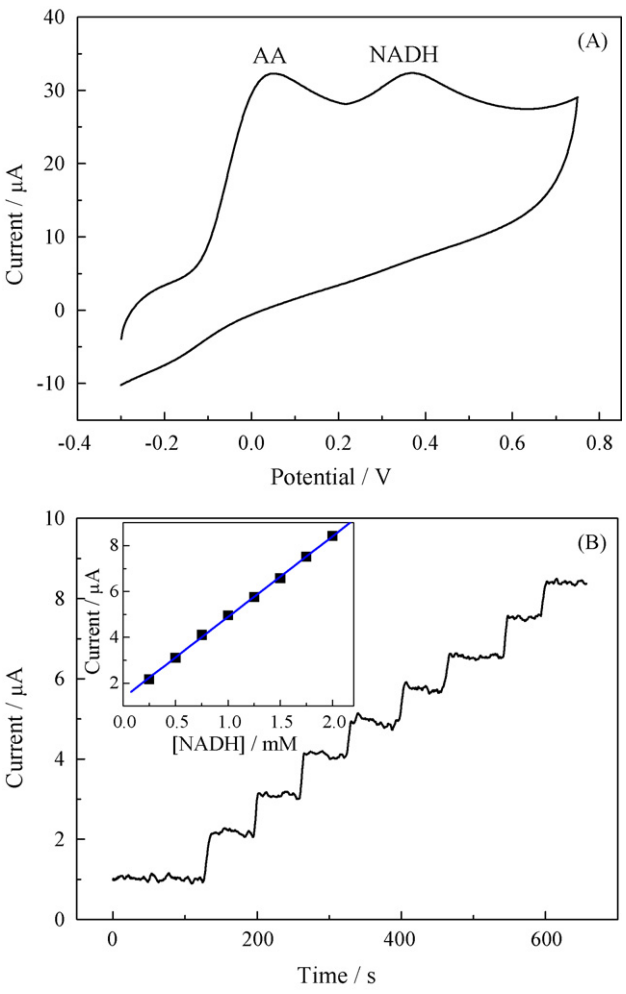
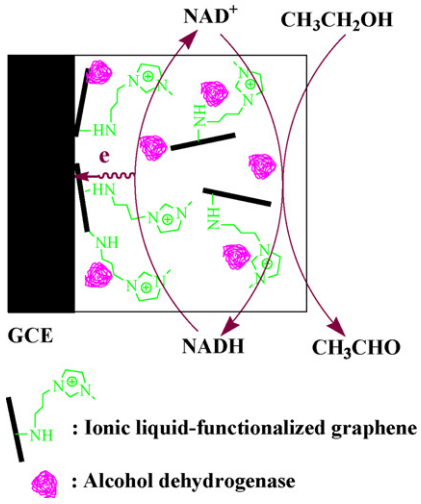


Fig. 4. (A) Cyclic voltammogram of IL-graphene/chitosan-modified electrodes in PBS (0.05 M, pH 7.4) containing 1 mM AA and 1 mM NADH. Scan rate: 0.05 V s^{-1}. (B) Chronoamperometric response of IL-graphene/chitosan-modified electrode in PBS (0.05 M, pH 7.4) on injecting the concentration of NADH in 0.25 mM steps at working potential of 0.45 V. Inset: amperometric response to NADH concentration.



Scheme 1. Schematic representation for the bioelectrocatalytic sensing of ethanol using IL-graphene/chitosan/ADH modified electrode.

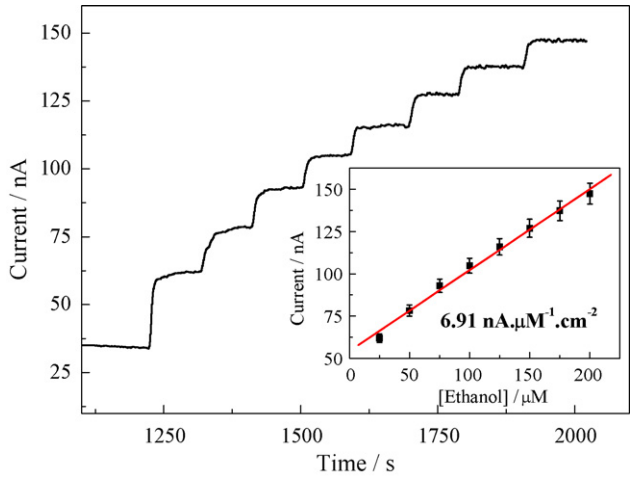


Fig. 5. Chronoamperometric response of IL-graphene/chitosan/ADH modified electrode in 5 mg mL^{-1} NAD^+ PBS (0.05 M, pH 7.4) on injecting the concentration of ethanol in 25 μM steps at working potential of 0.45 V. Inset: amperometric response to ethanol concentration. Error bars = ±standard deviation.

25 to 200 μM with a good sensitivity of 6.91 nA μM^{-1} cm^{-2}. The limit of detection was estimated at a signal-to-noise ratio of 3 to be 5.0 μM, which was much lower than those of 0.1 mM and 49 μM reported for sensors based on injection of the recognition element (Svensson et al., 2005) and Au nanoparticles (Xiao et al., 2005), respectively. The prepared ethanol biosensor also had good reproducibility. The relative standard deviation (RSD) of the current response to 100 μM ethanol at 0.45 V was 4.2% for six successive measurements. The stability of the IL-graphene/chitosan/ADH modified electrode was investigated when stored at 4 °C. After 5 days, the response current was still retained at 92.3% value of the initial response. Response current for 15 days remained at 82.8% of the initial response. This implied that the IL-graphene/chitosan composites film was efficient for retaining the bioactivity of ADH.

3.4. Determination of ethanol in real samples

As a simple application of the novel biosensor for the analysis of real samples, the proposed ethanol biosensor was used to determinate the ethanol concentrations in commercial beer (3.6%, V/V) and wine (38%, V/V). The results obtained were 3.3 ± 0.9 and 37 ± 1.2% (V/V) ethanol for beer and wine, respectively. It can be seen that the results obtained at the IL-graphene/chitosan/ADH biocomposites film modified electrode had good agreement with those certified by the supplier. These results indicate the great potential for practical application of the proposed ethanol biosensor for the analysis of ethanol in real samples.

4. Conclusion

The IL-graphene/chitosan-modified electrode has been prepared and shows a stable low-potential amperometric detection of NADH. The IL-graphene/chitosan film offers a remarkable decrease in the overvoltage for the NADH oxidation and eliminates surface fouling effects. A very simple ethanol biosensor has been constructed successfully, demonstrating potential application of the IL-graphene nanocomposites. The IL-graphene-based sensor for NADH and dehydrogenase substrates exhibits very good analytical performance with low cost, convenient preparation, and sensitive, rapid, and reproducible detection. Thus, such ionic liquid-functionalized graphene nanocomposite is an attractive amperometric transducer in fabrication of electrochemical biosensors. Moreover, the applicability of this biosensor to the rapid analysis of ethanol in real samples demonstrates the great potential for practical application.

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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.2009.11.009.

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