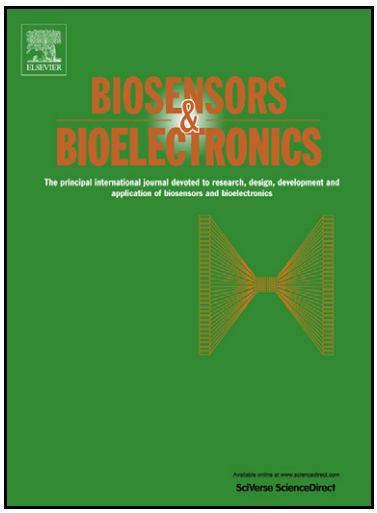

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Ensiyeh Sharifi, Abdollah Salimi, Esmaeil Shams

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Electrocatalytic activity of nickel oxide nanoparticles as mediatorless system for NADH and ethanol sensing at physiological pH solution

Ensiyeh Sharifi [c], Abdollah Salimi[a,b]*, Esmaeil Shams [c]

[a] Department of Chemistry, University of Kurdistan, P. O.Box 416, Sanandaj-Iran
[b] Research Center for Nanotechnology, University of Kurdistan, P. O.Box 416, Sanandaj Iran
[c] Chemistry Department, University of Isfahan, Hezar Jarib, Isfahan, 81746-73441, Iran.

Corresponding Author: Tel: +98-871-6624001, Fax, +98-871-6624008.
E-mail: absalimi@yahoo.com or absalimi@uok.ac.ir

Abstract

The glassy carbon (GC) electrode modified by nickel oxide nanoparticles (NiOxNPs) is proposed as a novel electrocatalytic system for the oxidation of NADH without using any electron transfer mediator. Here, chronoamperometry was used not only as a simple method for the deposition of NiOxNPs onto the GC electrode but also as an efficient tool in the controlling of nanoparticles size and efficient electrocatalytic activity. The surface morphology and electrochemical properties of the NiOxNPs/GC electrode was investigated using scanning electron microscopy and cyclic voltammetry techniques, respectively. The NPs are deposited uniformly across the GC surface and the size of NiOxNPs varies from 20 to less than 100 nm.

The NiOxNPs/GC electrode shows excellent electrocatalytic activity toward oxidation of NADH at reduced overvoltage. The detection limit and sensitivity of the modified electrode toward NADH were estimated to be 106 nM (S/N=3) and 0.052 $\mu A \mu M^{-1}$, respectively at a concentration range up to 1 mM. Due to the biocompatibility of NiOxNPs toward biomolecules, this modified electrode can be used as an efficient transducer in the design of an ethanol biosensor based on the coupled alcohol dehydrogenase enzyme(ADH). Hydrodynamic amperometric detection of ethanol on the ADH-Nafion/NiOxNPs/GC modified electrode gives linear responses over the concentration range of 0.2-6 mM with a detection limit of 6.4 μM and sensitivity of 36 nA mM^{-1}. Applicability of the proposed biosensor for ethanol detetion in real samples, easy and simple preparation, being mediator free, high sensitivity and biocompatibility are the major advantages of the proposed biosensor.

Keywords: NiOx Nanoparticles, Electrocatalytic activity, NADH oxidation, Alcohol dehydrogenase, Ethanol biosensor.

1. Introduction

Dihydronicotinamide adenine dinucleotide (NADH) and its oxidized form adenine dinucleotide (NAD^+) are vital cofactor systems for more than 300 dehydrogenase enzymatic reactions widely used for the construction of electrochemical biosensors. The NADH/NAD^+ redox couple plays an important role not only in the electron transfer of biological systems but also as a key central charge carrier in living cells (Saleh et al. 2011). Therefore, the electrochemical oxidation of NADH is important in the development of dehydrogenase based voltammetric or amperometric biosensors.

Alcohol dehydrogenase (ADH) based biosensors have been extensively employed for ethanol determination for many years due to their inexpensive, rapid, reliable and specific characteristics. In

these kinds of biosensors, ADH catalyzes the oxidation of ethanol to acetaldehyde in the presence of NAD^+ and generated NADH in the enzymatic reaction can be detected amperometrically after applying a suitable potential to the electrode.

However, the electrochemical determination of NADH at most of the unmodified metal and carbon surfaces suffers from large overpotentials which limit the selectivity of the detection in a real sample (Deore and Freund 2005). Furthermore determination at a high overpotential causes surface fouling associated with the accumulation of reaction products (Grundig et al. 1995), which decreases analytical sensitivity, reproducibility, and operational lifetime. A traditional approach for overcoming these problems is to use an appropriate mediator that can improve the kinetics of NADH oxidation. In this way, many compounds, such as dihydroxybenzene and quinone compounds (Fotouhi et al. 2010; Ghanem et al. 2009; Salimi et al. 2005), phenazine and phenoxazine dyes (Lates et al. 2011; Zhang and Gorski 2005), transition-metal complexes (Li et al. 2012; Walcarius et al. 2011), organic salts (Doumèche and Blum 2010), water soulble dys (Balamurugan et al. 2010), redox polymers films (Baskar et al. 2012; Saleh et al. 2011) and adenine derivatives (Teymourian et al. 2012a) have been used as electron-transfer mediators for the catalytic oxidation of NADH. However, most of such mediator-based electrodes show relatively low sensitivity due to the irreversibility of redox systems and slow diffusion of NADH within the immobilized matrixes. These kinds of modified electrodes also suffer from the low stability of mediators and their leaching from the electrode surfaces. In addition, due to the production of radical intermediates during NADH oxidation, the electrode fouling decreases the reproducibility of measurement results (Baskar et al. 2012).

Many nanomaterials, such as carbon nanotubes (CNTs) (Zhang and Gorski 2005), CdS nanoparticles (Vastarella and Nicastri 2005), nanostructured TiO_2 (Curulli et al. 2005), gold nanoparticles (Jena and Raj 2006), graphene nanosheets (Cao et al. 2011) and Fe_3O_4 nanoparticles (Teymourian et al.

2012b) have been employed to accelerate the kinetics of NADH oxidation and to minimize the electrode fouling. Furthermore, with the immobilization of different electron transfer mediators onto CNTs (Fotouhi et al. 2010), silver nanoparticles (Balamurugan et al. 2010), CNTs- Pd nanoparticles (You and Jeon 2011), graphite nanosheets (Zhu et al. 2010) and copper nanodoped mercury monolayers (Munteanu et al. 2012), sensitive electrochemical sensors for NADH detection have been fabricated.

NiOx is extensively applied in various fields such as, gas sensors (Steinebach et al. 2010), electrochromic films (Lin et al. 2012), magnetic materials (Guo and Bi 2006), fuel cell catalysts (Zhang et al. 2010) and as an electrocatalyst for organic synthesis (El-Safty et al. 2008). In addition, it has been used for the electrochemical sensing of H_2O_2 (Yan et al. 2012), glucose (Cao et al. 2011), methanol (Raoof et al. 2010), insulin and thiol derivatives amino acids (Salimi and Roushani 2006; Salimi et al. 2007a) and ethanol (Liu et al. 2010). The electrocatalytic effect is seen as arising from unpaired d-electrons or empty d-orbitals associated with the oxidized form of Ni (NiOOH), which are available for bond formation with adsorbed species (Hutton et al. 2011). Furthermore, the electrocatalytic activity of materials can be significantly increased by moving from bulk materials to nanosized structures (Welch and Compton 2006). Therefore, many efforts have been attempted to decorate supporting substrates with isolated nanostructures and create efficient electrocatalysts (Hutton et al. 2008). Due to the easy preparation, high porosity and electroinactivity of NiOx nanoparticles in physiological pH solutions we used them not only as efficient electrocatalytic system (Salimi et al. 2012) but also as an ideal platform for the entrapment of molecules and biomolecules and the fabrication of sensors and biosensors (Noorbakhsh and Salimi 2009; Salimi et al. 2007b). However, most of the electrocatalytic activities of NiOx were carried out in the alkaline media, which greatly limited its biological application.

The present study describes the development of a very simple NADH biosensor based on the strong electrocatalytic activity of NiOxNPs in physiological pH solutions without using any redox mediator. Finally, this NiOxNPs modified GC electrode was applied for the development of a novel amperometric biosensor for ethanol using alcohol dehydrogenase (ADH) as a model enzyme that can be simply immobilized on the electrode surface.

2. Experimental

2.1. Reagents

β-nicotinamide adenine dinucleotide reduced form (NADH) (disodium salt, 98%) and ADH (EC1.1.1.1) from baker's yeast in the form of lyophilized powder were obtained from Sigma and used as received. Nafion 117 solution (5%) was also obtained from Aldrich. β-nicotinamide adenine dinucleotide oxidized form (β-NAD^+) was purchased from Merck. Ethanol and all other chemicals such as, $Ni(NO_3)_2$, Na_2HPO_4, HCl, and NaOH were of analytical grade from Merck. All solutions were deaerated with high purity nitrogen prior to the experiments.

2.2. Apparatus

All electrochemical measurements were carried out with a three-electrode system comprising modified and unmodified glassy carbon as a working electrode, an Ag/AgCl (3M KCl) as a reference electrode and a platinum wire as an auxiliary electrode. Surface morphology was investigated using a Vega-Tesacn electron microscope (SEM). All electrochemical experiments were performed with a computer-controlled potentiostat, Autolab electrochemical analyzer model PGSTAT30 (Eco Chemie, Utrecht, The Netherlands) driven with GPES software (Eco Chemie). The electrochemical signals were obtained in PBS by using DPV with amplitude of 25 mV and pulse width of 50 ms.

2.3. Preparation of ADH-Nafion/ NiOxNPs nanobiocomposite modified GC electrode

The GC electrode (2 mm diameter) was carefully polished with 0.3 and 0.05 μm alumina, rinsed with doubly distilled water, and sonicated in a twice-distilled water bath to remove adsorbed particles. The in situ synthesis of metallic nickel on the surface of the GC electrode was carried out using a constant potential (-0.8V vs. reference electrode for 30 s) in pH 4 acetate buffer solution containing 1mM nickel nitrate. Then the Ni/GC electrode was immersed into a fresh PBS (pH 7) and cyclic potential were carried out in the range -0.50 to 1.0 V (30 scans) at a scan rate of 100 mVs^{-1} for the electrodissolution and passivation of a nickel oxide layer at a GC electrode (Giovanelli et al. 2003).

Enzyme-Nafion casting solution was prepared by dissolving 3 mg of ADH in 20 μL of 0.5% (w/w) Nafion and vortexed to produce a homogeneous solution. The ADH-Nafion/NiOxNPs/GC modified electrode was fabricated by casting 20 μL of ADH-Nafion solution onto the surface of a NiOxNPs/GC electrode and the solvent was allowed to evaporate at room temperature. The ADH-Nafion/NiOxNPs/GC electrode was thoroughly rinsed with distilled water for removing the loosely adsorbed ADH and stored in 0.1 M pH 7.0 PBS at 4 °C when it was not used.

3. Results and discussion

3.1. Characterization of the modified electrode

The SEM image of the NiOxNPs/GC electrode shows the isolated nanoparticles (from 20 to less than 100 nm) are distributed uniformly over the GC surface (Fig. 1A). The formation of electrodeposited NiOx layer on the GC surface was also investigated by recording cyclic voltammograms of the modified electrode in alkaline solution (Fig. 1B). The anodic peak at 0.54 V is observed due to the oxidation of the $Ni(OH)_2$ phase to NiOOH and the corresponding cathodic peak at 0.47 V represents the reduction of NiO(OH) to $Ni(OH)_2$ (Giovanelli et al. 2003).

Here Fig 1

The amount of the electrodeposited nickel oxide nanoparticles on the surface of the electrode can be estimated as an effective surface concentration, Γ:

$$\Gamma = Q_{ox} / nFA \qquad (1)$$

where F is the Faraday constant, n is the number of transferred electrons (n = 1), A is the surface area of the GC electrode (0.0314 cm^{-2}) and Q_{ox} is the charge associated with the oxidation of $Ni(OH)_2$. For a nickel oxide-modified GC electrode produced with changing deposition times (from 1 to 100 s), Γ values were estimated by integrating the area under the anodic peak. According to eq.1, Γ values were estimated to be (a) ~ 1.10 nmol cm^{-2}, (b) ~ 4.66 nmol cm^{-2}, (c) ~ 9.59 nmol cm^{-2}, (d) ~ 21.93 nmol cm^{-2} and (e) Γ ~ 44.18 nmol cm^{-2}, when depositing times for nickel formation at a constant potential of -0.8V (*vs*. Ag/AgCl) were 1, 5, 10, 30 and 100 s, respectively. These values clearly show that the amount of NiOxNPs deposited increase due to increase of deposition time. These results are similar to those previously reported by Hutton et al. (2011). In the present work, the controlling of the deposition time is an effective tool for controlling the amount of electrogenerated nickel oxide nanoparticles and construction of an efficient electrocatalytic surface.

3.2. Electrooxidation of NADH at NiOxNPs modified GC electrode

Fig. 2 shows the recorded cyclic voltammograms of GC and NiOxNPs/GC electrodes in the presence and absence of 0.5 mM NADH in physiological pH solution. At the bare GC electrode an ill-defined voltammetric peak is obtained at around 0.65V (Fig. 2b) for the oxidation of NADH. However, the modification of the GC electrode with NiOxNPs resulted in a dramatic shift of the oxidation peak potential of NADH to a more negative value, from ~0.65V to ~0.35 V *vs*. Ag/AgCl (Fig 2d). The substantially decreasing overvoltage and increasing oxidation peak

current of NADH at the NiOxNPs/GC electrode compared to the bare GC electrode confirms the excellent electrocatalytic properties of NiOxNPs toward the oxidation of NADH. For bare GC and NiOxNPs/GC modified electrodes no redox response were observed in the absence of NADH (Fig. 2a and c).

Here Fig 2

It seems that the mechanism of the catalytic activity of NiOxNPs toward NADH oxidation is based on the hypothesis that Ni-sites are the active sites for the oxidative dehydrogenation (ODH) of NADH to NAD^+. It has been reported in the literature that NiOx is a relatively active catalyst for the ODH of ethane to ethylene via a Mars and Van Krevelen mechanism (Grabowski 2006). Similar to this mechanism, interactions of NADH with the NiOxNPs modified GC electrode can promote the C–H bond rupture which is necessary for NADH oxidation. So, the oxidation potential of NADH decreases at the NiOxNPs modified GC surface. It should be noted that the catalytic activity of the NiOxNPs/GC electrode toward the electrochemical proton exchange of NAD^+/ NADH has been reported recently (Ali et al. 2012).

The effect of deposition time for nickel formation on the GC electrode surface and its electrocatalytic activity toward NADH oxidation was investigated. Fig 3 shows the electrocatalytic response of six electrodes to NADH, prepared with different deposition times and therefore different Γ values. It is clear from Fig. 3 that with the increasing Γ value, the oxidation potential of NADH decreases and reaches a constant value (0.3V) at Γ ~ 21.93 nmol cm^{-2} (30 s deposition). Thereafter, as the Γ value increases, the oxidation peak potential leads to a more positive value. The fact that the catalytic ability of the NiOxNPs/GC electrode was improved with the increasing amount of deposited NPs, suggests that for Γ values in the range of 1.10-9.59 nmol cm^{-2}, electrocatalytic oxidation of NADH might be under kinetic control. Thus,

in the present study, similar to that previously described by Hutton et al. (2001), the NiOxNPs/GC electrode with the smallest Γ value (1s deposition) is considered to be less able to catalyze the NADH than those with higher Γ values up to 21.93 nmolcm^{-2} (30s deposition). For Γ values more than 21.93 nmol cm^{-2} (>30 s deposition) electron-transfer kinetic is not a limiting factor; however, these longer deposition times led to aggregated NPs structures and resulted in decreases in the catalytic activity of NPs. The deposition condition of -0.8V (*vs*.Ag/AgCl) for 30 s was applied for modification of the GC electrode with NiOxNPs.

Here Fig 3

The stability and antifouling ability of the NiOxNPs/GC surface toward NADH oxidation was investigated by the differential pulls voltammetry (DPV) technique. Fig 4A shows the 10 successive DPV of the NiOxNPs/GC electrode in 0.1 M PBS (pH 7) containing 0.2 mM NADH. As can be seen the peak potential and peak current of NADH oxidation do not change in the successive sweeps, indicating that the surface fouling during the electrochemical oxidation of NADH has not occurred. Furthermore, the electrocatalytic activity of modified electrode toward NADH oxidation was 96% of its initial value after it was stored in 0.1 M pH 7.0 PBS for 48 h. So this modified electrode is a suitable sensor for the determination of NADH.

Ascorbic acid present in the physiological samples is one of the most important interferences in the measurement of NADH. In the present work, we studied the interference effect of AA on the voltammetric response of NADH. As is shown in Fig 4B, the NiOxNPs/GC electrode could separate the voltammetric signal of NADH from the voltammetric signal of AA and exhibits individual voltammetric peaks for NADH and AA. Only a minor change of the sensor sensitivity was detected for NADH in the presence of AA.

Here Fig 4

Cyclic voltammograms of the oxidation of NADH on the NiOxNPs/GC electrode at different scan rates were recorded. The oxidation peak current, increases linearly with the square root of the scan rate, from 5 to 600 mV/s (r = 0 .999), suggesting that the process is controlled by the diffusion of the analyte to the interface (Fig.1 supplement).

Square wave voltammograms of the NiOxNPs/GC electrode were recorded at different concentrations of NADH (Fig. 4C). Insets of Fig. 4C shows the resulting calibration plot of i_{cat} against NADH concentration. The catalytic peak current increases linearly with increasing NADH concentration from 0.3 μM to 1 mM (R^2= 0.998), demonstrating a wide linear response for the sensor. The detection limit (based on signal to noise ratio of 3) and sensitivity of the modified electrode toward NADH were calculated to be 106 nM and 0.052μAμM^{-1}, respectively. The analytical characteristics (such as applied potential, detection limit, sensitivity and linear range) of NiOxNPs/GC electrode toward the oxidation of NADH was compared to some of sensors (that includes mostly mediatorless sensors) previously reported for the electrocatalytic oxidation of NADH (Table.1). The high sensitivity of the NiOxNPs/GC electrode is attributed to the excellent catalytic ability of NiOxNPs.

Here Table 1

Owing to the high biocompatibility, the large specific surface area for enzyme loading and excellent electrocatalytic activity toward NADH, the NiOxNPs/GC electrode can be used as a promising platform for the development biosensors based on dehydrogenase enzymes.

3.3. Electrochemical studies of Ethanol biosensor

To confirm the analytical usefulness of the described electrocatalytic system, it was applied as a new platform to the construction of a very simple ethanol biosensor. This amperometric

biosensor is based on the electrochemical detection of NADH generated during the ADH enzymatic reaction in the presence of NAD^+. The cofactor (NAD^+) is reduced to NADH during the enzymatic oxidation of ethanol and the amount of generated NADH is directly proportional to the ethanol concentration.

3.3.1. Electrochemical impedance spectroscopy study

The electrochemical impedance spectroscopy (EIS) experiment was performed as a powerful tool for the investigation of the interface properties of the modified electrode. The Nyquist plots of bare GC, NiOxNPs/GC and ADH-Nafion/NiOxNPs/GC electrodes were recorded in a 0.1 M KCl solution containing 0.5 mM $[Fe(CN)_6]^{3-/4-}$ at a frequency range of 0.1 Hz to 100 kHz (Fig.2 supplement). The EIS at a bare GC electrode displays a very small semicircle and the charge transfer resistance (R_{ct}) was evaluated to be ~ 500 Ω. After the electrodeposition of NiOxNPs on the surface of the GC electrode, the value of R_{ct} increased to about 1500 Ω due to the semiconductor properties of NiOxNPs. Further increase in R_{ct} was observed with the covering of a layer of enzyme-Nafion solution on the NiOxNPs/GC electrode surface (~ 3000 Ω), due to the increase in the surface negative charge and also the blocking effects of the enzyme-Nafion membrain. These results confirm the successful stepwise preparation of the modified electrode.

3.3.2. Effects of the NAD^+ concentration

The NAD^+ cofactor plays a key role as an acceptor of electrons generated in enzymatic reaction. To obtain the best electrochemical response of the ethanol biosensor, NAD^+ concentration as an important experimental parameter was optimized. The amperometric responses of the ADH-Nafion/NiOxNPs/GC electrode to ethanol were obtained in the presence of different concentrations of NAD^+ (Fig.3 supplement). The electrocatalytic current response increases from 0.5 to 3 mM NAD^+ and reaches a maximum current at 3 mM NAD^+. Thereafter the current

response decreases due to increase in the NAD^+ concentration because of its inhibitory effects. Therefore the amperometric sensing of ethanol was carried out at a concentration of 3 mM of NAD^+.

3.3.3. Amperometric detection of ethanol

The electrochemical determination of ethanol was carried out at the modified electrode under optimum conditions. Fig. 5A shows the amperometric responses of the ADH-Nafion/NiOxNPs/GC electrode at an applied potential of +0.45 V (*vs*. Ag/AgCl) to the successive additions of 1 mM ethanol in a magnetically stirred pH 7.0 PBS containing 3.0 mM NAD^+. After each addition of ethanol to the PBS, the oxidation current increased and approached a steady-state within 10 s representing the fast response time of the ethanol sensor. The oxidation current of ethanol increased linearly with the concentration of ethanol over a range of 1 to 6 mM with a correlation coefficient of 0.9927 (Fig. 5B). The catalytic oxidation peak of ethanol at the NiOxNPs/GC electrode appeared at a potential of +1.3 V (vs. Ag/AgCl) in PBS (pH 7) (Fig. 4 of supplement). The amerometric response of the modified electrode was also recorded in PBS with successive injection of 10 μM of ethanol. The linear least square calibration graph over the range 10-80 μM is I/nA = 0.036 [ethanol] /μM+ 2.4 /nA with a correlation coefficient of 0.993 was obtained (Figure is not shown). The detection limit (S/N = 3) and sensitivity of the sensor were calculated to be 6.4 μM and 36 nA mM^{-1},respectively, which is comparable with those previously reported for other amperometric ethanol sensors (Table 1).

The ADH-Nafion/NiOxNPs/GC electrode shows high stability toward the oxidation of ethanol. Fig 5C shows the amperometric response of the modified electrode to 0.5 mM ethanol recorded in a magnetically stirred pH 7.0 PBS containing 3.0 mM NAD^+ over a continuous period of 950 s. The amperometric response of ethanol remained 95.1% of its initial response, indicating excellent stability of the developed ethanol biosensor.

3.3.4. Interference study and real sample analysis

To demonstrate the selectivity of proposed biosensor, the effects of a variety of common interfering compounds on amperometric responses of modified electrod toward NADH and ethanol were evaluated. At first , the interference effects of common interfering compounds exist in fruit juice, human serum sample and the effect of gallic acid on the voltammetric response of NiOxNPss/GC modified electrode was investigated (Fig.5 of supplement). As can be seen for AA exist in real sample (such as fruit juice) individual oxidation peak oserved without any overlap with NADH oxidation peak (Fig.5 A and B of supplement), while the oxidation peak of gallic acid overlap with NADH oxidation peak.The interference effect of gallic acid and otherr negatively charged compounds eliminated by covering of electrode surface with nafion thin film (Fig. 5C of supplement). Furthermore, for evaluation the applicability of the modified electrodes as amperommetric sensor the responses of the Nafion-NiOxNPs/GC and ADH-Nafion/NiOxNPs/GC electrodes toward NADH and ethanol were measures in the presence of some common species which are normally found in real samples (Fig. 6 of supplement). As observed in Fig.6A of supplement the amperometric response of NiOxNPs-Nafion/GC modified electrode for fruit juice, serum sample, non- alcoholic beverage, 0.1 mM glucose, 0.1 mM gallic acid, 0.1 mM oxalic acid, 20 μM Cys and 0.1 mM AA in compaired to 1 μM NADH were negiligiable. It should be noted that the concentration of Cys added is 20 μM, which is in the normal range of free cysteine in serum samples. The electrode response for Cys> 20 μM is comparable with its response toward NADH.

Furthermore, the amperometric response of ADH-Nafion/NiOxNPss/GCE biosensor for fruit juice, serum sample, non-alcoholic beverage, 0.1 mM glucose, 0.1 mM gallic acid, 0.1 mM oxalic acide, 20 μM Cys and 0.1 mM AA in comparison to biosensor response for 0.5 mM of

ethanol were undetectable. To evaluate the applicability and accuracy of the proposed biosensor for ethanol determination in real samples, recovery tests were performed in a biological and a food sample and results are presented in table 1 of supplement. The recovires values were in the range of 95-105% for the spiked ethanol samples. These results indicated the proposed sensor and biosensor can be used for detection of NADH and ethanol in real samples.

3.4. Conclusion

The application of NiOxNPs as an attractive electrocatalytic nanomaterial is proposed to fabricate a novel mediatorless biosensor for NADH determination. The NiOxNPs/GC electrode exhibits very efficient electrocatalytic behavior toward the oxidation of NADH. The excellent antifouling ability of the NiOxNPs modified GC electrode improves the operational stability of the sensor. Owing to the sensitive and stable response of the modified electrode to NADH, this electrocatalytic system is employed for the construction of a dehyddrogenase-based biosensor for simple and fast determination of ethanol. The low cost and fast response proposed sensor and biosensor for both NADH and ethanol detection prepared rapidly. With covering of modified electrode with a thin film of nafion the applicability of Nafion/NiOxNPs/GC and ADH-Nafion/NiOxNPs/GC modified electrodes as sensitive and selective amperometric sensor and biosensor was improved. Thus, this study suggests that the NiOxNPs/GC electrode is a promising candidate for other dehdrogenase based biosensing systems and it has great potential toward the development of miniaturized dehdrogenase based biosensors for clinical, pharmaceutical , environmental and forensics applications.

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Figure Captions

Table 1. Analytical characteristics of mediatorless based modified electrodes for NADH detection.

Fig.1. (A) SEM image of GC electrode modified with electrodeposited NiOxNPs, (B) cyclic voltammogram of the NiOxNPs/GC electrode in 0.1 M NaOH, deposition time 30 s and scan rate 100 mVs^{-1}.

Fig.2. Recorded cyclic voltammograms of GC electrode in the absence (a) and presence(b) of 0.5 mM NADH; (c) and (d) as (a) and (b) for NiOxNPs/GC electrode at scan rate of 20 mV s^{-1} and in 0.1 M PBS (pH 7).

Fig.3. Recorded Differential pulls voltammograms of GC electrode modified with electrodeposited NiOxNPs using different deposition times (a) 1s, (b) 5 s, (c) 10 s, (d) 30 s, and (e) 100 s in 0.1M PBS (pH 7), in the presence of 0.1 mM NADH, scan rate 20 mVs^{-1}. Inset: plots of oxidation potential and oxidation current of NADH vs. deposition time.

Fig.4. (A) Recorded differential pulse voltamograms (1^{st} and 14^{th} cycle) of NiOxNPs/GC electrode in the presence of 0.2 mM NADH and (B) Differential pulse voltamograms of NiOxNPs/GC electrode in the presence of (a) 50 μM NADH, (b) as (a) after the addition of 50 μM AA and (c) in the presence of 100 μM NADH and 100 μM AA, in 0.1 M PBS (pH 7) at scan rate 20 mVs^{-1}. (C) Differential pulse voltamograms of NiOxNPs/GC electrode in the presence of different concentration of NADH (A) 0.1 – 1.4 mM and 0.3-3 μM in 0.1M PBS (pH 7) at scan rate of 20 mVs^{-1}. Insets C: plots of catalytic current vs. NADH concentration.

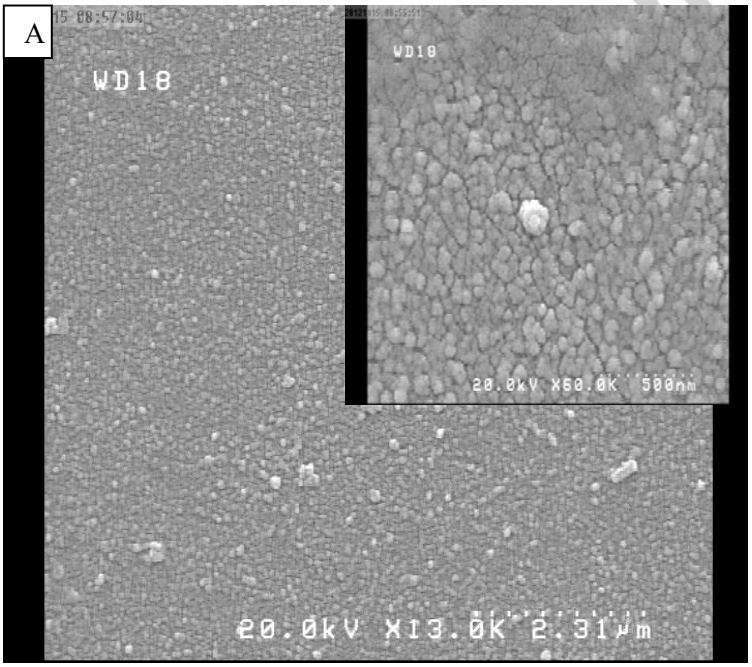
Fig.5. (A) Amperometric response of the ADH-Nafion/NiOxNPs/GC electrode to successive additions of 1 mM Ethanol in 0.1M PBS (pH 7) solution containin 3mM NAD^+ at an applied potential of 0.45V. (B) Plot of chronoamperometric currents vs. Ethanol concentrations. (C) Recorded chronoamperogram for the ADH-Nafion/NiOxNPs/GC electrode in 0.1M PBS (pH 7) solution contaning 3mM NAD^+ and 0.5 mM Ethanol during 950s at an applied potential of 0.45V.

Table 1: Analytical characteristics of mediatorless based modified electrodes for NADH detection

Electrode	E_p/V (vs.Ag/AgCl)	LOD (μM)	Sensitivity μA/mM	Linear range (mM)
	NADH/Ethanol	NADH/Ethanol	NADH/Ethanol	NADH/Ethanol
MWCNT-Pd NP/ poly 3,4-ethylenedioxypyrrole/GC (You and Jeon 2011)	0.42 / -	0.18 / -	-	0.001- 13 /-
GNSs/GC electrode (Zhu et al. 2010)	0.32 / -	-	535.0 / - cm^{-2}	0.002- 4.69 /-
DDF/MWCNT/CPE (Fotouhi et al, 2010)	0.22 / -	0.7×10^{-3} / -	0.31×10^{-3} / -	$(0.01\text{-}0.08) \times 10^{-3}$ / -
CNT–HA/GC (Filip et al, 2011)	0.4 / -	63 / -	6.86 / -	- / -
BGMC/GCE(You et al, 2009)	0.046 / -	1 / -	-	3×10^{-3} -1.4 / -

PP–MWCNT/GC (Yuan et al, 2011)	0.6 / 0.6	10 / 12	40.4 / 30 cm^{-2}	0.02- 0.8 / 0.03- 0.7
Nafion/ADH/graphene/GC (Guo et al. 2011)	0.5 / 0.5	20 / 25	12.6 / -	0.05–1.4 / 0.2- 21.0
ADH/graphene/GC (Guo et al, 2011)	0.7 (pH7)	20 / 25	12.6 / -	0.05–1.4 / 0.2- 21.0
Gaphene/chitosan/GC (Shan et al, 2010)	0.45 / 0.45	- / 5	37.43 / 6.91 cm^{-2}	0.25- 2 / 0.025- 0.2
ADH–Aucoll–MWCNTs-Teflon (Manso et al, 2008)	0.3 / 0.3	4.6 / 4.7	37.7 / 2.27	0.01-1.0 / 0.02- 1
ADH–MWCNTs–Teflon (Manso et al, 2008)	0.3 / 0.3	13 / 32	17.3 / 1.8	0.01-1.0 / 0.1- 1
MWCNT-CHIT-ADH/GC (Lee and Tsai 2009)	- / 0.7	- / 0.52	- / 164.6cm^{-2}	- / -
PVA–MWCNT–ADH/GC (Tsai et al, 2007)	0.6 / 0.7	- / 13	5.88 / 0.2	- / up to 1.5
Pt NPs/PPy composite hollow nanospindle/GC (Mao et al, 2011)	0.65 / -	0.7 / -	4.41 / -	0.04-0.8 / -
NiONPs/ADH-Nafion/GC (present study)	0.35 / 0.45	0.11 / 6.4	52 / 0.036	0.00011 - 1 .0 /0.0064 –6

MWCNT: multiwalled carbon nanotube.; GNSs : graphite nanosheet; PP–MWCNT= diphenylalanine peptide-covered;BGMC: bicontinuous gyroidal mesoporous carbon.; IL-graphene: Ionic liquid-functionalized grapheme; CNT–HA:carbon nanotube-hyaluronic acid bionanocomposite.; Pt NPs/PPy: spindle-like polypyrrole hollow nanocapsules containing Pt nanoparticles.; DDF/MWCNT/CPE: 6,7-dihydroxy-3-methyl-9-thia-4,4a-diazafluoren-2-one/multi-wall carbon nanotubes film-modified carbon paste electrode.



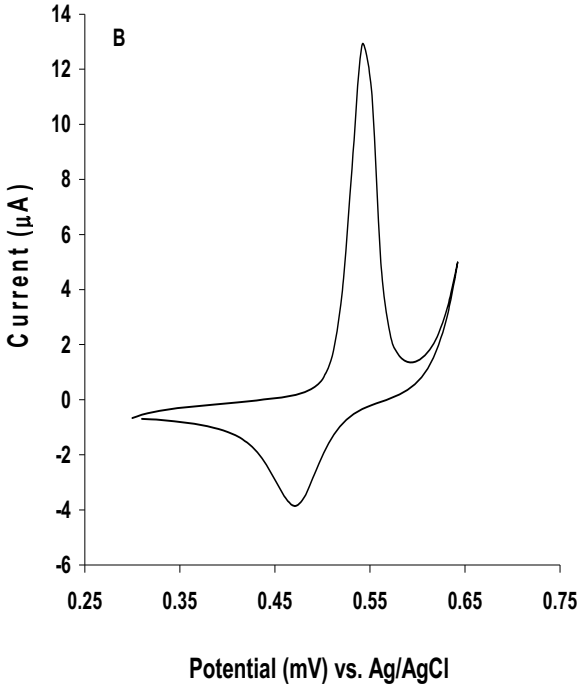


Fig. 1

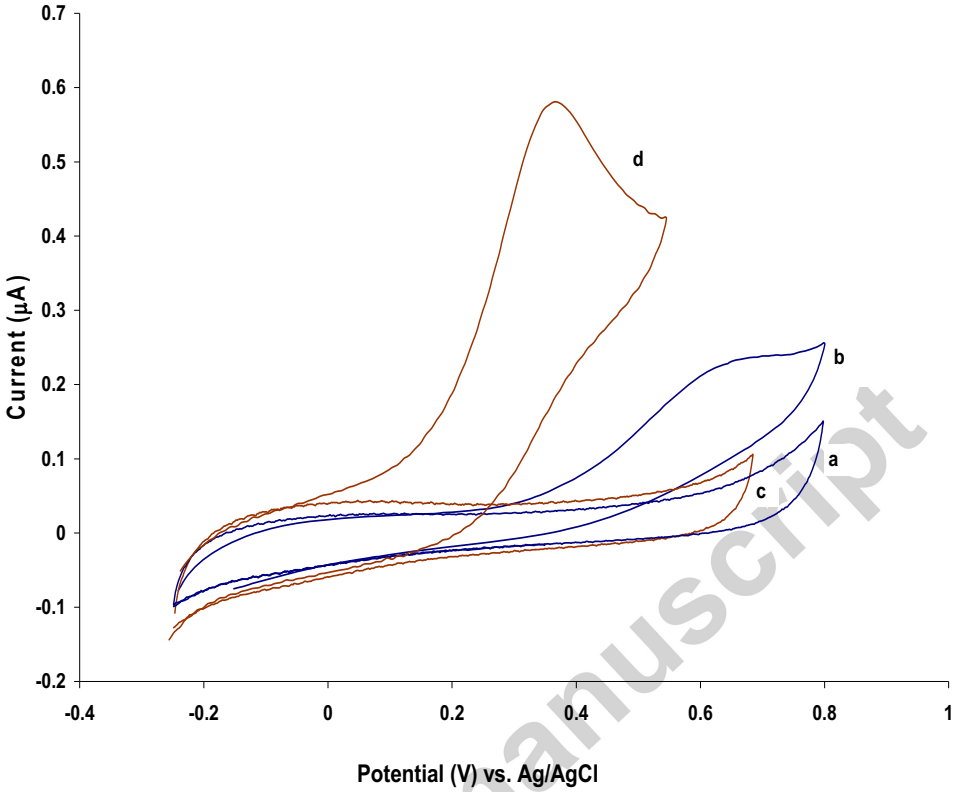


Fig.2

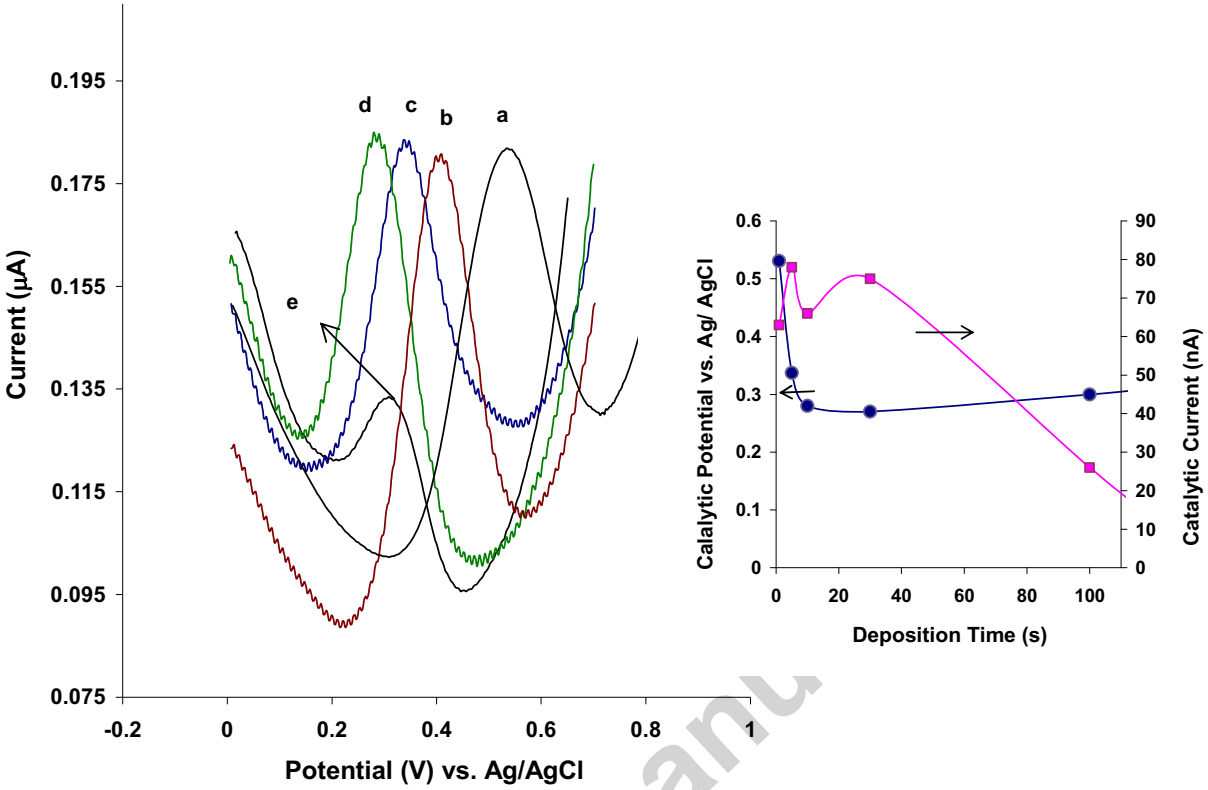


Fig. 3

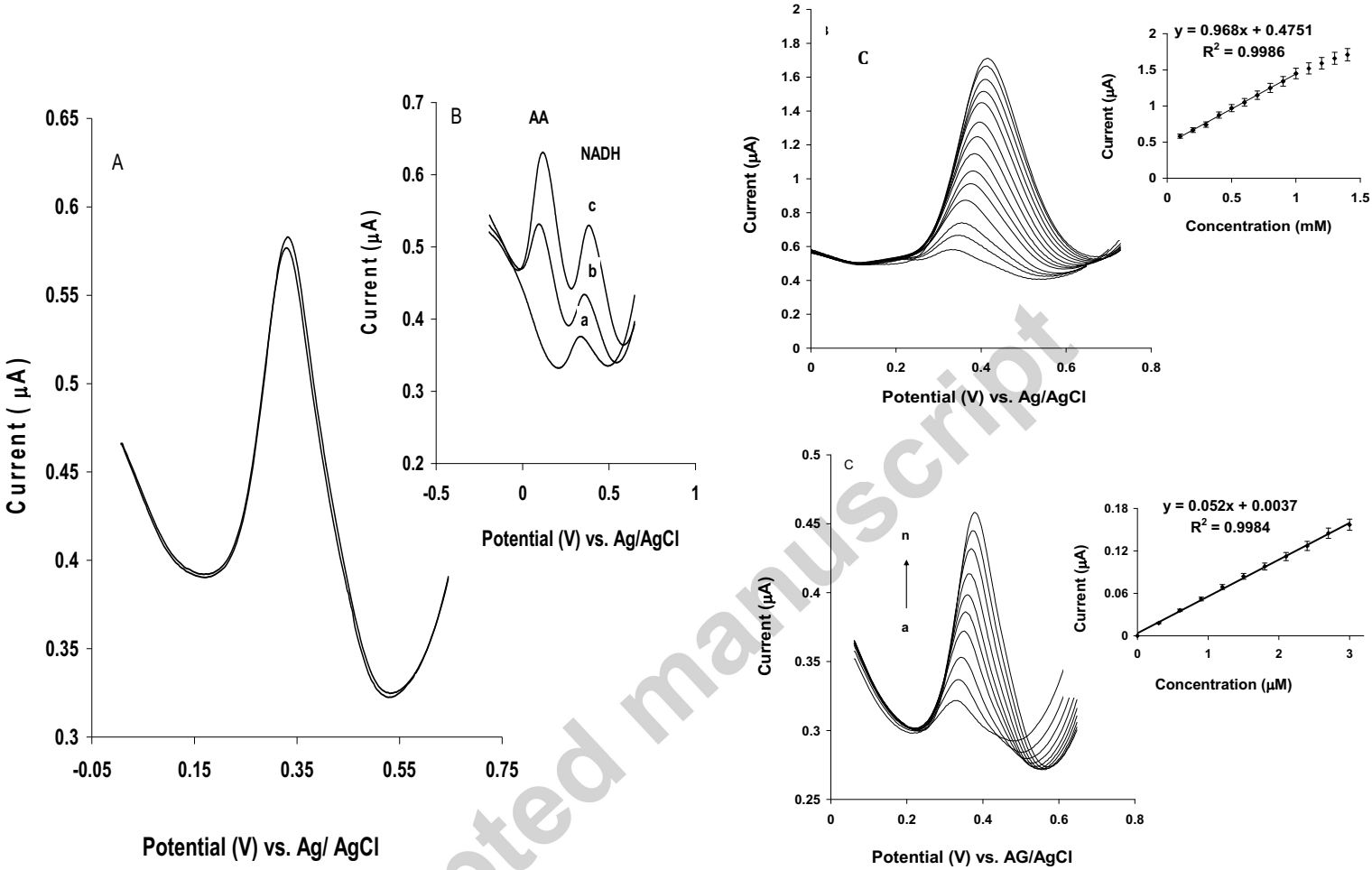


Fig. 4

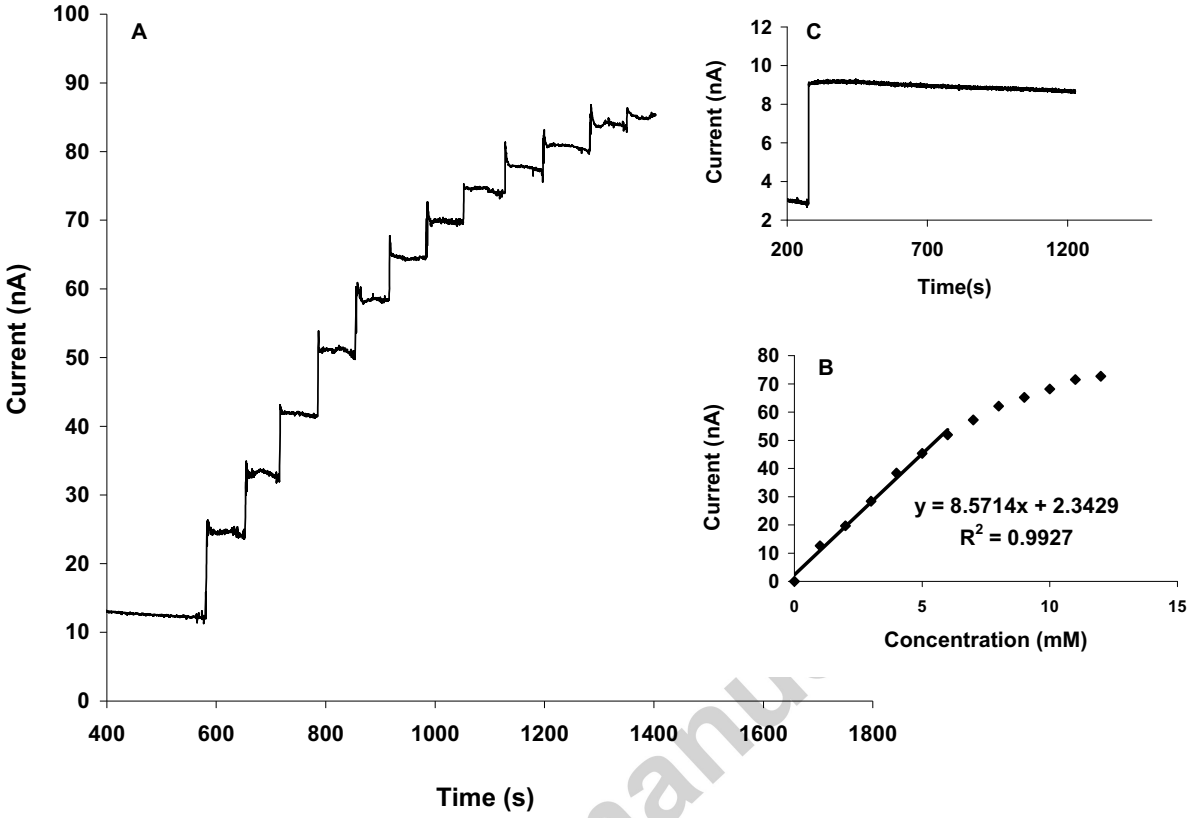


Fig. 5

Highlights for Review

Glassy carbon electrode modified with electrodeposited nickel oxide nanoparticles (NiOxNPs) ►The NiOxNPs was used as mediatorless system for electrocatalytic oxidatiob of NADH ► Detection limit and sensitivity of system toward NADH were 0.1 μM and 0.052 μA μM^{-1}►With immobilization of alchol dehydrogenase onto NiOxNPs a new ethanol biosensor was fabricated► This system have great potential toward the development of miniaturized dehdrogenase based biosensors