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Fabrication of bienzyme nanobiocomposite electrode using functionalized carbon nanotubes for biosensing applications

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Abstract

Mediated biosensors consisting of an oxidase and peroxidase (PO*x*) have attracted increasing attention because of their wider applicability. This work presents a novel approach to fabricate nanobiocomposite bienzymatic biosensor based on functionalized multiwalled carbon nanotubes (MWNTs) with the aim of evaluating their ability as sensing elements in amperometric transducers. Electrochemical behavior of the bienzymatic nanobiocomposite biosensor is investigated by Faradaic impedance spectroscopy and cyclic voltammetry. The results indicate that glucose oxidase (GOD) and horseradish peroxidase (HRP) are strongly adsorbed on the surface of the thionin (TH) functionalized MWNTs and demonstrate a facile electron transfer between immobilized GOD/HRP and the electrode via the functionalized MWNTs in a Nafion film. The functionalized carbon nanotubes act as molecular wires to allow efficient electron transfer between the underlying electrode and the redox centres of enzymes through TH. Linear ranges for these electrodes are from 10 nM to 10 mM for glucose and 17 nM to 56 mM for hydrogen peroxide with the detection limit of 3 and 6 nM, respectively. A remarkable feature of the bienzyme electrode is the possibility to determine glucose and hydrogen peroxide at a very low applied potential where the noise level and interferences from other electroactive compounds are minimal. Performance of the biosensor is evaluated with respect to response time, detection limit, selectivity, temperature and pH as well as operating and storage stability.

Keywords: Bienzyme; Nanobiocomposite; Biosensor; Thionin; Glucose; Hydrogen peroxide

1. Introduction

The integration of nanotechnology with biology is expected to produce major breakthrough in molecular diagnostics, detection of biological agents, chemical agents and in bioengineering (Li and Rothberg, 2004; Ray, 2006). Much effort is currently devoted to the development of surface-based bioanalytical sensors that enable selective detection of biorecognition reactions. It is highly desirable that these sensors allow rapid high-throughput analysis, while at the same time fulfilling the requirements on high specificity and sensitivity. Biosensors based on amperometric devices with immobilized enzymes have been described which recognize the analyte at the molecular level and generate an analytical signal by an electrochemical reaction (Guilbault, 1984). The major problem that appears in the operation of these devices is the electron transfer between the enzyme's active center and the electrode. The first developed, oxidase-based, amperometric biosensors relied upon the oxidation of H_2O_2 resulting from the enzyme's reaction with its natural cofactor, O_2 present in the solution. For this type of biosensors high potential must be applied on the interface that leads to high background currents and also to the non-selectivity of the electrodes. Since these drawbacks become very important in real sample analysis, many solutions to avoid high-applied potentials have been proposed, leading to the second and third generation of the amperometric enzyme-based biosensors (Cass, 1990). Peroxidases are among the very few enzymes which are able to directly transfer electrons to an electrode at relatively low applied potentials (Gorton et al., 1992) but in order to improve sensitivity, mediated electron transfer involving multitude of redox mediators was also attempted. To address the interference problem, Kulys et al. (1981) designed the first oxidase/peroxidase bienzyme electrode with a film containing HRP and GOD to determine glucose by measuring the amount of H_2O_2 produced through the peroxidase. Using oxidase/peroxidase bienzyme systems the detection principle

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switches from an electrochemical oxidation to a reduction process that takes place at much lower potentials, and therefore, is improving considerably the selectivity of the device.

There has been a growing interest to use carbon nanotubes (CNTs) in biosensor platforms (Wang et al., 2003) in order to develop a new class of biosensors with improved performances. Depending on CNTs electrochemical properties, the CNTs-based sensors have been used for detecting NADH, hydrogen peroxide at a lower working potential (Wang, 2005; Musameh et al., 2002). However, the widespread use of CNTs is hampered due to its limited solubility in solvents (Star et al., 2001). Considerable efforts have been made to solublize CNTs through covalent modification (Liu et al., 1998) and non-covalent functionalization (Riggs et al., 2000). A few polymers (Star et al., 2001) are known to effectively solublize CNTs and they have strong tendency to randomly wrap around CNT bundles unless they have specific binding interactions (Kim et al., 2003). CNTs have been effectively suspended and solublized in Nafion, a perfluorosulfonated polymer to facilitate the modification of electrode surfaces toward the development of an amperometric biosensor for glucose (Wang et al., 2003). Recent studies have demonstrated improved electrochemical responses of ethanol and glucose determination at MWNT–Nafion nanocomposite film modified electrode (Tsai et al., 2005; Liaw et al., 2006).

This study proposes a model biosensor system which consists of two enzymes immobilized with thionin functionalized MWNTs. One of the enzymes, GOD converts glucose to gluconolactone with hydrogen peroxide, which is the substrate for the second enzyme, HRP. HRP is in direct electronic communication with the electrode via thionin functionalized MWNTs thus bringing about the electrocatalytic reduction of hydrogen peroxide, which can be measured amperometrically at a moderate reducing potential such as −100 mV (vs. SCE). The bifunctional H_2O_2 electrode is used as an amperometric transducer in designing a glucose biosensor with GOD as enzyme for H_2O_2 generation. Cascade schemes, where an enzyme is catalytically linked to another enzyme, can produce signal amplification and therefore increase the biosensor efficiency. The fabricated nanobiocomposite system represents a simple and functional approach to the integration of enzymes and electrodes, which can provide analytical access to a large group of enzymes for a wide range of bioelectrochemical applications including biosensors and biofuel cells.

2. Experimental

2.1. Chemicals and reagents

Enzymes: glucose oxidase (GOD, E.C.1.1.3.4, activity 250 EU mg^{-1}, from *Aspergillus niger*) and horseradish peroxidase (HRP, E.C.1.11.1.7, activity 90 EU mg^{-1}) were from Sigma Chemical Co. (St. Louis, MO), and were used without further purification. The MWNTs sample used in this work has been prepared by the catalytic decomposition of acetylene over Ni/Cr hydrotalcite-type anionic clay catalyst and the purity is more than 95% (Shaijumon et al., 2005). Thionine from Himedia, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxy sulfo-succinimide (NHS) were from Fluka. Nafion (5 wt%) from Aldrich were diluted to 0.5 wt% in light alcohols. Hydrogen peroxide and D-glucose were purchased from Merck. Glucose stock solution in phosphate buffer was prepared at least one day before measurements for muta rotation. All other chemicals employed were of analytical grade and used without further purification. Doubly distilled water was used to prepare the solutions.

2.2. Apparatus

Amperometry, cyclic voltammetry (CV) and Faradaic impedance spectroscopy measurements were performed with a CHI 660B electrochemical workstation (CH Instruments, USA) in a conventional three-electrode cell. The working electrodes were modified and unmodified glassy carbon (GC) electrodes. A platinum wire was used as the counter electrode (CE) and a saturated calomel electrode (SCE) as the reference electrode (RE). All potentials were measured versus the SCE and all experiments were carried out at room temperature. The Faradaic impedance measurements were carried out in a background solution of 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in phosphate buffer (pH 7.4) at a normal potential. The alternating voltage was 5 mV and the frequency range was 0.1 Hz to 100 kHz.

2.3. Preparation of the bienzyme nanobiocomposite biosensors

The MWNTs were purified as reported previously (Valentini et al., 2003; Shobha Jeykumari and Narayanan, 2007). Briefly, 100 mg of MWNTs were oxidized at 400 °C for 30 min to remove amorphous carbon particles and to eliminate metal oxide catalyst. The oxidized carbon nanotubes were dispersed in conc. HCl for 1 h under ultrasonic agitation, and washed until the pH of the solution was neutral and then dried. The precleaned MWNTs were then treated with 12.8 M nitric acid and refluxed for 12 h, washed extensively with water and dried (Wang et al., 2005).

The redox mediator TH was attached to the MWNTs by immersing the activated MWNTs in a freshly prepared aqueous solution of EDC and sulfo-NHS and stirred as described elsewhere (Shobha Jeykumari et al., 2007). The reaction was allowed to occur at room temperature for 2 h and then the MWNTs were washed thoroughly with water, filtered and dried.

The bienzyme biosensor for glucose and H_2O_2 was fabricated as follows: 4 mg of GOD and 3 mg of HRP were dissolved in 0.3 ml of phosphate buffer (pH 7.0) and completely mixed. Enzyme immobilization with TH-functionalized MWNTs (1 mg) was achieved by mixing thoroughly with 20 μl of the enzyme mixture by stirring. Due to the ability of MWNTs to bind biomolecules, the two enzymes became electrostatically and hydrophobically adsorbed onto the surface of TH-functionalized MWNTs during mixing. The enzyme–MWNTs mixture was then dispersed in 1 ml of 0.5 wt% Nafion solution with the aid of ultrasonic agitation for 5 min to form a homogeneous nanobiocomposite colloidal solution. For comparison, the bienzyme electrode with mere MWNTs was also prepared.

Bienzyme nanobiocomposite electrodes were prepared by casting 10 μl of MWNT/TH/GOD/HRP/Nf or MWNT/GOD/HRP/Nf biocomposite colloidal solutions on the surface of GC electrode (3 mm diameter), followed by air drying for about 2–3 h, rinsed with water several times before use. When not in use all the modified electrodes were stored at 4 °C.

3. Results and discussion

The as prepared MWNTs could not be modified by covalent attachment due to the lack of reactive functional groups on the surface. Treatment of MWNTs with chemical reagents, such as oxidation with strong oxidative acids, is an effective way to generate –COOH groups on the MWNT surface. The mediator immobilization and enzyme loading on MWNTs were confirmed with FTIR spectra.

3.1. Electrochemical performance of the bienzyme electrode

3.1.1. Faradaic impedance spectroscopy

Impedance spectroscopy is an effective method to probe the features of the surface modified electrodes (Stoynov et al., 1991). These studies were employed for modification of the electrode with individual and mixed components used for the fabrication of the biosensor. The complex impedance can be presented as the sum of the real, Z' (ω), and imaginary Z'' (ω), components that originate mainly from the resistance and capacitance of the cell, respectively. The general electronic equivalent scheme (Randles and Ershler model, inset of Fig. 1), include the ohmic resistance of the electrolyte solution, R_s, the Warburg impedance, Z_w, resulting from the diffusion of ions from the bulk electrolyte to the electrode interface, the double-layer capacitance, C_{dl}, and the electron-transfer resistance R_{et}, that exists if a redox probe is present in the electrolyte solution. The electron-transfer resistance, R_{et}, controls the electron-transfer kinetics of the redox probe at the electrode interface. From the shape of an impedance spectrum, the electron-transfer kinetics and diffusional characteristics can be extracted from spectra.

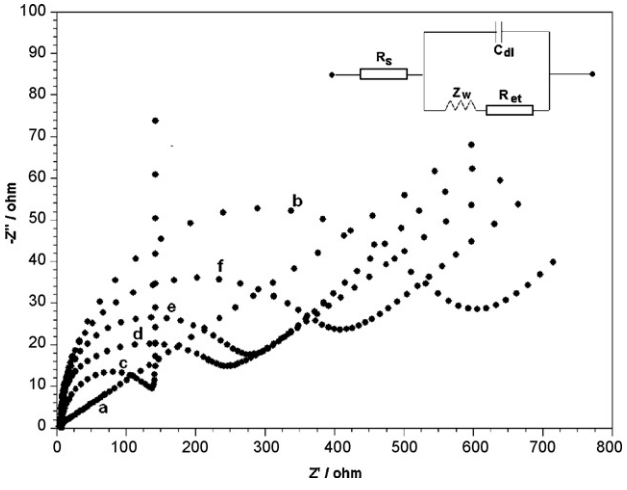

Fig. 1. Nyquist diagram (Z' vs. Z'') for the Faradaic impedance measurements in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ after the different steps of modification: (a) bare GCE, (b) MWNT/Nf modified, (c) MWNT/TH/Nf modified, (d) MWNT/TH/GOD/Nf modified, (e) MWNT/TH/HRP/Nf modified and (f) MWNT/TH/GOD/HRP/Nf modified GCE. The electrode potential was −0.5 V vs. SCE in 0.1 M PBS (pH 7.4). The inset is the Randles equivalent circuit for the modified electrodes.

Fig. 1 shows the Faradaic impedance spectra, presented as Nyquist plots (Z'' vs. Z') upon the modification of the electrodes with the functionalized and enzyme immobilized carbon nanotubes. The bare GC electrode exhibits an almost straight line (curve a) that is characteristics of the diffusionally limited electron-transfer process. After the MWNTs were treated with nitric acid, the –COOH groups present on the MWNTs surface showed the greatest semicircle with an interfacial resistance of $R_{et} = 602\ \Omega$, due to the electrostatic repulsion between negatively charged surface and probe molecule, $Fe(CN)_6^{3-/4-}$ (curve b). Upon covalent attachment of TH with MWNT (curve c), the electron-transfer resistance decreased dramatically to $R_{et} = 136\ \Omega$, due to the decrease in the negative charges, making it easier for the electron transfer to take place. After the immobilization of monoenzymes, GOD and HRP (curves d and e) separately over the surface of the nanotubes, which generates a hydrophobic insulating layer on the electrode, introduced a barrier to the interfacial resistance. As a result the electron transfer values increased to $R_{et} = 239\ \Omega$ and $R_{et} = 275\ \Omega$, respectively, for GOD and HRP. Upon immobilization of both the enzymes over the TH functionalized MWNTs, the electron-transfer resistance further increased to $R_{et} = 421\ \Omega$ (curve f). This is due to the attachment of each of the enzymes that increases the extent of insulation of the conductive support by the hydrophobic protein layers. As a result, the interfacial electron-transfer resistance gradually increases in their values upon the immobilization of the two biocatalysts.

3.1.2. Cyclic voltammetry

The cyclic voltammograms of different modified electrodes were obtained in the potential range of 0 to −0.7 V, except for the MWNT/GOD/HRP/Nf modified bienzyme electrode which was obtained in the range of 0.2 to −0.5 V in 0.1 M PBS (pH 7.4) solution. No redox peak was observed for the bare GC electrode and MWNT/Nf modified GC electrode. Compared with bare GC electrode, the background current of MWNT/Nf electrode is apparently larger, which indicates the effective electrode surface is significantly enhanced by use of MWNT to modify the electrode (not shown here). In CV MWNT/GOD/Nf modified electrode has shown a pair of well-defined and nearly symmetric redox peaks with the formal potential of −0.47 V. The redox peaks are attributed to GOD, which is similar to the results reported (Liu et al., 2005; Cai and Chen, 2004). The CV for MWNT/HRP/Nf modified GC electrode shows a pair of redox peaks with the formal potential of −0.29 V for the HRP. This is ascribed to the direct electron transfer between the HRP and the underlying electrode. The CV for MWNT/GOD/HRP/Nf modified electrode, which showed two pairs of redox couple with formal potentials at −0.36 and −0.51 V may be due to the presence of

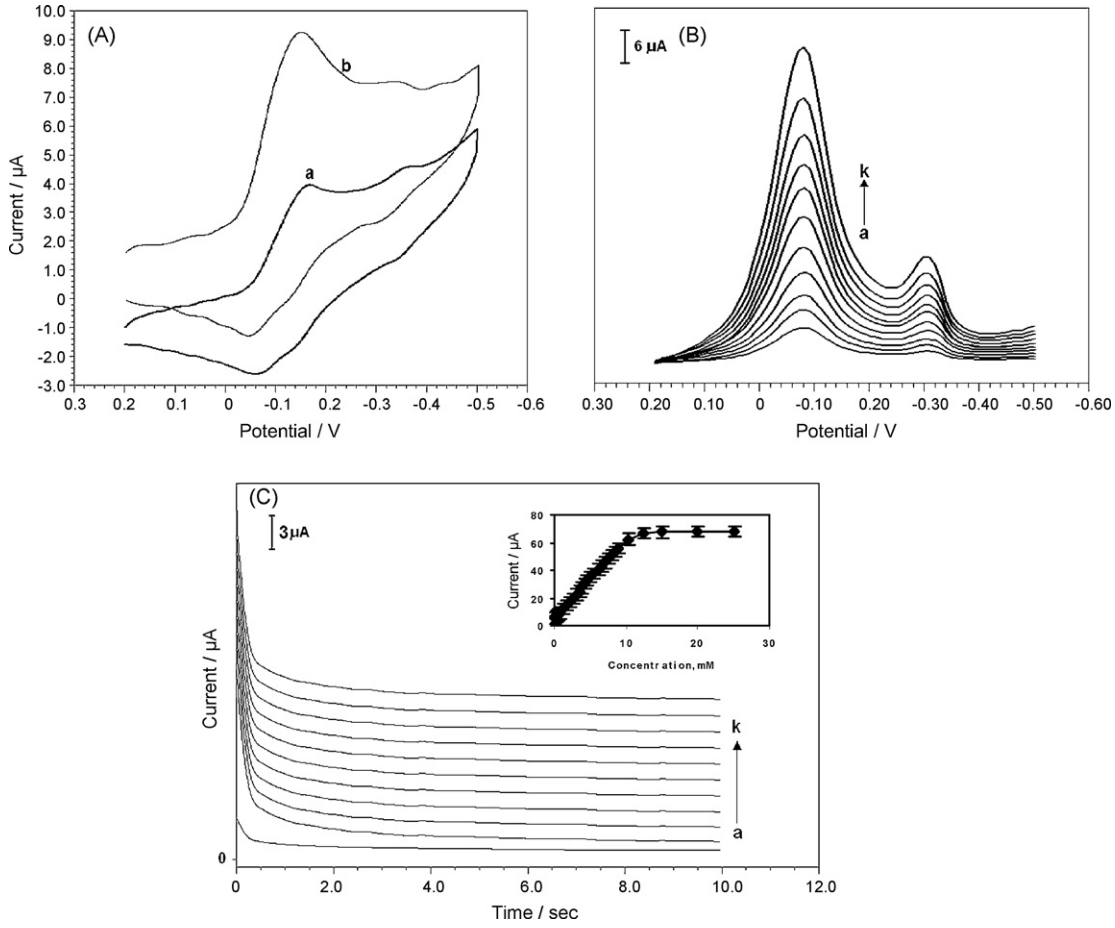

Fig. 2. (A) Cyclic voltammograms of MWNT/TH/GOD/HRP/Nf nanobiocomposite biosensor (a) in the absence and (b) in the presence of 1.0 μM glucose; (B) differential pulse voltammograms of nanobiocomposite biosensor (a) in the absence and (b–k) successive addition of 1.0 μM glucose; (C) amperometric response (a) in the absence and (b–k) successive addition of 0.1 μM glucose; inset shows the linear calibration plot.

HRP and GOD adsorbed over the surface of the nanotubes. However, MWNT/TH/GOD/HRP/Nf modified biocomposite electrode displayed the CV in a different form exhibiting two well defined reversible redox waves at very low potentials with formal potentials at −0.11 and −0.354 V, with higher reduction and oxidation peak currents when compared to electrodes, which should be attributed to the TH functionalized MWNTs.

The dependence of the peak current towards the scan rate has been also studied for the MWNT/TH/GOD/HRP/Nf modified bienzyme electrode. Good linearity in the plot of peak current versus scan rate in the range from 0.1 to 2.0 V s^{-1} was observed as expected for surface confined redox process. In addition, with increasing scan rates, the peak separations begin to increase, indicating the limitation arising from charge transfer kinetics. Based on Laviron theory (Laviron, 1979), the electron transfer rate constant (K_s) and charge transfer coefficient (α) can be determined by measuring the variation of peak potential with scan rate. The values of peak potentials were proportional to the logarithm of the scan rate for scan rates higher than 2.0 V s^{-1}. The calculated values for (K_s) and (α) were about 7.81 s^{-1} and 0.55, respectively. The surface concentration of electroactive species Γ can be calculated from the slope of the plot of I_{pa} versus scan rate and was 9.18×10^{-10} mol cm^{-2}.

3.2. Bioelectrocatalytic oxidation of glucose at the bienzyme-based biosensor

Electrocatalytic action of the bienzyme electrode was characterized by CV experiments. No electrocatalytic oxidation or reduction is found to occur at MWNT/TH/Nf modified electrode when glucose is added to the phosphate buffer solution (not shown). But even in the absence of glucose bienzyme modified electrode yields a voltammogram, which exhibits two redox peaks in the potential range from 0.2 to −0.5 V corresponding to the redox behavior of both the enzymes for the MWNT/TH/GOD/HRP/Nf modified nanobiocomposite electrode (Fig. 2A (curve a)). Upon addition of glucose, the bienzyme-based biosensor triggers bioelectrocatalytic reaction, which dramatically changes the cyclic voltammogram with a sharp increase in the cathodic current and a concomitant decrease in the anodic current (Fig. 2A (curve b)). Glucose oxidase catalyses the oxidation of glucose to gluconic acid, in the presence of natural co-substrate O_2, with production of H_2O_2 (Eq. (1)). HRP reduces H_2O_2 and in this process, is getting oxidized which is

then reduced by the mediator, which in turn is reduced electrochemically.

$$\beta\text{-D-glucose} + O_2 \rightarrow \delta\text{-glucono-}\beta\text{-lactone} + H_2O_2 \quad (1)$$

H_2O_2 formed by GOD undergoes a series of reactions (2)–(5).

In the first two electron step, H_2O_2 oxidizes ferriheme prosthetic group of HRP (Fe^{3+}), producing an unstable intermediate HRP-I consisting of a π cation radical of heme with Fe(IV) to which an oxygen atom is coordinated ([Fe(IV) = O]):

$$HRP\,(Fe^{3+}) + H_2O_2 \rightarrow HRP\text{-}I + H_2O \quad (2)$$

The reduction of HRP-I to HRP (Fe^{3+}) takes place through two successive one-electron steps via the electron transfer (TH)

$$HRP\text{-}I + TH_{red}\,(\text{or } e^-) \rightarrow HRP\text{-}II + TH_{ox} \quad (3)$$

$$HRP\text{-}II + TH_{red}\,(\text{or } e^-) \rightarrow HRP\,(Fe^{3+}) + TH_{ox} \quad (4)$$

where HRP-II, an intermediate state, possesses a heme with Fe(IV) to which OH is coordinated ([Fe(IV) OH]). TH_{ox} and TH_{red} represent oxidized and reduced forms of thionin, respectively. Oxidized mediator (TH_{ox}) is reduced at the electrode, bringing about a reduction current,

$$TH_{ox} + 2H + 2e^- \rightarrow TH_{red}\,(\text{at the electrode}) \quad (5)$$

The mechanism is similar to the report of Ruzgas et al. (1995). Therefore, the detection of H_2O_2 is based on the electrochemical reduction of the TH ion generated from the enzymatic reaction at −0.10 V (vs. SCE).

As the charging current contribution to the background current is the limiting factor in the analytical determination of any electroactive species, differential pulse voltammetry (DPV) experiments were carried out. When the DPV was performed instead of CV (Fig. 2B), an obvious improvement in the height of the peaks was observed, which was quite important in the detection of low levels of glucose. The linear range with DPV was from 10 nM to 10 mM. Fig. 2C shows the chronoamperometric response of the biosensor to successive increments (0.1 μM) of glucose to the phosphate buffer solutions under stirring at an applied potential of −0.1 V. The time required to reach 95% of the maximum steady-state current was 2 s. The response was also linear in the range from 10 nM to 10 mM. The linear regression equation was $I(A) = 0.52[\text{glucose}] + 4$ (A with the correlation coefficient of 0.9998). The detection limit was 3 nM when the signal to noise ratio is 3. When injecting standard solutions the accuracy (as R.S.D.%) was 2.3% for nine successive assays. The apparent Michaelis–Menten constant K_M^{app} was used to evaluate the biological activity of immobilized enzyme and it was calculated according to the Michaelis–Menten equation

$$i = i_{max} - K_M^{app}\left(\frac{i}{C}\right)$$

where i is the steady-state catalytic current, i_{max} the maximum current measured under saturated substrate conditions, C referred to the glucose concentration and K_M^{app} stands for the apparent Michaelis–Menten constant of the system. K_M^{app} in this work, was evaluated as 0.9 mM and is in agreement with the earlier reports (Kamin and Wilson, 1980). The results show that the biosensor possesses higher biological affinity to glucose. The working mechanism of the GOD/HRP bienzyme electrodes is depicted in Fig. 3.

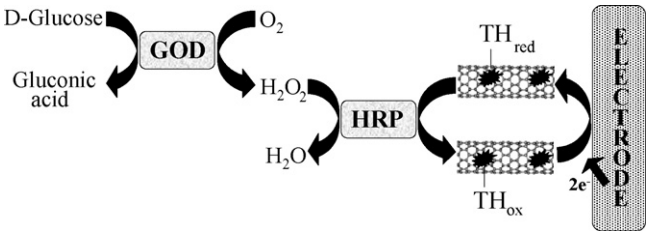

Fig. 3. Reactions occurring at the GC electrode modified with MWNT/TH/GOD/HRP/Nf nanobiocomposite.

3.3. Biosensing of hydrogen peroxide

The cyclic voltammograms of TH mediated MWNT bienzyme-based biosensor-containing HRP before and after addition of H_2O_2 are shown in Fig. 4A. In the absence hydrogen peroxide two typical reversible redox waves of MWNT/TH/GOD/HRP/Nf are observed (Fig. 4A, curve a). Nevertheless, addition of H_2O_2 to the solution brings about a significant increase in cathodic peak current and almost complete disappearance of the anodic peak current. Comparison of the voltammograms with and without hydrogen peroxide demonstrates that TH incorporated with MWNT effectively enhances electron transfer between the electrode and HRP through TH. For the mediatorless biosensor only a small enhancement of the cathodic peak current was observed upon addition of H_2O_2 into the electrolyte solution, which indicates that direct electron transfer from MWNT to HRP is slow. Comparison of the TH mediated biosensor showed 500-fold higher sensitivity to hydrogen peroxide than the mediatorless one, which indicates that electron transfer via TH incorporated MWNT is more efficient in the bioelectrocatalytic reduction of H_2O_2. The mechanism of the bioelectrocatalytic reaction between H_2O_2 and HRP has been given in Eqs. (2)–(5) (Ruzgas et al., 1995; Tatsuma et al., 1995).

Differential pulse voltammetric experiments were also carried out for the determination by varying H_2O_2 concentrations (Fig. 4B). The sensor showed a linear relationship for H_2O_2 from 17 nM to 56 mM. The chronoamperometric response of the biosensor to successive increments of 0.1 μM of hydrogen peroxide is shown in Fig. 4C. At an applied potential of −0.1 V, where H_2O_2 is added, the time required to reach the 95% steady-state response is less than 2 s. Steady-state cathodic currents are proportional to H_2O_2 concentration in the range from 17 nM to 50 mM. The linear regression equation was $I(A) = 0.5777[H_2O_2] + 5.256$ (A with the correlation coefficient of 0.9992). The biosensor provides an extremely low detection limit of 6 nM of H_2O_2 (based on signal-to-noise ratio of 3). When injecting standard solutions the accuracy (as R.S.D.%) was found to be 2.4%. The apparent Michaelis–Menten constant K_M^{app} is calculated to be 0.89 mM. This suggests that the present sensor exhibits a higher affinity for H_2O_2.

3.4. Influential factors on response characteristics of MWNT/TH/GOD/HRP/Nf nanobiocomposite electrode

3.4.1. Effect of pH and temperatures on the biosensor

The effect of pH on the response of MWNT/TH/GOD/HRP/Nf modified electrode was observed at different pHs from 4.5 to 8.5. Enzymatic activity varies with immobilization method and microenvironment around the enzyme. The optimal pH reported for GOD is in the range of 6.5–7.5 (Chen et al., 2003; Yu et al., 2003). Similarly the optimum pH for HRP ranges from 5.5 to 7.4 (Okawa et al., 1999; Yu and Ju, 2002; Delvaux et al., 2004). The proposed biosensor showed a maximum response at pH 7.4. A further increase of buffer pH led to decrease in the response, indicating that the catalytic response was controlled by the enzymatic activity of the enzymes. Decrease in the response at low and high pHs was due to the decrease of enzymatic activity which my be caused by denaturing of the enzymes.

The activity of enzyme is affected by change in temperature and hence the thermal stability of the biosensor was studied at various temperatures from 15 to 70 °C in the presence of 0.2 mM glucose or H_2O_2. The response current increased gradually with increasing temperature and reached its maximum at about 60 °C. This shows that the activity of the immobilized enzyme increases as the temperature increases upto 60 °C. At temperatures above 60 °C the catalytic current decreases. This is because the enzyme is partly denatured at high temperature and the result is in agreement with the earlier reports (Johnson, 2002). It was evident that the immobilized bienzymes had good thermal stability upto 60 °C because of the unchanging microenvironment. This indicated that this biosensor could be used over a wide range of temperatures. However, for practical convenience, all other experiments were carried out at room temperature.

3.4.2. Stability of the sensor performance

The long-term stability of the sensor has been studied with respect to its shelf-life (not in use) and during continuous operation (with applied potential). During the continuous operation studies, the analyte (glucose or H_2O_2) was changed periodically with freshly prepared solutions. Calibration curves were used to evaluate shelf-life stability and long-term operational stability. The sensor showed a good stability, which maintained >90% of maximum response for 90 days whether used continuously or kept without using.

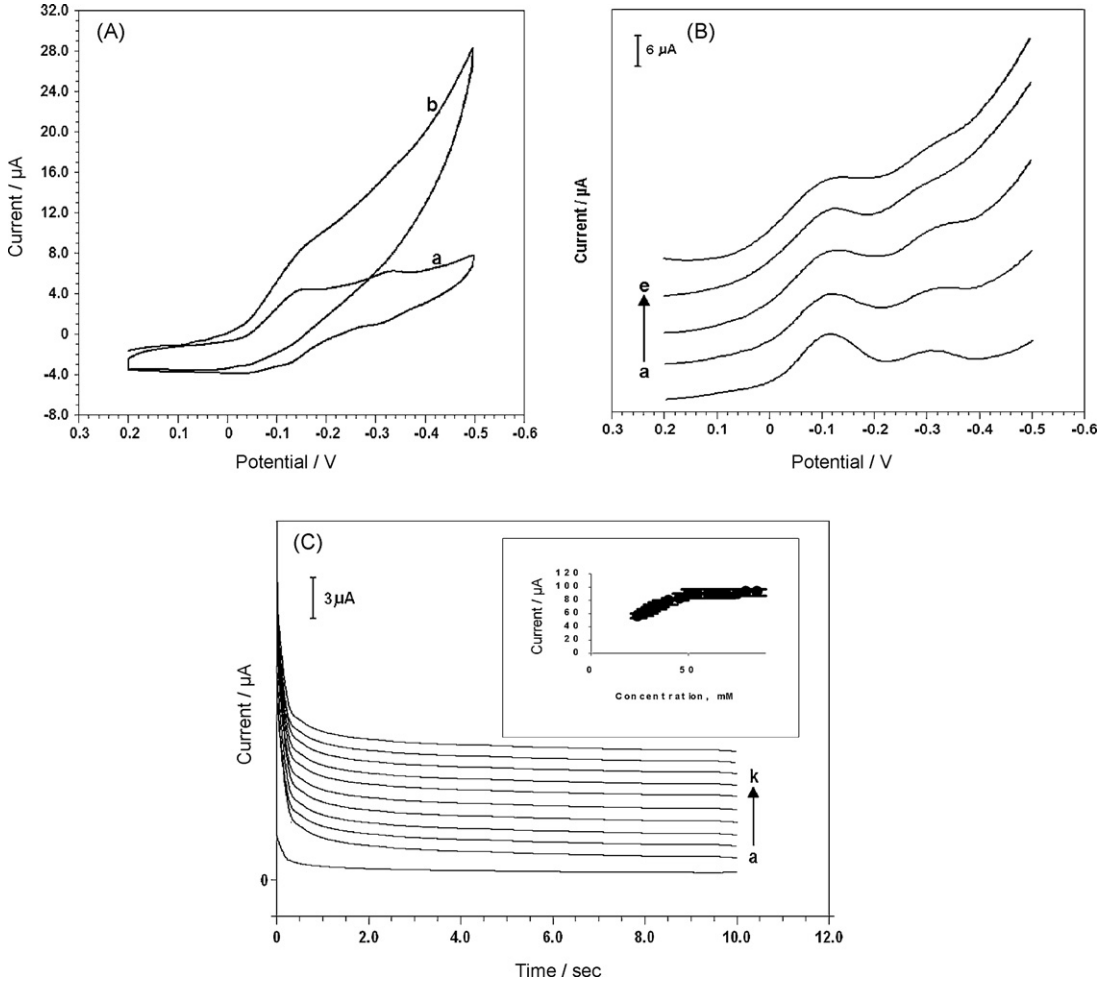

Fig. 4. (A) Cyclic voltammograms of MWNT/TH/GOD/HRP/Nf nanobiocomposite biosensor (a) in the absence and (b) in the presence of 1.0 µM H_2O_2; (B) differential pulse voltammograms of nanobiocomposite biosensor (a–e) at successive addition 1.0 µM H_2O_2; (C) amperometric response (a) in the absence and (b–k) successive addition of 0.1 µM H_2O_2 in 0.1 M PBS (pH 7.4); inset shows the linear calibration plot.

Table 1
Effect of interferants on analyte (0.1 mM glucose or H_2O_2) determination

Interferant compounds	Molar ratio of analytes:interferant compounds	Relative error (%)	
		Glucose	H_2O_2
L-Cysteine	1:2	1.7	1.2
L-Tyrosine	1:2	1.3	1.4
L-Leucine	1:2	1.8	1.6
L-Tryptophan	1:2	1.5	1.7
L-Histidine	1:2	1.4	1.5
L-Aspartic acid	1:2	1.9	1.8
L-Glutamic acid	1:2	1.2	1.5
Fructose	1:5	1.3	0.9
Galactose	1:5	1.7	1.1
Ascorbic acid	1:5	1.4	1.2
Uric acid	1:2	0.9	0.7
Acetaminophen	1:2	1.1	0.8

3.4.3. Selectivity of the bienzyme biosensor

An often underestimated aspect in amperometric biosensors is that of electroactive interferences which ultimately determines the usefulness of the device. The selectivity of the present sensor was examined by studying the effects of foreign species on the determination of 0.1 mM glucose or H_2O_2 (Table 1). As can be seen, the error caused by the interferants is less than 3% only. This is due to the selectivity of the fabricated bienzyme biocomposite electrode and low operating potential of the electrode. Further the nafion acts as an effective permselective membrane for the electroactive interferants.

3.4.4. Real sample analysis

The proposed bienzymatic biosensor was used for the measurement of glucose in human serum samples provided by the local hospital. The blood samples were diluted 10 times with phosphate buffer solution prior to measurements. The assay results determined by this method are shown in Table 2 and it can be seen that the values are consistent with those given by the hospital.

To assess the possible application of the sensor to assay hydrogen peroxide, the proposed biosensor was applied to pharmaceutical samples. The results showed an acceptable correlation between the claimed and measured values. The quantitative recoveries from 98.2 to 102.5% were obtained when a known amount of hydrogen peroxide was added to the sample.

The high sensitivity and stability of the MWNT-based biosensor can be attributed to its special structure. The magnitude of the response depends not only on the amount of GOD and HRP loading, but also the GOD and HRP's activity on the electrode surface (Harsanyi, 2000; Stefan et al., 2001). The structure of nanotubes consists of concentric cylinders made of graphene sheets (hexagonal lattice), which are closed at the ends by hemispherical or polyhedral caps due to the presence of (6 + 6) pentagonal defects. The nanotube caps can be opened when the nanotubes are treated with HNO_3. The open nanotube ends allow the enzymes GOD and HRP to enter the hollow MWNTs when they are immersed in the solution containing GOD and HRP. In addition, a large surface area per unit volume of the MWNTs enhances a large amount of GOD/HRP to be immobilized with the nanotubes, resulting in a CNT-based enzyme reservoir. In addition nanotubes can also promote electron transfer in bioelectrochemical reactions (Nugent et al., 2001). These properties consequently lead to a high sensitivity of the MWNT-based sensor to glucose and H_2O_2. In addition, the presence of TH attached to MWNTs through covalent immobilization enhances the electron transfer between the enzymes and the electrode, enhancing the sensitivity of the electrode further.

Table 2
Determination of glucose in human serum samples using bi enzymatic nanobiocomposite biosensor

	Sample no.		
	1	2	3
Determined by biosensor[a] (mM)	7.12 ± 0.091	6.79 ± 0.063	6.51 ± 0.047
Claimed value (mM)	7.10	6.72	6.50

[a] Mean ± S.D. of three measurements.

4. Conclusion

In conclusion, oxidase/PO*x* bienzyme mediated biosensors are very useful to construct a variety of sensing devices for biologically related analytes. First attempt to fabricate a bienzymatic biosensor for glucose and H_2O_2, based on nanobiocomposite-modified electrodes has been made. Two enzymes, GOD and HRP, were co-immobilized onto the surface of multiwalled carbon nanotubes modified with TH. Faradaic impedance, cyclic voltammetry and chronamperometric measurements have been employed to demonstrate the suitability of bienzyme-based nanobicomposite biosensor for the determination of glucose and H_2O_2. Because of the high efficacy of bioelectrocatalytic reduction of H_2O_2 via TH functionalized MWNTs as an electron transfer mediator, the sensor showed high performance characteristics with a broad detection range, a short measuring time, a good stability and an interference-free determination of glucose and hydrogen peroxide.

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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.2008.02.001.

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