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A novel nanobiocomposite based glucose biosensor using neutral red functionalized carbon nanotubes

D.R. Shobha Jeykumari, S. Sriman Narayanan *

Department of Analytical Chemistry, School of Chemical Sciences, University of Madras, Guindy Campus, Chennai 600 025, India

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Abstract

The performance of a new glucose biosensor based on the combination of biocatalytic activity of glucose oxidase (GOx) with the electrocatalytic properties of CNTs and neutral red (NR) for the determination of glucose is described. This sensor is comprised of a multiwalled carbon nanotubes (MWNTs) conduit functionalized with NR and Nafion (Nf) as a binder and glucose oxidase as a biocatalyst. Neutral red was covalently immobilized on carboxylic acid groups of the CNTs via carbodiimide reaction. The functionalized MWNTs were characterized by microscopic, spectroscopic and thermal methods. The MWNT–NR–GOx–Nf nanobiocomposite was prepared by mixing the GOx solution with NR functionalized CNTs followed by mixing homogeneously with Nafion. The performance of the MWNT–NR–GOx–Nf nanobiocomposite modified electrode was examined by electrochemical impedance spectroscopy and cyclic voltammetry. The catalytic reduction of hydrogen peroxide liberated from the enzymatic reaction of glucose oxidase upon glucose with NR functionalized CNTs leads to the selective detection of glucose. The excellent electrocatalytic activity and the influence of nanobiocomposite film result in good characteristics such as low potential detection of glucose with a large determination range from 1×10^{-8} to 1×10^{-3} M with a detection limit of 3×10^{-9} M glucose, a short response time (with 4 s), good stability and anti-interferent ability. The improved electrocatalytic activity and stability made the MWNT–NR–GOx–Nf nanobiocomposite biosensor system a potential platform to immobilize different enzymes for other bioelectrochemical applications.
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Keywords: Multiwalled carbon nanotubes; Nanobiocomposite; Nafion; Glucose biosensor

1. Introduction

The search for novel materials to design electrochemical biosensors is of significant at present. Biosensors are attractive in analytical applications due to their unique specificity (Harsanyi, 2000), and the development of composite electrodes leads to important advances in biosensor devices. There is always a demand for highly sensitive, stable and disposable biosensors in determining glucose in blood and urine for the diagnosis of diabetes. Enzyme immobilization is considered as an important factor in bioreactor and biosensor technologies (Albareda-Sirvent et al., 2000). The majority of biosensors for glucose utilize the enzyme glucose oxidase (GOx), which has been described as an ideal enzyme for biosensor development (Wilson and Turner, 1992).

Classical glucose biosensors were based on monitoring either the consumption of oxygen or the production of hydrogen peroxide (Wang, 2001). Unfortunately, the amperometric determination of hydrogen peroxide requires high anodic potential (Iannello and Iacynych, 1981). In addition the redox centre of the enzyme is well embedded in the protein centre core which gives a kinetic barrier for direct electron transfer between the enzyme redox centre and the electrode. In such cases, an electron transfer mediator (ETM) is often applied to shuttle electrons between the redox centres and the electrode. Hence integrated biosensors have been extensively fabricated in which selective ETM such as metals (Fang et al., 2003), metal hexacyanoferrates (Ricci et al., 2003), metal phthalocyanines (Shi et al., 2000) ferrocene (Cass et al., 1984), and osmium complex (Battaglini et

* Corresponding author. Tel.: +91 44 2220 2717; fax: +91 44 2235 2494.
E-mail addresses: sriman55@yahoo.com, sriman55@gmail.com (S. Sriman Narayanan).

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al., 2000), etc. were co-immobilized with glucose oxidase (GOx) on electrode surfaces.

Redox dyes are also employed in the construction of mediated amperometric biosensors. They exhibit fast reversible electrochemical response at negative potentials, which make them useful as redox mediators for numerous enzymatic reactions (Casero et al., 2006). The phenazine dye neutral red (NR) was found to be a convenient redox mediator for electrochemical investigations of biological system. It has a much lower redox potential than analogous phenothiazine and phenoxazines dyes due to the heteroatom, which is nitrogen instead of a divalent oxygen or sulfur (Karyakin et al., 1999).

Since the discovery of carbon nanotubes, several unique properties of these exciting materials were reported (Iijima, 1991). Its higher specific surface, the inherent physical and chemical properties and the introduction of abundant functional groups can significantly improve the catalytic properties of CNTs. The performance of CNTs has been found to be superior to other carbon electrodes and there has been a growing interest to use CNTs in biosensor platforms (Balasubramanian and Burghard, 2006). The immobilization of biomolecules with CNTs resulted in a new class of biosensors with improved platforms (Balavoine et al., 1999). However, manipulation and processing of CNTs has been limited by their solubility in most common solvents, although some dissolution has recently been reported (Pénicaud et al., 2007). Their chemical modification might pave the way to many useful applications, including the preparation of composite materials or the immobilization of biological molecules such as enzymes (i.e., for biosensors and electrochemical sensors). The nitric acid purification treatment is an oxidative method that can produce the functionalized carbon nanotubes (Dillon et al., 1999). This process introduces carboxylic acid groups on the surface and at the ends of the CNTs and provides sites for the covalent attachment of proteins/enzymes (Shim et al., 2002; Chen et al., 2001), or other kind of reagents.

Nafion, a perfluorinated ionomer because of its unique ion exchange, discriminative, and biocompatible properties, has been widely used to confine electrocatalysts and/or biomacromolecules (e.g. enzymes and proteins) on electrode surfaces to fabricate chemically modified electrodes (Fan and Harrison, 1992). CNT/Nafion nanocomposite electrodes were prepared and found to have excellent electrocatalytic activities toward carbohydrates, H_2O_2 and NADH (Lin et al., 2005). CNTs solubilized in Nafion were used to modify electrode surface for the development of an amperometric biosensor for glucose (Tsai et al., 2005). The catalytic activities toward H_2O_2 and NADH were further exploited for construction of oxidase- or dehydrogenase-based biosensors with a good reproducibility and high stability as well as minimized interference (Wang et al., 2003).

The key idea of this work is to develop a NR functionalized MWNTs–GOx–Nafion nanobiocomposite for bioanalytical application. MWNTs were functionalized with neutral red through carbodiimide reaction. By mixing NR functionalized MWNTs, GOx and Nafion, a novel nanobiocomposite film was produced by solvent casting process. Based on the flexibility and robustness of the composite film, a simple amperometric biosensor was developed for the determination of glucose.

2. Materials and methods

2.1. Chemicals and reagents

MWNTs with >95% purity was synthesized by chemical vapor deposition method (Shaijumon et al., 2005). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and *N*-hydroxy-succinimide (NHS) were obtained from Himedia, Neutral red was from SD-fine chemicals, glucose oxidase (GOx, EC 1.1.3.4, 24 U/mg) was from Fluka, α-D(+)-glucose, ascorbic acid, uric acid, paracetamol, glycine, urea and all solvents were from Merck. Nafion (5 wt% in lower aliphatic alcohols) was purchased from Aldrich. Phosphate buffer solution (PB, 0.1 M) served as the background electrolyte. The aqueous solutions were prepared with doubly distilled water. Glucose stock solutions were allowed to mutarotate at room temperature overnight before use.

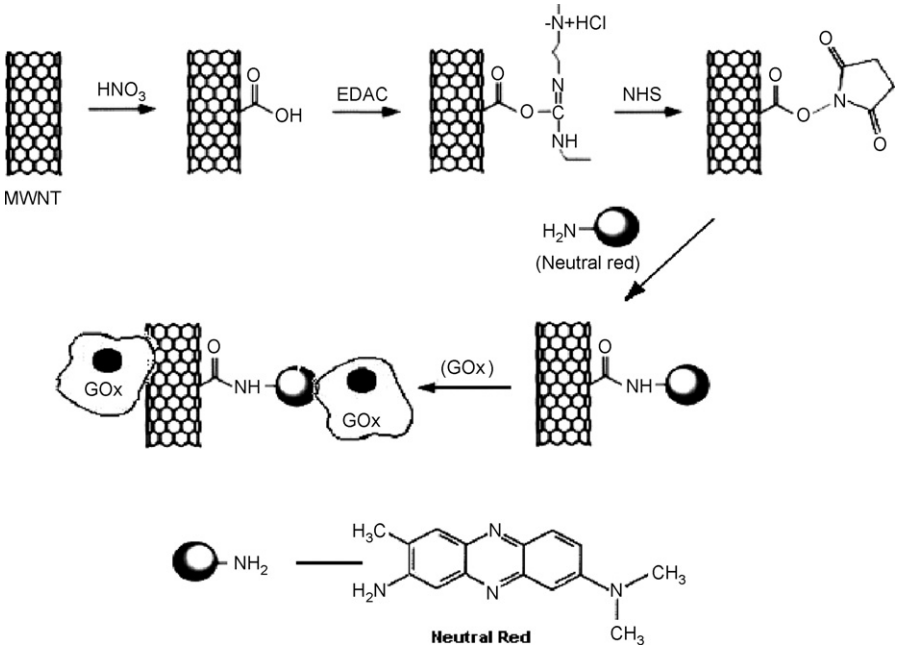
2.2. Apparatus and measurements

The morphology and microscopic structure of CNT and MWNT/NR/GOx/Nf nanobiocomposite were characterized by TEM (JEOL-JEM-200 FX II) and SEM (JEOL JSM 6360, Japan), FTIR spectra were recorded with a Shimadzu FT-IR 8300 spectrophotometer. The electrochemical measurements were performed at room temperature (27 °C except in experiments on temperature effects) in a conventional one compartment cell with a three-electrode configuration using a CHI 660B electrochemical workstation (CH Instruments, USA) linked to a personal computer for data acquisition and potential control. Paraffin impregnated graphite electrodes (PIGEs) were prepared as reported (Shobha Jeykumari and Narayanan, 2007a,b). The working electrodes were MWNT–NR–GOx–Nf nanobiocomposite film modified, MWNT–GOx–Nf film modified and unmodified PIGEs, the auxiliary electrode was a Pt wire and the saturated calomel electrode (SCE) served as the reference electrode. All potentials were measured and quoted versus SCE.

2.3. Functionalization of the MWNTs

The MWNTs were purified as reported previously (Shobha Jeykumari et al., 2007). Briefly, 50 mg of MWNTs was heated at 400 °C for 30 min and then dispersed in conc. HCl for 1 h under ultrasonic agitation, centrifuged, washed until the pH of the supernatant was neutral, and then dried in air at room temperature.

The MWNT sample was then treated with HNO_3 (12.8 M) and refluxed for 12 h. Then it was centrifuged and washed extensively with distilled water and dried. The activated MWNT sample (50 mg) was dispersed in a freshly prepared 10 ml aqueous solution of EDAC (10 mg/ml), followed by the addition of 100 mg of NHS to the solution with stirring. The pH of the solution was adjusted to 7 followed by the addition of 25 mg of NR. The reaction was allowed to proceed at room temperature for 2 h and then the MWNTs were washed thoroughly with water and filtered (Lin et al., 2004; Shobha Jeykumari and Narayanan, 2007a).



Scheme 1. Schematic representation of the covalent attachment of neutral red with MWNTs and physical adsorption of GOx over the functionalized MWNTs.

Enzyme immobilization with MWNTs was then achieved by mixing thoroughly with GOx (5 mg/ml in PB) solution. GOx molecules were physically adsorbed onto the surface of CNT during mixing. The reaction sequence for NR-functionalized MWNTs is shown in Scheme 1. The mixture was subsequently stored at room temperature for 30 min before fabricating the enzyme electrode. NR functionalized GOx adsorbed MWNTs (10 mg) were 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 enzyme electrode with mere MWNTs was prepared similarly, but in the absence of NR functionalized MWNTs.

2.4. Preparation of the modified electrodes

Prior to surface modification, the PIGEs (dia. 3 mm) were first polished with sand paper followed by 0.05 μm alumina slurry, respectively, then sonicated in a water bath. And then the oxidized MWNTs immobilized with GOx dispersed in Nafion modified electrode (MWNT–GOx–Nf) and the NR functionalized MWNTs immobilized with glucose oxidase modified electrode (MWNT–NR–GOx–Nf) were prepared by casting 25 μl volume of the MWNT–GOx solution or the nanobiocomposite colloid solution on the PIGE followed by air drying for about 2–3 h, rinsed by water several times for use. When not in use all the modified electrodes were stored at 4 °C.

3. Results and discussion

3.1. Structural and morphological characterizations of the electrode materials

TEM image of the purified MWNTs treated with conc. HCl, showed a very clean surface for all of the tubes. The MWNTs are very long and present as a highly entangled network structure. However after refluxing in 12.8 M HNO_3 the MWNTs were isolated and almost no impurities were observed. SEM image of the NR–MWNT–GOx–Nafion nanobiocomposite on the electrode surface were recorded. The random distribution of MWNTs on the electrode surface was observed which showed that the MWNTs were homogeneously dispersed into the Nafion polymer matrix.

The thermo gravimetric analysis showed that the thermal stability of MWNTs was remarkably decreased after their immobilization with neutral red, comparing pristine MWNTs and purified MWNTs, indicating the covalent immobilization of NR with MWNTs.

3.2. FTIR spectroscopy

Confirmation of NR functionalization of MWNTs and the attachment of glucose oxidase was accomplished by FTIR spectroscopy. The FTIR spectra of oxidized MWNTs showed peaks corresponding to the carboxylic group at 1588 cm^{-1}, which is in accordance with the previous reports (Shobha Jeykumari et al., 2007). This indicates that carboxylic and carboxylate groups are present on the surface of the CNT. The peaks at 3398 and 1166 cm^{-1} are due to stretching vibrations of –OH and C–OH, respectively. For the NR immobilized MWNTs the C=O stretching frequencies of the amide bond formed by the functionalization reaction appeared at 1685 and 1515 cm^{-1}. The bands at 2927 and 2864 cm^{-1} can be clearly assigned to the C–H stretching vibrations of the attached amine. After conjugation with the enzyme, new peak appeared at 3270 cm^{-1}, which is assigned, to N–H and O–H stretching vibrations from the enzymes tethered to MWNTs. Also peaks at 2961, 2925 and 2829 cm^{-1} in the spectrum correspond to different C–H bond stretching vibrations associated with the enzyme which

shows the successful attachment of GOx with functionalized MWNTs.

3.3. Electrochemical characterization of the nanobiocomposite glucose biosensor

3.3.1. Electrochemical impedance spectroscopy (EIS) analysis

Impedance spectroscopy is a highly effective means of probing the features of surface-modified electrodes (Patolsky et al., 1999; Ehret et al., 1997). To confirm the functionalization of MWNTs with NR and GOx, EIS was used to investigate the physical adsorption of the enzyme with the functionalized nanotubes. Fig. 1 shows the results of EIS, presented as Nyquist plots (Z'' versus Z'), for the bare graphite electrode and three modified electrodes at different modification steps, where, Z'' and Z' are the real variable and the negative value of the imaginary variable of impedance, respectively. The electrochemical impedance measurements in this report 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. Randles equivalent circuit was chosen to fit the obtained impedance data (Bard and Faulkner, 1980).

Significant differences in the impedance spectra were observed during stepwise modification. The respective semicircle diameter corresponds to the electron transfer resistance at the electrode surface (Pei et al., 2001). As shown in figure the bare PIGE exhibited an almost straight line (curve a), which is characteristic of a diffusional limiting step of the electrochemical process. When nitric acid treated MWNTs were incorporated on PIGE, the plot showed the greatest semicircle corresponding to the largest electron transfer resistance, due to the electrostatic repulsion between negatively charged surface and the probe molecule, $Fe(CN)_6^{3-/4-}$ (curve b). With further immobilization of MWNT with NR, the diameter of the Nyquist plot decreased due to the decrease in the surface negative charges (curve c), implying that after immobilization of NR with MWNT the interfacial resistance decreased due to decrease in the negative charges. With further adsorption of GOx, the surface turned negatively charged again, and the enzyme with negative charges also blocked the access of the probe molecules to the electrode surface, resulting in the increase of the electron transfer resistance (curve d). These data showed that the MWNT/NR/GOx/Nf were successfully attached to the electrode surface and formed a tunable kinetic barrier.

3.3.2. Cyclic voltammetry of the MWNT–NR–GOx–Nafion nanobiocomposite biosensor

The homogeneity of the MWNT–NR–GOx–Nafion nanobiocomposite is a key factor for biosensor fabrication. Cyclic voltammetric (CV) method was used to investigate the electrochemical properties characteristics of the enzyme-modified electrode. Fig. 2 illustrates the cyclic voltammograms of the bare PIGE, MWNT–GOx–Nf film modified electrode and MWNT–NR–GOx–Nf nanobiocomposite electrode in phosphate buffer solution (pH 7.4) at a scan rate of 20 mV/s. As can be seen from the figure, the bare graphite electrode (curve a) exhibits no voltammetric response. However, a significant enhancement in the background current with the ill-defined redox peaks was observed for the MWNT–GOx–Nf electrode (curve c). In the case of MWNT–NR–GOx–Nf nanobiocomposite electrode (curve e), redox waves appeared at −0.560 and −0.461 V, corresponding to a two-electron process of the NR_{ox}/NR_{red} pair (Shobha Jeykumari and Narayanan, 2007b). The formal potential estimated by $(E_{pc} + E_{pa})/2$ is −0.51 V. Although larger background current occurred in presence of MWNTs, the MWNT–NR–GOx–Nf composite film showed a better voltammogram, which was due to the closer proximity between MWNTs and conjugated mediator and the enzyme in

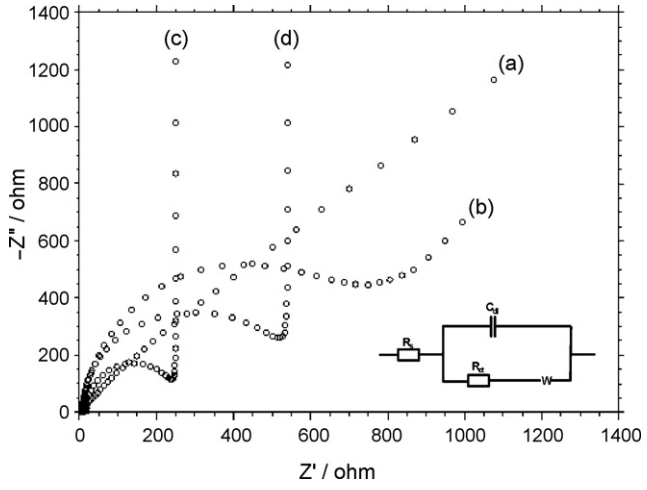


Fig. 1. The Nyquist plots for (a) bare PIGE, (b) MWNT–Nf film modified, (c) MWNT–NR–Nf film modified and (d) MWNT–NR–GOx–Nf composite film modified electrodes, respectively in the presence of 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ by applying an AC voltage with 5 mV amplitude in a frequency range from 0.1 Hz to 100 kHz. The electrode potential was −0.5 V vs. SCE. The inset is the Randles equivalent circuit for the modified electrodes.

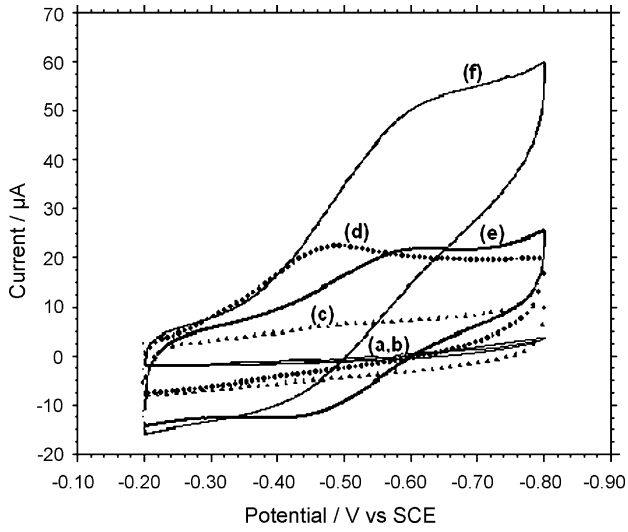


Fig. 2. Cyclic voltammograms of (a) bare PIGE, (c) MWNT–GOx–Nf and (e) MWNT–NR–GOx–Nf electrodes; (b), (d) and (f) are in presence of 1 mM glucose, respectively for the above electrodes. Background electrolyte: 0.1 M phosphate buffer solution; scan rate 20 mV/s.

the composite film. The doped MWNTs improved greatly the conductivity of the film and the electron shuttle between the mediator and electrode.

Experiments were carried out to study the dependence of the peak currents on the scan rate. A plot of peak current versus scan rate from 10 to 1000 mV/s showed a linear relationship as expected for a surface confined process. Moreover, ΔE_p, remains virtually constant with lower scan rates. The values of peak-to-peak potential separations were proportional to the logarithm of the scan rate for scan rates greater than 1000 mV/s. Based on the Laviron theory, the transfer co-efficient (α) and electron transfer rate constant (k_s) can be estimated by measuring the variation of peak potential with scan rate (Laviron, 1979a). The transfer coefficient and heterogeneous electron transfer rate constant were approximately 0.44 and 8.5 s^{-1}, respectively. The rate constant is much higher than that of direct adsorption of GOx on the CNTs (1.7 s^{-1}) (Guiseppi- Elie et al., 2002), GC electrode (1.6 s^{-1}) (Chi et al., 1994) and CNTs (1.53 ± 0.45 s^{-1}) (Cai and Chen, 2004) and also onefold higher than that of FAD adsorbed on the CNTs (3.1 s^{-1}) (Guiseppi- Elie et al., 2002). This suggests that the proposed nanobiocomposite matrix facilitates a facile electron transfer from GOx to the electrode through NR functionalized MWNTs.

For a surface process, surface coverage concentration (Γ) can be obtained according to Laviron's equation (Laviron, 1979b)

$$I_p = \frac{n^2F^2vA\Gamma}{4RT} = \frac{nFQv}{4RT}$$

where Q is the amount of charge; A is the electrode area, I_p is the peak current, F is the Faraday, R is the gas constant and T is the temperature. From the curves in Fig. 2, Q was calculated to be 4.338×10^{-5} C; A is 0.0708 cm^2 from our previous work (Shobha Jeykumari and Narayanan, 2007b). The average surface coverage of the mediator on the electrode surface, Γ was calculated to be 3.1746×10^{-10} mol/cm^2.

3.3.3. Effect of the pH on the biosensor response

A series of experiments were performed to study the dependence of current response on the pH of the solution for the fabricated glucose biosensor. The biosensor exhibited good response in 0.1 M phosphate buffer solution in the pH range from 5.0 to 8.0. The response decreased above and below the said pH range, as extreme pH conditions will denature the enzyme. However the sensor showed maximum response at pH 7.4, which was chosen for subsequent experiments. The immobilized GOx retained activity under the pH conditions studied indicating that the NR functionalized MWNTs and the Nafion provide a biocompatible microenvironment for GOx to withstand outside conditions.

3.4. Electrocatalytic activity of the nanobiocomposite biosensor

The principle of the glucose biosensor is based on the amperometric detection of H_2O_2, which is generated during the course of the GOx-catalyzed oxidation of glucose in the presence of dissolved oxygen. The following equations indicate the reaction mechanism for the determination of glucose by the proposed method

$$\beta\text{-D-glucose} + O_2 \xrightarrow{GOx} H_2O_2 + \text{gluconolactone}$$

$$M_{red} + H_2O_2 \rightarrow M_{ox} + H_2O$$

$$M_{ox} + e^- \xrightarrow{\text{electrochemical reaction}} M_{red}$$

The electrocatalytic activities of the modified electrodes were examined by cyclic voltammetry. Fig. 2 shows the CVs obtained at the modified electrodes in the absence and the presence of glucose in 0.1 M phosphate buffer solution (pH 7.4). As shown in Fig. 2, glucose does not undergo any electrochemical reaction with the unmodified PIGE (curves a and b). The MWNT–GOx–Nf modified electrode in the presence of 1 mM glucose, showed a reduction peak at about −0.5 V (curve d). This observation indicates the catalytic activity of MWNT for the reduction of H_2O_2. For the NR–MWNT–GOx–Nf modified electrode, upon the addition of 1 mM glucose, there is a dramatic enhancement of cathodic peak current and a decrease in anodic peak current (curve f), indicating the strong catalytic effect of the NR functionalized GOx immobilized MWNTs.

To determine the optimum potential for the sensor operation, hydrodynamic amperometry was carried out. Fig. 3 illustrates the comparison of hydrodynamic amperograms for 1 mM glucose at (a) unmodified PIGE, (b) MWNT–GOx–Nf modified and (c) MWNT–NR–GOx nanobiocomposite film modified electrode. As shown in figure (curve b), the MWNT–GOx–Nf modified PIGE displayed a current response, reaching maximum around −0.6 V, while for the MWNT–NR–GOx–Nf modified PIGE, an enhanced current response with a significant decrease in the over potential was observed for the catalytic reduction of H_2O_2 (curve c). The response was insignificant for the unmodified electrode for the entire potential range studied. An operating potential of −0.5 V was chosen to demonstrate the applicability of this sensor for the sensitive detection of glucose.

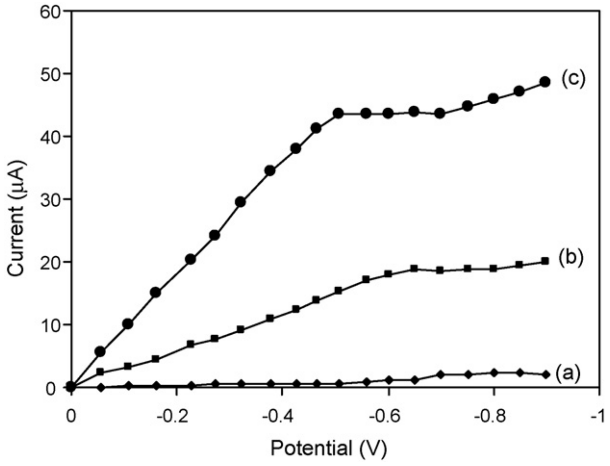


Fig. 3. Hydrodynamic voltammograms for 1 mM glucose in 0.1 M phosphate buffer solution (pH 7.4) at (a) unmodified electrode, (b) MWNT–GOx–Nf modified electrode and (c) MWNT–NR–GOx–Nf nanobiocomposite film modified electrode.

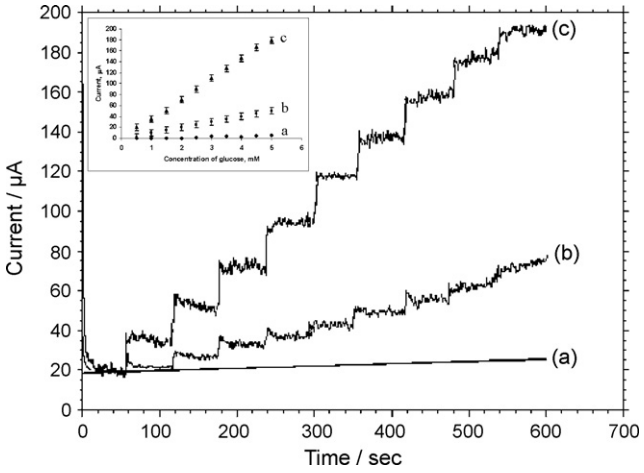


Fig. 4. The amperometric response at an applied potential of −0.5 V vs. SCE from (a) unmodified PIGE, (b) MWNT–GOx–Nf based biosensor (c) NR functionalized MWNT based nanobiocomposite glucose biosensor to successive addition of 0.5 mM glucose in 0.1 M phosphate buffer solution (pH 7.4) at room temperature of 27 °C, respectively.

Chronoamperometric measurements were also carried out for the determination of glucose. Fig. 4 compares the amperometric response of the unmodified (a), MWNT–GOx–Nf modified (b) and MWNT–NR–GOx–Nf modified (c) PIGE under stirred conditions (300 rpm), where the potential was kept at −0.5 V in phosphate buffer solution of pH 7.4, with the successive addition of 0.5 mM of glucose. As expected, the unmodified electrode gave insignificant signal to the addition of glucose and the MWNT–GOx–Nf modified PIGE produced a small increase in the current response. In contrast, the nanobiocomposite biosensor exhibits excellent bioelectrocatalytic activity for glucose determination with a remarkable increase in current, and the time required to reach the 95% steady-state response is only 4 s. A plot of current versus glucose concentration is shown in the inset. The biosensor shows a linear response over the wide concentration range from 1×10^{-8} to 1×10^{-3} M with a correlation coefficient of 0.9998, and a detection limit of 3×10^{-9} M. These analytical parameters are comparable to or better than, results reported for electroanalytical determination of glucose with different sensors and biosensors (Ghica and Brett, 2005; Bharathi and Nogami, 2001; Rubianes and Rivas, 2007).

3.4.1. *Effect of the temperature on the biosensor response*

It is well known that the activity of enzyme depends on the temperature. The response of the biosensor to 1 mM glucose in 0.1 M PB (pH 7.4) at different temperatures was studied. The current increases with the increase of temperature and a maximum response were observed at about 50 °C. This may result from the enzyme reaction rate increasing with the increase of temperature below 50 °C. The decrease in current above 50 °C may be due to the denaturing of enzyme. This optimal temperature of the biosensor is little higher than that of another GOx-based biosensor (Singhal et al., 2002), which indicates the merits of the proposed method for enzyme immobilization. Such enhancement in stability of immobilized enzyme is due to the covalent linkage between NR and the MWNTs in the Nafion film and GOx, which would lead to reduced conformational freedom with respect to change in environmental parameters such as pH and temperature. However, for practical convenience, all other experiments were carried out at room temperature.

3.5. *Interference studies*

The interferences from electroactive compounds commonly present in physiological samples of glucose were studied by monitoring biosensor's response to the interferences in the absence of glucose (background interference) and in the presence of glucose.

Fig. 5 presents the results of the interference test carried out by amperometric measurements with the fabricated biosensor. Fig. 5a corresponds to the experiment when interfering substrates (8.5 mM urea, 0.5 mM glycine, 0.125 mM ascorbic acid, 0.13 mM paracetamol and 0.33 mM uric acid) were added to the solution in the absence of glucose. Fig. 5b corresponds to the test of the interference effects in the presence of 1 mM glucose, where the same amount and sequence of interfering substrates were added to the solution. It can be seen from the figures that, in both cases, the interference is less than <4% (ascorbic acid: 3.8%, uric acid: 3.8%, paracetamol: 3.2%, glycine: 1.5% and urea: 1.6% of glucose response) and hence considered as insignificant.

It can be concluded from these tests that the biosensor can be used in the physiological conditions without the significant

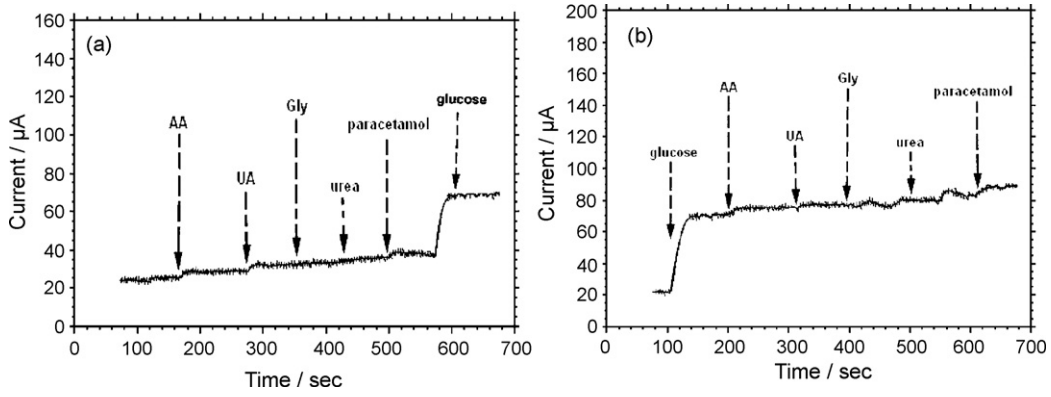


Fig. 5. Interference test protocols of the nanobiocomposite glucose biosensor to the interferences in the absence of glucose (curve a), and in the presence of glucose (curve b).

interference from the substances present in the body fluids. This indicates that the Nafion-modified CNT electrode has good anti-interferent ability to these interferents.

3.6. Repeatability, reproducibility and long-term stability of the biosensor

The repeatability of the biosensor was investigated from five successive measurements with 1 mM glucose. The relative standard deviation (R.S.D.) of the current responses is 0.5%. The fabrication reproducibility of five biosensors showed an acceptable reproducibility with a relative standard deviation of 2.8%. No significant change in current response was observed over the 3-month period studied, when the biosensors were stored at 4 °C. Such an excellent stability is crucial for any commercial biosensor device. The high stable nature of this system is considered to result from the intimate integration of the enzyme within the electrode material.

The response of the biosensor to glucose in human serum of hospitalized patients and recovery tests to added glucose were carried out. The values obtained are comparable to the values obtained by hospital and the recovery of added glucose was found to be 97–103%.

The high sensitivity and stability of the MWNT-based biosensor can be attributed to its special structure. The magnitude of glucose response depends not only on the amount of GOx loading, but also the GOx's activity on the electrode surface (Yang et al., 2003). The nanotubes are closed at the ends by hemispherical or polyhedral caps due to the presence of pentagonal defects. The caps are opened when the nanotubes are treated with a mixture of HNO_3 and functionalized with NR. The opened nanotube ends allow the enzyme of GOx to enter the hollow MWNTs when they are mixed with GOx solution. In addition, a large surface area per unit volume of the MWNTs enhances a large amount of GOx to immobilize within the nanotubes, resulting in a CNT-based enzyme reservoir.

4. Conclusion

In summary, we have developed and characterized the performance of a new glucose biosensor based on a MWNT–NR–GOx–Nafion nanobiocomposite. Neutral red has been shown to be an effective mediator when integrated in the biocomposite matrix. The resulting MWNT–NR–GOx–Nafion nanobiocomposite film alleviates the electrode surface-fouling problem. The MWNT–NR–GOx–Nafion nanobiocomposite film combines the advantages of the coupling efficient electrocatalytic activity of MWNTs with the excellent properties of composite materials. It is exploited to fabricate the glucose biosensors with excellent performances, such as high sensitivity, fast response and long-term stability, which originate from the structure of biocomposite making the substrate accessible to the active centre of GOx. The linear range for glucose determination is 1×10^{-8} to 1×10^{-3} M with a detection limit of 3×10^{-9} M glucose. The nanobiocomposite film eliminates potential interference through the preferential detection of glucose. The glucose biosensor based on the MWNT–NR–GOx–Nafion nanobiocomposite film is thus suitable for highly selective detection of glucose in a variety of biological fluids. With the rapid developments in nanoparticle applications, these functional nanocomposites would be useful in the areas of oxidase-based biosensors and bioelectronic devices.

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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.2007.12.007.

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