Fabrication of an amperometric bienzyme biosensing system with neutral red functionalized carbon nanotubes

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Novel nanomaterials for biosensor applications represent a rapidly progressing field of nanotechnology. An exploration of functionalized multi-walled carbon nanotubes (MWNTs) as a platform for amperometric determination of glucose employing the enzymes glucose oxidase (GOx) and horseradish peroxidase (HRP), which were immobilized with neutral red (NR) functionalized MWNTs is presented. The fabrication and analytical characterization of the glucose biosensor for the measurement of low concentration of glucose is described. Owing to the electrocatalytic effect of carbon nanotubes, the measurement of faradic responses resulting from enzymatic reactions has been realized at low potential with acceptable sensitivity. Experimental parameters affecting the sensitivity of biosensors, *e.g.*, applied potential, pH, temperature *etc.*, were optimized and potential interferences were examined. The response time for the glucose biosensor was very fast (within 2 seconds) and it showed good storage stability at 4 °C over a 5-month period. The proposed biosensor exhibited short response time, high sensitivity, easy operation, and simple sensor assembly. The biosensor was successfully applied to the determination of glucose in human blood samples and acceptable results were obtained.

1. Introduction

Nanomaterials have become the focus of scientific research in the past few years because of their unique electronic, optical, and catalytic properties.[1] Among the numerous active nanomaterials, carbon nanomaterials are at the forefront of research in a variety of chemical and physical disciplines. In particular carbon nanotubes and carbon nanofibers have specific chemical and physical characteristics that made them ideal for the development of biosensors with unique analytical characteristics.

It has been reported that carbon nanotubes can be used to promote electron transfer reactions when used as an electrode material.[2,3] Chemical functionalization of CNTs can be used to attach almost any desired compound to them, which allows—for instance—to enhance the solubility and biocompatibility of the nanotubes. This has permitted the realization of composite electrodes comprising CNTs well dispersed in an appropriate polymer matrix.[4] To strengthen the mechanical attachment of the nanotubes to the underlying electrode, nafion is commonly used. Nafion (Nf), a perfluorinated sulfonic acid ionomer with good ion exchange and biocompatible properties, has proven to be very effective as a protective coating for glucose sensors.[5]

Water soluble dyes such as methylene blue, methylene green, meldola blue, Azure-A and B, neutral red, and other azines have been used as electron transfer mediators for electrocatalytic reduction or oxidation of different substances.[6] Neutral red (NR) is an azine dye, which is similar to the other planar dyes in the chemical structure belonging to the acridine, thiazine and xanthene groups having nitrogen as the hetero atom instead of divalent oxygen or sulfur.[7] Due to significant mechanical strength, excellent electrical conductivity and good chemical stability, carbon nanotubes are promising platforms for immobilization of these electron transfer mediators[8,9] to fabricate chemical and biosensors.

Nowadays an ingenuous combination of two enzymes is widely used to develop biosensors with improved characteristics. As the detection principle switches from an electrochemical oxidation to a reduction process that is happening at much lower potentials, the selectivity in these devices is improved considerably. The construction of bienzymatic oxidase/peroxidase amperometric biosensors has been applied for glucose detection.[10] In this work, by combining the advantageous features of CNTs with glucose oxidase (GOx) and horseradish peroxidase (HRP), a novel glucose biosensor has been constructed by integrating MWNTs with neutral red. In the fabrication process, Nf is used to disperse NR/MWNTs and to immobilize GOx and HRP. By the combination of NR/MWNTs, GOx, HRP and Nf, a novel nanobiocomposite thin film has been produced by a simple solvent casting process. The MWNTs are electrochemically active and act as efficient conduits for electrons, Nf is an electron transfer promoting polymeric binder, and GOx and HRP are the biological catalysts that facilitate the conversion of substrate into product by lowering the activation energy of the reaction. The biosensor exhibits high sensitivity, rapid response, good reproducibility, long-term stability and freedom from other potentially interfering species.

2. Experimental

2.1 Reagents and materials

Multiwalled carbon nanotubes (MWNTs) with >95% purity were synthesized by the chemical vapor deposition method.[11]

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1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and *N* -hydroxy sulfosuccinimide (sulfo-NHS) were obtained from Himedia, neutral red (3-amino-7-dimetylamino-2-methylphenazine hydrochloride) was from SD-fine chemicals, glucose oxidase (GOx, 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. 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 (PBS, 0.1 M) served as the background electrolyte. The D-glucose stock solution (5×10^{-3} M in phosphate buffer, pH 7.0) were allowed to mutarotate at room temperature for 24 h before use. Other chemicals were of analytical reagent-grade and used as received. All the experiments were performed at room temperature. Doubly distilled water was used throughout.

2.2 Apparatus

Electrochemical measurements were carried out in a CHI 660B electrochemical workstation (CH Instruments, USA) linked to a personal computer for data acquisition and potential control. The electrochemical measurements were carried out in a conventional three-electrode cell with a saturated calomel electrode (SCE) as the reference electrode, a Pt wire as the counter electrode and glassy carbon electrodes (GCEs) with a diameter of 3 mm (modified and unmodified) were used as working electrodes, respectively. All electrodes were from CH Instruments and all the potentials are given against SCE.

The EIS measurements were performed in a solution containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 0.1 M phosphate buffer (pH 7.4) at a normal potential. An AC amplitude of 5 mV and a frequency range from 0.1 Hz to 100 KHz were applied.

The amperometric data were obtained by measuring the current for successive addition of aliquots of glucose solution in the cell at a constant potential (0.1 μM glucose solution to the phosphate buffer, pH 7.4) with a time interval of 10 s. The time to reach a steady-state value of the current did not exceed 2 seconds. All the results reported here were obtained as an average of at least three independent measurements. A magnetic stirrer (rotation speed 300 rpm) provided the convective transport during the amperometric measurement.

2.3 Preparation of the bienzyme nanobiocomposite

The MWNTs (50 mg) were functionalized to have carboxylic acid groups by sonication and refluxion in a 12.8 M nitric-acid (80 ml) for 8 h, in accordance to a previously described protocol.[12] Subsequently, the pretreated CNTs were washed with water, then with 0.1 M NaOH (to reach neutrality of pH 7.0), filtered, and dried for 1 h at 100 °C. The redox mediator, NR was attached to the MWNTs by immersing the activated MWNTs in a freshly prepared aqueous solution of EDC and sulfo-NHS and then stirred with NR. 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 enzyme mixture was prepared by dissolving 4 mg of GOx and 3 mg of HRP in 0.3 ml of PBS (pH 7.4). Enzyme immobilization was achieved by mixing thoroughly NR-functionalized CNTs (1 mg) with 20 μl of the enzyme mixture. The enzyme-CNTs mixture was then dispersed in 1 ml of 0.5 wt% Nf solution with the aid of ultrasonic agitation for 5 mins to form a homogeneous biocomposite colloidal solution.

2.4 Fabrication of MWNTs/NR/GOx/HRP/Nf nanobiocomposite film coated GCE

To prepare a MWNTs modified electrode, a GCE was polished with emery paper followed by alumina (0.1 and 0.5 μm) and then thoroughly washed with double-distilled water. Then the electrode was placed in 1:1 nitric acid solution, alcohol and redistilled water, sequentially, and subjected to sonication to remove adsorbed particles. Bienzyme nanobiocomposite electrodes were prepared by casting 10 μl of MWNT/NR/GOx/HRP/Nf or MWNT/GOx/HRP/Nf nanobiocomposite colloidal solutions on the surface of GC electrode followed by air drying for about 2–3 h, rinsed with water several times before use. For comparison, a bienzyme electrode with mere MWNTs (without NR) was also prepared. When not in use all the modified electrodes were stored at 4 °C.

3. Results and discussion

3.1 Electrochemical impedance spectroscopy (EIS)

Electrochemical impedance spectroscopy is an effective method for examining the interfacial properties of electrodes, especially the modified surfaces, and is frequently used for realizing electrochemical transformation and processes associated with conductive supports.[13,14] Fig. 1 shows the Faradaic impedance spectra, presented as Nyquist plots (*Z′ vs. Z″*) with the

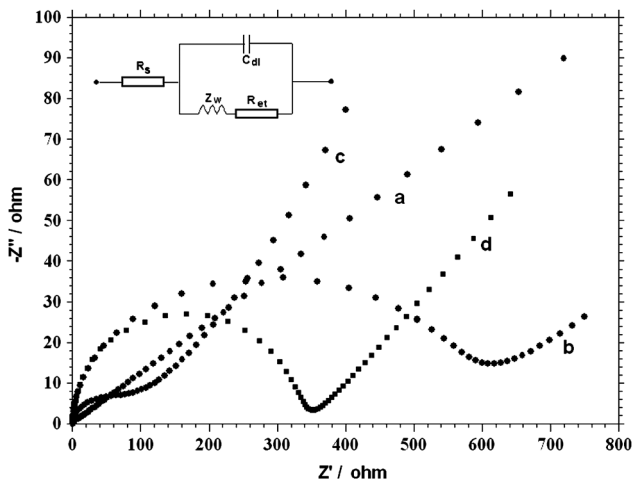


Fig. 1 Nyquist plots of impedance spectra obtained in 0.10 M PBS (pH 7.4) containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ at (a) bare GCE; (b) MWNT/Nf modified GCE; (c) MWNT/NR/Nf modified GCE and (d) MWNTs/NR/GOx/HRP/Nf modified GCE. The electrode potential was −0.5 V *vs.* SCE. The frequency was from 100 kHz to −0.1 Hz and the amplitude was 5.0 mV. The inset shows the equivalent circuit applied to fit the impedance spectroscopy.

functionalized and enzyme immobilized carbon nanotubes. The Randles circuit (inset of Fig. 1) is chosen to fit the obtained impedance data. The bare GC electrode exhibits an almost straight line (curve a) that is characteristics of the diffusion limited electron-transfer process. After the MWNTs were immobilized, the GCE showed the greatest semicircle with an interfacial resistance of $R_{et} = 602\ \Omega$, due to the electrostatic repulsion between negatively charged surface due to the –COOH groups which are present on the MWNTs and the probe molecule, $Fe(CN)_6^{3-/4-}$ (curve b). Upon covalent attachment of NR with MWNT (curve c), the electron-transfer resistance decreased dramatically to $R_{et} = 126\ \Omega$, due to the decrease in the negative charges, making it easier for the electron transfer to take place. Upon immobilization of the enzymes GOD and HRP over the NR functionalized MWNTs, the electron-transfer resistance further increased to $R_{et} = 343\ \Omega$ (curve d). This is due to the attachment 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 upon the immobilization of the two biocatalysts. These results demonstrate that NR can form a good electron pathway between the electrode, enzymes and electrolyte and the presence of NR made the electron transfer easier compared with that of MWNTs.

3.2 Electrochemical behavior of MWNT/NR/GOx/HRP/Nf nanobiocomposite biosensor

The cyclic voltammograms of the biosensor were obtained in the potential range of 0 to −0.7 V for the MWNT/NR/GOx/HRP/Nf modified electrode and from 0 to −0.8 V for the bare electrode in 0.1 M PBS (pH 7.4) solution and are shown in Fig. 2. No redox peak was observed for the bare GC electrode and MWNT/Nf modified GC electrode (not shown here) even at higher potential. Compared with bare GC electrode, the background current of MWNT/GOx/HRP/Nf electrode was apparently larger, which indicates the effective electrode surface is significantly enhanced by use of MWNT to modify the electrode and also due to the presence of HRP and GOx adsorbed over the surface of the nanotubes (Inset). In contrast, MWNT/NR/GOx/HRP/Nf modified biocomposite electrode displayed the CV exhibiting a well defined reversible redox waves at very low potentials with a formal potential at −0.41 V, also with higher reduction and oxidation peak currents when compared to other electrodes, which should be attributed to the NR functionalized MWNTs.

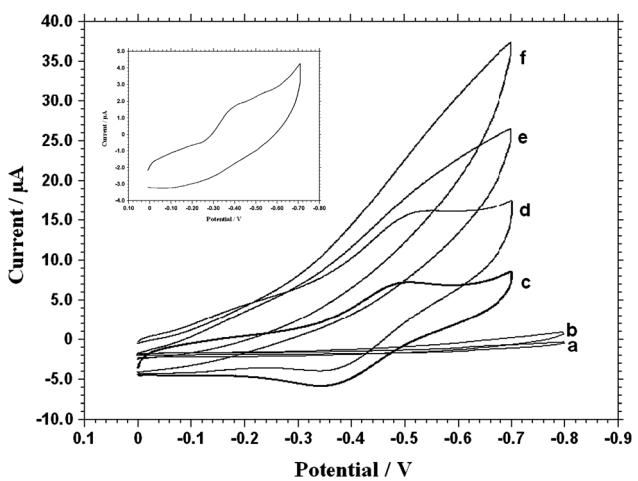
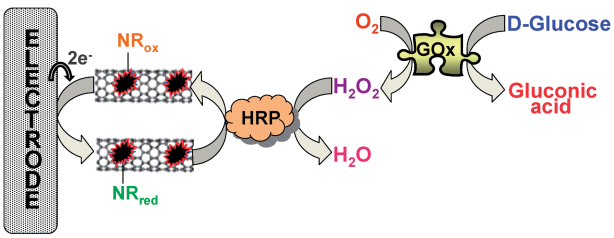


Fig. 2 Cyclic voltammograms of bare GCE (a) without glucose (b) with 3.0 μM glucose and (c,d,e,f) MWNTs/NR/GOx/HRP/Nf modified GCE in 0.1 M 7.4 PBS containing 0, 1.0, 2.0 and 3.0 μM glucose at 0.02 V s^{-1}. Inset: cyclic voltammogram of MWNT/GOx/HRP/Nf modified GCE in 0.1 M 7.4 PBS at 0.02 V s^{-1}.

The dependence of the peak current towards the scan rate has been also studied for the MWNT/NR/GOx/HRP/Nf modified bienzyme biosensor. Good linearity in the plot of peak current *vs.* scan rate in the range of 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,[15] the electron transfer rate constant (k_s) and charge transfer coefficient (α) were determined by measuring the variation of peak potential with scan rate. The calculated values for (k_s) and (α) were 7.91 s^{-1} and 0.57 respectively. The surface concentration of electroactive species Γ can be calculated from the slope of the plot of I_{pa} *vs.* scan rate and was found to be 9.75×10^{-10} mol cm^{-2}.

3.3 Bioelectrocatalytic oxidation of glucose at bienzyme-based biosensor

The biocatalytic scheme used for the determination of glucose involved the reduction of H_2O_2, generated in the enzyme reaction with GOx, and regeneration of HRP with neutral red which is a mediator, functionalized with MWNTs. Therefore, the amperometric signal employed for monitoring the process corresponded to the electrochemical reduction of neutral red. The reaction scheme is as given below



Reactions occurring at the MWNT/NR/GOx/HRP/Nf nanobiocomposite biosensor.

With glucose oxidase and HRP co-immobilized with NR functionalized carbon nanotubes, glucose is detected through the following scheme

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + 2H^+ + \text{Med}_{red} \xrightarrow{\text{HRP}} 2H_2O + \text{Med}_{ox}$$

$$\text{Med}_{ox} + e^- \rightarrow \text{Med}_{red}$$

Cyclic voltammograms of the biosensor were recorded in 0.1 M PBS (pH 7.4) in the absence and presence of glucose (Fig. 2). Curve (c) shows the cyclic voltammogrm of the biosensor in the absence of glucose. Upon addition of glucose, the shape of the cyclic voltammogram of the biosensor changed with an increase in reduction current displaying a pronounced electrocatalytic behavior of the immobilized HRP to the reduction of H_2O_2 (curve d). With increasing concentration of glucose the electrocatalytic response also increased (curve e and f). In contrast no significant response was observed for the bare electrode in the presence of glucose (curve b). This indicates that the proposed biosensor exhibits good response for the detection of glucose.

3.4 Effecting factors

The effect of applied potential on the amperometric response of the biosensor to glucose was examined. Upon varying the potential from 0 to −0.7 V, a higher steady-state current was observed from −0.3 to −0.7 V due to the increased driving force for the fast reduction of H_2O_2. Considering the interference of many coexisting foreign species at high negative potentials −0.35 V was selected as the working potential for the determination of glucose.

The effect of pH on the response of the biosensor was observed in the pH range 4.0 to 9.0. The proposed biosensor showed a maximum response at pH 7.5. The response decreased above and below the pH range studied. This may be due to the influence of pH on protein denaturation. A high pH value was disadvantageous with the electrochemistry of HRP also. Therefore PBS (pH 7.4) was used as the supporting electrolyte for glucose detection, considering the physiological pH condition.

With increasing temperature from 15 to 60 °C the activity of the immobilized enzymes increased, leading to a gradual increase in the amperometric response of the biosensor and reached its maximum at about 60 °C. When the temperature was higher than 60 °C the amperometric response decreased slightly. The amperometric detection of glucose was performed at 25 °C (room temperature), at which the response was 91% of that at 60 °C.

3.5 Performance of the biosensor

The performance of the biosensor was investigated by chronoamperometric method under the optimum conditions. Fig. 3A shows a typical current–time response for successive addition of 0.1 μM of glucose to the PBS under stirring condition. The time required to reach 95% of the maximum steady-state current was 2 s. The calibration curve in Fig. 3B shows a linear range for the concentration of glucose from 12 nM to 16 mM. The linear regression equation was I(A) = 0.52[glucose] + 4 (A with the correlation coefficient of 0.9999). The detection limit was 4 nM when the signal to noise ratio is 3. Deviation from linearity was observed at higher concentration indicating a typical Michaelis–Menten kinetics. The Michaelis–Menten constant (K_m) was found to be 0.89 mM using the Lineweaver–Burk equation (Fig. 3B) and is in agreement with the earlier report.[16] These results show that the biosensor possesses higher biological affinity to glucose.

The fabrication reproducibility of six biosensors, made independently, showed an acceptable reproducibility with a RSD of 5.1% for the determination of 0.1 μM glucose The reproducibility was ascertained by monitoring the current response for 10 replicate injections of 0.1 μM glucose with an applied potential of −0.35 V. The RSD was 4.5%, indicating a good reproducibility of the biosensor, suggesting both enzymes did not leach out of the composite in the detection process, and the biosensor could repeatedly be used for the determination of glucose.

3.6 Operational lifetime and biosensor selectivity

The stability of the biosensor was examined by checking periodically their relative responses (the ratios of the catalytic currents detected at different times to the initial current value) in 0.1 M PBS (pH 7.4) containing 0.1 μM glucose (Fig. 4). The sensors could retain 98% of their initial activity to glucose within a storage period of 60 days when stored in 0.1 M PBS (pH 7.4) or in air at 4 °C. After this time there was a decrease of 3% of the initial value. Good long-term stability was attributed to the following aspects. The construction process is mild, and no damage is inflicted on the enzyme molecules. Also the interaction

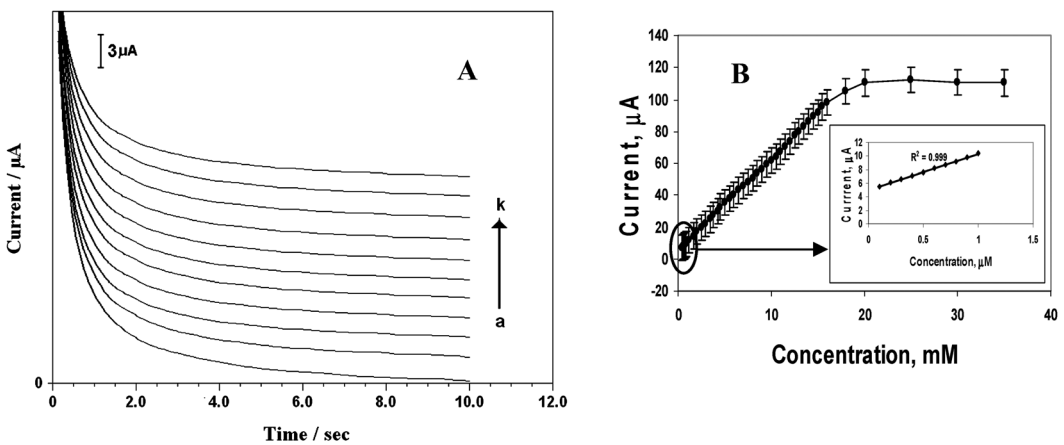


Fig. 3 **(A)** Amperometric response of the MWNT/NR/GOx/HRP/Nf modified electrode (a) in the absence and (b–k) successive addition of 0.1 μM glucose. **(B)**. Plot of catalytic current *vs.* glucose concentration for glucose oxidation. Inset shows the linear calibration plot for lower concentration of glucose, stirring rate: 300 rpm.

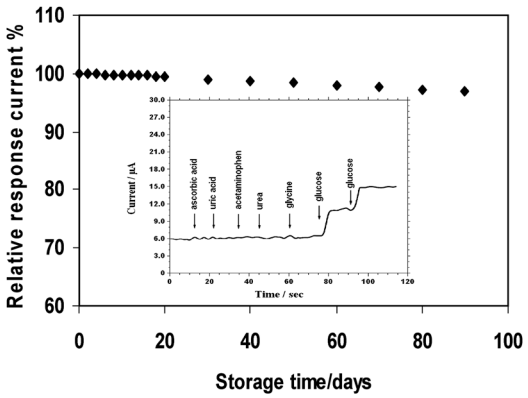


Fig. 4 Stability of the biosensor stored in PBS (pH 7.4) at 4 °C. The solution is 0.1 μM glucose in PBS. The inset shows the influence of electroactive interferences (0.5 mM AA, UA, urea, gly and AAP) in 0.1 μM glucose.

Table 1 Determination of glucose in human serum samples using bienzymatic nanobiocomposite biosensor

Sample no.	Provided by local hospital (mM)	Determined by biosensor[a] (mM)
1	6.98	6.97 ± 0.09
2	7.51	7.53 ± 0.04
3	6.47	6.43 ± 0.08
4	5.49	5.50 ± 0.09
5	7.32	7.31 ± 0.06

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

between NR immobilized MWNTs and GOx and HRP is strong and not affected by the changes of pH and temperature. Finally, the good biocompatibility of NR immobilized MWNTs maintains the biological activity of the enzymes immobilized on the electrode.

As selectivity is another important parameter to analyze when developing an electrochemical biosensor, the interferences from electroactive compounds, such as ascorbic acid (AA), uric acid (UA), urea, glycine (gly) and acetaminophen (AAP) which are commonly present in physiological samples of glucose were also studied. The inset in Fig. 4 shows that the response signals of AA, UA, urea, gly and AAP (0.5 mM) were negligible compared to glucose (1.0 μM). Good selectivity of the biosensor can be attributed to the lower working potential and the permselective nature of nafion. Thus the Nafion-modified biosensor would eliminate the interferences and enhance the selectivity of the biosensor.

3.7 Real sample analysis

To illustrate the feasibility of the modified electrode in practical analysis, the MWNT/NR/GOx/HRP/Nf biosensor was used to detect glucose in human blood samples. Fresh human blood samples were analyzed in a local hospital by a spectrophotometric method. Then the samples were assayed with the proposed biosensor. A rapid and stable amperometric response was acquired at −0.35 V with the addition of 0.2 ml of sample into 5 ml of PBS. The content of glucose in blood was calculated from the calibration curve and the results are listed in Table 1. The good agreement between the value provided by the hospital and the value found in this method indicated that the determination of glucose by the proposed biosensor was effective and sensitive for biological samples.

4. Conclusions

The present study has demonstrated the development of a new type of bienzymatic biosensor, which is based on the immobilization of GOx and HRP with NR functionalized carbon nanotubes. The results demonstrate that a MWNT/NR/GOx/HRP/Nf nanobiocomposite constitutes a robust amperometric biosensor suitable for the reliable analysis of glucose. The bienzyme biosensor also presented a fast response, preferable linear range accompanied with favorable sensitivity and detection limit. The fabricated bienzyme sensor provides a promising strategy to construct fast, sensitive, stable and anti-interferential amperometric biosensors.

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References

1 L. He and C. S. Toh, *Anal. Chim. Acta*, 2005, **556**(1), 1–15.
2 J. Wang, *Electroanalysis*, 2005, **17**(1), 7–14.
3 J. Wang, M. Musameh and Y. H. Lin, *J. Am. Chem. Soc.*, 2003, **125**(9), 2408–2409.
4 K. B. Wu and S. S. Hu, *Microchim. Acta*, 2004, **144**(1–3), 131–137.
5 R. F. B. Turner, D. J. Harrison and R. V. Rajotte, *Biomaterials*, 1991, **12**(4), 361–368.
6 A. Salimi, A. Noorbakhas and F. S. Karonian, *Int. J. Electrochem. Sci.*, 2006, **1**, 435–446.
7 A. A. Karyakin, E. E. Karyakina and H. L. Schmidt, *Electroanalysis*, 1999, **11**, 149–155.
8 M. Zhang and W. Gorski, *J. Am. Chem. Soc.*, 2005, **127**(7), 2058–2059.
9 M. Zhang and W. Gorski, *Anal. Chem.*, 2005, **77**(13), 3960–3965.
10 L. Zhu, R. Yang, J. Zhai and C. Tian, *Biosens. Bioelectron.*, 2007, **23**(4), 528–535.
11 M. M. Shaijumon, N. Bejoy and S. Ramaprabhu, *Appl. Surf. Sci.*, 2005, **242**(1–2), 192–198.
12 S. C. Tsang, Y. K. Chen, P. J. F. Harris and M. L. H. Green, *Nature*, 1994, **372**, 159–162.
13 K. Rosserg, G. Paasch, L. Dunsch and S. Ludwig, *J. Electroanal. Chem.*, 1998, **443**(1), 49–62.
14 S. Komaba and T. Osaka, *J. Electroanal. Chem.*, 1998, **453**(1–2), 19–23.
15 E. Laviron, *J. Electroanal. Chem.*, 1979, **101**(1), 19–28.
16 R. A. Kamin and G. S. Wilson, *Anal. Chem.*, 1980, **52**(8), 1198–1205.

