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Immobilization of horseradish peroxidase and nile blue into the ormosil nanocomposite for the fabrication of hydrogen peroxide biosensor based on MWCNT modified glassy carbon electrode

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ABSTRACT

A novel approach to construct a second-generation amperometric biosensor is described. The classical redox dye nile blue (NB) as mediator and horseradish peroxidase as a base enzyme were coimmobilized into the multiwalled carbon nanotubes (MWCNTs) modified ormosil matrix. Nafion was dispersed into the matrix to enhance the rate of the electron transfer and prevent the cracking of the ormosil film. The surface morphology of MWCNT/NB/NAF/HRP nanocomposite was characterized by scanning electron microscopy (SEM), atomic force microscopy (AFM) and electrochemical impedance spectroscopy (EIS). Cyclic voltammetry and amperometry measurements were used to study and optimize the performance of the resulting peroxide biosensor. The apparent Michaelis–Menten constant was determined to be 1.1 mM. The effect of pH, applied potential and amount of the HRP enzyme on the electrochemical biosensor has been systematically studied. The fabricated biosensor demonstrated significant electrocatalytic activity for the reduction of hydrogen peroxide with wide linear range from 2×10^{-7} to 3.8×10^{-4} M, and low detection limit 1×10^{-7} M (S/N = 3) with fast response time <3 s. The facile procedure of immobilizing HRP and MWCNTs into the ormosil used in the present work can promote the development of electrochemical research for enzymes, proteins, biosensors, biofuel cells and other bioelectrochemical devices.

1. Introduction

Carbon nanotubes have emerged as promising nanomaterials for the fabrication of electronic devices and sensor due to their extraordinary physical and electrical properties such as high tensile strength, high elastic modules, high thermal conductivity and electrical conductivity [1,2]. The carbon nanotubes usually have high surface area to weight ratio of 300 $m^2 g^{-1}$, and most of this surface area is accessible to both electrochemistry and immobilization of the biomolecules [3]. Unique electrical properties together with significant surface enlargement make them an important component in sensing applications. MWCNTs have been extensively used in the fabrication of electrochemical biosensors due to their excellent electrocatalytic activity and antifouling properties [4–7]. Their use in these devices is based on the fact that MWCNTs can play dual roles. They can be easily used as immobilization platform for biomolecules, while at the same time they can relay the electrochemical signal acting as transducers. As electrode materials, MWCNTs can be used for promoting electron transfer between the electroactive species and the electron and provide a novel platform for designing electrochemical biosensors.

The determination of hydrogen peroxide (H_2O_2) is extensively studied in clinical diagnostics, chemical and pharmaceutical industry and environmental control. Amperometric biosensors based on horseradish peroxidase (HRP) have emerged as the most convenient tools for H_2O_2 determination due to the simplicity, high sensitivity and selectivity [8,9]. Several numbers of MWCNTs modified amperometric peroxide biosensors have been fabricated based on MWCNT/TH/Nf modified PIGE [10], CNT/CHIT/HRP/SG modified GCE [11], MWCNT/HRP/Au modified electrode [12], poly(TB)/HRP/MWCNT/CHIT modified electrode [5], MWCNT/BSA/HRP/Fc modified electrode [13], MWCNT/HRP/MB modified electrode [14] and MWCNT/TTF-TCNQ/HRP modified gold electrode [15].

A series of water soluble organic dyes such as brilliant cresyl blue [16], methylene green [17], meldola blue [18], prussian blue [19] and celestine blue [20] have been used for the modification of electrode surface and they exhibit excellent mediating ability for the electrocatalytic reduction of H_2O_2. We used nile blue (NB) a phenoxazine dye in our investigation due to its higher sensitivity and mediating ability toward H_2O_2 determination. The leaching out of the biomolecules/dyes from the electrode surface is a crucial prob-

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lem in the fabrication of electrochemical biosensor. To overcome this issue, we used MWCNTs modified organically modified sol–gel glasses (ormosil) as a biocompatible matrix to the immobilization of HRP and NB. Ormosils are the unique matrices to encapsulate the biomolecules [21,22], due to their inert chemical nature, high mechanical strength, excellent optical properties, tunable porosity, strong adhesion property to its surface support and ease of modification. Ormosils are more biocompatible and it increases the long-term stability of the developed biosensor. The intersection of ormosil with multiwalled carbon nanotube provides better nanoporous matrix with high electrical conductivity for the immobilization of enzymes in its native state. The ormosil/multiwalled carbon nanotubes modified electrodes possess large potential window, low and almost constant background current over a large potential window and fast kinetics for a large number of electrochemical mediators.

In present work, for the first time we coimmobilized a phenoxazine dye nile blue with HRP into the multiwalled carbon nanotubes modified nanoporous ormosil matrix (3-aminopropyltrimethoxysilane and 2-(3,4-epoxycyclohexyl)-ethyltrimethoxysilane) for the development of an amperometric hydrogen peroxide biosensor. Because of the unique ion-exchange, discriminative and biocompatible properties, nafion was dispersed into the matrix to prevent the cracking and brittleness of the modified film [23]. In addition, the selectivity of the biosensor against several possible interfering species like ascorbic acid could be improved because the nafion polymer contains negatively charged sulfonate groups, thus preventing the negatively charged interfering species from partitioning into an electrode surface. The widely present amino groups in nanocomposite ormosil matrix provide a hydrophilic environment, which is compatible with the biomolecules [24]. The experimental results showed that the MWCNT/NB/NAF/HRP modified GCE exhibited excellent electrocatalytic activity toward the reduction of H_2O_2. The proposed hydrogen peroxide biosensor exhibited better wide linear range and low detection limit toward to H_2O_2, compare to the other biosensors earlier reported in the literature.

2. Experimental

2.1. Reagents and materials

Peroxidase from horseradish (POD, EC 1.11.1.7 type VI) was obtained from Sigma; nile blue and nafion (perfluorosulfonated ion-exchange resin, 5% (w/v) solution in a solution of 90% aliphatic alcohol and 10% water mixture) were purchased from Fluka. 3-Aminopropyltrimethoxysilane, 2-(3,4-epoxycyclohexyl) ethyltrimethoxysilane, *N*,*N*-dimethylformamide (DMF) and multiwalled carbon nanotubes (10–15 nm diameter) were purchased from Aldrich Chemical Co. Hydrogen peroxide (30%, w/v solution) was purchased from Wako Pure Industrial Co., Japan. The concentrations of more diluted hydrogen peroxide solutions were determined by titration with cerium (IV) to a ferroin end point. Phosphate buffer solutions (PBS) of various pH were prepared with 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4 with supporting electrolyte 0.1 M KCl. All other chemicals employed were of analytical grade. All the solutions were prepared with doubly distilled water.

2.2. Apparatus

The electrochemical measurements were performed with a computer controlled CHI750A (TX, USA) electrochemical system. Cyclic voltammetry and amperometric measurements were done with a three electrode system comprising the NB/NAF/HRP/GCE and MWCNT/NB/NAF/HRP/GCE as a working electrode, an Ag/AgCl reference electrode and platinum wire as counter electrode. All electrochemical measurements were carried out in 5 ml (0.1 M, pH 7.0) phosphate buffer solution. All experimental solutions were thoroughly deoxygenated by bubbling nitrogen through the solution for at least 15 min. Hitachi scientific instruments (Japan) Model S-3000H scanning electron microscope, CPSM model (USA) atomic force microscope and Hitachi scientific instruments (Japan) Model U-3300 UV–vis spectrophotometer were used for surface image and UV–vis spectra measurements respectively.

2.3. Fabrication of MWCNT based H_2O_2 biosensor

A glassy carbon electrode (GCE, 3 mm in diameter) was polished with 1.0, 0.3 and 0.05 μm Al_2O_3 slurry successively followed by rinsing thoroughly with double distilled water until a mirror-like surface was obtained. Then it was washed ultrasonically in 1:1 nitric acid, absolute ethanol and double distilled water, each for 5 min, and allowed to dry at room temperature. Two types of ormosil modified electrodes (OMEs) were fabricated based on the composition given in Table 1.

2.3.1. MWCNT/NB/NAF/HRP/GCE

The sol–gel process was used for the fabrication of hydrogen peroxide biosensor. The advantages of the strategy for fabricating hydrogen peroxide biosensor come from the following two aspects; the proposed strategy was simple and mild and the reactivity between amino groups and epoxy groups offered simple and convenient methodology for encapsulation of HRP into the inorganic–organic hybrid frame work [25]. 60 μl of Aminopropyltrimethoxysilane, 20 μl of nile blue, 5 μl of nafion and 175 μl DD water were mixed in a cell for 5 min. 40 μl of MWCTs (dissolved in DMF) and 50 μl of HRP were mixed in same solution. Lastly 10 μl 2-(3,4-epoxycyclohexyl) ethyltrimethoxysilane, and 10 μl HCl were added in the solution to complete hydrolysis. The whole mixture was stirred for 10 min to get homogenous solution of MWCNT modified ormosil matrix.10 μl of prepared homogeneous solution was dispersed on the surface of glassy carbon electrode and was dried at room temperature for 6 h.

2.3.2. NB/NAF/HRP/GCE

MWCNT was not incorporated in this modified electrode and the rest of the process was same as that for the modified electrode A.

3. Result and discussion

3.1. Physical characterization

3.1.1. Surface characterization of MWCNT modified ormosil nanocomposite

SEM and AFM are the excellent tools to study of surface characterization of modified nanocomposite. A scanning electron

Table 1
Composition of ormosil modified electrodes.

OME*	APTMOS[a] (μl)	ETMOS[a*] (μl)	DD** Water (μl)	NB[a**] (1 mM) (μl)	HRP[b] 1.2 mg/ml (μl)	NAF[b*] (μl)	MWCNT[b**] (2 mg/ml in DMF) (μl)	HCl (μl)
1	60	10	175	20	50	5	–	10
2	60	10	175	20	50	5	40	10

*Ormosil modified electrode, **double distilled water, [a]3-amino propyltrimethoxysilane, [a*]2-(3, 4-epoxycyclohexyl) ethyltrimethoxysilane; [a**]nile blue, [b]horseradish peroxidase; [b*]nafion, [b**]multiwalled carbon nanotube.

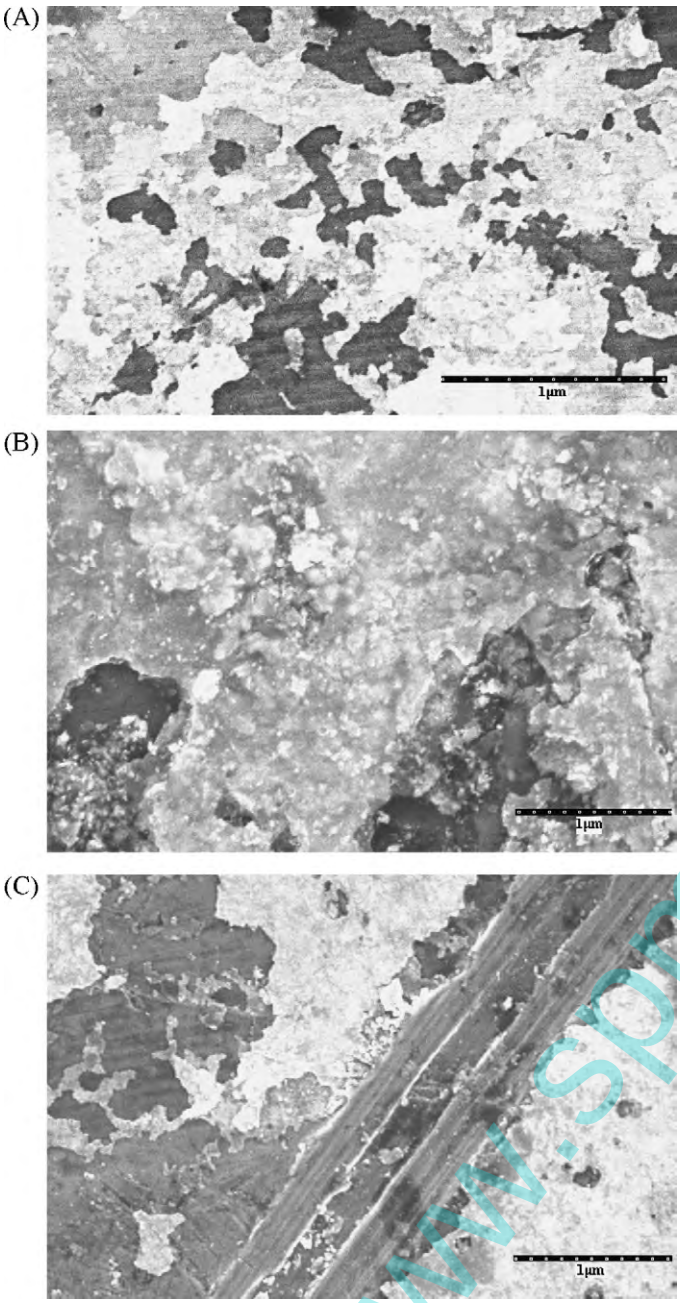

Fig. 1. Typical SEM images of NB/NAF/ormosil nanocomposite film (A) ormosils/NB/NAF nanocomposite film with HRP (B) ormosils/NB/NAF/HRP nanocomposite with MWCNT (C) on the surface of ITO glass.

micrograph (SEM) of the NB/NAF nanocomposite film, NB/NAF/HRP nanocomposite film and MWCNT/NB/NAF/HRP nanocomposite film are shown in Fig. 1(A, B and C). We can see the nanoporous structure of ormosil nanocomposite film with nafion, indicating that the ormosil matrix is highly porous in nature Fig. 1(A). Therefore, the faster diffusion of analytes into the matrix is possible through the interconnected porous channels in the nanocomposite films. The uniform porous structure of the NB/NAF ormosil nanocomposite films provided significantly enhanced effective electrode surface for high enzyme loading. Fig. 1(B) shows the SEM of HRP into the nanoporous ormosil matrix. The bright dense globular shaped HRP molecules uniformly distributed into the ormosil matrix, suggesting that HRP was successfully immobilized on the electrode surface. Fig. 1(C) showed the immobilized MWCNTs into the ormosil matrix with the dimension of 150–200 nm. Incorporation of MWCNTs into the ormosil matrix enhances the electrokinetic properties and porosity of the film, which will lead to fast diffusion of the analytes. So, the uniform and open nanoporous structure facilitates substrate access to the enzyme and thus results in a good biosensor response.

AFM technique was employed to confirm the self-assembled process of the peroxide biosensor fabrication. Typical AFM images (tapping mode) of the NB/NAF/HRP and MWCNT/NB/NAF/HRP in ormosil nanocomposite were shown in Fig. 2(A and B). The bright globular images of HRP were uniformly dispersed in the ormosil matrix with smooth surface. A large bright spherical shape also showed up due to aggregation of HRP molecules Fig. 2(A). Nafion was equally spread out into the film as shown in figure and was not prominent due to immobilization of HRP into the matrix. The thickness of the NB/NAF/HRP layer was ~43 nm with R_{rms} 0.843. After incorporation of MWCNTs in film the R_{rms} changed to 5.99 nm, indicated that MWCNT was successfully immobilized on the modified electrode surface. The roughness of the MWCNT/NB/NAF/HRP was increased due to coimmobilization of HRP and MWCNT in the matrix and the thickness of the MWCNT/NB/NAF/HRP modified film was ~69 nm (estimated by AFM image) as shown in Fig. 2(B). The height difference between the bright region and the dark ground was about 10 nm. Such morphological characteristics might result in high loading of enzyme and fast response to the substrate.

3.2. Electrochemical impedance spectroscopy (EIS) study of ormosil nanocomposite film

EIS is a convenient and effective tool to provide useful information on the impedance changes of the electrode surface during the fabrication process. The Nyquist plot of impedance spectra includes a semicircle portion and a linear portion. The semicircle portion at higher frequencies corresponds to the electron transfer limiting process and its diameter is equal to the electron transfer resistance and linear portion at lower frequencies corresponds to diffusion process [26]. Fig. 3 displays the Nyquist plot of NB/NAF/GCE (a), NB/NAF/HRP/GCE (b) and MWCNT/NB/NAF/HRP/GCE (c) in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) containing 0.1 M KCl. The R_{et} (2248 Ω) of NB/NAF/HRP/GCE was much larger than that of NB/NAF/GCE (R_{et} 544 Ω), suggesting that the HRP immobilized in ormosil film obstructs the electron transfer of the electrochemical probe Fig. 3(b). After the modification of MWCNTs (MWCNT/NB/NAF/HRP/GCE), the R_{et} decreased unceasingly to about 1092 Ω Fig. 3(c), indicating that the MWCNTs formed high electron conduction pathway between the electrode and electrolyte, and obviously improved the diffusion of $[Fe(CN)_6]^{3-/4-}$ toward the electrode surface. These results also demonstrated that HRP and MWCNTs had been successfully coimmobilized on the surface of modified electrode.

3.3. Electrochemical properties of MWCNT/NB/NAF/HRP and NB/NAF/HRP modified glassy carbon electrodes

Cyclic voltammetry was used to study the main electroanalytical properties of the fabricated peroxide biosensor. Fig. 4 shows the cyclic voltammograms of NB/NAF/HRP and MWCNT/NB/NAF/HRP modified GCE in 0.1 M PBS (pH 7) at different scan rate in potential range 0.0 to −0.6 V. Fig. 4(B) shows the symmetrical and good redox behavior of nile blue in ormosil matrix and contributing to the significance of MWCNTs. Multiwalled carbon nanotubes not only increased the current response, they also improved the reversible character of nile blue in ormosil nanocomposite film because it increases the effective surface area of modified electrode and provides the sufficient space for fast electrochemical phenomenon and maximum enzyme loading on the modified electrode surface. The oxidation (~100 mV) and reduction peak (~50 mV) shifts toward

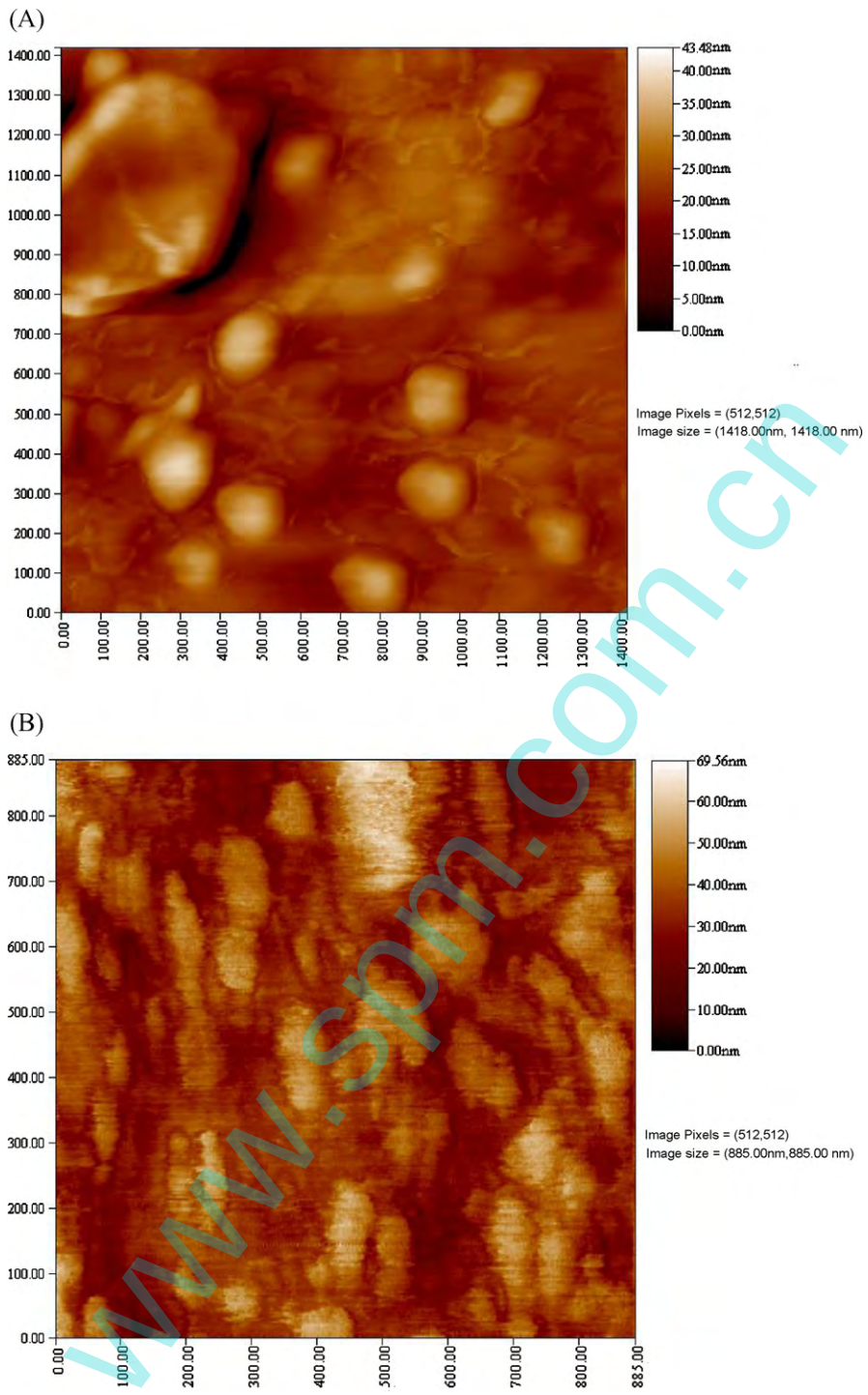

Fig. 2. AFM images of the ormosil/NB/NAF/HRP nanocomposite film (A) ormosil/NB/NAF/HRP nanocomposite film with MWCNTs (B) coated on ITO glass.

more positive potential for MWCNT modified electrode than without MWCNT modified electrode, which helps the electrocatalysis of hydrogen peroxide at low over potential with the removal of possible interferences during the analytical determination of H_2O_2. The cathodic peak potential and anodic peak potential obtained at MWCNT/NB/NAF/HRP/GCE were −400 mV and −240, respectively. The formal potential $[E^{\circ} = (E_{pa} + E_{pc})/2]$ was −320 mV. The peak separation (ΔE_p) was ~140 mV at the scan rate of 50 mV/s and the anodic peak currents ($I_{pa}/I_{pc} \sim 1$) were almost the same as the corresponding cathodic peak currents and the peak potential did not change with increasing the scan rate for MWCNT modified electrode, as shown in Fig. 4(B). As shown in Fig. 4(C), the peak currents increased linearly with the scan rate between 5 and 200 mV/s as expected for a surface confined process. Thus it is clear that NB immobilized with MWCNTs undergoes electron transfer reactions and multiwalled carbon nanotubes facilitated the reversible electrochemical behavior of NB. On the other hand, NB/NAF/HRP modified glassy carbon electrode showed the poor reversible electrochemistry for NB Fig. 4(A). An ill defined and insignificant redox peaks were observed for NB/NAF/HRP/GCE. The anodic and cathodic peaks were rather broad and the magnitude of the peak current was significantly less than that observed on

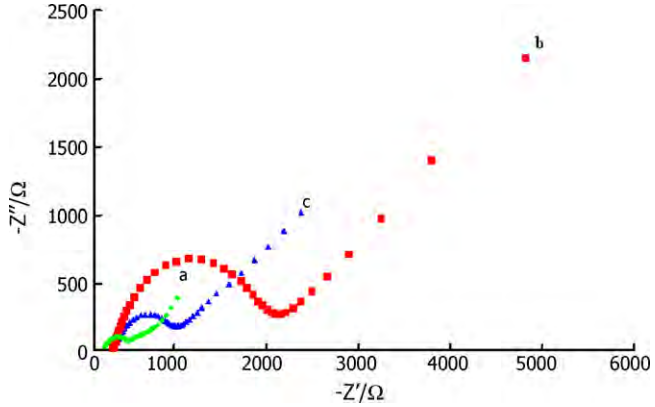

Fig. 3. Electrochemical impedance spectroscopy for NB/NAF/GCE (a) NB/NAF/HRP/GCE (b) and MWCNT/NB/NAF/HRP/GCE in a solution of 5.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ with Ag/AgCl as reference electrode.

the MWCNT/NB/NAF/HRP composite electrode. Furthermore, the ΔE_p was relatively large (~170 mV, scan rate-50 mV/s), suggesting a sluggish electron transfer kinetics. From this result, we confirmed that MWCNT/NB/NAF/HRP nanocomposites have excellent reversible electrochemical properties.

Surface coverage (Γ) for the electroactive species was estimated by using Eq. (1)

$$\Gamma = \frac{Q}{nFA} \quad (1)$$

where A (0.70 cm^2) is the area of the working GCE, n ($n = 2$) the number of electron per reactant molecule, Q (2.27×10^{-6} C) the charge obtained by integrating the anodic peak at low voltage scan rate (5 mV/s), and F is the Faraday Constant. In the present case, the calculated surface coverage for the electroactive species was 1.68×10^{-11} mol cm^{-2}.

3.4. Electrocatalysis of H_2O_2 on MWCNT/NB/NAF/HRP/GCE and NB/NAF/HRP/GCE

Fig. 5 shows the cyclic voltammograms of plane NB modified electrode and NB/NAF/HRP/GCE and MWCNT/NB/NAF/HRP/GCE in the presence of 0.2 mM hydrogen peroxide in PBS (pH 7) at the scan rate of 50 mV/s. A small cathodic current response was observed for H_2O_2 reduction at NAB/NAF/HRP/GCE in the presence of 0.2 mM hydrogen peroxide at potential range 0.0 to −0.6 V Fig. 5 (curve b). In contrast in the same operating conditions at the MWCNT/NB/NAF/HRP/GCE, a cathodic peak with higher current appears at more positive potential for the reduction of H_2O_2 and this dramatic increase of current indicates the significant catalytic activity of NB immobilized MWCNT to H_2O_2 reduction Fig. 5 (curve c). The reduction catalytic current of hydrogen peroxide started at −0.10 V and obviously catalytic reduction peak appeared at the potential of −0.4 V. This demonstrates that the MWCNT could act as a promoter to enhance the electrochemical reaction and to increase the rate of electron transfer [27]. MWCNT not only increased the electrocatalytic current, also lowered the overpotential (~100 mV than NB/NAF/HRP/GCE) for the electrocatalytic reduction of hydrogen peroxide to reduce the interferences in the measurements. There was no significant effect of H_2O_2 on the plane NB modified GCE electrode Fig. 5 (curve a). Fig. 6 presents the CVs of addition of various concentrations of hydrogen peroxide at the scan rate of 20 mV/s in (0.1 M, pH 7.0) PBS for MWCNTs modified electrode. This shows a typical electrocatalytic reduction process. The cathodic peak current was dramatically increased and anodic peak current disappeared, indicating the

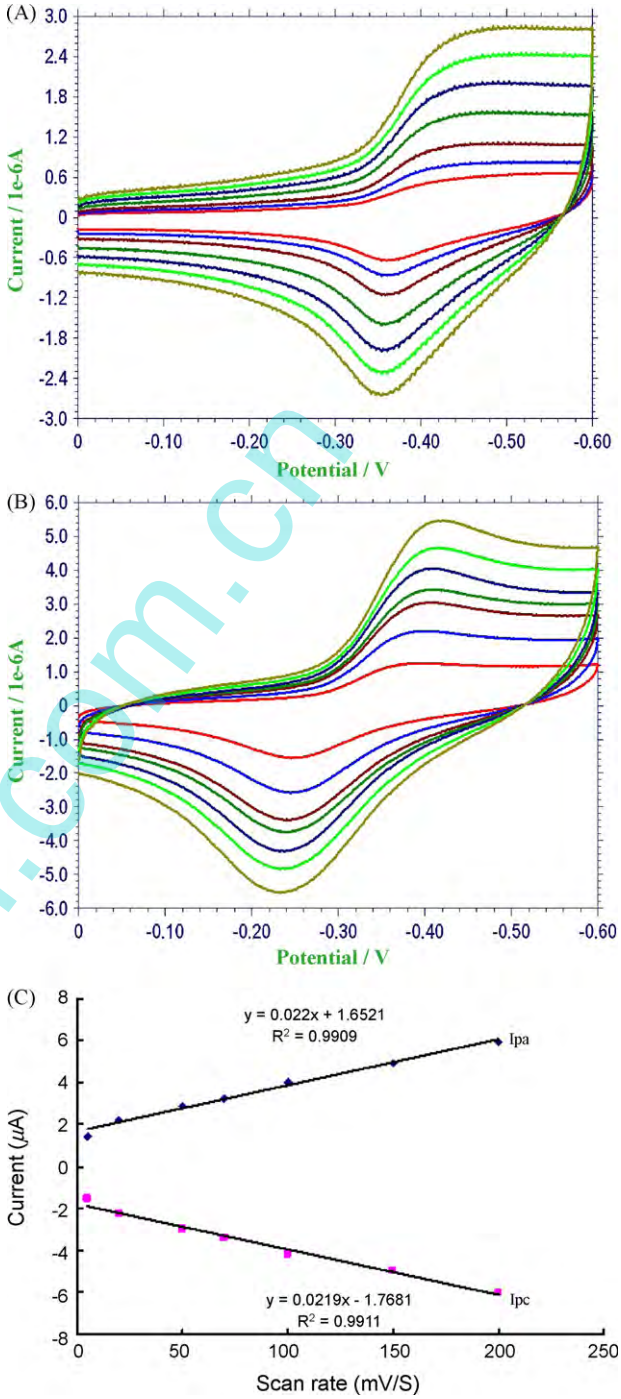

Fig. 4. Cyclic voltammograms of Nile blue immobilized in ormosil in the absence (A) and presence (B) of MWCNTs in 0.1 M PBS (pH 7.0) at the scan rate of 5, 20, 50, 70, 100, 150 and 200 mV/s. (C) Plots of peak current vs. the scan rate of MWCNT/NB/NAF/HRP/GCE.

fast electrocatalytic reduction of peroxide on the MWCNTs modified GCE electrode. The overall summary of the reduction of H_2O_2 catalyzed by HRP and mediated by NB can be described as follows:

$$H_2O_2 + 2H^+ + HRP_{red} \rightarrow HRP_{ox} + 2H_2O$$

$$HRP_{ox} + NB_{red} \rightarrow HRP_{red} + NB_{ox}$$

$$NB_{ox} + H^+ + 2e \rightarrow NB_{red}$$

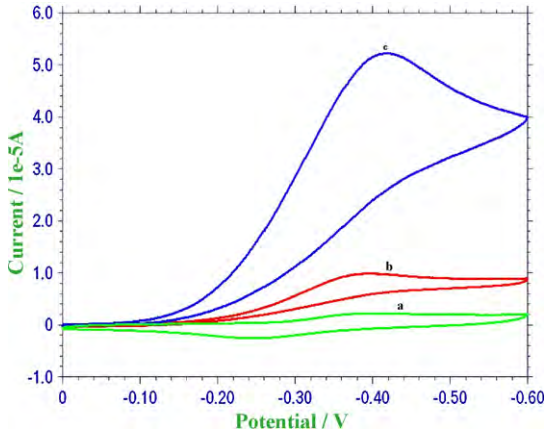

Fig. 5. Cyclic voltammograms of (A) plane NB incorporated in ormosil (B) and (C) are the CVs of NB/NAF/HRP/GCE and MWCNT/NB/NAF/HRP/GCE respectively in the presence of 0.2 mM H_2O_2 in 0.1 M PBS (pH 7.0) at the scan rate of 50 mV/s.

where HRP_{ox} and HRP_{red} represent the oxidized and reduced form of HRP, and NB_{ox} and NB_{red} represent the oxidized and reduced form of NB, respectively.

3.5. Optimization of experimental conditions

The experimental variables such as enzyme concentration, operating potential and pH were explored for optimum analytical performance of the hydrogen peroxide biosensor. The effects of these experimental variables were investigated using steady-state amperometry. The effect of the operational potential on the biosensor response was studied over the potential range of −0.24 to −0.45 V with 0.2 mM H_2O_2 in 0.1 M PBS at pH 7 Fig. 7(A). The steady-state reduction current increases gradually as the applied potential decreases from −0.24 to −0.38 V. The maximum reduction current was achieved at around −0.38 V. At more negative potentials, there may be interfering reactions from other electroactive species in the solution. Therefore, we chose the applied potential of −0.38 V as the working potential for this proposed biosensor. This potential is superior to the previous reported work [7].

The performance of a biosensor also strongly depends on the pH value of electrolytes. Effect of the solution pH on the electrolytic reduction of H_2O_2 was investigated in the pH range of 5.0–9.0. Fig. 7(B) presents the amperometric response of the MWCNT/NB/NAF/HRP/GCE at different pH in the presence of 0.2 mM H_2O_2. The steady-state reduction current is increases with the increasing of the pH from 4.0 to 7.0; however, the amperometric response decreases, when the pH further increases from 7.0 to 9.0. As the maximum response current was observed at pH 7.0. So, this pH was selected as optimum pH for the fabrication of hydrogen peroxide biosensor.

The amount of peroxidase enzyme in the biosensor fabrication process strongly affects the biosensor performance in terms of sensitivity and dynamic range. Therefore, the effect of the amount of enzyme doped into the MWCNT modified ormosil composite film on the biosensor response was examined for 0.2 mM H_2O_2 in 0.1 M PBS at pH 7. As seen in Fig. 7(C), the biosensor

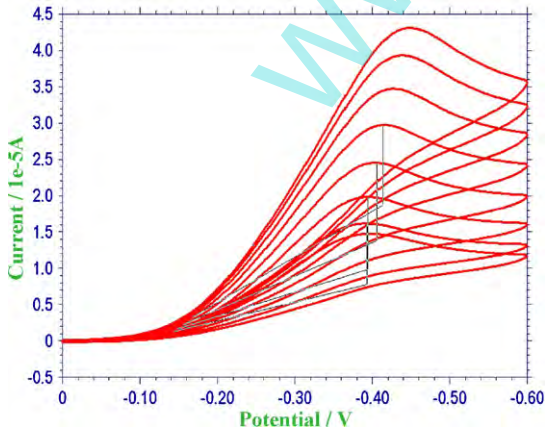

Fig. 6. Cyclic voltammograms of MWCNT/NB/NAF/HRP/GCE in the presence of various concentrations of H_2O_2: 0.05, 0.1, 0.3, 0.5, 0.7, 1.0, 1.2, 1.5 mM in 0.1 M PBS (pH 7.0) at the scan rate of 20 mV/s.

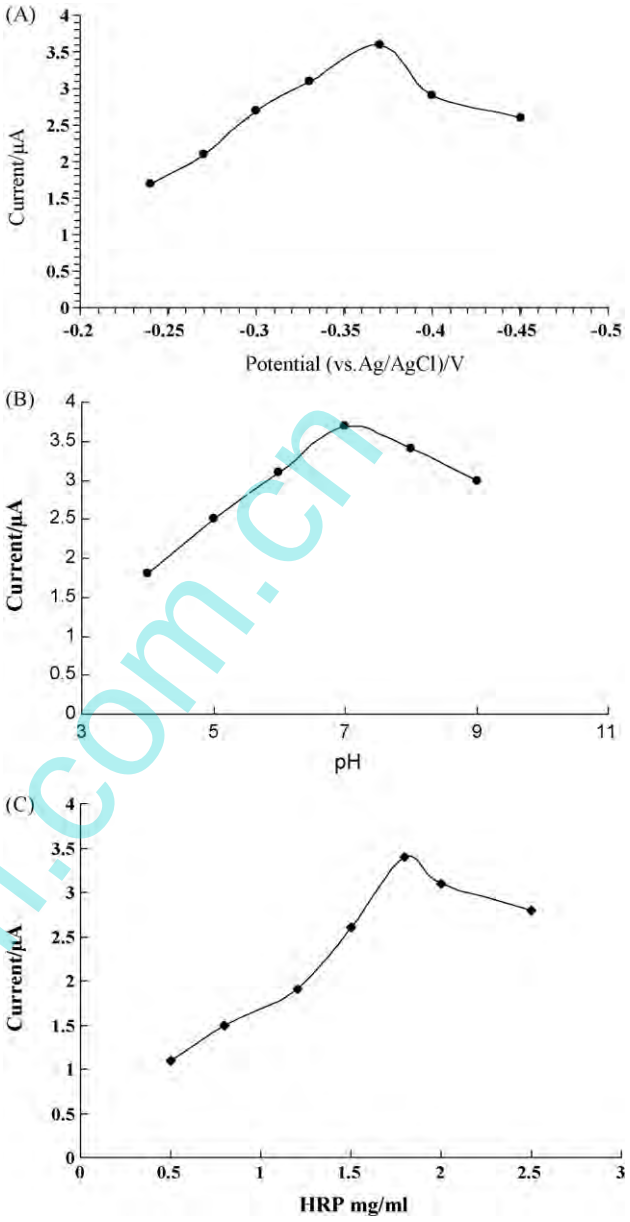

Fig. 7. (A) Effect of potential on the sensor response for 0.2 mM H_2O_2 in 0.1 M PBS (pH 7.0). (b) Influence of pH on the H_2O_2 sensor, study-state current measured in the presence of 0.2 mM H_2O_2 in 0.1 M PBS (pH 7.0) at applied potential of −0.38 at 25 °C. (c) Influence of the amount of HRP on the fabrication of biosensor.

response increases as the amount of enzyme loading increases up to 1.8 mg/ml. However, a slight decrease in the biosensor response was observed with additional enzyme loading above 1.8 mg/ml. The further increase in the amount of enzyme loading might be leading to the increase of the diffusional resistance for the enzymatically reduced substrate to arrive to the electrode surface and contributes the decrease in the biosensor response [28]. So, an optimal loading of 1.8 mg/ml was used for subsequent experiments.

The effect of temperature on the sensor was examined between 15 and 60 °C. The response signal of the H_2O_2 sensor increased as the temperature varied from 20 to 40 °C. But at temperature lower than 20 °C, the activity of the enzyme was rather lower and the response time was relatively longer. On the other hand, at temperatures higher than 40 °C, the activity of enzyme decreased rapidly due to the partial denaturation of the enzyme. Taking both the lifetime and response time into consideration, 25 °C was the selected temperature for the fabrication of biosensor.

3.6. Kinetic analysis

The apparent Michaelis–Menten constant (K_M), a reflection of the enzyme affinity, was evaluated from the electrochemical version of the Lineweaver–Burk equation,

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M}{I_{max}} \times \frac{1}{C}$$

where I_{ss} is the steady-state current after the addition of substrate, C is the bulk concentration of the substrate and I_{max} is the maximum current measured under saturated substrate condition. The K_M value was determined by analysis of the slope and intercept for the plot of the reciprocals of the cathodic current versus H_2O_2 concentration. The K_M value of the H_2O_2 sensor was determined by steady-state amperometric response and found to be 1.1×10^{-3} M. This value was lower than those of 2.1 mM for sol–gel/nafion/MG electrode [17], 4.6×10^{-3} M for HRP immobilized in sol–gel/hydrogel modified electrode [29], 4.51×10^{-3} M for HRP on AuNP/CHIT/SPCE [30], 4.04×10^{-3} M for HRP on TTF/TCNQ/MWCNT modified electrode [15], 2.5×10^{-3} M for HRP immobilized on a ferrocene containing polymer electrochemically deposited onto a Pt electrode [31], 5.12×10^{-3} M for SBP (soybean peroxidase) immobilized in sol–gel thin film [32], 2.0 mM for HRP immobilized on FMC-BSA/MWCNT ormosil composite-modified GC electrode [13] and was close to 0.9×10^{-3} for HRP/SiO_2/MB/Gelation/GCE [33] and 1.3×10^{-3} for PDDA/HRP/DNA-Ag/Au modified electrode [34]. The low value of K_M means that HRP immobilized into the MWCNT/NB/NAF nanocomposite retains well its bioactivity and has a high affinity to H_2O_2.

3.7. Amperometric response of the hydrogen peroxide biosensor and calibration plot

Fig. 8(A) records the Amperometric current–time curve of different ormosil modified electrodes toward H_2O_2 upon successive addition of H_2O_2 to a continuous stirred 0.1 M PBS (pH 7) under optimized conditions. The amperometric response curve of the MWCNT/NB/NAF/HRP/GCE was displayed in Fig. 8(A) (curve a). With the addition of hydrogen peroxide, drastic increase in the response current was observed (curve a). Moreover, this biosensor exhibited a rapid response for the addition of H_2O_2, and achieved 95% of the steady-state current within 3 s. Fig. 8(B) (curve a) pictured the calibration plot of the MWCNT/NB/NAF/HRP/GCE for H_2O_2 determination. A good linear relationship was found between the chronoamperometric current and H_2O_2 concentration from 2×10^{-7} to 3.8×10^{-4} M with a correlation coefficient of 0.9917 ($n = 10$). From the slope of the calibration curve, the detection limit of 1.0×10^{-7} was estimated at signal-to-noise ratio of three. The higher sensitivity of the sensor may result from the biocompatible microenvironment around the enzyme [35]. As controlled experiments, the amperometric response was also measured at NB/NAF/HRP/GCE Fig. 8(A) (curve b) to discern the role of MWCNTs in biosensor fabrication. For NB/NAF/HRP modified electrode Fig. 8(B) (curve b), the linear range for the determination of H_2O_2 was 3.5×10^{-6} to 4×10^{-4} M with a detection limit of 3×10^{-6} M. On the basis of comparative study of above two modified electrodes, it can be seen that the proposed biosensor, i.e. the MWCNT/NB/NAF/HRP/GCE exhibited a broader liner range and a lower detection limit than the NB/NAF/HRP/GCE. This observation demonstrated clearly that the presence of MWCNTs into ormosil matrix facilitated the electron transportation in the film and remarkably improved the catalytic activity of the biosensor for the determination of hydrogen peroxide at lower over potential. The linear range, response time and limit of detection observed with MWCNT/NB/NAF/HRP modified electrode is in general comparable with most of the modified electrode reported in the literature (Table 2).

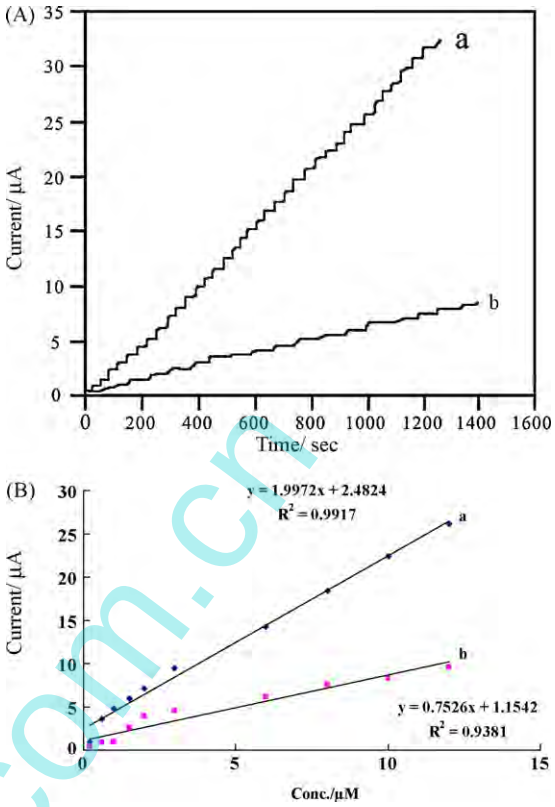

Fig. 8. Amperometric response of (curve a) MWCNT/NB/NAF/HRP/GCE and (curve b) NB/NAF/HRP/GCE in a stirred 0.1 M PBS (pH 7.0) after successive hydrogen peroxide additions at applied potential −0.38 V vs. Ag/AgCl Fig. 8(A). The linear calibration plot of catalytic currents vs. hydrogen peroxide concentrations for MWCNT/NB/NAF/HRP/GCE (curve a) and NB/NAF/HRP/GCE (curve b), Fig. 8(B).

3.8. Reproducibility, stability and interference determination

The biosensor showed a good reproducibility for the determination of H_2O_2 in its linear range. The concentration of 0.2 mM H_2O_2 was measured consecutively for 10 times and relative standard deviation (R.S.D.) of 3.1% was obtained. The storage stability of the proposed sensor was also studied. When not in use, the biosensor was stored dry at 4 °C and measured at intervals of a week, and it lost

Table 2
Comparison of the efficiency of MWCNT/NB/NAF/HRP modified GCE used in determination of H_2O_2.

Electrode	Method	Electrolyte	LOD	LCR (M)	RT (s)	Ref.
MWCNT/MB/HRP/GCE	CV	pH 7 PBS	1 μM	4×10^{-6} to 2×10^{-3}	<30	[14]
MWCNT/PS/HRP/SPE	CV	pH 7 PBS	25 μM	2×10^{-5} to 5×10^{-4}	–	[36]
MWCNT/FMC-BSA/HRP/GCE	CV, FIA	pH 6.8 PBS	5 μM	2×10^{-5} to 4.5×10^{-3}	20	[13]
MWCNT/TB/HRP	CV	pH 7 PBS	1.7 μM	Up to 4×10^{-4}	8	[5]
SG/MG/Nafion/HRP/GCE	CV	pH 7 PBS	0.1 μM	5×10^{-7} to 1.6×10^{-3}	20	[17]
MWCNT/NB/HRP/CHIT	CV	pH 7 PBS	0.12 μM	1×10^{-6} to 2.4×10^{-4}	2	[7]
MWCNT/NB/NAF/HRP/GCE	CV	pH 7 PBS	0.1 μM	2×10^{-7} to 3.8×10^{-4}	<3	Present work

LCR, linear concentration range; LOD, limit of detection; RT, response time.

Table 3
Interferences studies.

Interferents	Current ratio
Ascorbic acid	1.00
Oxalic acid	1.00
NADH	1.00
Citric acid	1.02
Uric acid	1.00
Glucose	0.97
Cysteine	1.00
Dopamine	0.98

only 4.9% of the initial response after 4 weeks and maintained more than about 91.6% of the initial values after storage for 50 days. Such a good stability of the biosensor was attributed to the excellent film forming ability of nafion and MWCNT with ormosil and this MWCTs modified ormosil matrix provided a favorable microenvironment for retaining the biological activity of enzymes.

The selectivity and anti-interference ability of the fabricated biosensor was also studied. In order to investigate the effect of some possible interfering substances on the biosensor, 8 interfering substances were used for measurement in our experiments. The results are listed in Table 3. The current ratio were calculated by reading the current of the biosensor in the assay solution containing 0.2 mM interfering species and 0.2 mM H_2O_2 and comparing with it with the current from the biosensor in the same assay solution containing only 0.2 mM H_2O_2. According to the results of current ratio, we can find that the influence of interfering species tasted on the H_2O_2 response was negligible, indicating a high selectivity of the proposed peroxide biosensor.

4. Conclusions

We have developed a new approach to fabricated hydrogen peroxide biosensor based on MWCNTs/ormosil modified electrode. Nile blue (redox mediator) and HRP were coimmobilized into the ormosil matrix with nafion. Nafion not only promoted the electron transfer rate, it also reduced the brittleness of the film. Result showed that the presence of the MWCNTs could effectively increase the amount of immobilized enzyme and greatly enhance the electrical conductivity of the resulting films. The fabricated biosensor based on MWCNT/N/NAF/HRP ormosil nanocomposite-modified electrode showed excellent linear range with significant low limit of detection (1×10^{-7} M), good operational stability and anti-interference ability toward H_2O_2 determination. The response time of developed biosensor was very prompt (<3 s). This method is simple and easy to control. It is hopeful that this method could be used to immobilize other enzymes to construct a range of biosensors.

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