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Bienzyme system for the biocatalyzed deposition of polyaniline templated by multiwalled carbon nanotubes: A biosensor design

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ABSTRACT

A novel method based on covalent attachment of two enzymes, glucose oxidase (GOD) and horseradish peroxide (HRP), onto carboxylic-derived multiwalled carbon nanotubes (MWNTs) for the deposition of electroactive polyaniline (PANI) under ambient conditions is described. Ultraviolet–visible spectroscopy, Fourier-transform infrared (FTIR) spectroscopy, and transmission electron microscopy were used to characterize the assembling of bienzyme and the morphology of PANI|MWNTs. Under the bienzyme biocatalytic condition, a head-to-tail structure of PANI templated by MWNTs was formed. The voltammetric characteristics of the resulting biosensor were investigated by cyclic voltammetry in the presence of glucose. The current response of PANI was linearly related to glucose concentration between 0.05 and 12.0 mM with a correlation coefficient of 0.994. The synergistic performance of bienzyme, highly efficient polymerization, and templated deposition provide a general platform for the synthesis of nanowires and nanocircuits, the construction of bioelectronic devices, and the design of novel biosensors.

1. Introduction

Integration of individual building block materials to functional materials is the principal challenge for development of the next generation of biosensor or nanodevice with excellent chemical, electrochemical and optical properties (Ma et al., 2004; Willner et al., 2007). Recently, tremendous interests have been focused on the application of biocatalysts to the synthesis of metallic nanoparticles (NPs) and the enlargement of metallic NPs, especially the integration of NPs into biocatalytic reactions (Katz and Willner, 2004; Medintz et al., 2005; Möller et al., 2005; Wang and Arribas, 2006). Willner and co-workers have reported the quantitative optical monitoring of several enzymatic reactions for amplified optical enzymatic assays in connection with biocatalytically induced particle-growth processes (Willner et al., 2006; Basnar et al., 2006; Baron et al., 2007; Shlyahovsky et al., 2006). The concept of integration of biomolecules with nanomaterials has opened a new way in developing new electronic and optical biosensors, in synthesizing nanowires and nanocircuits, and in fabricating new devices.

Polyaniline (PANI), which is an intrinsically and versatilely conductive polymer, has been studied extensively due to its environmental stability, redox properties and reversible nature of electrical conductivity (Ho et al., 2005; Gao et al., 2007). Generally, electrically conducting PANI is commonly synthesized by oxidizing aniline either electrochemically or chemically (Wu and Lin, 2006; Rao et al., 2002) in a strong acid environment to initiate the polymerization reaction. The final electroactive polymer can exist in various oxidation states (Kobayashi et al., 2001; Xu et al., 2002) and are typically a mixture of at least two structurally different types, an *ortho*- and a *para*-substituted PANIs. The presence of these highly branched PANIs severely limits the degree of conjugation and hence the electrical and optical properties of the resulting polymers. Therefore, improvements have been made regarding the molecular weight, organization, and processing of these polymers. Recently, the use of enzymatic approach in the synthesis of PANIs has attracted great interest (Gao et al., 2007; Thiyagarajan et al., 2003; Caramyshev et al., 2005), due to its advantages of simple, environmentally benign, higher degree of control over the kinetics of the reaction, and the high efficiency of the enzyme catalyst. However, there is a major drawback that, as soon as the polymer begins to form in aqueous solutions, only very low molecular weight polymers and a mixture of at least two structurally different types of PANIs were formed (Liu et al., 1999; Nagarajan et al., 2001). To overcome this problem, a variety of modified enzymatic polymerizations using biological based materials (Gao et al., 2007; Ma et al., 2004) or polyelectrolyte (Liu et al., 1999; Thiyagarajan et al., 2003) as templates have been proposed and investigated. It has been proved that an intramolecular complex was formed between PANI and templates, and PANI in the complex retains the

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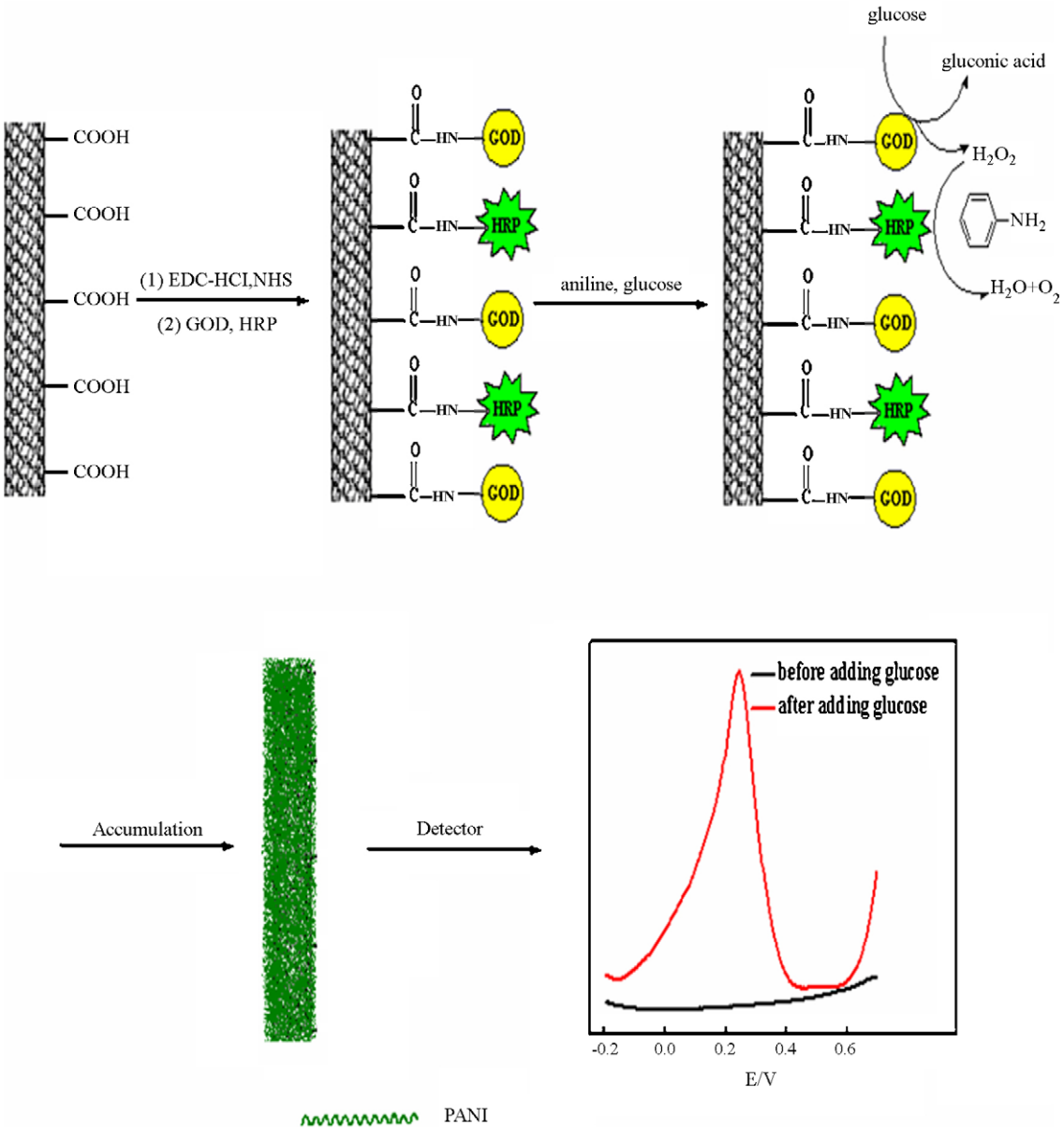
electrochemical activity with mostly the desired head-to-tail structure and minimal branching (Nagarajan et al., 2001; Thiyagarajan et al., 2003).

Multiwalled carbon nanotubes (MWNTs) have been widely used in the fabrication of electrochemical sensing devices and biosensors during the past decade because of their unique electronic, chemical, and mechanical properties (Baughman et al., 2002; Wang et al., 2004). Although the combinations of MWNTs and PANI have been reported previously (Wu and Lin, 2006; Sainz et al., 2005), no work has been reported on studies of PANI chains growing on a MWNT surface under biocatalytic conditions. Recently, the possible cooperation of two enzymes (HRP coupled with other oxidases) for the construction of biosensors to detect glucose, lactate, alcohol, and others has already been reported (Delvaux et al., 2005; Castillo et al., 2003; Shi et al., 2003). Among the multienzymatic systems, it seems that the coupling of several enzymes through substrate or co-substrate regeneration is quite promising for amplifying the electrochemical responses in biosensing applications (Chang et al., 2002; Limoges et al., 2006; Baron et al., 2006). On the basis of this observation, it is likely that an electrochemical procedure for the detection of glucose can be achieved using MWNTs as a template for PANI polymerization. With these guiding principles in mind, we have been interested in the study of bienzyme to initiate the synthesis of conducting-polymer and for the construction of novel electrochemical device and biosensors. In the present study, two enzymes, HRP and GOD were covalently immobilized on carboxylic MWNTs. Then, deposition of PANI on the MWNTs surface occurred after addition of glucose into the system. Glucose acts as the substrate and reacts with GOD, in the presence of the natural co-substrate O_2, to produce H_2O_2. Then, H_2O_2 serves as a substrate and reacts with HRP for the deposition of PANI, in the presence of aniline (Caramyshev et al., 2005). The whole process is illustrated in Scheme 1 and the reaction scheme is

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 + \text{aniline} \xrightarrow{\text{HRP}} \text{PANI} + O_2 \quad (2)$$



Scheme 1. Scheme diagram of the glucose biosensor based on bienzyme-catalyzed deposition of PANI on MWNTs.

In this paper, we presented a novel scheme for the detection of glucose involving bienzyme-catalyzed deposition of conducting-polymer. In this biosystem, MWNTs can act not only as an enzyme immobilization reagent but also the template for the formation of head-to-tail structure PANI. Moreover, it can accelerate the electron transfer between electroactive species and electrode substrates. The proposed method holds great promise for the application of enzymatic polymerizations by bienzyme, the construction of nanocircuits and nanodevices, and the development of simpler, more sensitive and selective biosensors.

2. Experimental

2.1. Reagents

MWNTs (Shenzhen Nanotech Port Co., Ltd., China) were used without further purification. Aniline (99.5%) was obtained from Sigma–Aldrich (St Louis, MO). GOD from *Aspergillus niger* (EC 1.1.3.4, type VII-S), HRP (EC 1.11.1.7, type VI), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC·HCl) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma (Aldrich). Ferrocene monocarboxylic acid (FMCA, 97%, Aldrich) was used as received. D-Glucose, aniline and other regents were used as received without further purification. Glucose stock solutions were allowed to mutarotate for at least 24 h prior to use and stored at 4 °C. All solutions were prepared with ultrapure water (>18 MΩ cm) obtained from a Millipore Milli-Q water purification system.

2.2. Apparatus

Fourier-transform infrared (FTIR) spectra of MWNTs, HRP, GOD, HRP–GOD|MWNTs, and PANI|HRP–GOD|MWNTs samples were recorded on KBr disk using a Nicolet 380 FTIR Spectrometer (Thermo Electron Corporation, USA). Transmission electron microscopic (TEM) images were acquired with a JEOL JEM-3010 high-resolution transmission electron microscope using an accelerating voltage of 200 kV. TEM images were obtained at different parts of the grid and with different magnifications. UV–vis spectra were recorded on a Shimadzu UV-2501PC spectrophotometry at room temperature. A Model CHI660A Electrochemistry Workstation (Chenhua Instruments in Shanghai, China) was employed for all electrochemical experiments. A conventional three-electrode system was used. A saturated calomel electrode (SCE) and a platinum wire electrode were used as the reference electrode and the auxiliary electrode, respectively. An enzyme-carbon nanotube-modified glassy carbon electrode (GCE, 3 mm in diameter) (see Section 2.3) was used as a working electrode. The SCE and GCE used in electrochemical measurements were purchased from Chenhua Instruments in Shanghai (China). After modifying with MWNTs, the sensing area of the MWNTs modified GCE was determined using cyclic voltammetry. This was based on the Randles–Sevčik equation (Gooding et al., 1998): $I_p = 2.69 \times 10^5 (n)^{3/2} (v)^{1/2} A(D)^{1/2} C_o$ where I_p is the peak current (A), n is the number of electrons transferred, A is the electrode area (cm^2), D is the diffusion coefficient of the electro-active species ($cm^2\ s^{-1}$), C_o is the bulk concentration of the same species ($mol\ cm^{-3}$), and v is the scan rate ($V\ s^{-1}$). In this paper, cyclic voltammetric experiments were performed in 1.0 mM $K_3[Fe(CN)_6]$ and 0.1 M KCl solution. We assume for our purposes the value of $D = 7.6 \times 10^{-6}\ cm^2\ s^{-1}$ in the same supporting electrolyte reported previously (von Stackelberg et al., 1953). The sensing area of the MWNTs modified GCE was determined as 0.165 cm^2 (the standard deviation was 0.014 cm^2 for five repeated analyses). All electrochemical experiments were conducted at room temperature.

2.3. HRP–GOD|MWNTs modified electrode preparation

In general, the preparation of HRP–GOD|MWNTs was based on an acylation reaction between carboxylic acid functionalized MWNTs and the amine-terminated HRP and GOD. Carboxylic acid functionalized MWNTs were prepared by refluxing the as-received MWNTs in acid solution. Briefly, 50 mg MWNTs was dispersed in 50 mL of a nitric acid and sulfuric acid (7:3) mixture solution. The mixture solution was ultrasonically agitated for 0.5 h and then refluxed for 3 h. The MWNTs were washed with double-distilled water until the filtrate became neutral, then washed with acetone and dried in air. Prior to use, the GCE was polished with 0.3 and 0.05 μm alumina slurries and rinsed with double-distilled water. Then, a 10 μL aliquot of a 2 $mg\ mL^{-1}$ carboxylic acid functionalized MWNTs in *N*,*N*-dimethylformamide solution was applied on the electrode surface. The coating was dried at room temperature. After drying, the electrode was rinsed thoroughly with double-distilled water and then transferred to a solution containing 10 mM NHS and 10 mM EDC (set to pH 6.0 with 0.1 M HCl), for 1 h at room temperature. Following activation, the electrode was transferred to a solution containing 2 $mg\ mL^{-1}$ HRP and 1 $mg\ mL^{-1}$ GOD. The reaction was allowed to proceed for 1 h at 4 °C. As for comparison, two monoenzymatic electrodes were also constructed in a similar way by incubating the pre-actived MWNTs modified electrodes in 2 $mg\ mL^{-1}$ HRP and 1 $mg\ mL^{-1}$ GOD solutions for 1 h, respectively. The resulting electrodes were described as (1) HRP|MWNT and (2) GOD|MWNT modified electrode, respectively.

2.4. Enzymatic synthesis of PANI and electrochemical measurements

The polymerization of PANI under enzymatic condition was carried out by immersing the HRP–GOD|MWNTs modified electrode in a 0.1 M HAc–NaAc solution (pH 4.3) containing 20 mM aniline and different amount of glucose for a desirable accumulation time (about 10 min). After that, the electrode was removed from the above solution and transferred into a 0.1 M HAc–NaAc solution (pH 4.3), after which square wave voltammetry (SWV) was conducted.

3. Results and discussion

3.1. Characterization of the assembly of bienzyme on MWNTs

In a biocatalytic system, MWNTs can act not only as an electron transfer accelerator between electroactive species and electrode substrates, but also an enzyme immobilization reagent. The assembly of bienzyme on MWNTs is based on an acylation reaction between carboxylic acid functionalized MWNTs and the amine-terminated HRP and GOD, with the help of EDC and NHS. The assembly of HRP and GOD on MWNTs surface can be monitored by UV–vis spectroscopy. As can be seen from Fig. 1(A) and the inset of Fig. 1(A), the UV–vis spectrum of free GOD shows one sharp peak at about 285 and two characteristic peaks for the oxidized form of flavin groups at 380 and 450 nm (Fig. 1(A), trace a). As for HRP (trace b), two typical peaks (285 and 405 nm) are observed. The position and shape of adsorption bands are almost the same as those reported in previous study (Quinn et al., 1984). The sharp peak located at about 285 nm for GOD and HRP is the characteristic peak for polypeptide chains in protein structures, which is consistent with previous reports (Pahari et al., 1995; Li et al., 2001). The absorption spectrum of carboxylic MWNTs shows a wide and sharp peak at about 287 nm (trace c). The position and shape of absorption bands (285 and 405 nm) for HRP and GOD after covalent immobilization (trace d) are almost the same as those of free

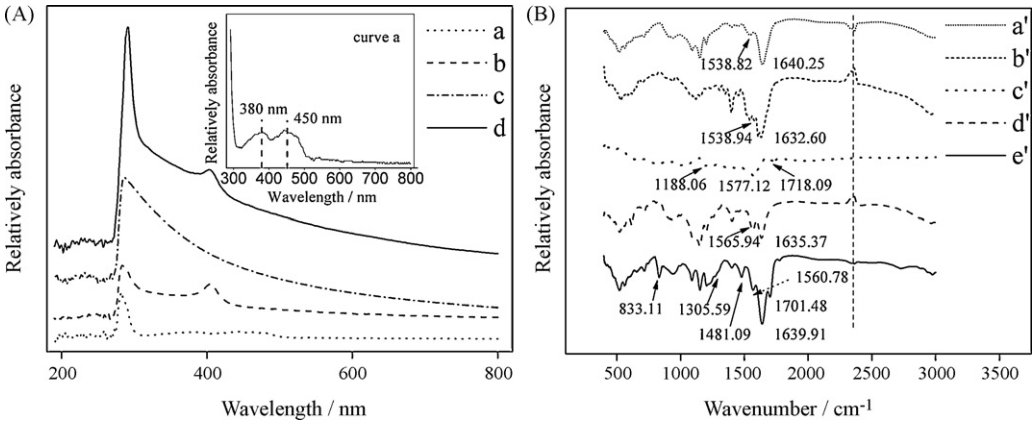


Fig. 1. (A) The UV–vis spectra of (a) GOD, (b)HRP, (c) MWNTs, and (d) HRP–GOD|MWNTs. Inset graph: amplified UV–vis spectra of GOD; (B) the FTIR spectra of (a′) free HRP, (b′) free GOD, (c′) MWNTs, (d′) HRP–GOD|MWNTs and (e′) the deposition of PANI induced by bienzymatic reaction.

HRP and GOD in solution, suggesting that the native structures of HRP and GOD do not change during the immobilization process. Moreover, a close inspection of the spectrum of free HRP, GOD and the HRP–GOD|MWNTs adsorptive nanostructure revealed that sufficient amount of HRP and GOD was immobilised on MWNTs and they maintained their activities.

The successful covalent attachment of HRP and GOD to MWNT is also examined by FTIR. Traces a′ and b′ in Fig. 1(B) are the FTIR spectra of free HRP and GOD, respectively. The bands of amide I (appear at 1640 cm^{-1} for HRP and 1633 cm^{-1} for GOD) and II (appear at 1539 cm^{-1} for HRP and 1539 cm^{-1} for GOD) are assigned to the C=O stretching vibrations of peptide linkages. The appearance of the peaks (1718, 1577, and 1190 cm^{-1}) in the FTIR spectrum of MWNTs (Fig. 1(B), trace c′) indicates the presence of carboxylic and carboxylate oxygen-containing groups on the surface of the MWNTs (Hou et al., 2002). This provides precondition of covalent immobilization of enzymes. Trace d′ shows the FTIR spectrum of HRP and GOD immobilized on the surface of MWNTs. The amide I and II bands in trace d′ have similar shapes to that of free HRP (trace a′) and GOD (trace b′). The similarities of the three spectra of free HRP, GOD and HRP–GOD|MWNTs (traces a′, b′ and d′) indicate that HRP and GOD are successfully immobilized on MWNTs and retain the essential feature of their original secondary structures. The characteristic FTIR peaks of the resulting component of PANI|HRP–GOD|MWNTs are also recorded (Fig. 1(B), trace e′). The bands at 1481 cm^{-1} is due to benzene ring deformation (Furukawa et al., 1988), and the band at 1306 cm^{-1} is assigned to C–N stretching of a secondary aromatic amine (Tang et al., 1988). The C–H out-of-plane bending located at 833 cm^{-1} in both spectra is due to a *para*-substitution pattern, indicating a head-to-tail coupling of aniline occurs during the polymerization (Liu et al., 1999; Tang et al., 1988).

In order to further ensure that HRP and GOD immobilized on the surface of MWNTs have retained their bioactivities, the electrocatalytic reduction of H_2O_2 and the electrocatalytic oxidation of glucose in the presence of FMCA as an electron mediator were studied by cyclic voltammetry, respectively. As shown in Fig. 2(A), the reduction peak current of 2.0 mM H_2O_2 at the HRP|MWNTs composite film increases and the oxidation peak current decreases, indicating an obvious electrocatalytic process of FMCA to the enzymatic reduction of H_2O_2 by the immobilized HRP. The mechanism can be summarized as follows:

$$H_2O_2 + HRP(red) \rightarrow H_2O + HRP(ox) \quad (3)$$

$$HRP(ox) + FMCA \rightarrow HRP(red) + FMCA^+ \quad (4)$$

$$FMCA^+ + e^- \rightarrow FMCA \quad (5)$$

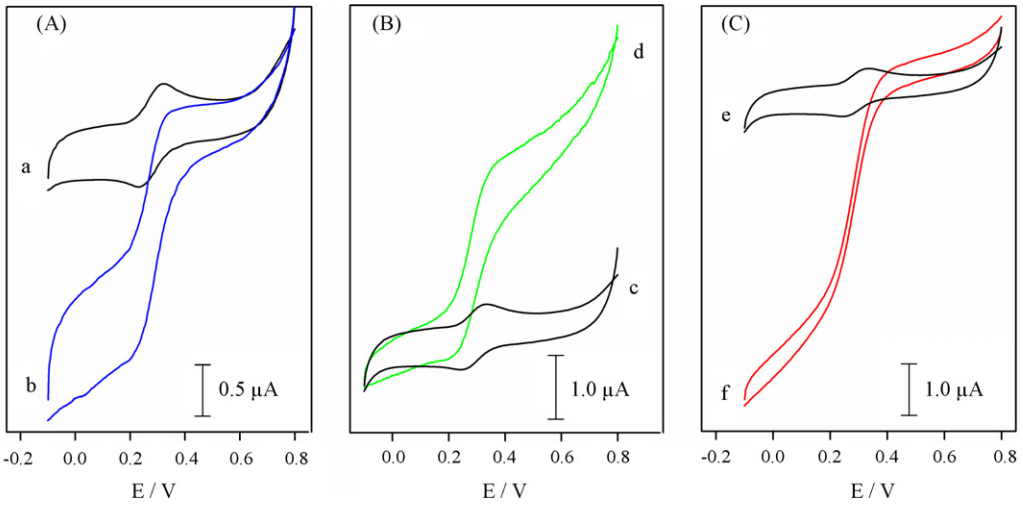


Fig. 2. (A) Cyclic voltammograms for 0.2 mM FMCA in the absence (a) and presence (b) of 2.0 mM H_2O_2 at HRP|MWNTs modified GCE; (B) cyclic voltammograms for 0.2 mM FMCA in the absence (c) and presence (d) of 10.0 mM glucose at GOD|MWNTs modified GCE; (C) cyclic voltammograms for 0.2 mM FMCA in the absence (e) and presence (f) of 20.0 mM glucose at HRP–GOD|MWNTs modified GCE. Electrolyte, pH 4.3 0.1 M HAc–NaAc solution, scan rate: 50 mV s^{-1}.

The FMCA acted as an electron transfer mediator and $FMCA^+$ is reduced at the sensor, resulting in a cathodic current. As for GOD|MWNTs, the anodic peak current increases rapidly in the presence of 10.0 mM glucose concentration and the cathodic peak current decreased (Fig. 2(B)), indicating an obvious electrocatalytic process of FMCA to the enzymatic reduction of glucose by the immobilized GOD. The mechanism can be summarized as follows:

$$\text{glucose} + \text{GOD(ox)} \rightarrow \text{gluconolactone} + \text{GOD(red)} \quad (6)$$

$$\text{GOD(red)} + 2\text{FMCA}^+ \rightarrow \text{GOD(ox)} + 2\text{FMCA} \quad (7)$$

$$2\text{FMCA} \rightarrow 2\text{FMCA}^+ + 2e^- \quad (8)$$

Here, the FMCA acted as an electron transfer mediator and FMCA is oxidized at the sensor, resulting in an anodic current. These observations illustrate that HRP and GOD are immobilized on MWNTs efficiently and can catalyze the reduction of H_2O_2 and the oxidation of glucose in presence of FMCA, respectively. Subsequently, the bioelectrocatalytic activity of HRP–GOD|MWNTs for the oxidation of glucose is examined in the presence of 20.0 mM glucose and 0.2 mM FMCA. As can be seen from Fig. 2(C), the steady state cathodic current increases rapidly, which can be ascribed to the electrocatalytic reduction of H_2O_2 in presence of FMCA generated from the oxidation of glucose catalyzed by GOD. The result further supports that the assembling process for HRP and GOD on MWNT is simultaneously and efficiently.

3.2. The performance of HRP–GOD|MWNTs modified electrode for the deposition of PANI

Prior to assessing the performance of the bienzymatic glucose biosensor, we firstly demonstrate the polymerization of PANI on (1) HRP|MWNTs and (2) GOD|MWNTs modified electrodes. After incubation in a 0.1 M HAc–NaAc solution (pH 4.3) containing 20.0 mM aniline and 10.0 mM glucose for 10 min, cyclic voltammograms are recorded. Results show that no redox peaks were observed for (1) and (2), suggesting that the polymerization of PANI did not occur when mono-enzyme (HRP or GOD) was present in the system.

As mentioned in Section 2, HRP and GOD are covalently conjugated to the carboxylic-derived MWNTs coated electrode (HRP–GOD|MWNTs), and consequently the deposition of PANI occurred after incubating the electrode in 0.1 M HAc–NaAc solution (pH 4.3) containing 20.0 mM aniline and different concentration of glucose. To demonstrate this concept, we first evaluate the response of the biosensor to 10.0 mM glucose in 0.1 M HAc–NaAc solution (pH 4.3) containing 20.0 mM aniline. Before PANI deposition, no redox peaks can be observed at the MWNTs modified GCE (Fig. 3, trace a) and the HRP–GOD|MWNTs modified GCE (trace b) in the potential range of interest. After an accumulation time of 10 min, the biosensor was examined voltammetrically. Fig. 3, trace c depicts a typical cyclic voltammogram of the bienzyme modified electrode after accumulation in the glucose/aniline solution for 10 min. One pair of well-defined redox peaks with the reduction and oxidation peak potentials at 0.202 and 0.281 V (*vs.* SCE) is observed, ascribing to the first redox process of PANI (leucoemeraldine/emeraldine transition) (Liu et al., 1999; Nagarajan et al., 2001; Thiyagarajan et al., 2003). The reason that only one pair of redox peaks appears in cyclic voltammogram is believed to be caused by the high stability of PANI or the inability of PANI to be further oxidized (Gao et al., 2007). Additional experiments show that no obvious changes occur after extensive washing and potential cycling once the electroactive PANI is formed on the surface of MWNTs.

For comparison, electrodeposition of PANI film on MWNTs modified GCE is also tested by potential cycling from −0.2 to 0.8 V in a solution contains 1.0 M HCl and 10 mM aniline. It has been demonstrated previously that two sets of redox peaks can be observed with

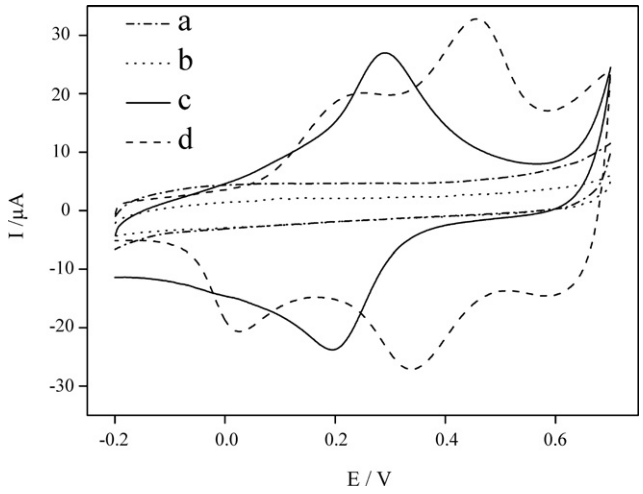


Fig. 3. Cyclic voltammograms obtained at HRP–GOD|MWNTs modified GCE (a) before and (c) after bienzyme-catalyzed deposition of PANI. Reaction solution: 10 min incubation in pH 4.3 0.1 M HAc–NaAc solution containing 20 mM aniline and 10.0 mM glucose. Cyclic voltammograms obtained at MWNTs modified GCE (b) before and (d) after electrodeposition of PANI. Electrolyte, pH 4.3 0.1 M HAc–NaAc solution, scan rate: 50 mV s^{-1}.

electrochemically grown and chemically prepared PANI (Wei et al., 1989). As can be seen from Fig. 3 (trace d), the electrodeposited PANI film on MWNTs modified electrode shows two pairs of redox peaks at about 0.022, 0.232 and 0.342, 0.456 V (*vs.* SCE), respectively, at a scan rate of 50 mV s^{-1} in 0.1 M HAc–NaAc solution (pH 4.3). The above voltammetric results suggest that the protocol for the deposition of PANI under bienzyme catalytic conditions is feasible.

The morphologies of MWNTs before and after the deposition of PANI induced by bienzymatic reaction are characterized by high-resolution transmission electron microscopy (HRTEM). Fig. 4(a) is the high quality individual MWNTs with a diameter of about 30 nm. HRTEM of MWNTs (Fig. 4(b)) reveals that the distance *d* (the distance between two parallel neighboring graphite sheets) is 0.34 nm, which corresponds to the interlayer spacing of graphite and is consist with the previous report (Dresselhaus et al., 1996). After polymerization, a tubular layer of a highly uniformly coated PANI film is present on the MWNTs surface (Fig. 4(c)). The diameter of the resulting composites becomes larger than that of the original MWNTs after the bienzyme initiated polymerization. Closer inspection of HRTEM images (Fig. 4(d) and (e)) reveals that the resulting PANI|MWNTs composite has a coaxially tubular structure. This PANI|MWNTs composite is the typical core-shell structure and the PANI is uniformly formed on the surface of the MWNTs with a thickness of about 4 nm. Thus, the formation of desired head-to-tail structure templeted by MWNTs can be attributed to the fact that MWNTs are able to transfer electrons (Kocherginsky et al., 2005). That is, the molecules of aniline and oxidant are expected to meet, and consequently react. This fact dramatically increases the probability for an aniline molecule to be oxidized to a head-to-tail structure PANI constitutional unit. On the other hand, the PANI chains growing at the MWNTs surface are also conductive and thus participate in the electron transfer from the aniline unit that is being added to the PANI chain-end and MWNTs (Konyushenko et al., 2006).

3.3. Effects of HRP–GOD, incubation time, aniline concentration and solution pH on the performance of the biosensor

It is expected that the performance of the biosensor would mainly be affected by the incubation time, the ratio of HRP–GOD,

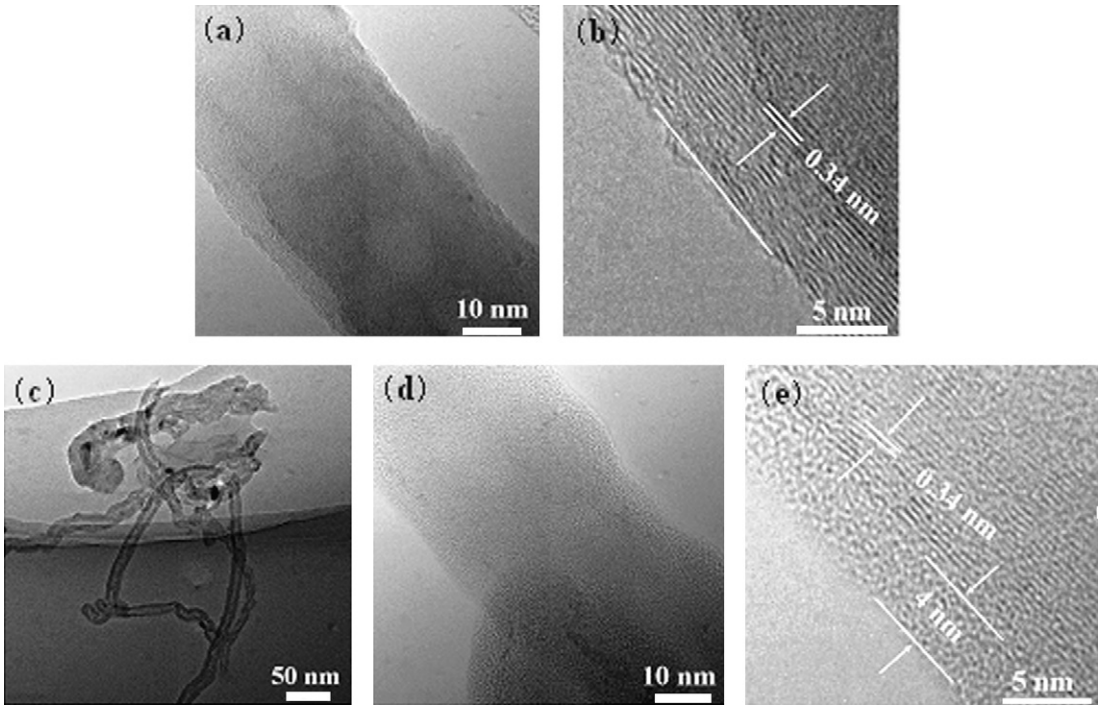


Fig. 4. TEM images of MWNTs with (a) low magnification and (b) high magnification; TEM images of PANI formed on MWNTs with (c) low magnification, (d) high magnification and (e) HRTEM image after bienzyme catalytic reaction.

the amounts of HRP and GOD for immobilization on carboxylic MWNTs surface, and the aniline concentration for polymerization. Supplementary information of Fig. 1S(A) shows the effect of the concentration ratio of HRP–GOD for immobilization on the voltammetric response of the biosensor. Under the optimized incubation time, with the increasing concentration ratio of HRP–GOD used for preparation of the biosensor by the bienzyme catalytic process, the current response increases and reaches a constant value at the concentration ratio of 2. Further, the total concentration of enzymes used in the system is evaluated by increasing the amount of enzymes from 1 to 5 mg mL^{-1} and recording the current responses. Results show that the obtained biosensor gave the best performance for the polymerization of PANI at a total concentration of 3 mg mL^{-1}. Thus, the biosensor was prepared by adjusting the concentration ratio of HRP–GOD to 2 and the total concentration of enzymes to 3 mg mL^{-1}. The optimum incubation time is obtained by recording the current response of the biosensor with different incubation time. Results show that the current response increases with the increasing incubation time, then more slowly, and levels off above 10 min (supplementary information of Fig. 1S(B)).

The effect of aniline is first studied by varying the aniline concentration in the mixture solution from 0.5 to 30.0 mM while keeping the enzymes concentrations and the incubation time unchanged. As illustrated in supplementary information of Fig. 1S(C), with the aniline concentration increases from 0.5 to 30.0 mM, the current response of PANI formed on MWNTs surface first increases and then attains a steady value at 20.0 mM. It is known that the rate of enzyme-catalyzed reaction increases with increasing substrate concentration until is high enough to saturate the enzyme. 20.0 mM of aniline is therefore selected to perform PANI deposition, which is high enough to facilitate the longer polymer chain growth and avoid short chain segments formation (Nagarajan et al., 2001).

The pH of the solution is critical in controlling the alignment of the aniline molecules in this system. Previous results show that the enzymatic polymerization of aniline is strongly pH dependent (Liu et al., 1999). A lower pH (4.0–4.5) is required to produce the conductive PANI, whereas a pH of 6.0 or higher results in a more branched, insulating form of PANI. Previous studies have confirmed that under conditions with a solution pH of 4.3, the enzyme activities (HRP and GOD) are sufficient to provide adequate bioactivities of the enzymes to form the conducting PANI.

3.4. Application for the glucose determination

Because of the superior sensitivity, SWV, rather than cyclic voltammetry, is used in quantification experiments. As illustrated in Scheme 1, the peak current is related to the concentration of glucose added to the incubate solution. Thus, the proposed biosensor can be used for the determination of glucose. The PANI electrooxidation current in SWV is measured in pH 4.3 0.1 M HAc–NaAc solution. Fig. 5(A) shows the SWVs of the HRP–GOD|MWNTs modified electrode in the incubation solution with different concentrations of glucose (0–20.0 mM). The peak current originated from oxidation of PANI increased as the concentration of glucose increased. Therefore, it indicates that the amount of deposited PANI on MWNTs surface is related with the glucose concentrations. The calibration plot corresponding to amperometric responses (Fig. 5(B)) is linear *vs.* the concentrations of glucose ranging from 0.05 to 12.0 mM. The linear regression equation is I_p (μA) = 0.55 + 0.94*C* (mM) with the correlation coefficient of 0.994. The error bars shown in Fig. 5(B) represent standard deviations. The detection limit is determined to be 0.02 mM with the signal-to-noise ratio of 3. The sensitivity of the present biosensor (0.94 μA mM^{-1}) is not improved drastically limited by the efficiency for PANI deposition on the MWNTs surface, but it is still higher than that of 0.065 μA mM^{-1} (Pan et al., 2004) and 0.15 μA mM^{-1} (Shi et al., 2004) prepared by electropolymerized PANI. A structural alignment of the enzymes on electrodes by using MWNTs as electrical connectors between the enzymes and the electrode may improve the sensitivity of the biosensor. Moreover,

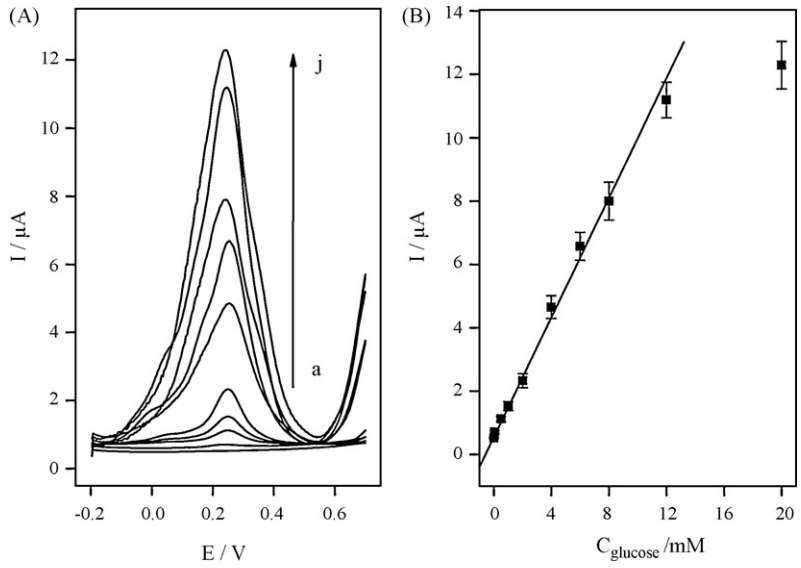


Fig. 5. (A) SWVs obtained at the biosensor after incubation in 0.1 M HAc–NaAc solution (pH 4.3) containing 20 mM aniline and different glucose concentrations for 10 min. Glucose concentration ranges: (a) 0 mM, (b) 0.05 mM, (c) 0.5 mM, (d) 1.0 mM, (e) 2.0 mM, (f) 4.0 mM, (g) 6.0 mM, (h) 8.0 mM, (i) 12.0 mM and (j) 20.0 mM; (B) calibration plot of peak current *vs.* glucose concentration.

the linear response obtained with the biosensor covers the clinical region for a range of 3.5–6.5 mM glucose (Heider et al., 1990), indicating that the biosensor can be possibly used in the analysis of real samples. The reason that the calibration plot deviated from linearity when the glucose concentration was above 12.0 mM can be ascribed to the deposition of PANI on top of the MWNTs would have retarded the production of H_2O_2, and hence the polymerization of aniline.

The stability and reproducibility of the bienzyme (HRP and GOD) assembly on MWNTs for the deposition of PANI are also investigated. Results show that the peak currents change slightly with a relative standard derivation of 9.5% ($n = 8$). This suggests that the assembly of bienzyme and the deposition of PANI are satisfactory and meet the requirements for biosensing.

4. Conclusion

In summary, we have proposed a novel biosensor for glucose detection that utilizes the MWNTs as a template for the deposition of PANI initiated by bienzyme (HRP–GOD). The coupled enzymes, HRP and GOD, were assembled on the surface of MWNTs simultaneously. A desired head-to-tail structure of PANI deposited on MWNTs was obtained under the biocatalytic system. The bioactivities of immobilized enzymes were retained and the electrochemical detection of glucose could be achieved by the proposed biosensor. The optimization results showed that the proposed method by using bienzyme for the deposition of PANI is fixable and holds great promise for the construction of bioelectronic devices and the design of novel electrochemical biosensors with other bio-systems.

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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.2008.08.029.

References

Baron, R., Lioubashevski, O., Katz, E., Niazov, T., Willner, I., 2006. Org. Biomol. Chem. 4, 989–991.
Baron, R., Willner, B., Willner, I., 2007. Chem. Commun. 4, 323–332.
Basnar, B., Weizmann, Y., Cheglakov, Z., Willner, I., 2006. Adv. Mater. 18, 713–716.
Baughman, R.H., Zakhidov, A., de Heer, W.A., 2002. Science 297, 787–792.
Caramyshev, A.V., Evtusheko, E.G., Ivanov, V.F., Barcelo, A.R., Roig, M.G., Shnurov, V.L., van Huystee, R.B., Kurochkin, I.N., Vorobiev, A.K., Sakharov, I.Y., 2005. Biomacromolecules 6, 1360–1366.
Castillo, J., Gáspár, S., Sakharov, I., Csöregi, E., 2003. Biosens. Bioelectron. 18, 705–714.
Chang, S.C., Rawson, K., McNeil, C.J., 2002. Biosens. Bioelectron. 17, 1015–1023.
Delvaux, M., Walcarius, A., Demoustier-Champagne, S., 2005. Biosens. Bioelectron. 20, 1587–1594.
Dresselhaus, M.S., Dresselhaus, G., Eklund, P.C., 1996. Science of Fullerenes and Carbon Nanotubes. Academic Press, San Diego.
Furukawa, Y., Ueda, F., Uyodo, Y., Harada, I., Nakajima, T., Kawagoe, T., 1988. Macromolecules 21, 1297–1305.
Gao, Z.Q., Rafea, S., Lim, L.H., 2007. Adv. Mater. 19, 602–606.
Gooding, J.J., Praig, V.G., Hall, E.A.H., 1998. Anal. Chem. 70, 2396–2402.
Heider, G.H., Sasso, S.V., Huang, K.M., Yacynych, A.M., Wieck, H.J., 1990. Anal. Chem. 62, 1106–1110.
Ho, H.A., Dore, K., Boissinot, M., Bergeron, M.G., Tanguay, R.M., Boudreau, D., Leclerc, M., 2005. J. Am. Chem. Soc. 127, 12673–12676.
Hou, P.X., Bai, S., Yang, Q.H., Liu, C., Cheng, H.M., 2002. Carbon 40, 81–85.
Katz, E., Willner, I., 2004. Angew. Chem. Int. Ed. 43, 6042–6108.
Kobayashi, S., Uyama, H., Kimura, A., 2001. Chem. Rev. 101, 3793–3818.
Kocherginsky, N.M., Lei, W., Wang, Z., 2005. J. Phys. Chem. A 109, 4010–4016.
Konyushenko, E.N., Stejskal, J., Trchová, M., Hradil, J., Kovářová, J., Prokeš, J., Cieslar, M., Hwang, J.Y., Chen, K.H., Sapurina, I., 2006. Polymer 47, 5715–5723.
Li, Q.W., Luo, G.A., Feng, J., Zhou, Q., Zhang, L., Zhu, Y.F., 2001. Electroanalysis 13, 413–416.
Limoges, B., Marchal, D., Mavré, F., Savéant, J.-M., 2006. J. Am. Chem. Soc. 128, 6014–6015.
Liu, W., Kumar, J., Tripathy, S., Senecal, K.J., Samuelson, L.A., 1999. J. Am. Chem. Soc. 121, 71–78.
Ma, Y.F., Zhang, J.M., Zhang, G.J., He, H.X., 2004. J. Am. Chem. Soc. 126, 7097–7101.
Medintz, A.L., Uyeda, H.T., Goldman, E.R., Mattoussi, H., 2005. Nat. Mater. 4, 435–446.
Möller, R., Powell, R.D., Hainfeld, J.F., Fritzsche, W., 2005. Nano Lett. 5, 1475–1482.
Nagarajan, R., Liu, W., Kumar, J., Tripathy, S.K., Bruno, F.F., Samuelson, L.A., 2001. Macromolecules 34, 3921–3927.
Pahari, D., Patel, A.B., Behere, D.V., 1995. J. Inorg. Biochem. 60, 245–255.
Pan, X.H., Kan, J.Q., Yuan, L.M., 2004. Sens. Actuators B 102, 325–330.

Quinn, R., Mercer-Sitnth, J., Burstyn, J.N., Valentine, J.S., 1984. J. Am. Chem. Soc. 106, 4136–4144.
Rao, P.S., Sathyanarayana, D.N., Palaniappan, S., 2002. Macromolecules 35, 4988–4996.
Sainz, R., Benito, A.M., Martínez, M.T., Galindo, J.F., Sotres, J., Baró, A.M., Corraze, B., Chauvet, O., Maser, W.K., 2005. Adv. Mater. 17, 278–281.
Shi, L.X., Lu, Y.X., Sun, J., Zhang, J., Sun, C.Q., Liu, J.Q., Shen, J.C., 2003. Biomacromolecules 4, 1161–1167.
Shi, L.X., Xiao, Y., Willner, I., 2004. Electrochem. Commun. 6, 1057–1060.
Shlyahovsky, B., Pavlov, V., Kaganovsky, L., Willner, I., 2006. Angew. Chem. Int. Ed. 45, 4815–4819.
Tang, J.S., Jing, X.B., Wang, B.C., Wang, F.S., 1988. Synth. Met. 24, 231–238.
Thiyagarajan, M., Samuelson, L.A., Kumar, J., Cholli, A.L., 2003. J. Am. Chem. Soc. 125, 11502–11503.
von Stackelberg, M., Pilgrim, M., Towne, V., 1953. Z. Electrochem. 57, 342–349.
Wang, J., Arribas, A.S., 2006. Small 2, 129–134.
Wang, J., Liu, G.D., Jan, M.R., 2004. J. Am. Chem. Soc. 126, 3010–3011.
Wei, Y., Focke, W.W., Wnek, G.E., Ray, A., MacDiarmid, A.G., 1989. J. Phys. Chem. 93, 495–499.
Willner, I., Basnar, B., Willner, B., 2007. Adv. Funct. Mater. 17, 702–717.
Willner, I., Baron, R., Willner, B., 2006. Adv. Mater. 18, 1109–1120.
Wu, T.M., Lin, Y.W., 2006. Polymer 47, 3576–3582.
Xu, P., Kumar, J., Samuelson, L., Cholli, A.L., 2002. Biomacromolecules 3, 889–893.