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# A sensitive and stable biosensor based on the direct electrochemistry of glucose oxidase assembled layer-by-layer at the multiwall carbon nanotube-modified electrode

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ABSTRACT

A novel strategy for fabricating the sensitive and stable biosensor was present by layer-by-layer (LBL) self-assembling glucose oxidase (GOD) on multiwall carbon nanotube (CNT)-modified glassy carbon (GC) electrode. GOD was immobilized on the negatively charged CNT surface by alternatively assembling a cationic poly(ethylenimine) (PEI) layer and a GOD layer. And the direct electrochemistry of GOD in the self-assembled $\{GOD/PEI\}_n$ film was investigated. CNT as an excellent nanomaterial greatly improved the direct electron transfer between GOD in $\{GOD/PEI\}_n$ film and the electrode. And the ultrathin $\{GOD/PEI\}_n$ film on the CNT surface provided a favorable microenvironment to keep the bioactivity of GOD. Moreover, PEI used as an out-layer was adsorbed on the top of the $\{GOD/PEI\}_n$ film to form the sandwich-like structure ($PEI/\{GOD/PEI\}_n$), improving the stability of the enzyme electrode. On basis of these, the developed $PEI/\{GOD/PEI\}_n$/CNT/GC biosensor has a high sensitivity of 106.57 $\mu A\,mM^{-1}\,cm^{-2}$, and can measure as low as 0.05 mM glucose. In addition, the biosensor has excellent operational stability with no decrease in the activity of enzyme over a 1-week period. Therefore, the developed strategy making use of the advantages of CNT and LBL assembly is ideal for the direct electrochemistry of the redox enzymes and the construction of the sensitive and stable enzyme biosensor.



## 1. Introduction

Since the first concept of an enzyme-based biosensor was proposed by Clark and Lyons (1962), many researches about biosensors have been developed. The long-term goal of these researches is to explore the biosensor for practical applications. However, it is well-known that the most critical issues in the application of in vivo biosensor systems are the stability of the biological sensing element (e.g. protein or enzyme) and the efficient and facile signal mediation and transduction for improved sensitivity and elimination interference (Vamvakaki et al., 2008). Therefore, to resolve these difficulties, the enzyme biosensor has been developed and experienced three stages in past years: the first, second and third generations. Recently, the third-generation biosensor based on the direct electrochemistry of protein/enzyme has been developed widely (Kang et al., 2009; Liu and Ju, 2003; Wu et al., 2008). This can be ascribed to these as follows: (1) comparing the first/second-generation biosensor, the third-generation biosensor has better selectivity and stability because it overcomes some drawbacks such as the possible interference from electrochemically oxidable compounds in real samples, and the un-stability of mediators (Battaglini et al., 2000; Garjonyte and Malinauskas, 1999; Yan et al., 2008); (2) the direct electron transfer between a protein/enzyme and the electrode can be considered as a mimetic process of the electron transfer through redox molecules chains in biological systems (Shen and Hu, 2005). Therefore, the study of direct electrochemistry of redox proteins/enzymes at electrodes may provide a working model for the mechanistic study of electron transfer in living systems (Gooding et al., 2003; Zhang and Hu, 2007). However, it is generally difficult to establish direct electron transfer between protein/enzyme and conventional electrodes because of the inaccessibility of the redox sites of protein/enzyme seated in the large three-dimensional structure (Hamachi et al., 1997; Shi et al., 2007). Therefore, the establishment of a fast electron transfer becomes a significant challenge in designing enzyme-based sensors. In 2008, we produced boron-doped carbon nanotubes (BCNTs) and investigated the direct electrochemistry of glucose oxidase (GOD) at the BCNTs/GC electrode. And it was found that the direct electron

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transfer between GOD and the electrode was improved obviously (Deng et al., 2009). However, it should be pointed that the biosensor based on the CNT-modified electrode may be more typical and popular than that based on the BCNTs-modified electrode, because multi-wall carbon nanotubes can be obtained more easily than BCNTs. In this case, un-doped carbon nanotube (CNT) as a classic new nanomaterial may be still attractive in the direct electrochemistry of proteins/enzymes because CNT can promote the direct electron transfer between the redox sites of protein/enzyme and the electrode (Gooding et al., 2003; Zhang et al., 2007), and improving the sensitivity of the related biosensor.

On the other hand, a novel technique of layer-by-layer self-assembly which is based on the alternate adsorption of oppositely charged species from their solutions (Decher, 1997), has aroused more interests among researchers and has been developed into a general approach for fabricating ultrathin films on solid surfaces. In recent years, the layer-by-layer assembly has been extended to build up protein or other biomacromolecule films (Lvov, 2001) and has been successfully employed for the design and construction of biodevices. Moreover, it was reported that layer-by-layer films not only provide a favorable nanoenviroment for the enzymes or proteins (Hu, 2001), but also enhance the direct exchange between the proteins and underlying electrode (Shen and Hu, 2005). Therefore, the technique of layer-by-layer assembly has been employed to achieve the direct electrochemistry of proteins/enzymes and fabricate the related biosensor. For example, nanostructured biosensors were built by layer-by-layer electrostatic assembly of enzyme-coated single-walled carbon nanotubes and redox polymers (Wang et al., 2006). Shi et al. investigated the electrochemistry and electrocatalytic properties of hemoglobin in layer-by-layer films of $SiO_2$ with vapor-surface sol–gel deposition (Shi et al., 2007). The direct electrochemistry of electroactive hemoglobin was carried out by layer-by-layer assembly of hemoglobin and surfactant didodecyldimethylammonium bromide (Hu et al., 2007).

In this present paper, based on the direct electrochemistry of redox enzyme, we try to integrate the excellent properties of CNT with the advantages of layer-by-layer self-assembly to fabricate a sensitive and stable biosensor. GOD was immobilized on the negatively charged CNT surface by alternatively assembling cationic poly(ethylenimine) (PEI) layers and GOD layers. Herein, CNT as an excellent nanomaterial can improve the direct electron transfer between redox sites of glucose oxidase and the electrode. The $\{GOD/PEI\}_n$ film played an important role in retaining the bioactivity of the immobilized glucose oxidase. Moreover, PEI used as a out-layer was adsorbed on the top of the $\{GOD/PEI\}_n$ film to form the sandwich-like structure ($PEI/\{GOD/PEI\}_n$), improving the stability of the enzyme electrode. And also the fabrication of the enzyme biosensor was controllable, time saving and convenient. Based on the ($PEI/\{GOD/PEI\}_n$)/CNT-modified electrode, the biosensor has high sensitivity and good stability, which is due to the advantages of nanostructured materials like CNT and the layer-by-layer assembly.

## 2. Experimental

### 2.1. Reagents and apparatus

Glucose oxidase (211 U $mg^{-1}$) was purchased from Amresco, and used without further purification. The solution of GOD was prepared in ultrapure water at a concentration of 10 mg $mL^{-1}$. Poly(ethylenimine) (PEI, MW 60,000) was dissolved in ultrapure water at a concentration of 3 wt.%. Stock solution of 1 M glucose was allowed to mutarotate for 24 h so that the α and β forms of D-glucose could reach a final stable ratio (Wang et al., 2006). Carbon nanotube with multiwall and diameter of about 20–30 nm was obtained from Shenzhen Nanotech Port Company. 0.1 M phosphate buffer solution (PBS, pH 7.0) was used as the supporting electrolyte. All other reagents were of analytical grade.

Electrochemical measurements were performed on a CHI660A electrochemical workstation (Chenhua Instrument Company of Shanghai, China) with conventional three-electrode cell. A bare glassy carbon (GC) electrode or the enzyme-modified electrode was used as the working electrode. A saturated calomel electrode (SCE) and a platinum wire were used as the reference and counter electrodes, respectively. All the potentials in this paper were in respect to SCE. Scanning electron microscopy (SEM) images were obtained on a GSM-6400F (Japan). Except the specific statement, the electrochemical measurements were carried out in a phosphate buffer solution at room temperature.

### 2.2. Preparation of the CNT-modified GC electrode

The multiwall CNT was first shorten and functionalized by sonicating CNT in a mixture of concentrated sulfuric acid–nitric acid (3:1, v/v) for about 3 h followed by extensive washing in deionized water until the filtrate was neutral. The treated CNT was dried in vacuum at 60 °C. The resulting black powder were sonicated in *N*,*N*-dimethylformamide (DMF) for about 1 h with a concentration of 1 mg $mL^{-1}$.

The glassy carbon electrode (3 mm diameter) was polished to a mirror-like surface with 1.0 and 0.3 μm alumina slurry and washed thoroughly with ultrapure water. Afterwards, a 5 μL of 1 mg $mL^{-1}$ CNT suspension was dropped to fully cover the surface of the pretreated GC electrode and dried under an infrared lamp. Thus a thin film of CNT was formed to generate the CNT-modified electrode, and the surface was then washed carefully with ultrapure water.

### 2.3. Self-assembling GOD on the CNT-modified electrode

Glucose oxidase was immobilized on the negatively charged CNT surface by alternatively assembling PEI layers and glucose oxidase layers, as shown in Fig. 1A. Prior to it, the preparation conditions were optimized. And it was found that the immersing time of 10 min and 30 min was reasonable and optimal for the adsorption of positively charged PEI and negatively charged GOD, respectively. Based on the optimal preparation conditions, the positively charged polycation was first adsorbed by dipping the negatively charged CNT/GC electrode in PEI solution for 10 min (Fig. 1A-a). Then, the electrode was rinsed with water and dried in nitrogen to form the PEI/CNT/GC electrode. Secondly, the resulted PEI/CNT/GC electrode was immersed into a glucose oxidase solution (10 mg $mL^{-1}$ at pH 7.0) for 30 min, where the negatively charged GOD (p*I* = 4.6) was adsorbed to the positively charged PEI surface through electrostatic interactions. The loosely attached GOD molecules were removed from the surface by a thorough rinse with ultrapure water, and the GOD/PEI/CNT/GC electrode was obtained (Fig. 1A-b). The desired number of bilayers ($n$) for $\{GOD/PEI\}_n$ films on the CNT/GC electrode can be controlled by repeating the above steps (Fig. 1A-c). Another PEI layer was adsorbed on the top of the GOD layer using the same procedure to improve the stability of the enzyme modified electrode (Fig. 1A-d). Thus, it may take ca. 2.5 h to obtain the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode, which was time saving and convenient comparing with reports in literatures (Deng et al., 2009; Liu and Ju, 2003). The modified electrodes were kept at 4 °C in a refrigerator for storage when not in use. And for comparison, the GOD/CNT/GC electrodes were fabricated by immersing the CNT/GC electrodes in 10 mg $mL^{-1}$ glucose oxidase solution for 30 min or 90 min, respectively.

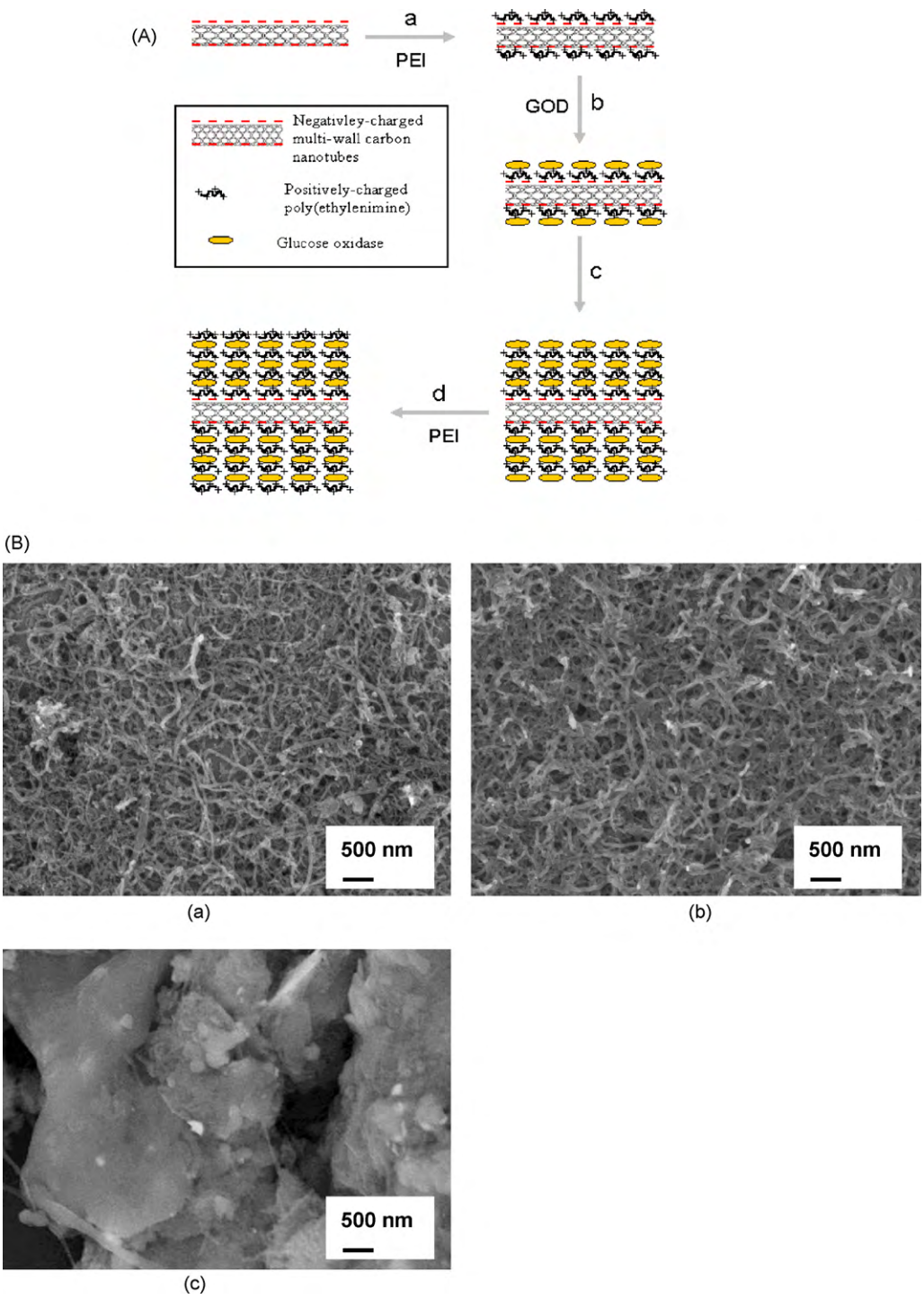


**Fig. 1.** (A) Scheme of layer-by-layer electrostatic self-assembly of GOD on multi-wall carbon nanotubes. (a) assembling positively charged PEI on negatively charged CNT; (b) assembling negatively charged GOD; (c) repeating the two steps (a and b) to control the desired number of layers (n); (d) assembling PEI as out-layer. (B) SEM images of (a) CNT film, (b) PEI/CNT film, and (c) GOD/PEI/CNT film on the GC electrodes, respectively.

## 3. Results and discussion

### 3.1. Characterization of the modified electrode

Cyclic voltammetric measurements were performed using the $[Fe(CN)_6]^{3-/4-}$ redox couple to monitor the formation of each adsorption layer on the GC electrode, and the cyclic voltammograms of 5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.1 M KCl solution at a bare GC (a), CNT/GC (b), PEI/CNT/GC (c) and GOD/PEI/CNT/GC (d) electrodes were investigated, respectively (see supplemental figure S.1). Comparing the bare GC electrode with the CNT/GC electrode, it can be obtained that the peak currents of $[Fe(CN)_6]^{3-/4-}$ at the CNT/GC electrode increase and a peak-to-peak potential separation ($\Delta E_p$) decrease (curve b). It can be due to the high conductivity of CNT (Llopis et al., 2005). Once positively charged PEI was adsorbed on the CNT/GC electrode, the peak currents of $[Fe(CN)_6]^{3-/4-}$ also increased greatly (curve c). It can be ascribed to that more redox probe-$[Fe(CN)_6]^{3-/4-}$ anions was adsorbed onto the positively charged modified electrode surface resulting from the adsorption of PEI onto the CNT/GC electrode. However, the peak

currents of $[Fe(CN)_6]^{3-/4-}$ decreased and a peak-to-peak potential separation ($\Delta E_p$) increased after the PEI/CNT/GC electrode was immersed in the glucose oxidase solution (p$I$ = 4.6) (curve d). All these results indicate that electrostatic interactions between PEI and glucose oxidase happened and the glucose oxidase molecules were immobilized successfully on the modified electrode.

In order to further prove the assembling and immobilization of PEI and GOD, scanning electron microscopy (SEM) was employed to gain insights into the surface morphology of CNT (a), PEI/CNT (b) and PEI/GOD/PEI/CNT (c) films on the GC electrode, as shown in Fig. 1B. From Fig. 1B-a, it can be obtained that multiwall carbon nanotubes film was formed and displayed a well-dispersed one-dimensional structure on the whole surface of the GC electrode. Fig. 1B-b demonstrates that after PEI was adsorbed on the surface of the CNT/GC electrode, and CNTs were efficiently entrapped within the polymeric net and homogenously distributed on the surface. This uniform nanostructure may provide a significant increase of effective area for enzyme loading (Wang and Musameh, 2005). From Fig. 1B-c, it was found that GOD molecules adsorbed on the surface of PEI/CNT/GC electrode and tended to aggregate into island-like structures. In addition, it can be observed that CNT was almost hardly visualized, which indicates that CNT was totally wrapped by the huge protein molecules and PEI. It is favorable for the CNT to be in close contact with the active centre of GOD and act as electron conducting wires to establish direct electron communications. Such kind of morphology is consistent with reports in literatures (Wang and Musameh, 2005; Zhu et al., 2007).

### 3.2. Direct electrochemistry of glucose oxidase in layer-by-layer assembled films

Fig. 2A shows the cyclic voltammograms of the bare GC (a), CNT/GC (b), and PEI/CNT/GC (c) electrodes in 0.1 M deoxygenated phosphate buffer solution (pH 7.0) at the scan rate of 50 mV s$^{-1}$, respectively. It can be seen that voltammograms for the bare GC, CNT/GC and PEI/CNT/GC electrodes contain only nonfaradic current in the applied potential window. And the background of the CNT/GC electrode is higher than that of the bare GC electrode, which can be ascribed to the large specific area of CNT (Liu and Lin, 2006). And once PEI was adsorbed onto the CNT/GC electrode, the background current decreased, it may be due to the non-conductivity of PEI. Therefore, these experimental results further prove that the electrostatic adsorption between positively charged PEI and negatively charged CNT takes place.

On the other hand, $\{PEI/GOD\}_n$ films was also monitored by cyclic voltammetry (CV) and the corresponding results were shown in Fig. 2B. And a-c corresponds to that the number of bilayers ($n$) is from 1 to 3. In Fig. 2B, a pair of well-fined, nearly-reversible redox peaks is observed at about −0.45 V, characteristic of FAD/$FADH_2$ redox couples (Wang et al., 2006; Shuangguan et al., 2008). This demonstrates that by self-assembling GOD on the carbon nanotube surface, a good electron transfer between the redox center of GOD and the electrode can be achieved without the help of the electron transfer mediator. However, no redox peaks of GOD are observed at the GOD/PEI/GC electrode (not shown in plot). The above experimental results demonstrate that CNT plays a critical role in the direct electron transfer of GOD in the self-assembled (Zhang et al., 2007; Wang et al., 2005). In addition, it can be observed that the current of the cathodic peak at ca. −0.45 V also increases linearly with the number of bilayers ($n$) until $n$ = 3. At $n$ > 3, no further increase in the peak currents is observed (not shown in plot), indicating the elecctroactive GOD could extend to only three GOD/PEI bilayers. And therefore, $\{GOD/PEI\}_3$ film was studied in detail by electrochemistry. From Fig. 2B-c, it can be observed that

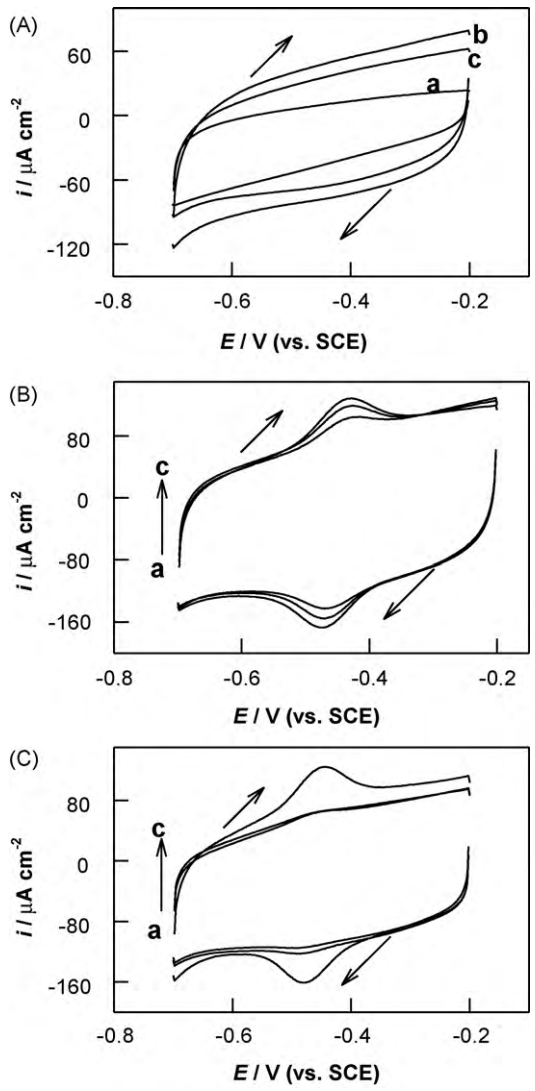


**Fig. 2.** (A) Cyclic voltammograms of different electrodes in 0.1 M deoxygenated phosphate buffer solution (pH 7.0) at the scan rate of 50 mV s$^{-1}$. (a) bare GC, (b) CNT/GC, and (c) PEI/CNT/GC electrodes; (B) (a) GOD/PEI/CNT/GC, (b) $\{GOD/PEI\}_2$/CNT/GC, (c) $\{GOD/PEI\}_3$/CNT/GC electrodes. (C) GOD/CNT/GC electrodes obtained by immersing the CNT/GC electrodes in GOD solution for 30 min (a) or 90 min (b), respectively, (c) the PEI/$\{GOD/PEI\}_3$/CNT/GC electrode.

the anodic peak potential ($E_{p,a}$) and cathodic peak potential ($E_{p,a}$) are −0.436 and −0.476 V with a small peak potential separation ($\Delta E_p$ = 40 mV), proving a fast electron transfer process. Additionally, the formal potential ($E^0 = 1/2\ (E_{p,a} + E_{p,c})$) can be calculated and equals to −0.45 V, which is close to the standard electrode potential of −0.46 V (vs. SCE) for FAD/$FADH_2$ at pH 7.0 (25 °C) (Liu and Ju 2003; Tinoco et al., 1978), indicating that most GOD molecules retain their native structure after the immobilization process. According to $\Gamma = Q/nFA$, (where $Q$ is the charge, $\Gamma$ is the electroactive glucose oxidase amount, $n$ is the electron transfer number, $F$ the Faraday constant, and $A$ is the geometric area of the working electrode) (Bard and Faulkner, 2001), the amount of electroactive glucose oxidase ($\Gamma$, mol cm$^{-2}$) in the $\{GOD/PEI\}_3$ film was estimated to be $4.7 \times 10^{-10}$ mol cm$^{-2}$. This value is much higher than the theoretical value ($2.86 \times 10^{-12}$ mol cm$^{-2}$) for the monolayer of GOD on the bare electrode surface (Xu et al., 2003) and reports in literature (Huang et al., 2005; Liu and Ju 2003). This might be ascribed to LBL self-assembly of more GOD molecules and CNT with good conductivity and high surface area. Therefore, it can be believed that by self-assembling GOD on the carbon nanotubes

surface, the fast electron transfer of more GOD molecules can be achieved at the $\{GOD/PEI\}_n$/CNT/GC electrode, which is significant for the improvement of the sensitivity of the enzyme modified electrode.

Additionally, in order to investigate the role of PEI, the comparison between the GOD/CNT/GC electrode and the GOD/PEI/CNT/GC electrode was carried out. Therefore, the cyclic voltammograms of the GOD/CNT/GC electrode and the GOD/PEI/CNT/GC electrode were investigated, and corresponding results are shown in Fig. 2B-a and C-a, respectively. It can be observed that redox peaks of GOD almost cannot be observed at the GOD/CNT/GC electrode. This can be explained that because GOD was immobilized on the CNT/GC electrode by physical adsorption, immersing the CNT/GC electrode in GOD solution for 30 min leaded to that only a small amount of GOD molecules were adsorbed on the CNT/GC electrode (Deng et al., 2009). However, the redox peaks of GOD at the GOD/PEI/CNT/GC electrode are obvious (Fig. 2B-a). This can be ascribed to that many GOD molecules were adsorbed on the surface of the PEI/CNT/GC electrode in a short time because glucose oxidase was immobilized on the PEI/CNT/GC electrode by the electrostatic reaction between positively charged PEI and negatively charged glucose oxidase, which is consistent with literatures (Liu and Lin, 2006; Shi et al., 2007). Based on the above results, it can be believed that functionalization of CNT with positively charged PEI leaded to the fast immobilization of GOD, and thus the fabrication of the enzyme-based biosensor would be time saving and convenient.

### 3.3. Effect of out-layer on the stability of the enzyme modified electrode

Besides the sensitivity, the stability of the enzyme modified electrode is another important issue. Considering this point, poly(ethylenimine) layer as out-layer was adsorbed on the top of the $\{GOD/PEI\}_3$ film to form the sandwich-like structure ($PEI/\{GOD/PEI\}_3$) on the CNT surface. It was expected that the stability of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode was improved comparing with the $\{GOD/PEI\}_3$/CNT/GC electrode. Therefore, both the operational stability and storage stability of the $\{GOD/PEI\}_3$/CNT/GC electrode and the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode were investigated, respectively. The operational stability was investigated by examining the cyclic voltammetric peak currents of GOD after continuously scanning for 30 cycles. There was nearly no decrease of the voltammetric response for the $PEI/\{GOD/PEI\}_n$/CNT/GC electrode. And, in the case of the $\{GOD/PEI\}_3$/CNT/GC electrode, it was found that the cyclic voltammetric peak currents of GOD retain 89%. On the other hand, it was found that the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode was stable in a week and the electrochemical response of GOD at the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode decrease less than 5% after stored in buffer solution for 20 days. In comparison, the cyclic voltammetric peak currents of GOD at the $\{GOD/PEI\}_3$/CNT/GC electrode retained 82% of their initial response values after stored for 20 days. The improvement in the stability of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode can be ascribed to the use of the PEI out-layer which effectively prevents GOD leaking from the electrode surface (Liu and Lin, 2006; Yemini et al., 2005). Therefore, to obtain a stable biosensor for glucose sensing, the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode may be employed.

### 3.4. Electrochemical properties of $PEI/\{GOD/PEI\}_3$/CNT/GC electrode

The cyclic voltammogram of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode was investigated, as shown in Fig. 2C-c. It can be seen that the redox peaks of GOD at the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode is much higher than those of the GOD/CNT/GC electrode

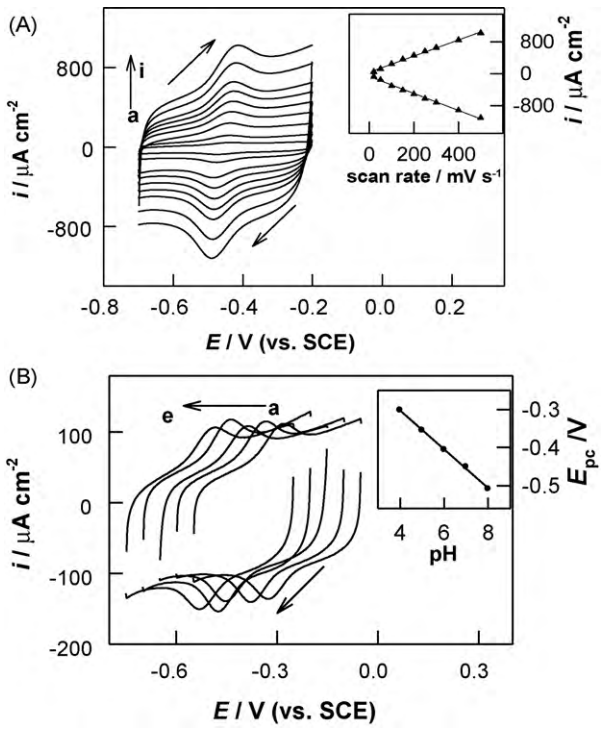


**Fig. 3.** (A) Cyclic voltammograms of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode in 0.1 M deoxygenated phosphate buffer solution (pH 7.0) at the different scan rates. Scan rates (a–i): 20, 50, 100, 150, 200, 250, 300, 400 and 500 mV $s^{-1}$. Inset: the dependence of the anodic (a) and cathodic (b) peak currents of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode on the scan rate. (B) Cyclic voltammograms of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode in the deoxygenated solution with different pH values of (a–e): 4.0, 5.0, 6.0, 7.0, 8.0. Scan rate: 50 mV $s^{-1}$. Inset: plot of the formal potential of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode as function of pH value.

(Fig. 2C-b), which further proves that PEI plays an essential role in the fast immobilization of GOD. Additionally, comparing the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode with the $\{GOD/PEI\}_3$/CNT/GC electrode (Fig. 2B-c), it can be obtained that the poly(ethylenimine) layer as out-layer has no obvious influence on the redox peaks of GOD. According to literatures, it may be ascribed to that CNT incorporated in PEI film facilitated the charge transport (Granot et al., 2006; Rubianes and Rivas, 2007; Yan et al., 2008) and the layer-by-layer films enhance the direct exchange between the proteins and underlying electrode (Shen and Hu, 2005). Therefore, considering the above experimental results and literatures, it can be concluded that functionalization of CNTs with PEI does not block the electron transfer between GOD and the modified electrode although PEI is non-conductive.

Based on these, the effect of scan rate on the voltammetric response of immobilized GOD was studied. Fig. 3A shows the cyclic voltammograms of the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode at different scan rates from 20 to 500 mV $s^{-1}$. It can be observed that the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode displays a pair of symmetric and well-defined redox peaks at different scan rates. From the inset of Fig. 3A, it can be seen that the anodic and cathodic peak currents increase with the increase of the scan rate and a linear relationship can be observed from 20 to 500 mV $s^{-1}$. This demonstrates that the redox reaction of GOD at the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode is a surface-controlled process, not a diffusion-controlled process (Dai et al., 2004; Liu et al., 2007; Shi et al., 2007).

The pH value of the solution affects the electrochemical behavior of GOD at the $PEI/\{GOD/PEI\}_3$/CNT/GC electrode. As shown in Fig. 3B, the cyclic voltammograms with stable and well-defined peaks are observed from pH 4.0 to 8.0, and the redox peak potential shifts negatively with the increase of pH. The inset of Fig. 3B

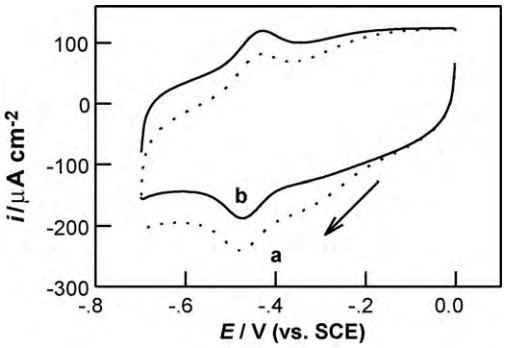


**Fig. 4.** Cyclic voltammograms of the PEI/{GOD/PEI}$_3$/CNT/GC electrode in 0.1 M (a) air-saturated and (b) deoxygenated phosphate buffer solution (pH 7.0) at a scan rate of 50 mV s$^{-1}$.

shows the effect of solution pH on the formal potential of the PEI/{GOD/PEI}$_3$/CNT/GC electrode, and it can be seen that the formal potential of the PEI/{GOD/PEI}$_3$/CNT/GC electrode depends linearly on the pH value in the range of 4.0–8.0 with the slope of −51.6 mV/pH, which is close to the expected value of −58.6 mV/pH for a two-electron coupled with two-proton reaction, and the reaction mechanism can be written as (Huang et al., 2005; Liu et al., 2007):

$$\text{GOD–FAD} + 2e^- + 2H^+ \leftrightarrow \text{GOD–FADH}_2 \quad (1)$$

### 3.5. Electrocatalytical behavior of the PEI/{GOD/PEI}$_3$/CNT/GC electrode

The effect of the dissolved oxygen on the electrochemical behavior of the PEI/{GOD/PEI}$_3$/CNT/GC electrode was investigated, as shown in Fig. 4. Although a pair of well-defined, quasi-reversible redox peaks is observed in both deoxygenated and air-saturated PBS (pH 7.0), respectively, the reduction peak current of the PEI/{GOD/PEI}$_3$/CNT/GC electrode in air-saturated PBS is larger than that in deoxygenated PBS and the oxidation peak current is in reverse. The corresponding results are consistent with these reports in literatures (Huang et al., 2005; Liu et al., 2007). It confirms that dioxygen dissolved in the solution is catalytically reduced at the PEI/{GOD/PEI}$_3$/CNT/GC electrode:

$$\begin{array}{l} \text{GOD-FAD} + 2e^- + 2H^+ \leftrightarrow \text{GOD-FADH}_2 \\ \quad\uparrow \qquad\qquad\qquad\qquad \downarrow \\ \text{GOD(FADH}_2) + O_2 \rightarrow \text{GOD(FAD)} + H_2O_2 \end{array} \quad (2)$$

More importantly, when glucose was added into air-saturated PBS, the reduction peak currents at the PEI/{GOD/PEI}$_3$/CNT/GC electrode decreased gradually with the increase of the concentration of glucose, as shown in Fig. 5A. According to the following enzyme-catalyzed reaction:

$$\text{Glucose} + \text{GOD(FAD)} \rightarrow \text{gluconolactone} + \text{GOD(FADH}_2) \quad (3)$$

It can be explained that glucose is the substrate of GOD, when it was added to the air-saturated PBS, the enzyme-catalyzed reaction occurred and the concentration of the oxidized form of GOD decreased. Thus, the addition of glucose restrained the electrocatalytic reaction and leaded to the decrease of the reduction current (Deng et al., 2009; Huang et al., 2005; Liu et al., 2007). Based on the decrease of the reduction current, the concentration of glucose can be detected.

Fig. 5B shows the relationship between the glucose concentration and the decrease of the reduction peak current at the PEI/{GOD/PEI}$_3$/CNT/GC electrode. The current values of the PEI/{GOD/PEI}$_3$/CNT/GC electrode change linearly with the

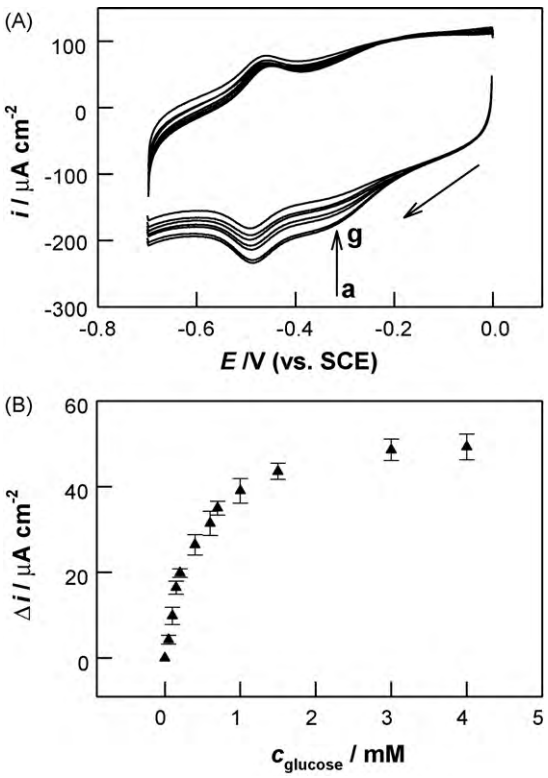


**Fig. 5.** (A) Cyclic voltammograms of the PEI/{GOD/PEI}$_3$/CNT/GC electrode in 0.1 M air-saturated phosphate buffer solution (pH 7.0) before (a) and after adding (b) 0.05 mM, (c) 0.2 mM, (d) 0.4 mM, (e) 0.7 mM, (f) 1.5 mM, and (g) 3.0 mM glucose. (B) Dependence of the decreased reduction peak current of the PEI/{GOD/PEI}$_3$/CNT/GC electrode on the concentration of glucose in 0.1 M air-saturated phosphate solution (pH 7.0).

concentration of glucose up to 0.3 mM with a correlation coefficient ($r$) of 0.993. The sensitivity of the PEI/{GOD/PEI}$_3$/CNT/GC electrode was calculated to be 106.57 μA mM$^{-1}$ cm$^{-2}$, much higher than those previously reported, for example, the CNT-based multi-layer biosensor (5.6 μA mM$^{-1}$ cm$^{-2}$) (Yan et al., 2007) and the Nafion–CNTs–CdTe–GOD/GC based biosensor (14.41 μA mM$^{-1}$ cm$^{-2}$) (Liu et al., 2007). The higher sensitivity of this enzyme biosensor can be ascribed to more GOD molecules in assembled film and CNT with high conductivity, which provides a novel protocol for the construction of enzyme-based biosensors.

In addition, the apparent Michalis–Menten constant ($K_m^{app}$), an indicator of enzyme–substrate reaction kinetics, was used to evaluate the biological activity of the immobilized enzyme. ($K_m^{app}$) can be calculated using the Lineweaver–Burk equation (Kamin and Wilson, 1980):

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_m^{app}}{I_{max} C_{glucose}} \quad (4)$$

where $I_{ss}$ is the steady-state response current after the addition of substrate, $I_{max}$ is the maximum current under saturated substrate conditions, and $C_{glucose}$ is the bulk concentration of glucose. In this work, ($K_m^{app}$) of the PEI/{GOD/PEI}$_3$/CNT/GC electrode was estimated to be ~0.95 mM, which is smaller than those reported the literatures (Shuangguan et al., 2008; Kang et al., 2009). The smaller ($K_m^{app}$) value indicates that the immobilized GOD possesses higher enzymatic activity and the PEI/{GOD/PEI}$_3$/CNT/GC electrode exhibits a higher affinity for glucose than that reported in literatures (Liu and Ju, 2003; Liu et al., 2007). Therefore, it can be believed that the sandwich-like structure PEI/{GOD/PEI}$_3$ film on the carbon nanotube surface provides favorable microenviroment for retaining the bioactivity of GOD.

**Table 1**
Determination of glucose in human plasma samples by the PEI/{PEI/GOD}$_3$/CNT/GC electrode.

| Sample | Found (μM) | Added (μM) | Found (μM) | Recovery (%) |
|---|---|---|---|---|
| 1 | 30.0 | 3.0 | 32.9 | 96.7 |
| 2 | 30.0 | 5.0 | 34.1 | 102.0 |
| 3 | 30.0 | 8.0 | 37.8 | 97.5 |
| 4 | 12.5 | 2.0 | 14.6 | 105.0 |
| 5 | 12.5 | 6.0 | 18.7 | 103.3 |

The effect of possible interfering species such as uric acid and ascorbic acid, on glucose detection was also examined. It was found that the addition of 0.01 mM uric acid or ascorbic acid in 0.1 mM glucose almost did not cause the interference to the reduction current of glucose, respectively. These results suggest that the proposed PEI/{PEI/GOD}$_3$/CNT/GC electrode has high selectivity, and no interference from coexisted electroactive substances due to the low operating potential.

On the other hand, the reproducibility of the glucose biosensor was investigated by successive detection of 0.1 mM glucose for 10 times, and an acceptable reproducibility with a relative standard deviation (RSD) of 3.6% was obtained. In addition, the RSD of current signals for measurement of 0.1 mM glucose at six independently prepared biosensors was 4.5%. These experimental results demonstrate a good reproducibility of the biosensor.

The PEI/{PEI/GOD}$_3$/CNT/GC electrode has been employed for the detection of blood sugar concentration in human plasma utilizing standard addition method. The analysis of glucose was carried out by transferring 20 μL plasma samples to the cell with 10 mL 0.1 M PBS (pH 7.0). And the corresponding results were listed in Table 1. It can be obtained that the recoveries for the determination of glucose are between 96.7% and 105.0% for five times determination. This demonstrates the positively charged PEI film as out-layer does not interfere the determination of glucose in real sample and the detection of glucose using the enzyme biosensor is reliable and effective.

## 4. Conclusion

We described a high sensitive and stable biosensor for glucose sensing based on LBL self-assembling glucose oxidase on CNT-modified GC electrode. The formation of LBL nanostructure on the CNT surface provides a favorable microenvironment to maintain the bioactivity of GOD, and CNT as an excellent material greatly improve the direct electron transfer between GOD and the electrode. Moreover, PEI layer as out-layer was used to prevent enzyme molecule leaking. Therefore, based on the sandwich-like structure PEI/{GOD/PEI}$_3$ film at the carbon nanotube-modified electrode, the fabrication of the PEI/{GOD/PEI}$_n$/CNT/GC electrode is simple, controllable and fast and the designed biosensor for glucose sensing possesses the high sensitivity and high stability. The present strategy making use of the advantages of nanostructured materials like CNT and the layer-by-layer assembly may have potential significance in the development of the selective and stable biosensor.

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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.2010.06.013.

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