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A Glucose Biosensor based on Horseradish Peroxidase and Glucose Oxidase Co-entrapped in Carbon Nanotubes Modified Electrode

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In this work, glucose oxidase (GOD) and horseradish peroxidase (HRP) were co-assembled onto carbon nanotubes (CNTs) modified glassy carbon electrode (GCE) to prepare a novel bi-enzyme electrode. The electrocatalytic performance and electron transfer of GCE/CNTs/GOD-HRP were explored by Cyclic voltammetry (CV). The UV-vis absorption spectroscopy showed that the HRP and GOD formed a protein complex and their structure was well kept. The CVs results also showed that the CNTs enhanced the immobilization of GOD-HRP and facilitated the electron transfer. GOD's biological activity was well kept due to the biocompatible microenvironment provided by the surrounding HRP. The effects of electrolyte pH and scan rate on the catalytic performance of the bi-enzyme were investigated. The GCE/CNTs/GOD-HRP electrode is used to construct an electrochemical glucose sensor. The sensor showed satisfactory results with a wide linear range from 0.022 to 7.0 mM, a high sensitivity of 5.14 μA mM^{-1} cm^{-2}, and a low detection limit of 7 μM. The as-prepared sensor also display advantages of good selectivity, high repeatability and reproducibility, and relatively high stability.

Keywords: Glucose oxidase; Horseradish peroxidase; Carbon nanotubes; Glucose; Electrochemical biosensor.

1. INTRODUCTION

Quantitative determination of glucose concentration is very important in clinical analysis, food industry, environmental analysis and biological analysis [1]. Glucose oxidase (GOD) as one of the most popular enzymes in biological research has been widely applied to construction of electrochemical glucose biosensor [2-4]. GOD, as a kind of enzyme, is able to specially catalyze oxidation glucose into glucose acid [5]. During the catalytic process, O_2 as an essential reactant will be

reduced into H_2O_2. Therefore, O_2 and H_2O_2 are usually used for the quantitative detection of glucose [6]. Most of the glucose biosensors based on O_2 limits the detection of large concentration of glucose because of the great O_2 consumption [7].

According to previous results [8-10], the horseradish peroxidase (HRP) can catalyze the oxidation of H_2O_2 into O_2 in the disproportionation reaction, so the HRP and GOD were constructed into the bi-enzyme interface for constructing electrochemical glucose biosensors. When the glucose is oxidized by GOD, the disproportionate reaction of H_2O_2 catalyzed by HRP could produce O_2 to partly replenish the consumption of O_2 so that reduce the consumption of O_2, remove the H_2O_2 at the same time and protect the activity of enzyme. As a result, a high concentration of glucose can also be sensitive and accurate detection based on the bi-enzyme biological sensors. It has been reported that glucose sensor constructed by GOD and HRP has superior performance. The biggest bright spot is the application of catalytic principle of cascade based on the signal amplification step by step so as to improve the sensitivity of biosensor. These results have inspired us with GOD and HRP bi-enzyme modified electrode to construct the excellent performance of bi-protein biosensor [11,12].

In recent years, the direct electron transfer (DET) of redox enzyme electrode aroused the interest of many researchers [13]. However, GOD's redox center - FAD buried deep inside the thick protein shell to hinder the electronic transmission between GOD and the electrode surface, which will affect the GOD in the application of enzyme sensor [14-16]. Many efforts have been put on the enhancement effect of the electron transfer between the electrode surface and protein [17-19]. For example, the introduction of nanomaterials is becoming more and more popular because of the good conductivity of nanomaterials, the special nanometer structure, high catalytic activity, more active sites on the surface and good adsorption and so on. In recent years, many researchers take advantage of nanometer materials to immobilize GOD onto the electrode surface to construct the glucose biosensor. Many GOD biological sensors based on nanomaterials were also developed based on the DET of GOD. Carbon nanotubes (CNTs) [20-23] has the unique mechanical strength, high specific surface area and fast electron transfer effect and chemical stability. The CNTs modified electrode can not only improve the electron transfer speed but also serve as a good catalyst for the reduction of O_2, which makes the CNTs are widely used in the construction of biological electrochemical sensor.

In this work, the CNTs were assembled onto the surface of the glassy carbon electrode (GCE), and then used for the construction of a new type of bi-enzyme biological interface of GOD and HRP complexes. The experimental results show that the bi-enzyme interface successfully achieved the direct electrochemistry of GOD. The as-prepared electrode could be used to detect glucose based on the consumption of O_2. The novel glucose sensor exhibited wide linear range from 0.022 to 7.0 mM. Thus, this study not only successfully achieved direct electron transfer of GOD, but also constructed a novel biosensor for glucose detection.

2. EXPERIMENTAL

2.1. Materials

GOD (EC 1.1.3.4, 140 U mg^{-1}), HRP (EC 1.11.1.7 250 U mg^{-1}) and Nafion(5 wt.%) were purchased from Sigma-Aldrich. Glucose was purchased from Tianjin Chemical Reagent factory

(Tianjin, China). CNTs were purchased from Cheap Tubes Inc. Other reagents were obtained from Beijing Chemical Reagent Factory (Beijing, China) and were of analytical reagent grade. The solution was stored in a refrigerator (4 °C) prior to use. Phosphate buffer solution (PBS) was obtained by mixing 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4. The GOD solution (65 μM) and HRP solution (250 μM) were prepared by dissolving GOD and HRP in 0.2 M PBS (pH 7.0) and stored at 4 °C. All reagents were used as received. All solutions were prepared with ultra-pure water, produced by a Millipore-Q System (18.2 MΩ cm^{-1}).

2.2 Preparation of the GCE/CNTs electrode

Firstly, the GCE was first carefully polished with 1.0 μm and 0.3μm alumina slurry on felt pads, and then washed ultrasonically in ultrapure water and ethanol for 1 minutes, respectively. After that, 10 μL CNTs solution (1 mg mL^{-1}) was transferred onto polished GCE surface and dried at 4 °C to obtain the GCE/CNTs. Briefly, CNTs were dispersed in water by an ultrasonication instrument to achieve mixture. After being dried, the resulting electrode was washed by ultra-pure water to remove those weakly bound molecules, and carefully drying with nitrogen.

2.3 Fabrication of the GCE/CNTs/GOD-HRP *modified electrode*

The GCE/CNTs/GOD-HRP modified electrode were prepared by first dropping 5 μL of the mixed protein mixture which consist of 4 μL of 65 μM GOD and 1 μL of 250 μM HRP onto the surface of the GCE/CNTs electrodeand then dried at 4 °C. Next, 1.0 μL of 0.05 % nafion solution were dropped onto the GCE/CNTs/GOD-HRP surface and subsequently dried at 4 ºC for 4 h. Put it in the refrigerator and stored at 4 ºC for further use. The GCE/CNTs/GOD-HRP was successfully obtained and its preparation process as shown in the Fig.1.

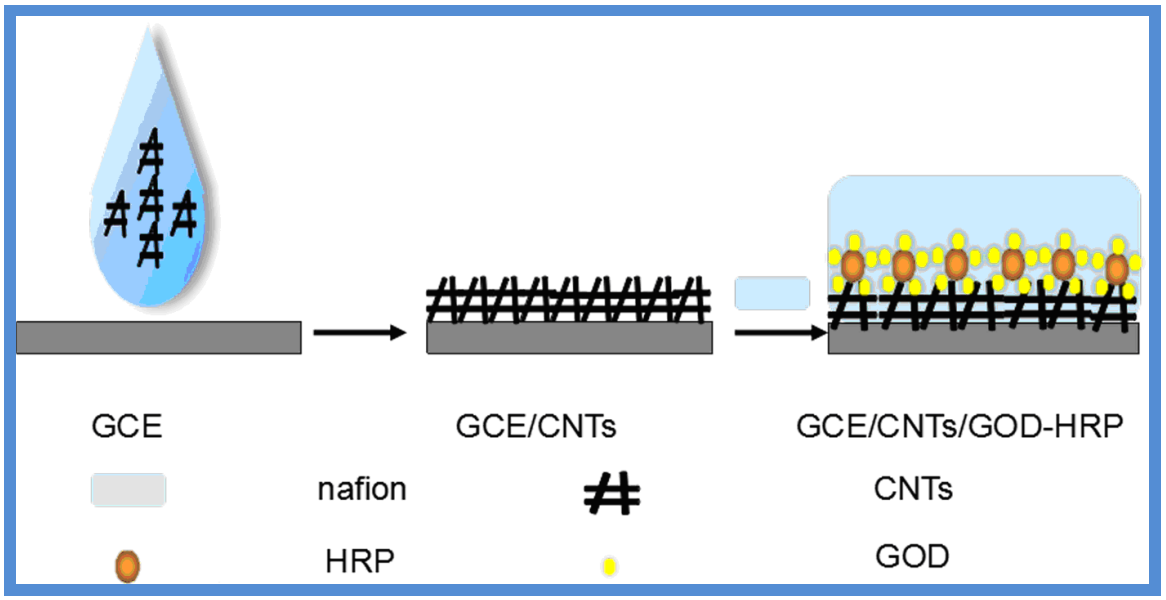

Figure 1. Schematic illustrating preparation process of the GCE/CNTs/GOD-HRP.

2.4 Instruments

All electrochemical experiments was operated on CHI750D electrochemical workstation (CH Instruments, China). In the present work, we use a platinum electrode as auxiliary electrode, a saturated calomel electrode (SCE) as the reference electrode and bare or decorated glassy carbon electrode (GCE) as working electrode to consists of the traditional system of three electrodes. Cyclic voltammetry (CV) were performed in 10.0 mL 0.2 M PBS (pH=7.0) solution at room temperature. Electrolyte solutions were purged with high purity nitrogen before starting experiments and nitrogen atmosphere was kept over the solution during measurements. Detection of glucose was carried out by using CV in O_2-saturated PBS (pH 7.0) solution. SEM images of CNTs/GCE were obtained using a HITACHI S-3400N scanning electron microscope (SEM) at an accelerating voltage of 15 kV. UV-vis spectra were obtained on a Lambda 35 UV-vis spectrometer.

3. RESULTS AND DISCUSSION

3.1 Interaction between HRP and GOD

UV-vis spectroscopy is a kind of technology used for characterization of the interaction between proteins and other substances, such as protein conformation changes. In this work, the interaction between GOD and HRP in 0.2 M PBS (pH 7.0) solution was studied by UV-vis spectroscopy. As can be seen from Fig. S1 (ESI^+), GOD has an obvious characteristic absorption peak at 276 nm, and the HRP showed characteristic absorption peak at 403 nm. The position and shape of adsorption bands are almost the same as those reported in previous study [24-25]. After the two proteins were mixed, The position and shape of absorption bands (276 and 403 nm) for GOD and HRP after covalent immobilization are almost the same as those of free HRP and GOD in 0.2 M PBS (pH 7.0) solution, suggesting that the native structures of HRP and GOD do not change during the immobilization process. This results showed that the HRP and GOD formed a complex and their bioactivity was well kept. Apart from that, It is reported that the isoelectric point of GOD is about 4.8 [26], and the isoelectric point of HRP is about 7.2 [27].So, in the solution of pH 7.0, GOD has negative charges on the surface, HRP has positive charges on the surface. negatively charged GOD and positively charged HRP could combine through electrostatic interaction to form a positively charged complex. The results confirmed successful construction of the GOD- HRP bi-protein.

3.2 Characterization of CNTs/GCE

The pattern of CNTs/GCE was investigated by SEM. As shown in Fig. S2 (ESI^+), due to the strong van der Waals force between CNTs and CNTs, large length to diameter ratio and single span of his defects, the CNTs tend to integrate beam. As can be seen from Fig. S2 (ESI^+), the CNTs are tubular structure, intertwined with each other on GCE surface. Since its specific surface area is large and the electron transfer ability is good, the CNTs can be used as a kind of very good enzyme carrier[21].

More detailed information can be obtained from further magnified SEM images of the CNTs, we also can clearly see that CNTs exhibit a root of the filamentous morphology and uniformly and compactly wrapped around together. The specific surface area of CNTs is very large because of this morphology, which provides a good environment for the GOD-HRP complex. Besides, it can also promote effective electron transfer between GOD and the electrode[21,23].

3.3 Electrochemical behaviors of the GCE/CNTs/GOD-HRP

Fig. 2A showed the Cyclic Voltammograms (CVs) of various modified electrodes in a 0.2 M nitrogen- saturated PBS (pH =7) at a scan rate of 100 mV s^{-1}. As shown in Fig. 2A, obvious electrochemical redox peak of the bare GCE electrode and GCE/CNTs in the PBS couldn't be observed. While, a reduction peak at about -0.450 V and an oxidation peak at -0.509V appeared at the GCE/CNTs/GOD-HRP electrode. The peak resulted from the DET process between FAD and GCE/CNTs/GOD-HRP electrode. The peak-to-peak potential separation(ΔEp = Epa - Epc) is 59 mV, showing that the GOD realized a fast electron transfer process in the electrode [28,29]. It is because the HRP itself is also a kind of common electron transfer protein, and HRP wrapped around the GOD in a certain extent can promote electron transfer between GOD and the electrode. Thereby, the redox peak current was enhanced and ΔEp was reduced. From what has been shown, we can see that the modified electrode electrochemical behavior associated with the preparation of electrode. The good electron transport material CNTs played an important role in promoting direct electrochemistry of GOD. At the same time, the CNTs is a good support material and biocompatible materials and can load a large number of GOD molecules and keep its activity, so the CNTs played a positive role on the electronic transfer of GOD.

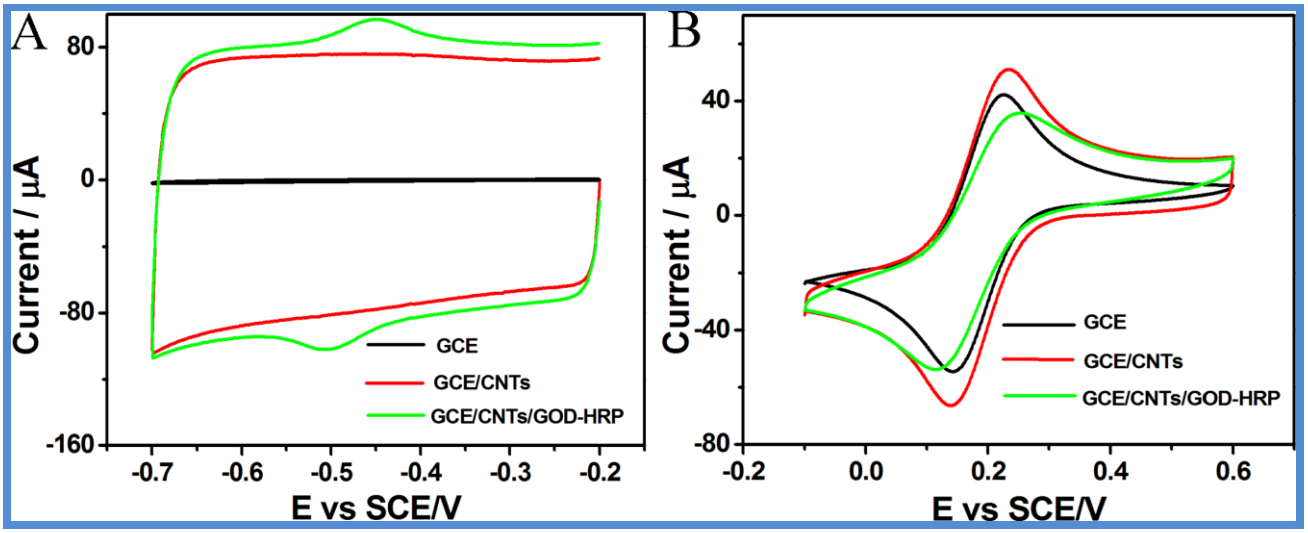

Figure 2. (A) CVs of the GCE (a) GCE/CNTs (b) and GCE/CNTs/GPD-HRP (c) in 0.2 M N_2-saturated PBS (pH 7.0). The scan rate is 100 mV s^{-1} (B)CVs of the GCE (a)GCE/CNTs (b) and GCE/CNTs/GPD-HRP (c) in 0.1 M KCl solution containing $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ with concentration of 5.0 mM, The scan rate is 100 mV s^{-1}.

The electron transfer behavior of the GCE/CNTs/GOD-HRP electrode was evaluated by CVin 0.1 M KCl solution containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ under scan rate of 100 mV $s^{-1.}$ As shown in Fig. 2B, the peak current increased significantly at the GCE/CNTs electrode as compared with the bare GCE electrode because the CNTs loaded on the GCE enlarged its specific surface area greatly. Furthermore, the peak-to-peak potential separation of GCE/CNTs electrode was estrange to that bare GCE electrode, indicating the CNTs have an important effect on the electron transfer of the GCE/CNTs electrode due to the excellent electrical conductivity of CNTs. When GOD-HRP complex further assembled into the surface of the CNTs/GCE electrode, and the redox peak current decreased significantly because GOD and HRP were protein molecules and had a barrier to the electron transfer. The results demonstrated the GOD-HRP were successfully assembled on the electrode.

Fig. 3A showed the CVs of the GCE/CNTs/GOD-HRP (n_{GOD}/n_{HRP} = 5:l) in 0.2 M PBS (pH 7.0) at various scan rates. As shown in Fig. 3A, the peak current enhanced gradually as the scan rate increased. The peak currents displayed good linear correlations with scan rate ranging from 100 mV s^{-1} to 1000 mV s^{-1}, indicating a quasi-reversible surface-confined electrochemical process (Fig. 3B). The electron transfer rate (k_s) could be estimated with the Laviron's equation [30]:

$$k_s = mnF\upsilon/RT \quad (1)$$

where n is the electron transfer number (n =2), F is the Faraday constant (F =96 493 C mol^{-1}), υ is the scan rate, R is the gas constant (R =8.314 J mol^{-1} K^{-1}), T is the temperature in Kelvin (T =298 K) and m is a constant which relates to ΔEp. The k_s of GCE/CNTs/GOD-HRP was calculated to be 1.518 s^{-1} at the scan rate of 100 mV s^{-1}. As a comparison, the k_s of GOD/MWCNTs modified electrode and PDDA/MWCNTs/AuNPs/GOD electrode were calculated to be 1.08 s^{-1} [31] and 1.01 s^{-1} [32] respectively, which were smaller than the k_s of 1.518 s^{-1}.

The average surface concentration of electroactive protein (Γ*, mol cm^{-2}) could be estimated by Faraday's law [33]:

$$I_P = \frac{nFvQ}{4RT} = \frac{n^2F^2A\Gamma^*v}{4RT} \quad (2)$$

which can come to the expression as follows:

$$\Gamma^* = \frac{Q}{nFA} \quad (3)$$

where Γ* is surface coverage of the redox materials, $\upsilon$is the potential scan rate, Q is the charge consumed in the CVs, F is the Faraday constant (F = 96485 C mol^{-1}), A is the electrode surface geometrical area, n is the overall number of electrons transferred in oxygen reduction and other symbols have their usual meaning. The value of Γ* which is calculated by Randles-Sevcik equation was about 2.08 x 10^{-10} mol cm^{-2} for GCE/CNTs/GOD-HRP， This surface coverage is far more than the coverage of the theoretical molecular monolayer(1.4 x 10^{-12} mol cm^{-2}), which attributable to large specific surface area of CNTs.

Fig. 3C showed the CVs of GCE/CNTs/GOD-HRP in 0.2 M N_2-saturated PBS at a scan rate of 100 mV s^{-1} over the pH from6.0 to 8.0 with a step of 0.5. With the increase of pH from 6.0 to 8.0, the anode and cathode peak potentials are shifted to the negative direction. As can be seen from Fig. 3D, it was proved that the standard potential E^0 (E^0 =1/2E_{pa}+1/2E_{pc}) of the GCE/CNTs/GOD-HRP shows a linear relationship with pH value, and the slope is -52.8mV pH^{-1} (R =0.999). As this value is very close

to the theoretical value of -58.6 mV pH^{-1} [34] for two-electron and two-proton transportation, the DET of this GCE/CNTs/GOD-HRP should also involve two electrons and two protons process.

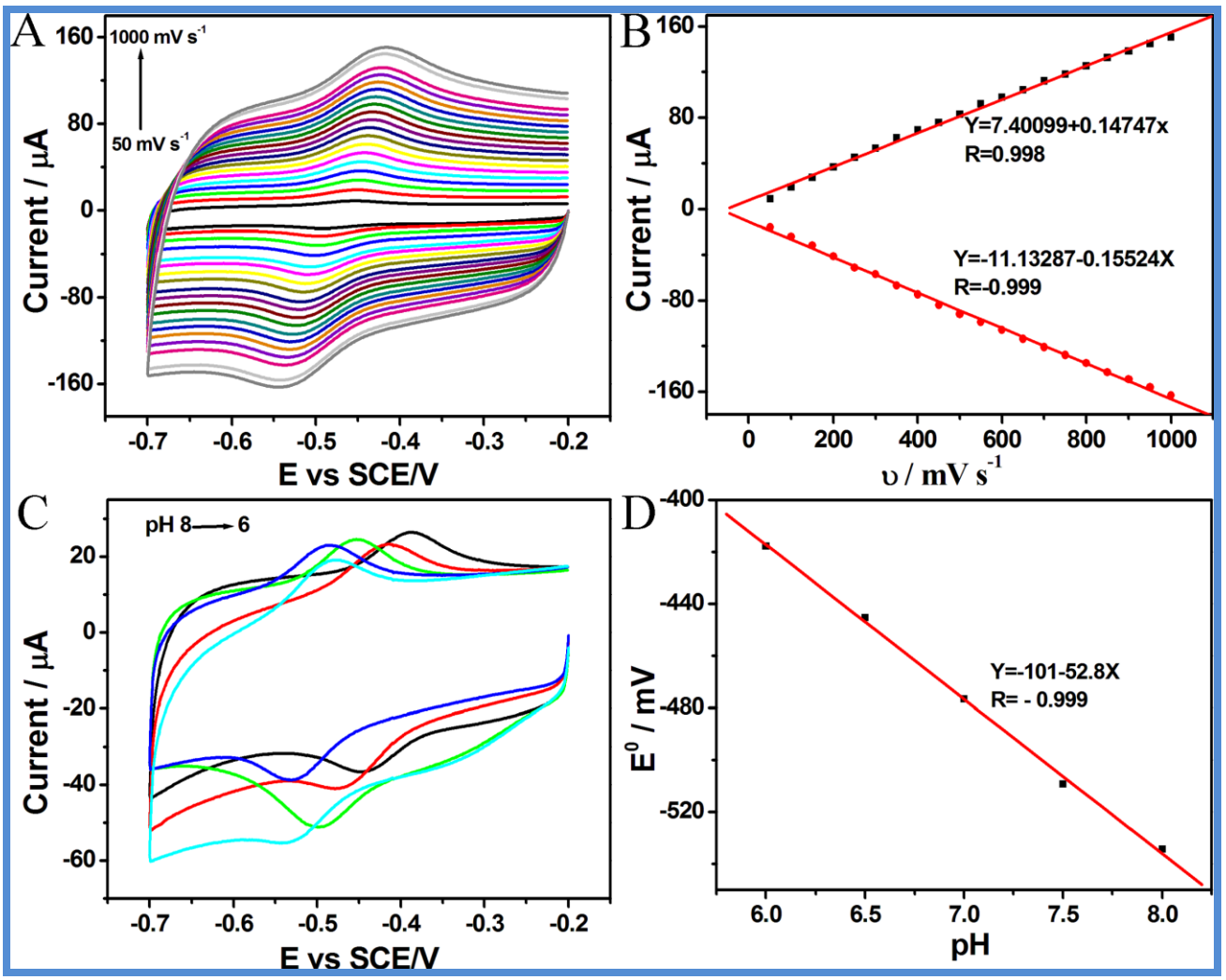

Figure 3. (A) CVs of the GCE/CNTs/GOD-HRP at different scan rates varied from 50 mV s^{-1} to 100 mV s^{-1} with a step of 50 mV s^{-1} in 0.2 M N_2 -saturated PBS (pH 7.0). (B) plot of peak current versus scan rate. (C) CVs of the GCE/CNTs/GOD-HRP in 0.2 M N_2 -saturated PBS with different pH values at 100 mV s^{-1}. (D) plot of E^0 ($E^0 = (E_{pa} + E_{pc})/2$) versus pH.

The molar ratio of GOD/HRP (n_{GOD}/n_{HRP}) play an important role in the electrochemical performance of the GCE/CNTs/GOD-HRP in 0.2 M N_2-saturated PBS at a scan rate of 100 mV s^{-1}. As shown in Fig. 4, when the n_{GOD}/n_{HRP} varied from 1:1 to 7:1, obvious change of peak currents were observed. The peak current increased gradually with the increase of n_{GOD}/n_{HRP} from 1:1 to 5:1, and the maximum value appeared at about 5:1. With the n_{GOD}/n_{HRP} further increased, the peak current tended to be reduced gradually. Previous result clearly demonstrated that two kinds of enzyme protein interactions could change the conformation of the protein, thus changed the symmetry properties of the porphyrin ring, such as cytc and GOD. In conclusion, these results revealed the structure of bi-protein

which is determined by the molar ratio of GOD and HRP plays an important role in the electron transfer.

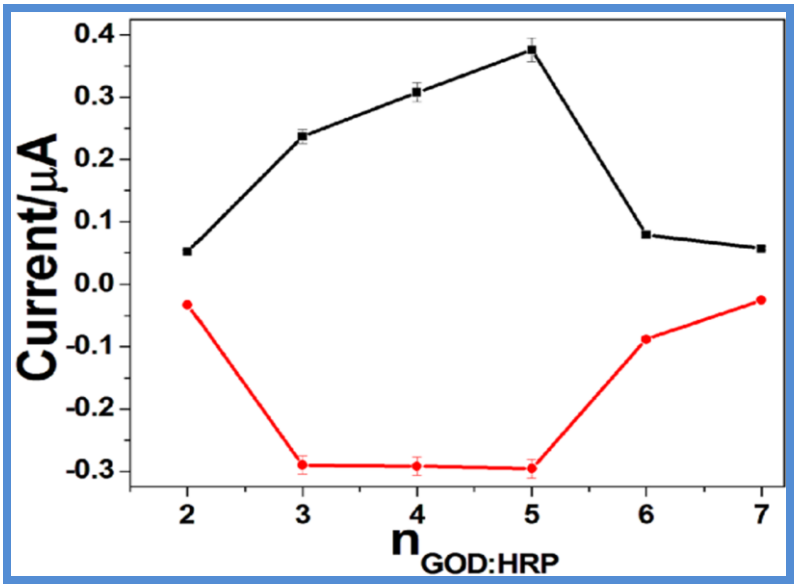

Figure 4. (A) Plot of oxidation peak current and reduction peak current of the GCE/CNTs/GOD-HRP versus molar ratio of n GOD/n HRP in a solution.

3.4. Electrocatalytic oxidation of glucose at the GCE/CNTs/GOD-HRP

Fig. 5A showed the CVs of the GCE/CNTs/GOD-HRP in in 0.2 M O_2-saturated PBS (pH 7.0) with various concentrations of glucose. As the adding of glucose, the reduction peak decreased gradually. As shown in Fig. 5B, and a detection limit of 7 mM was obtained based on the criterion of a signal-to-noise ratio of 3. The mechanism could be expressed as following equations [35-37].

$$\text{glucose} + \text{GOD (FAD)} \longrightarrow \text{gluconolactone} + \text{GOD (FADH}_2\text{)} \quad (4)$$

$$\text{GOD (FADH}_2\text{)} + O_2 \longrightarrow \text{GOD (FAD)} + H_2O_2 \quad (5)$$

$$H_2O_2 \xrightarrow{HRP} H_2O + \frac{1}{2}O_2 \quad (6)$$

In total:

$$\text{glucose} + O_2 \xrightarrow{GOD} \text{gluconolactone} + H_2O_2 \quad (7)$$

$$\text{glucose} + \frac{1}{2}O_2 \xrightarrow{GOD+HRP} \text{gluconolactone} + H_2O \quad (8)$$

Therefore, the cooperative interaction between GOD and HRP on the biointerphase leads to a glucose biosensor with a linear range from 0.022 to 7.0 mM (R =0.998, n=7) and a sensitivity of 5.14 μA mM^{-1} cm^{-2}. In order to show the advantages of the designed biosensor, the electrochemical performance of other glucose sensors in table 1 were compared with our designed biosensor. According to the results, the designed biosensor exhibited a lower detection limit(0.022 to 7.0 mM) and excellentsensitivity（5.14 μA mM^{-1} cm^{-2} ）toward glucose detection. This suggests that the development of bi-protein glucose biosensor is better than that of some previously reported ones.

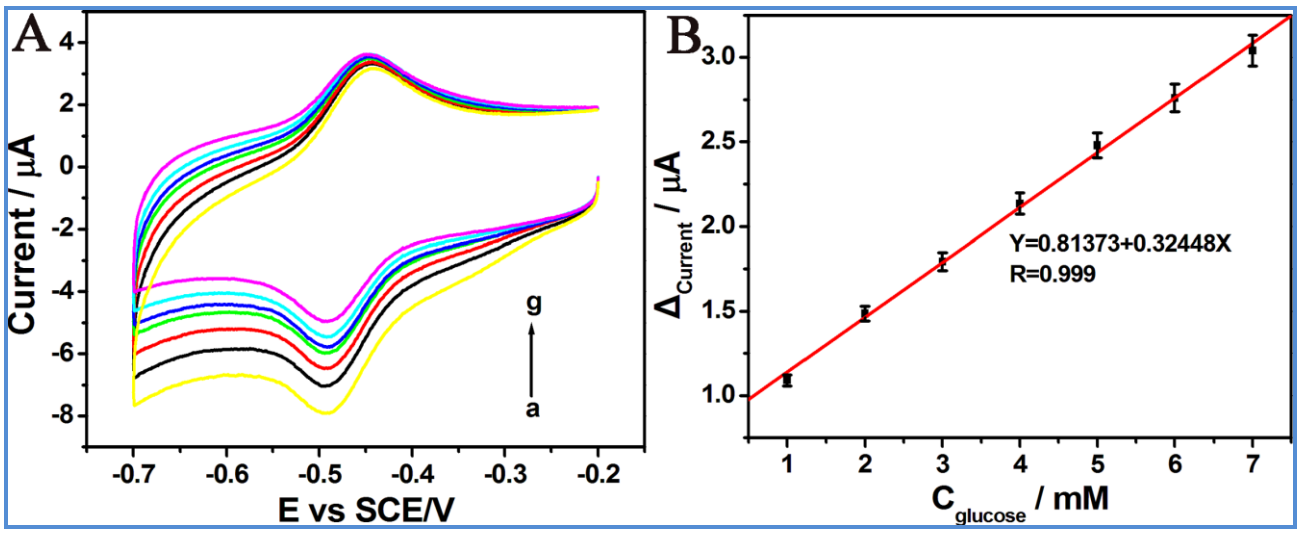

Figure 5. (A) the GCE/CNTs/GOD-HRP in 0.2 M O_2-saturated PBS (pH 7.0) at scan rate of 100 mV s^{-1} in the presence of (a) 1mM, (b) 2 mM, (c) 3 mM, (d) 4 mM, (e) 5 mM, (f) 6 mM, (g) 7mM glucose. (B) Plots of *ΔI* versus glucose concentration.

Table 1. Comparison of analytical performance of some GOD-based glucose biosensors

Glucose biosensors	Linear range(mM)	Detectin limit(μM)	Ref.
graphere/GOD	0.08-12.00	20	[38]
GCE/AuNPs/GOD	2-18	25	[39]
GCE/CHIT-AuNPs/GOD	2-12	0.37	[30]
GCE/CNTs/GOD	0-7.8	_____	[40]
Au/MUA-MCH/GOD-HRP/AuNP/4-Aminothiophenol	0.0165-10	5.4	[11]
Nafion/41-MCM/GOD	0.32-15.12	180	[41]
ITO/AuNP/cysteamine/GOD	0.04-4.8	15	[42]
GCE/CNTs/GOD-HRP	0.022-7	7	This work

3.5. Selectivity, repeatability and stability of the sensor

We evaluated the selectivity of the sensor, repeatability, stability. Interference from chemicals of uric acid (UA), ascorbic acid (AA), L-glutamic acid and L-cysteine in a 2-fold concentration was negligible. In addition, the relative standard deviation (RSD,n=5) was about 4.82% for five individual measurments, showing a good repeatability. After the as-prepared electrode was stored in N_2-saturated PBS (pH 7.0) at 4 ºC in a refrigerator for 7 days, the current response only decreased by 8.37% of the original response. Fifteen days later, the biosensor was still retained 89.35% of the initial response and superior to our previous work, indicating a good stability.

4. CONCLUSIONS

The CNTs/GOD-HRP was successfully assembled onto the GCE electrode to construct a glucose biosensor. It was found that the ratio of GOD to HRP has an important effect on electrochemical responses of GCE/CNTs/GOD-HRP. Biological activity of GOD was greatly retained due to the biocompatible micro-environment generated by the HRP which surrounded GOD. The results showed that the CNTs could be used to effectively load a large number of GOD-HRP for sensitively detect glucose. Thanks to the good supporting matrix CNTs and the cooperation of mixed proteins, electrochemical detection of glucose could be achieved with a wide linear range and low detection limit. The present work may provide new insights for studying electron transfer of multi-proteins system and developing advanced bio-interphase biosensors.

SUPPORTING INFORMATION:

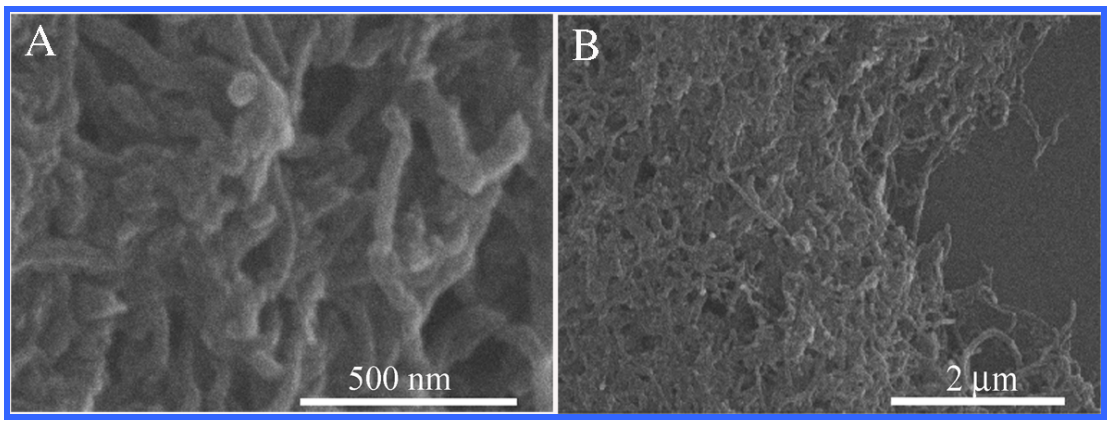

Figure S1. (A) High-magnification and (B) Low-magnifaction SEM images of CNTs.

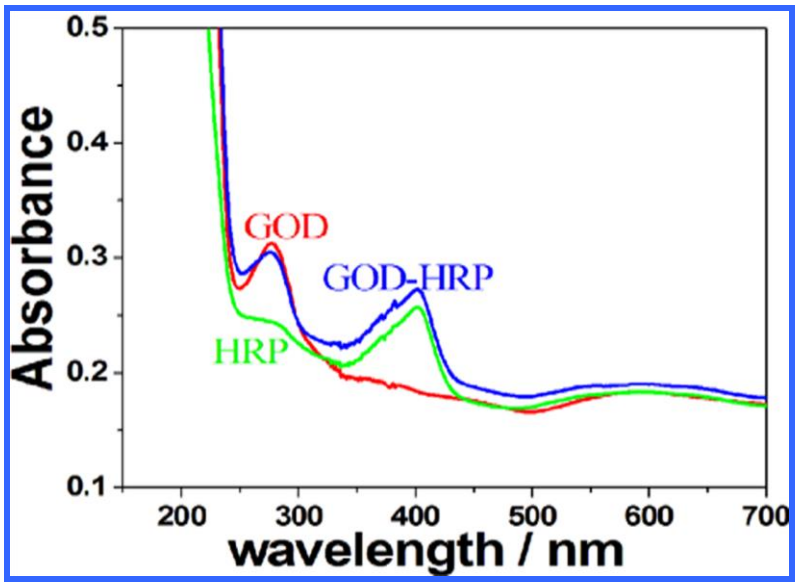

Figure S2. UV-vis spectra of GOD in a solution, HRP in a solution and GOD-HRP in a solution.

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