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Facile and controllable preparation of glucose biosensor based on Prussian blue nanoparticles hybrid composites

Lei Li, Qinglin Sheng, Jianbin Zheng *, Hongfang Zhang

Institute of Analytical Science / Shaanxi Provincial Key Lab of Electroanalytical Chemistry, Northwest University, Xi'an, Shaanxi 710069, China

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ABSTRACT

A glucose biosensor based on polyvinylpyrrolidone (PVP) protected Prussian blue nanoparticles (PBNPs)-polyaniline/multi-walled carbon nanotubes hybrid composites was fabricated by electrochemical method. A novel route for PBNPs preparation was applied in the fabrication with the help of PVP, and from scanning electron microscope images, Prussian blue particles on the electrode were found nanoscaled. The biosensor exhibits fast current response (< 6 s) and a linearity in the range from 6.7×10^{-6} to 1.9×10^{-3} M with a high sensitivity of 6.28 μA mM^{-1} and a detection limit of 6×10^{-7} M (S/N=3) for the detection of glucose. The apparent activation energy of enzyme-catalyzed reaction and the apparent Michaelis–Menten constant are 23.9 kJ mol^{-1} and 1.9 mM respectively, which suggests a high affinity of the enzyme-substrate. This easy and controllable construction method of glucose biosensor combines the characteristics of the components of the hybrid composites, which favors the fast and sensitive detection of glucose with improved analytical capabilities. In addition, the biosensor was examined in human serum samples for glucose determination with a recovery between 95.0 and 104.5%.

1. Introduction

For rapid response, high sensitivity and instinctual selectivity, enzymatic based biosensors have been studied extensively. Research in this field is still focused on the improvement of sensor properties by new sensing approaches. The glucose oxidase (GOD)-based biosensor for glucose sensing continues to be the primary model system in the development of new sensing materials and methods [1–5]. Owing to the usefulness in diagnostic analysis of diabetes, GOD-based biosensors have been studied greatly [6–8]. In this protocol, the glucose concentration can be obtained indirectly by amperometric detection of the oxidation current of hydrogen peroxide (H_2O_2), a side product during the enzymatic reaction. However, a great drawback for the model is represented by high over-potential (ca. 0.6 versus Ag/AgCl) [9] required for H_2O_2 oxidation which leads to interferences caused by the oxidization of many electroactive substances (i.e., ascorbate, urate, acetaminophen, etc.) in real samples. And several approaches have been employed to solve the problem, for example, utilizing negatively charged membranes on the electrode tip to expel the interference [10] or electron mediators to lower the oxidation potential for H_2O_2, such as horseradish peroxidase [11,12] and ferrocene [13–15].

Due to its structure, Prussian blue (PB), ferric hexacyanoferrate, has a perfect catalytic activity for H_2O_2 reduction with a rate constant of 3×10^3 M s^{-1}. Because of this outstanding catalytic property, PB was eulogized as an "artificial peroxidase" by Karyakin [16–18] and has been widely used in the biosensor construction as an attractive alternative electrocatalyst for the reduction of H_2O_2 to suppress the interference [19,20]. Compared to those of bulk materials, nanosized PB with small size, large surface-to-volume ratio and the increased surface activity qualify its use in catalysis and sensing. Recently, several groups have reported the marvelous electrocatalytic character of inorganic Prussian blue nanopaticles (PBNPs) towards the reduction of H_2O_2 compared with that of the conventional PB microparticles or polycrystalline film [21–23]. Zhang et al. developed PB nanoclusters from an acidic solution of ferricyanide by potentiodynamic technique [24]. Vaucher et al. synthesized PBNPs and nanocrystal superlattices in reverse microemulsions [25]. Zhai et al. developed a new nanocomposite by combination of PBNPs and multi-walled carbon nanotubes (MWNTs) in the matrix of biopolymer chitosan (CS). The electrode modified with this nanocomposite can extensively amplify the response current towards H_2O_2. The glucose biosensor based on the modified electrode also exhibits good analytical character [26]. On the other hand, composite materials based on integration of carbon nanotubes (CNTs) and some other materials to possess properties of the individual components with a synergistic effect have gained growing interest also. Materials for such purposes include conducting polymers [27,28], redox mediators [29], metal and metal oxide nanoparticles [30,31]. For its facile preparation, high conductivity and good environmental stability, polyaniline (PANI), a conducting polymer, become the most attractive one in formation of CNTs based

* Corresponding author. Tel.: +86 29 88302077; fax: +86 29 88303448.
E-mail address: zhengjb@nwu.edu.cn (J. Zheng).

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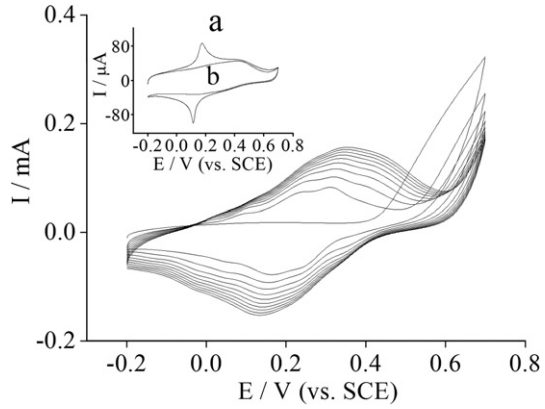

Fig. 1. Cyclic voltammograms recorded during the electropolymerization of PANI at a MWNTs/GCE in 0.1 M aniline + 0.1 M HCl by potential cycling between −0.2 and 0.7 V (vs. SCE) at a scan rate of 0.1 V s^{-1}. Inset shows the cyclic voltammograms of PBNPs-PANI/MWNTs/GCE (a) and PANI/MWNTs/GCE (b) obtained in pH 7.0 0.067 M PBS + 0.1 M KCl.

composites. PANI/CNTs composites synthesized chemically [28] or electrochemically [32] have improved the electrical conductivity, electrochemical capacitance and electrocatalytic properties of the polymers [33,34].

In this paper, we fabricated PBNPs-PANI/MWNTs composites modified glassy carbon electrode (GCE) electrochemically and a glucose biosensor was prepared by immobilizing GOD in crosslinked CS network on the modified electrode. Firstly, PANI was electropolymerized onto the MWNTs modified GCE. Then, a novel route for PBNPs fabrication was employed by depositing PBNPs electrochemically onto the surface of PANI/MWNTs/GCE from the $Fe_2(SO_4)_3$–$K_3[Fe(CN)_6]$ solution containing polyvinylpyrrolidone (PVP). Scanning electron microscope (SEM) was used to characterize the surface morphology of the modified electrode. The applied condition and performance of the resulting biosensors were further investigated in detail. Additionally, the resulting glucose biosensor was tested in human serum samples.

2. Experimental

2.1. Reagents

Carboxyl-functionalized MWNTs (>95 wt.%, 30–50 nm, Chengdu Organic Chemical Research Institute, Chengdu, China), Aniline (≥99.5%, Tianjin Kermel Chemical Reagents Development Centre, Tianjin, China), GOD (>300 u/g, *Aspergillus niger*, Sigma, America), CS (deacetylation>90%, Yuanju Bio-tech Co., Ltd, Shanghai, China), PVP (K30, Jinyu Fine Chemical Co., Ltd, Tianjin, China).

Glucose stock solutions were allowed to mutarotate at room temperature overnight before use. Other reagents were all of analytical grade and used as received without purification. The 0.067 M phosphate buffer solution (PBS) made from Na_2HPO_4 and KH_2PO_4 was applied as supporting electrolyte. All solutions were prepared in doubly distilled water.

2.2. Apparatus

All electrochemical experiments were carried out in a three-electrode cell controlled by a CHI 660 electrochemical workstation (Chenhua Instruments, Shanghai, China). A glucose sensor acted as the working electrode. A saturated calomel electrode (SCE) and a platinum wire served as reference and counter electrode, respectively. SEM images were taken by using JSM-6460 SEM (JEOL, Japan).

2.3. Fabrication of the biosensor

2.3.1. Electropolymerization of PANI onto the MWNTs/GCE

With the help of ultrasonic agitation, 2.5 mg pretreated MWNTs was well dispersed into 5 mL *N*,*N*-dimethylformamide. Before modification, the GCE was carefully polished with 0.05 μm alumina slurry, then ultrasonicated in double-distilled water and the anhydrous ethanol and dried in the open air. After the ultrasonication, the pretreated GCE was coated with a suspension (8 μL) of MWNTs and allowed to be dried under an infrared lamp.

First, PANI was electrochemically polymerized onto MWNTs coated GCE. The detail procedure was described as follow. The PANI/MWNTs modified electrode was fabricated in the solution containing of 0.1 M aniline in 0.1 M HCl by cycling between −0.2 and 0.7 V (vs. SCE) at a scan rate of 0.1 V s^{-1} for 10 cycles. The modified electrode was rinsed thoroughly with double-distilled water and dried in air.

2.3.2. Electrodeposition of PBNPs onto the PANI/MWNTs/GCE

The electrodeposition of PVP protected PBNPs onto the PANI/MWNTs/GCE was carried out secondly. A mixture of aqueous solutions was prepared by adding the solution of $K_3[Fe(CN)_6]$ into the solution of $Fe_2(SO_4)_3$ containing PVP, KCl, and HCl, and the final concentration was 1 mM $K_3[Fe(CN)_6]$ + 0.5 mM $Fe_2(SO_4)_3$ (PVP 10 mM) with 0.1 M KCl and 0.01 M HCl [35,36]. The PBNPs-PANI/MWNTs modified electrode was fabricated in the resulting solution by potential cycling between −0.2 and 0.7 V (vs. SCE) for 10 cycles at a scan rate of 0.1 V s^{-1}. After rinsed with double-distilled water, the PBNPs-PANI/MWNTs modified electrode was dried in air and later under the infrared lamp for approximately 2 h allowing the so-called zeolitic water removed from the PB polycrystal irreversiblely [37].

2.3.3. Fabrication of biosensor based on PBNPs-PANI/MWNTs/GCE

5 mg of GOD was dissolved in 1 mL of CS solution (1 wt.%), then 40 μL of glutaraldehyde solution (2.5%) was added into the GOD-CS solution. The mixture was hand-mixed completely. The glucose biosensor was constructed by coating a drop of 10 μL the resulting solution onto the modified electrode, and the biosensor was allowed

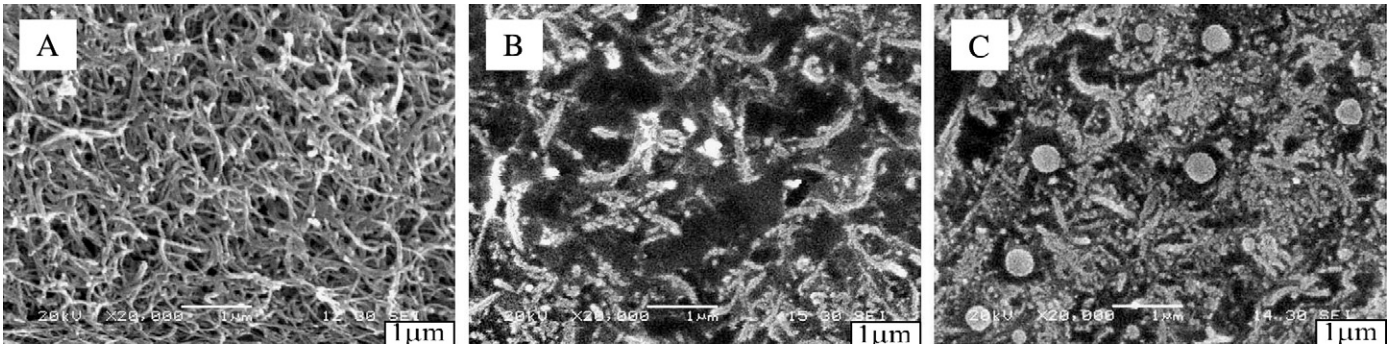

Fig. 2. SEM images of the modified GCEs: MWNTs/GCE (A), PANI/MWNTs/GCE (B) and PBNPs-PANI/MWNTs/GCE (C).

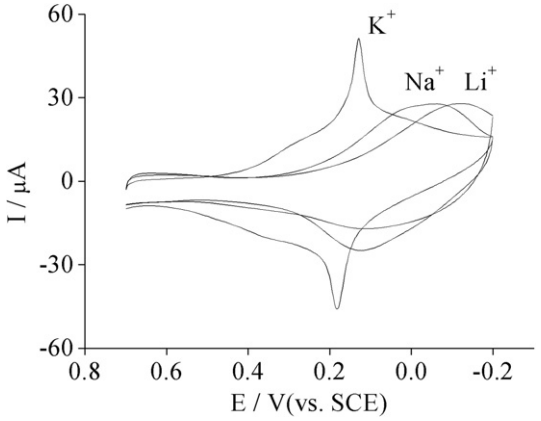

Fig. 3. Cyclic voltammograms of PBNPs-PANI/MWNTs/GCE in different cation solutions.

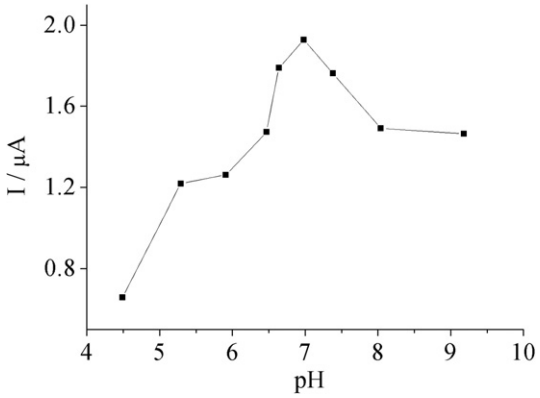

Fig. 5. Effect of the pH value applied on the current response in 0.067 M PBS+0.1 M KCl at 0.0 V (vs. SCE).

to be left for about 1 day at 4 °C in a refrigerator [38]. The biosensor was stored at 4 °C in a refrigerator when not in use.

3. Results and discussion

3.1. Preparation and characterization of PBNPs-PANI/MWNTs/GCE

In the present work, PANI was electrochemically polymerized on the MWNTs/GCE in a 0.1 M HCl solution containing 0.1 M aniline by potential cycling between −0.2 and 0.7 V (vs. SCE) at a scan rate of 0.1 V s^{-1} for 10 cycles to obtain a compact and relatively thin film. From the Fig. 1, three pairs of peaks can be observed indicating the presence of discrete electroactive regions in the film. Before and after the electrodepostion of PBNPs, the modified electrode was studied using cyclic voltammetry (the inset). It can be seen that curve a almost tallies with curve b and has a couple of reversible redox peaks, which suggests PB was deposited on the PANI/MWNTs/GCE successfully.

SEM was used to characterize the morphologies of modified electrodes, MWNTs/GCE, PANI/MWNTs/GCE and PBNPs-PANI/MWNTs/GCE (Fig. 2). As shown in Fig. 2A, the MWNTs with large surface area are well distributed on the surface with a diameter ranging from 30 to 50 nm in the form of small bundles and single tubes which is believed to be very beneficial for the performance of the modified electrode. After the polymerization of PANI on MWNTs/GCE, the majority of MWNTs has been entrapped in the PANI film (Fig. 2B). As shown in Fig. 2C, PB is electrochemically deposited on the surface of PANI/MWNTs with the diameter nanoscaled. Some larger particles can also be seen on the surface with diameter about 400 nm which seems to be the accumulation of the PBNPs. So from these SEM images, it can be indicated that the electrochemically synthesis of PVP protected PBNPs is applicable.

3.2. Effect of applied parameters on the biosensor response

The effects of Li^+, Na^+, K^+ in solution on the electrochemical behavior of PB mediator was estimated by cyclic voltammetry method in PBS solutions containing 0.1 M Li^+, Na^+, K^+ respectively. As shown in Fig. 3, the modified electrode showed a pair of ill-defined peaks with large peak width and peak to peak separation in Li^+ and Na^+, while it showed a pair of well-defined and sharp peaks in K^+. This behavior has been explained in terms of the hydrated ionic radius and the channel radius of the PB lattice [19]. The results indicate that the solution containing K^+ suit the use of PB mediator.

Choosing the applied potential at the working electrode gives fundamental contribution to achieve the lowest detection limit and avoid the electrochemical interferences. The applied potential changed from 0.2 to −0.2 V (vs. SCE) and the effect of the applied potential to the sensitivity of the enzyme sensor was illustrated in Fig. 4. The maximum current response was obtained at −0.1 V. However, taking sensitive responses, effective avoiding interference and operational stability into consideration, 0.0 V was selected as the applied potential in subsequent experiments.

Investigation of the pH value of the detection solution on the performance of the biosensor is of great importance, because the activity of the immobilized GOD is pH dependent [39]. The current response increased in the pH range 4.49 to 7.0, then decreased, a small flat appeared at about pH 5.0 to 6.0, and the background current increased at higher pH values (Fig. 5). The current response reached its

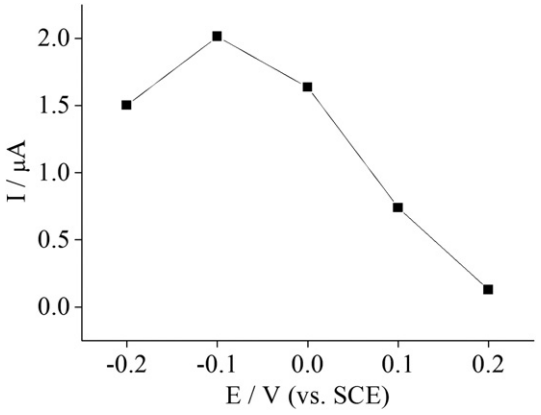

Fig. 4. Effect of applied potential on the current response in pH 7.0 0.067 M PBS + 0.1 M KCl.

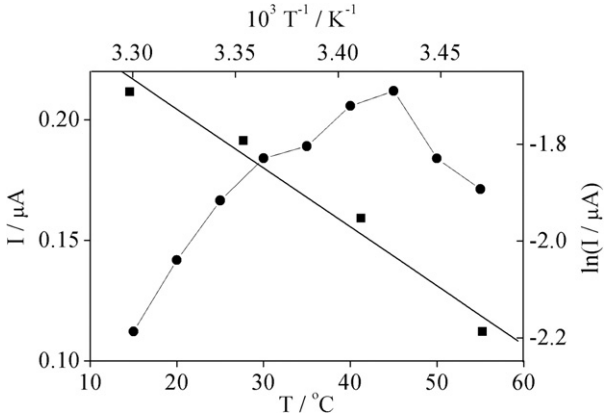

Fig. 6. (a) The influence of temperature on the current response in pH 7.0 0.067 M PBS + 0.1 M KCl at 0.0 V (vs. SCE). (b) Plots of ln*I* against T^{-1} according to the data in curve a.

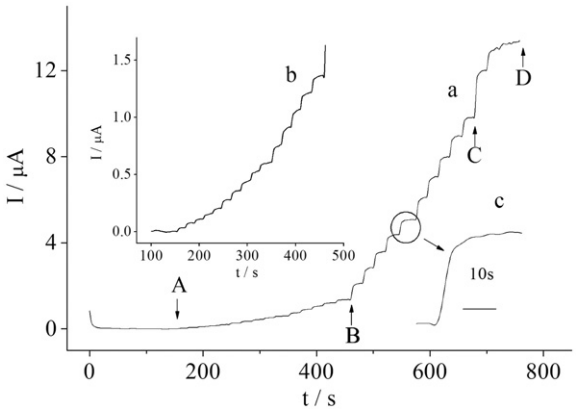

Fig. 7. (a) Current–time response curves obtained at the glucose biosensor for successive addition of glucose:, 2 mM steps; B→C, 20 mM steps; C→D, 0.2 M steps pH 7.0 0.067 M PBS+0.1 M KCl at 0.0 V(vs. SCE). (b) A→B part of the current–time response curves. (c) Steady-state current–time response of the biosensor.

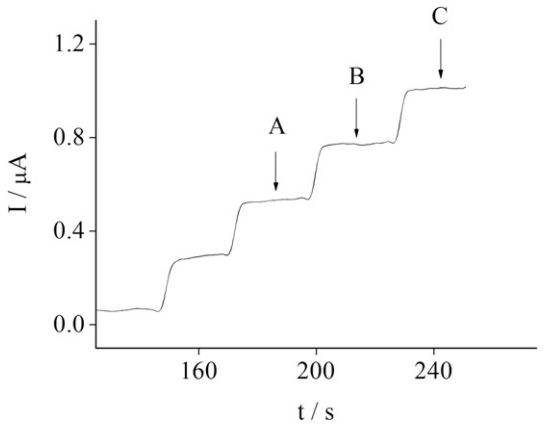

Fig. 9. Current–time recording at the biosensor for addition of (A) 0.2 mM uric acid, (B) 0.2 mM L-cysteine and (C) 0.2 mM acetaminophen to each addition of 1 mM glucose.

maximum at pH 7.0 which may be due to the immobilization of GOD in CS matrix making GOD more active in neutral medium. For the maximum current response of the free glucose oxidase was reported at pH 5.6 [40,41], the flat indicates the optimum pH of GOD has not changed. Moreover, PB is unstable at alkaline pH [19] and it would lead to the slow decrease of the signal, pH 7.0 was selected so as to ensure higher sensitivity and stability of the biosensor.

The effect of temperature on the biosensor based on PBNPs-PANI/MWNTs modified electrode has been evaluated from 15 to 55 °C. Before the amperometric detection, the biosensor was immersed in the buffer solution at different temperatures until thermal equilibrium. The result is shown in Fig. 6a. The current response increased with the temperature and a maximum current value was obtained at 45 °C. When the temperature was over 45 °C, the response decreased for the denaturation of the enzyme. As the optimum temperature of free GOD at around 30 °C [42], it is about 45 °C for the immobilized GOD which may be due to the good microenvironment provided by CS matrix to GOD.

According to the Arrhenius formula [43],

$$\ln(I/\mu A) = \ln(I/_0\mu A) - Ea/rt \quad (1)$$

where I represents the steady-state current response, I_0 stands for a collection of currents and Ea is the apparent activation energy. The relationship between $\ln(I/\mu A)$ and T^{-1} was plotted by using the data in Fig. 6b, and one straight line was obtained. The apparent activation energy can be calculated from the slope and the value of Ea in the phosphate buffer is 23.9 kJ mol^{-1}, which is similar to 25.3 kJ mol^{-1} obtained by previous work in phosphate buffer [39].

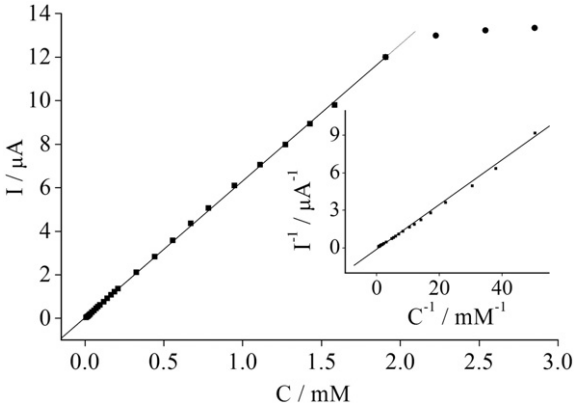

Fig. 8. Calibration curve of glucose on the biosensor in pH 7.0 0.067 M PBS+0.1 M KCl at 0.0 V (vs. SCE). Inset shows the determination of the apparent Michaelis–Menten constant.

3.3. The response characteristics of the glucose biosensor

As well known, the quantification of glucose can be achieved via electrochemical detection of the enzymatically liberated H_2O_2. The response of the glucose biosensor based on the PBNPs-PANI/MWNTs modified electrode to H_2O_2 was tested. The calibration curve of H_2O_2 at the biosensor showed a linearity in the range of 2.0×10^{-6} to 3.2×10^{-3} M (r=0.9954).

Fig. 7a illustrates a typical current-time curve of the glucose biosensor upon the addition of different concentration of glucose at 0.0 V in a 0.067 M PBS buffer solution containing 0.1 M KCl. The 95% of the steady-state current can be obtained at about 4 to 6 s by using the biosensor (Fig. 7c) which is faster than other glucose sensors [44–46]. The current response increased along with glucose concentration. The resulting calibration curve was linear in the range of 6.7×10^{-6} to 1.9×10^{-3} M (r=0.9998) as shown in Fig. 8. The sensitivity of 6.28 μA mM^{-1} is higher than other glucose biosensor based on PB or composites formed with other materials [47–51]. Even at low concentration of glucose, obvious current response can be observed (Fig. 7b). The detection limit of the biosensor is 6×10^{-7} M at the S/N ratio of 3.

The apparent Michaelis–Menten constant K_M is generally used to evaluate the biological activity of immobilized GOD, and it could be calculated according to the Michaelis–Menten equation [52],

$$1/I = 1/I_{max} + K_M/(I_{max} + C) \quad (2)$$

where I is the steady-state catalytic current, I_{max} is the maximum current measured under saturated substrate conditions, C refers to the glucose concentration and K_M stands for the apparent Michaelis–Menten constant of the system in this work. Based on the experimental data from the inset of Fig. 8, K_M was evaluated as 1.9 mM and the I_{max} is 10.9 μA which revealed that the whole system was controlled by the catalytic kinetic process of the enzyme. The value of K_M is lower than the reported 33 mM for GOD in solution phase [53], 11.09 mM for polyaniline glucose biosensor prepared with

Table 1
The determination of glucose in human serum

Sample no.	Concentration of glucose/mM		R.S.D. (%)[a]
	Glucose test meter	Biosensor	
1	5.1	5.4	2.2
2	5.0	5.2	1.8
3	4.6	4.4	2.4

[a] The R.S.D. of the five determinations by glucose biosensor.

Table 2
Recovery of glucose in human serum

Sample no.	Concentration of glucose in serum SD[a]/mM	Added/mM	Found[a]/mM	Recovery /%
1	1.43±0.02	1.60	1.67	104.5
2	2.87±0.09	3.20	3.04	95.0

[a] Average of five determinations, using the glucose biosensor applied at 0.0 V (vs. SCE).

template process [54], 6.7 mM for sol–gel method immobilized GOD on PB modified electrode [50] and 5.1 mM for GOD immobilized in Nafion on PB/PANI/MWNTs modified electrode [51]. It can be seen that high affinity can be accessed after the construction of the biosensor. The good microenvironment due to the immobilization method and the synergistic effect of the hybrid composites might contribute to the improvement of the affinity and good performances of the biosensor.

3.4. Interference tests

The most common electrochemical interfering species such as ascorbic acid, uric acid, L-cysteine and acetaminophen were evaluated. As shown in Fig. 9, 0.2 mM uric acid, 0.2 mM L-cysteine and 0.2 mM acetaminophen were added to each addition of 1.0 mM glucose and did not produce observable interference in the response, respectively, which may be attributed to the low potential applied. According to the real ratio of the ascorbic acid concentration to glucose, the interference of ascorbic acid was also tested by adding the mixture containing 0.3 mM ascorbic acid and 20 mM glucose to each addition of 20 mM glucose. And the current reduced 6.3% of original response of glucose solution. The results show the glucose biosensor has good anti-interferent ability.

3.5. Human serum samples detection

Human serum samples were assayed to demonstrate the practical usage of the glucose biosensor. Fresh serum samples were supplied by the Affiliated Hospital of Northwest University in China. The samples of whole blood were first analyzed by blood glucose test meter (SUPER GLUCOCARD II GT-1640) for the detection of glucose and centrifuged as soon as possible. After centrifugation, serum samples were separated and assayed by the enzyme modified electrode. As shown in Table 1, the results show a good agreement with those assayed by glucose test meter. Recoveries of added glucose in serum sample were also estimated. The recovery is between 95.0 and 104.5% (Table 2). From these results, it can be indicated that the detection of glucose using the enzyme biosensor based on PBNPs-PANI/MWNTs modified electrode is effective.

3.6. Reproducibility and stability of the biosensor

The reproducibility of glucose biosensor construction was estimated from the response to 0.5 mM glucose at five different sensors. The results revealed that the biosensor has satisfied reproducibility with a R.S.D. of 5.1%. The operational stability of the biosensor was tested at 0.0 V by five addition of 20 mM glucose to the supporting electrolyte solution. A good operational stability of the biosensor can be accessed with less than 1.1% relative deviation for five times continuous determinations of the same sample (R.S.D.=3.2%). The storage stability of the glucose biosensor was also estimated every three days. It was found that the biosensor retained about 90% of its original response after three weeks. The good stability could be due to the good biocompatibility and stabilizing microenvironment around the enzyme provided by the organically modified CS composite matrix.

4. Conclusions

In this work, a new glucose biosensor with GOD immobilized in cross-linked CS network was constructed on PBNPs-PANI/MWNTs hybrid composites modified GCE simply and reliably. A novel route for nanosized PB preparation is proposed. In contrast to the chemical method of preparation of PB and PANI, the electrochemical method is more easy and controllable. And because of this cause, the hybrid composites film is relative thin and thus the enzyme may be close to the surface of the electrode. The short distance of enzyme-to-electrode and the synergic effect of the nanosized materials may be the reason why this glucose biosensor exhibits fast response, high sensitivity and wide linearity. Good reproducibility and stability can be attributed to the suitable microenvironment for the enzyme immobilization. The glucose biosensor was successfully applied in human serum samples determination. Moreover, the potential use of this approach in other oxidase enzyme biosensor can be expected.

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