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A glucose biosensor based on graphene-Prussian blue-chitosan composite film fabricated by electrodeposition

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Abstract: The layer-by-layer structured composite film of graphene-Prussian blue (PB)-chitosan (CS) was fabricated on a glassy carbon electrode by three-step electrodeposition method and used for glucose sensing. Graphene nanosheets were directly deposited onto the electrode through reduction of graphene oxide by cyclic potential scanning. Then, a glucose biosensor was fabricated by electrodepositing PB nanoparticles and glucose oxidase (GOD)-chitosan hybrid film (GOD-CS) on the graphene modified electrode successively. The surface of the resulting modified electrode was characterized by electrochemical methods and scanning electron microscopy. Under optimal conditions, the biosensor shows high sensitivity (50.29 mA · L · mol^{-1} · cm^{-2}), low detection limit (12 μmol · L^{-1}) and fast response time (3 s). A linear dependence of the catalytic current upon glucose concentration is obtained in a wide range from 0.02 to 10 mmol · L^{-1}. In addition, the sensor also performs well for measuring glucose concentrations in human blood serum samples without any pretreatment.

Key words: glucose biosensor; graphene; Prussian blue; chitosan; electrochemical deposition

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基于电沉积石墨烯/普鲁士蓝/壳聚糖复合薄膜的葡萄糖生物传感器

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摘要:在玻碳电极表面通过三步电沉积法制备了石墨烯/普鲁士蓝/壳聚糖复合薄膜葡萄糖生物传感器.通过循环伏安法将氧化石墨烯电化学还原,在电极表面直接得到石墨烯纳米层,在石墨烯纳米层上成功电沉积得到普鲁士蓝纳米粒子和葡萄糖氧化酶-壳聚糖复合薄膜,制备的修饰电极通过电化学方法以及扫描电镜分析了其性能与结构.在最优条件下,该生物传感器表现出了灵敏度高(50.29 mA · L · mol^{-1} · cm^{-2})、检测限低(12 μmol · L^{-1})、响应时间短(3 s)等特点.电极响应电流与葡萄糖溶液浓度在0.02~10 mmol · L^{-1}范围内具有较好的线性关系.此外,在对人体血清样本的检测中,该传感器同样表现出优异的性能,对血清中

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常见物质具有较强的抗干扰能力.

关键词：葡萄糖生物传感器；石墨烯；普鲁士蓝；壳聚糖；电化学沉积

0 Introduction

Diabetes has become one of the most common public health problems now days. Therefore, the research on the detection technique of the human body glucose is of great significance and numerous studies have been reported. Amperometric enzyme electrodes, based on glucose oxidase (GOD), have been playing a leading role in the move towards simple easy-to-use blood sugar testing. Usually, the detection is accomplished by monitoring hydrogen peroxide generated by the GOD catalyzed reaction of glucose with oxygen[1]. However, the higher positive working potentials might lead to interferences from reducing species such as ascorbic acid and uric acid. To solve this problem, the electroactive mediators have been adopted to improve the electron transfer of hydrogen peroxide at the substrate electrode which allows monitoring glucose at lower operational potentials.

Graphene has recently attracted a tremendous amount of attention. Its novel physicochemical properties that arise from the strictly two-dimensional (2D) and one-atom-thick geometry[2], such as high surface-to-volume ratio, superior thermal conductivity, excellent electrical conductivity and robust mechanical properties, which make it a strong contender for potential applications in synthesis of nanocomposites, fabrication of various sensors and microelectronic devices[3-6]. One of the promising applications of graphene is electrochemical sensing, since the graphene modified electrode exhibits wonderful electrochemical behaviors. So far, most of the graphene films on electrodes have been obtained by drop-coating of the chemically reduced graphene oxide (GO) sheets[7]. However, the prepared graphene film by drop-coating method is not stable, and the method is also not efficient. Recently, electrochemical reduction of GO has drawn great attention due to its facile and green nature, and the thickness of films can transform with deposition time and deposition potentials. In this study, we prepared graphene film on electrode directly in a GO dispersion solution by electrodeposition, according to Ref. [8].

Prussian blue (PB) is an important inorganic polycrystal with well-known electrochromic and electrocatalytic properties. Since it was deposited on the surface of electrode by Neff in 1978[9], PB has been widely employed in many research areas, such as electrochemistry[10], electrochromics[11], magnetics[12] and potential analytical applications[13-14]. As a most advantageous transducer, PB has rapid catalytic reaction rate toward reduction of hydrogen peroxide at lower overpotential[14], leading to many researches focused on PB-based sensors and biosensors, which can exclude the interference from the coexisting substances such as ascorbic acid (AA), dopamine (DA) and uric acid (UA) in biological samples[14-15]. Furthermore, the nanocomposites of PB and graphene, which can produce signal amplification and increase the sensitivity of biosensor, have also been investigated in recent years.

Also, chitosan (CS) is a kind of natural porous biopolymer with attractive characteristics such as biocompatibility, nontoxicity, high mechanical strength and excellent film-forming ability. In most cases, CS membranes containing GOD were often manually coated on the modified electrode to fabricate biosensors[16-17]. However, it may result in uncontrollable thickness of the film and poor reproducibility. Recently, electrochemical deposition has been reported as a simple and controllable method for the formation of CS film. Specifically, CS is a water-soluble polyelectrolyte at lower pH (< 6.3). When the pH value is high than 6.3, it becomes insoluble. This property allows CS hydrogel to be deposited onto the electrode by increasing the local pH at electrode

surface and the film layer can tightly attached to the electrode and retain its natural properties. More importantly, other substances such as metal nanoparticles and enzymes such as GOD can be incorporated to form the biocomposites during the electrodeposition process[18-19].

Herein, a layer-by-layer structured nanocomposite film glucose biosensor was constructed successfully based on electrodepositing of graphene (EG), PB and GOD-CS hybrid film onto the surface of a glassy carbon electrode, respectively. GOD was mixed with chitosan and immobilized in situ in the deposited chitosan hydrogel during the electrodeposition process. As a result, this glucose biosensor showed good performances and was applied to detect glucose in human blood serum samples. The characteristics of the biocomposite biosensor were investigated in detail.

1 Experimental

1.1 Chemicals and reagents

All chemicals were of analytical grade and used as received. Oxidized graphite was synthesized from natural flake graphite by the Hummers method. Potassium ferricyanide ($K_3Fe(CN)_6$), potassium chloride (KCl), ferric chloride ($FeCl_3$), L-cysteine, L-cystine, L-histidineand and hydrogen peroxide (H_2O_2, mass fraction 30%), were all purchased from GUOYAO Group Co. (Shanghai, China). The exact concentration of hydrogen peroxide was determined by titration with potassium permanganate. Uric acid and ascorbic acid were from Shanghai Chemical Reagents (Shanghai, China). Chitosan from crab shells (85% deacetylated), dopamine and glucose oxidase (GOD, EC 1.1.3.4, lyophilized powder, 245 U · mg^{-1}) from Shanghai YUANJU Co. (Shanghai, China) were produced by Sigma. Glucose stock solutions (0.1 mol · L^{-1}) were prepared and allowed to mutarotate at room temperature for 24 h before use. All stock solutions were prepared with deionized water.

1.2 Instruments

All electrochemical experiments were performed on a LK2005 Electrochemistry Work Station (LANLIKE Co. Ltd., Tianjin, China). A conventional three-electrode configuration was employed, consisting of the modified glassy carbon electrode (GCE, 3 mm in diameter) as the working electrode, a saturated calomel electrode as the reference electrode and a platinum wire as the counter electrode. Phosphate buffered saline (0.025 mol · L^{-1} KH_2PO_4/K_2HPO_4, pH 6.86) with 0.1 mol · L^{-1} KCl was used as the supporting electrolyte. Different pH values were adjusted by adding the diluent of HCl or KOH into the supporting electrolyte solution. All electrochemical measurements were carried out at room temperature. Scanning electron micrographs (SEM) of graphene, Prussian blue and GOD-CS hybrid films were obtained from a JEOL JSM-6700F SEM system. TEM image analysis was carried out on a Joel JEM 2010 microscope.

1.3 Electrode preparation

1.3.1 Direct electrodeposition of graphene

The prepared graphite oxide powder was exfoliated in a phosphate buffer solution (PBS, pH 9) by ultrasonication to form a 1.0 mg · mL^{-1} GO colloidal dispersion solution. Before modification, the GCE was polished first on a wet fine emery (SiC) abrasive paper and then with alumina slurries (0.3 μm) until a mirror finish was observed. After that, it was rinsed thoroughly with deionized water and dried with N_2. Electrolysis in the GO dispersion solution was carried out by using cyclic voltammetry in the potential range from −1.5 to 0.5 V at the scan rate of 20 mV · s^{-1}, and then the EG modified electrode was dried in the air at room temperature.

1.3.2 Deposition of PB

Electrodeposition of the PB film was accomplished as follows: the EG modified GCE was inserted in a solution containing 2.0 mmol · L^{-1} $K_3Fe(CN)_6$, 2.0 mmol · L^{-1} $FeCl_3$, 0.1 mol · L^{-1} KCl and 10 mmol · L^{-1} HCl, then electrodeposited at a constant potential of 0.4 V (*vs.* SCE) for 120 s[14-15]. After being washed, the PB/EG modified electrode was transferred into another solution containing 0.1 mol · L^{-1} KCl

and 10 mmol • L^{-1} HCl and electrochemically activated by potential cycling scan from 0.35 to −0.05 V at a rate of 50 mV • s^{-1} until the CV curves were stable. Finally, the electrode surface was rinsed with deionized water and dried in the air. For comparison, PB was also electrodeposited on the bare GCE in the absence of graphene.

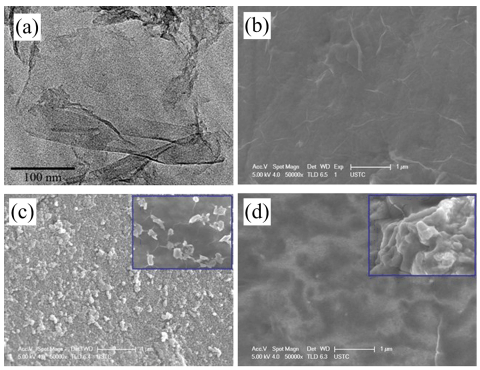
1.3.3　Preparation of GOD-CS hybrid film

A 0.5% chitosan solution was prepared by dissolving 0.5 g of chitosan power into 100 mL of 2.0% acetic acid and stirred at room temperature until complete dissolution. The GOD-chitosan solution was prepared by dissolving 25 mg GOD in 5 mL chitosan solution, then the PB/EG modified electrode was immersed in GOD – chitosan solution, and electrodeposited again at a constant potential of −2.0 V for 300 s. As a result, the GOD-CS hybrid film was deposited on the EG/PB modified electrode. More remarkably, the whole procedure for preparation could be limited in 1 h. Finally, the obtained electrode was dried in the air at room temperature and then stored in a refrigerator at 4 ℃ before use.

2　Results and discussion

2.1　TEM and SEM characterization of the nanocomposite films

The TEM and SEM images of the EG, PB/EG and GOD-CS/PB/EG films are shown in Fig. 1. Fig. 1(a) presents the representative TEM image of GO colloidal dispersion in a phosphate buffer solution (pH 9). It reveals that GO was entirely exfoliated as individual nano-sized GO sheets which are a few layers thick, and that the sheets contain a large amount of expanding graphitic edge planes that may facilitate the improvement on both the synthesis of other nanoparticles and its electrochemical behaviors. As confirmed by the SEM image shown in Fig. 1(b), the EG sheets electrodeposited on the electrode look like silk waves with high specific surface area, and the crumpled structure is beneficial for the further immobilization of PB nanoparticles. Fig. 1(c) demonstrates that the PB nanoparticles were deposited on the surface of EG to form EG/PB composite nanosheets. In addition, the inset shows that the PB nanoparticles were deposited on the kinked and wrinkled areas firstly, which implies that the wrinkled areas exhibit higher electroactivities. When GOD-CS was combined with the EG/PB composite film, as shown in Fig. 1(d), the obtained hybrid film was uniform and the thickness is controllable, which implies the successful attachment of GOD-CS hybrid film to the EG/PB modified electrode which provided an excellent platform for GOD immobilization. However, while the film was obtained by manual drop-coating method, it was rough and uneven with some agglomerate on the surface (see the inset in Fig. 1(d)). It indicated that the GOD-CS hybrid film obtained by electrodeposition was preferable.



(a) TEM image of GO dispersed in PBS (pH 9); (b) SEM images of EG film; (c) SEM images of PB/EG film, inset shows the PB deposition; (d) GOD-CS/PB/EG film modified on the GCE, inset shows the film obtained by manual drop-coating method.

Fig. 1　TEM and SEM characterization of the nanocomposite films

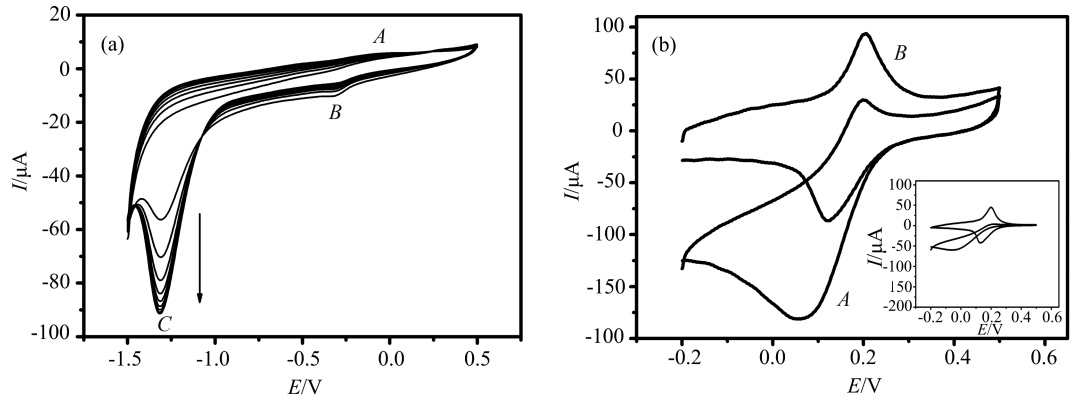
2.2　Electrodeposition process of EG modified electrode

The cyclic voltammograms (CVs) of GO reduction on GCE are given in Fig. 2(a). One anodic peak (A) and two cathodic peaks (B and C) were observed during the redox reaction of GO. The gradual increase of the peak currents with successive potential scans (direction of arrow) indicates that the electrodeposition of graphene on the GCE has been achieved successfully. The strong cathodic current peak C is attributed to the irreversible electrochemical reduction of GO, and

the anodic peak A and cathodic peak B are attributed to the redox pair of some electrochemically active oxygenic groups on graphene planes. In addition, the electrodeposition of graphene may be achieved on other conducting surfaces, and the graphene film modified on the electrode is very stable due to its insolubility in common solvents and compatiblity with the glassy carbon structure, and this makes it possible to immobilize other particles or films.

2.3 Electrocatalysis of PB/EG film to reduction of H_2O_2

To investigate the effect of EG on the catalytic action, we compared the responses of the biosensors newly prepared with and without EG to H_2O_2. As can be seen from Fig. 2(b), with the addition of 10 mmol · L^{-1} H_2O_2 into PBS (pH 6.86), the cathodic peak and the anodic peak on PB/EG/GCE electrode, measured with its cyclic voltammogram, increased and decreased distinctly, respectively (Fig. 2(b), curve A). Compared with the CV curve in the absence of H_2O_2(Fig. 2(b), curve B), it is characteristic of an electrochemically catalytic reaction of PB to H_2O_2. Correspondingly, only small current response to H_2O_2 on PB/GCE electrode can be observed (inset in Fig. 2(b)). It is reasonable to consider that the larger catalytic peak current comes from the cooperation between PB nanoparticles and EG film. This result shows that the PB/EG film can act as an effective catalyst to the reduction of H_2O_2.



(a) Cyclic voltammograms depicting electrochemical reduction of GO in PBS (pH 9) on GCE at 20 mV · s^{-1}, the arrow indicate the increase of peak current during potential cycles; (b) Cyclic voltammograms of PB/EG modified electrode (A) in the presence and (B) absence of 10 mmol · L^{-1} H_2O_2 at 50 mV · s^{-1} in PBS (pH 6.86). Inset shows CVs at PB/GCE electrode in the same condition

Fig. 2 Electrodeposition and electrocatalysis of PB/EG film

2.4 Optimization of working conditions

The layer-by-layer structured GOD-CS/PB/EG composite film modified electrode was constructed as a glucose biosensor. Detailed experiments on the influences of the applied potentials of the modified electrode and the pH of the testing solution were investigated. The effect of the applied potentials on the amperometric responses of the biosensor was studied between −0.3 V and +0.3 V (see Fig. 3(a)). The results reflected that the maximum response was reached at 0.0 V and this potential was selected as the working potential. Moreover, the biosensor can also avoid the interference of some electrochemical active substances when applied to serum sample analysis at this lower working potential. Fig. 3(b) gives the effect of the pH value of the working solution on the performance of the biosensor. The optimum response of the biosensor upon the acidity of the buffer solution was obtained in pH range from 5.0 to 7.0, which is lower than the optimum pH observed for free GOD. This phenomenon may be explained by the buffer effect of CS because it has a great deal of amino groups, which can provide a suitable pH microenvironment for GOD. It revealed that the CS is an excellent immobilizing

matrix and activity holder for enzyme. However, the response current decreased at pH 8.0, due to PB particles being unstable and dissolvable in neutral and alkalescent solutions[20]. Considering that the optimum acidity for many enzymes was within the pH range from 6.5 to 8.0, and that PB is instable at alkaline pH, it would lead to the slow decrease of the signal, so phosphate buffered saline of pH 6.86 was chosen for the detection of glucose so as to ensure higher sensitivity and stability of the biosensor.

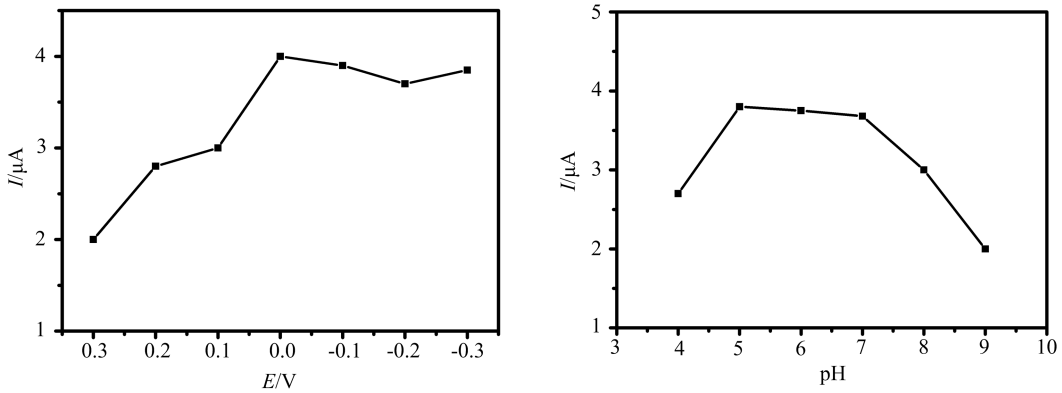
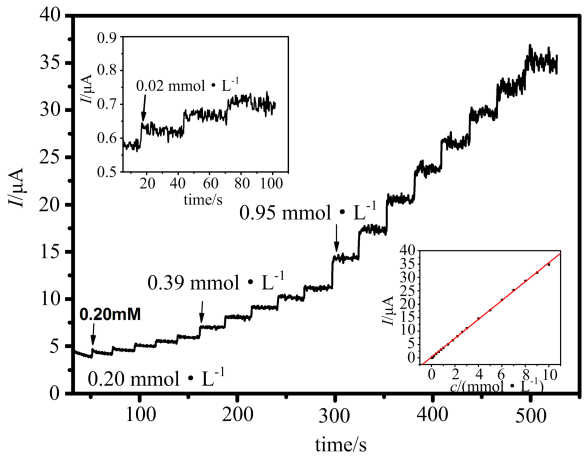


Fig. 3　Effects of (a) working potential, and (b) pH value on the current responses of the GOD-CS/PB/EG biosensor to 1.0 mmol · L^{-1} glucose

2.5　Amperometric response of the biosensor to glucose



Top inset displays the response to low concentration of glucose. Bottom inset shows the calibration curve of the biosensor as a function of glucose concentration

Fig. 4　Amperometric responses of the GOD-CS/PB/EG modified electrode at potential 0.0 V in phosphate buffer saline (pH 6.86) for successive addition of glucose

Since amperometry is much more sensitive than cyclic voltammetry, it was employed to estimate the low detection limit. Fig. 4 displays the amperometric responses of the biosensor upon the successive injection of glucose into the electrochemical cell. Under optimal experimental conditions, the reduction current increased steeply and then reached 95% of the steady-state current in less than 3 s. The results implied that the response of as-prepared biosensor was very fast. The current responses of the biosensor exhibited a linear dependence on glucose concentration, with the calibration equation of I (μA) = 0.31+3.52c (mmol · L^{-1}). The linear response range of the biosensor is from 0.02 to 10 mmol · L^{-1} with a correlation coefficient of 0.9970. The sensitivity of this biosensor was 50.29 mA · L · mol^{-1} · cm^{-2}, which was much higher than other reported glucose biosensors based on PB modified electrodes, such as GOD-CS/PB/Bi_2Se_3 biosensor (24.55 mA · L · mol^{-1} · cm^{-2})[21] and poly(3,4-ethylenedioxythiophene)/PB/MWCNTs glucose biosensor (2.67 mA · L · mol^{-1} · cm^{-2})[22]. The detection limit of 12 μmol · L^{-1} was estimated at a signal/noise ratio of 3.

2.6　Stability, reproducibility and interferences of the biosensor

The stability of the biosensor was determined by storing in 4 ℃ for 20 d. After 10 d and 20 d, the current response retain 97.3% and 94.8%, respectively. The current responses to 1.0 mmol ·

L^{-1} glucose were measured 6 times using the same modified electrode with a relative standard deviation (RSD) of 3.2%. For three different and freshly made modified electrodes, under the same conditions, the RSDs were obtained less than 3.6%. These results indicated that the as-prepared biosensor has a high stability as well as good reproducibility, which makes it applicable for practical use.

It is well known that some typical electroactive substances such as ascorbic acid (AA), uric acid (UA) and dopamine (DA) etc. may affect the amperometric response of glucose, so we also investigated the interference effects of AA, UA and DA, which normally coexist with glucose in real samples. The physiological glucose level is 3 to 8 mmol · L^{-1} and the other oxidizable interferents are present at levels as low as 0.1 mmol · L^{-1} in human blood serum samples, therefore, interference tests were carried out by adding 0.6 mmol · L^{-1} glucose, followed by 0.2 mmol · L^{-1} common interferents. The results demonstrate the negligible effects of the interferents (Fig. 5).

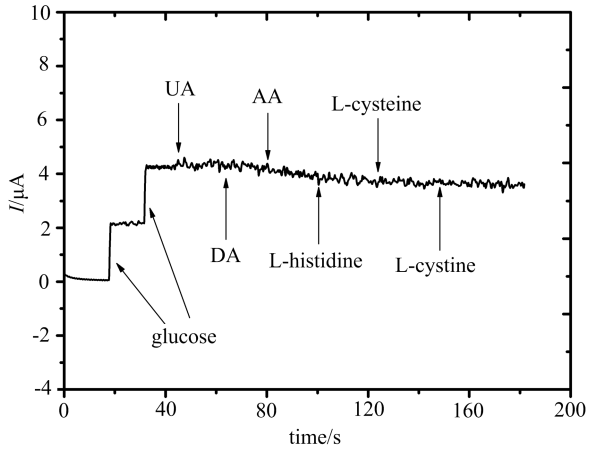


Fig. 5 Interference tests of the biosensor in phosphate buffer saline (pH 6.86) containing 0.6 mmol · L^{-1} glucose and 0.2 mmol · L^{-1} other common interferents

2.7 Glucose determination in serum samples

Finally, in order to illustrate the practical utility of the resulting glucose biosensor, human serum samples were assayed. Three fresh serum samples were supplied by a local hospital, which were firstly analyzed with a standard clinical assay based on glucose dehydrogenase electrode method. Then every 100 μL of serum samples were successively added into 10.0 mL phosphate buffer (pH 6.86) under the operation potential of 0.0 V. As shown in Tab. 1, very good agreement between the two sets of data allows us to ascertain the practical applicability of the proposed biosensor, which suggested that the proposed biosensor is promising for use in detection of glucose in serum samples.

Tab. 1 Amperometric determination of glucose in human blood serum samples

Blood sample	Given by hospital/ (mmol · L^{-1})	Proposed nonezymatic sensor/ (mmol · L^{-1})	RSD[a] /%
1	4.1	4.2±0.15	2.6
2	6.0	6.2±0.17	3.0
3	10.0	9.8±0.13	2.3

[Note] [a]RSD (%) calculated from three separate experiments.

3 Conclusion

In summary, a novel glucose biosensor with good sensitivity, selectivity and stability was constructed by three-step electrochemical deposition method. As the electron transfer mediators, graphene and PB films were electrodeposited successively on the substrate GCE, then GOD was immobilized by depositing a biocomposite film composed of CS and GOD. The method is simple and easy to control, and the whole process needs only a short period of time. In addition, the resulting CS film containing GOD keeps good bioactivity, which makes the biosensor possess of good bioactivities and high stability. The interferences from common interferents such as AA, UA and DA can be avoided due to the lower working potential being used. Moreover, the presence of the EG film increased the conductivity and electroactive sites of the biosensor. Combined with the advantages of graphene, PB nanoparticles and chitosan membrane, the layer-by-layer structured biosensor exhibits low detection limit, wide linear range, fast response, good operational stability and anti-

interference ability. The results indicate that the proposed three-step electrodeposition has great potential for further development of other enzyme-based electrochemical biosensors.

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