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ORIGINAL PAPER

Construction of an amperometric glucose biosensor based on the immobilization of glucose oxidase onto electrodeposited Pt nanoparticles-chitosan composite film

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Abstract One-step construction of Pt nanoparticles-chitosan composite film (PtNPs-CS) was firstly proposed as a novel immobilization matrix for the enzymes to fabricate glucose biosensor. This novel interface embedded in situ PtNPs in CS hydrogel was developed by one-step electrochemical deposition in solution containing CS and chloroplatinic acid (H_2PtCl_6). Several techniques, including scanning electron microscopy (SEM), cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and chronoamperometry were employed to characterize the assembly process and performance of the biosensor. Under the optimized experimental conditions, the resulting biosensor exhibited excellent linear behavior in the concentration range from 1.2 μM to 4.0 mM for the quantitative analysis of glucose with a limit of detection of 0.4 μM at a signal-to-noise ratio of 3. The apparent Michaelis–Menten constant (K_M^{app}) was evaluated to be 2.4 mM, showing good affinity. The proposed biosensor offered good amperometric responses to glucose due to the nanostructured sensing film provided plenty of active sites for the immobilization of glucose oxidase (GOD).

Keywords Glucose oxidase · Glucose biosensor · One-step electrodeposition · Pt nanoparticle · Chitosan · Composite film

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Introduction

As all we know, glucose is a keen metabolite for living organisms, especially in the case of patients suffering from diabetes. In order to treat and control diabetes, the amount of blood glucose has to be monitored. For this reason, there is increasing need to develop an electrochemical glucose biosensor with high performance by immobilizing enzymes on an electrode surface. Since Clark and Lyons [1] reported the first glucose sensor in 1962, there has been considerable attention paid to developing novel biosensors for the rapid, reproducible, sensitive, and selective or other potential applications. Numerous studies used GOD, which catalyzes the oxidation of glucose to gluconic acid in the presence of oxygen and generates hydrogen peroxide. This can be represented as follows: Glucose + O_2 → gluconolactone + H_2O_2.

One key step in the fabrication of an amperometric glucose biosensor is the immobilization of an enzyme, which is usually GOD as a model enzyme due to its being inexpensive, stable, and of practical use [2, 3], onto the surface of electrodes. However, they tend to suffer from instability and are easily denatured. Thus, the development of simple and reliable procedures to immobilize and stabilize reactive enzymes on the electrode has received considerable attentions [4]. Moreover, immobilization of enzymes onto a solid support provides an effective way for improving enzymatic performance. In particular, electrode materials with a large surface-to-volume ratio can increase the amount of immobilized enzyme, minimize the barriers for mass transportation between the substrate and the product, and provide a chemically and mechanically robust system.

In recent years, electrodeposition technology, as an effective method for the preparation of biocomposite film using controllable and simple procedure, is of great significance and still being practiced [5, 6]. Compared with

the traditional techniques, electrodeposition method provided a facile way under moderate conditions and the thickness of the resulting biocomposite film could be controlled. What's more, functional substances such as gold nanoparticles, carbon nanotubes and ionic liquid could be efficiently incorporated in the electrodeposition process. For example, Yang et al. [7] reported that MnO_2/Au composite film can be deposited onto carbon electrodes by a CV deposition process. Moreover, electrodeposition technique has been widely applied to construct glucose sensors, which exhibited high performance including simple operation, wide linearity, excellent stability and short response time [8–10]. Zeng et al. [11] fabricated a glucose biosensor based on DNA–Cu^{2+} complex onto the surface of electrode by using the one-step electrodeposition technique. An electrochemical non-enzymatic glucose sensor was developed by a simple and controllable electrodeposition method for the preparation of a CS-gold nanoparticles (GNPs) nanocomposite sensing film on a GCE surface [12]. Furthermore, Li et al. [13]. reported that a HRP–GOD bienzyme biosensor based on the electrodeposition approach by in situ formation of HRP–GOD/Concanavalin A (Con A)/CS biocomposite film on electrode.

CS is a type of natural cationic biopolymer, which has shown attractive characteristics such as film-forming ability, permeability and good adhesion. Therefore, CS can be used as a matrix for enzyme immobilization through glutaraldehyde or other reagent [14–19] and then construct biosensors. The major drawback of currently used CS film biosensor is the loss of electrical conductivity due to nonelectrical activity of CS layer. To overcome this problem, a interface embedded in situ PtNPs in CS hydrogel was developed by one-step electrochemical deposition in solution containing H_2PtCl_6 and CS. In view of the merits of PtNPs-CS composite film, this study reports a simple and new method to construct a novel glucose biosensor. First, GCE was modified with positively charged PtNPs-CS composite film by electrodepositing the compound of CS and H_2PtCl_6. The good electron-conductive ability of PtNPs-CS membrane has more active sites which provided a large loading capacity for GOD. The prepared glucose biosensor showed good analytical performance including simple operation, low detection limit, wide linearity, excellent stability and accepted repeatability.

Materials and methods

Chemicals

Glucose oxidase (EC 232-601-0, 155,000 U/g) and chitosan (deacetylating grade 70–85%) were purchased from Sigma (St. Louis, MO, USA). H_2O_2 (30%, m/v solution) was purchased from Shanghai Chemical Reagent Co. (Shanghai, China), and stock solutions of H_2O_2 were prepared daily. Hydrogen hexachloroplatinate (IV) hexahydrate ($H_2PtCl_6{\cdot}6H_2O$, 99%) and β-D-glucose were bought from Chongqing Chemical Reagent Co. (China). Double distilled water was used throughout this study. Other chemicals and solvents used were of analytical grade and were used as received. The stock solution of CS (1.00% m/V) was prepared daily by dissolving CS in 0.05 M acetic acid and stored at 4 °C.

Apparatus and measurements

Cyclic voltammetric (CV) and amperometric measurements were performed using a CHI 660D electrochemical work station (Shanghai Chenhua Instrument Co., China). All the electrochemical measurements were performed in the three-electrode system consisting of a modified GCE ($\Phi = 4$ mm) as working electrode, a Pt wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference electrode. All potentials were measured versus the SCE. The scanning electron micrographs were taken with a scanning electron microscope (SEM, S-4800, Hitachi, Tokyo, Japan) at an acceleration voltage of 20 kV.

Preparation of the glucose sensor

The GCE ($\Phi = 4$ mm) was, respectively, polished with 0.3 and 0.05 μm alumina slurry, and successively cleaned by sonicating in ethanol and water. PtNPs-CS film was modified on GCE by one-step electrochemical deposition in solution containing H_2PtCl_6 and CS at an applied potential of −1.00 V for 60 s (PtNPs-CS/GCE). Following that, the modified GCE (PtNPs-CS/GCE) was dried at room temperature. Then the fresh solution of GOD (10 mg/mL) was prepared in phosphate buffer solution (PBS, 0.1 M, pH 7.0) and 8 μL solution of freshly prepared GOD was mechanically spread onto the desired PtNPs-CS/GCE, and allowed to dry slowly at 4 °C. For comparison, PtNPs-CS/GCE and GOD/CS/GCE were prepared similarly. When these sensors were not in use, they were stored at 4 °C in a refrigerator. The fabricated procedure of the biosensor was shown in Fig. 1.

Results and discussion

SEM characterization

The SEM micrographs of different membranes were shown in Fig. 2. The typical morphology of the PtNPs-CS composite film could be observed that PtNPs were well fixed and

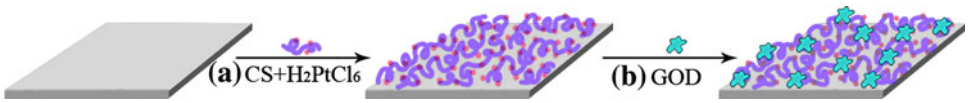

Fig. 1 The stepwise fabrication processes of the modified electrode

uniformly dispersed in the CS hybrid film, confirming this method of embedding in situ PtNPs in CS hydrogel was preferable (Fig. 2a). After GOD was assembled onto the PtNPs-CS composite film, the corresponding SEM image obviously changed (Fig. 2b), which indicated a large number of GOD immobilized onto the composite film surface.

Electrochemical characterization of the biosensor

Figure 3a shows CVs of modified electrodes in different composites in 5 mM $[Fe(CN)_6]^{4-/3-}$, with a scan rate of 50 mV/s. As can be seen, a well-defined oxidation and reduction peaks of $[Fe(CN)_6]^{4-/3-}$ were observed with the bare GCE (curve a). A remarkable peak current increase was observed (curve b) after PtNPs-CS composite film was immobilized on bare GCE. The increase was attributed to the PtNPs which played an important role similar to a conducting wire or electron conduction tunnel. Compared with curve b, peak current decrease was observed (curve c) when GOD was spread onto the PtNPs-CS composite film-modified electrode, owing to the non-conductive GOD was incorporated on the PtNPs-CS/GC electrode. The above results could clearly confirm the success of the assembly of the electrode.

In order to further confirm the interface properties of the surface-modified electrode more, EIS experiments were performed. As shown in Fig. 3b, curve a have a small semicircle, implying very low R_{et} bare GCE to the redox probe dissolved in 5 mM $[Fe(CN)_6]^{4-/3-}$ solution (curve a). After PtNPs-CS composite film was modified on the bare GCE, the EIS of the resulting film displayed an interfacial R_{et} (curve b), implying a decrease of R_{et} in the redox probe. The decrease was ascribed to the excellent conductivity of the PtNPs. Subsequently, the GOD immobilized on the surface of the PtNPs-CS composite film (curve c), the interfacial resistance increased, suggesting the non-conductive properties of GOD can obstruct electron transfer of the electrochemical probe.

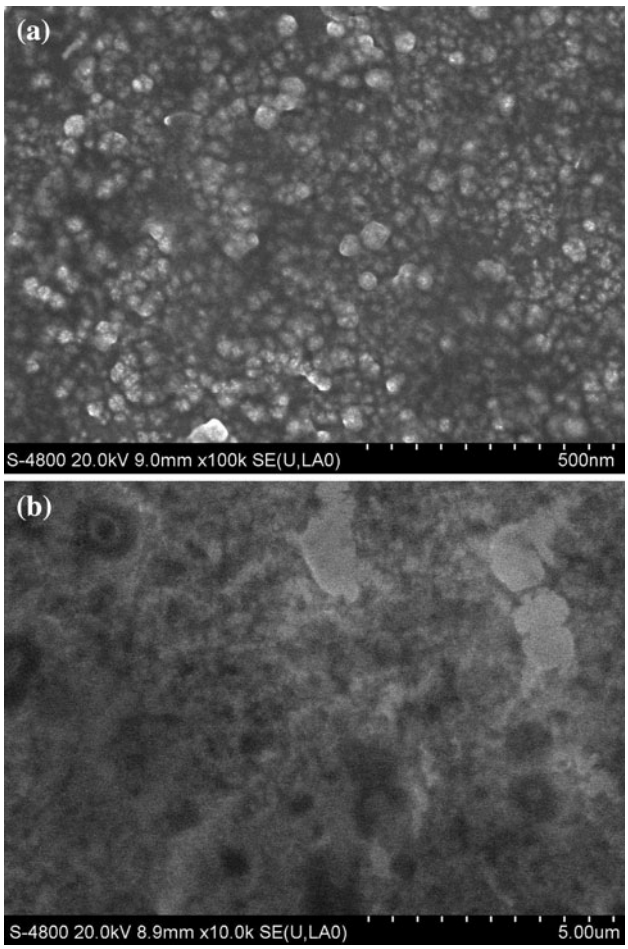

Fig. 2 SEM images of **a** PtNPs-CS composite film and **b** GOD/PtNPs-CS composite film

Electrochemical behavior of sensor

The electrocatalytic behavior of PtNPs-CS modified electrode toward the electrochemical reduction of H_2O_2 was investigated by CV. Figure 4a shows electrocatalytic behavior of the PtNPs-CS/GCE in 0.1 M PBS of pH 7.0. In the absence of H_2O_2, no obvious current was observed (curve a), but with the addition of H_2O_2, the catalytic current increased obviously (curve b). It demonstrated that PtNPs exhibited excellent electrocatalytic activity toward the reduction of H_2O_2.

The electrocatalytic reactivity of GOD/PtNPs-CS/GCE toward glucose was also investigated by CV. Figure 4b shows the bioelectrocatalytic behavior of the glucose biosensor in the absence (curve a) and the presence of glucose (curve b). Upon addition of 0.3 mM glucose, the reduction peak current and oxidation peak current increased clearly (curve b), indicating a typical electrocatalytic process of glucose. So, it can be concluded that GOD immobilized on PtNPs-CS composite film displayed good catalytic activity toward glucose.

Optimum conditions of glucose biosensor

In order to obtain an efficient biosensor for glucose, the influence of pH, applied potential and deposition time on

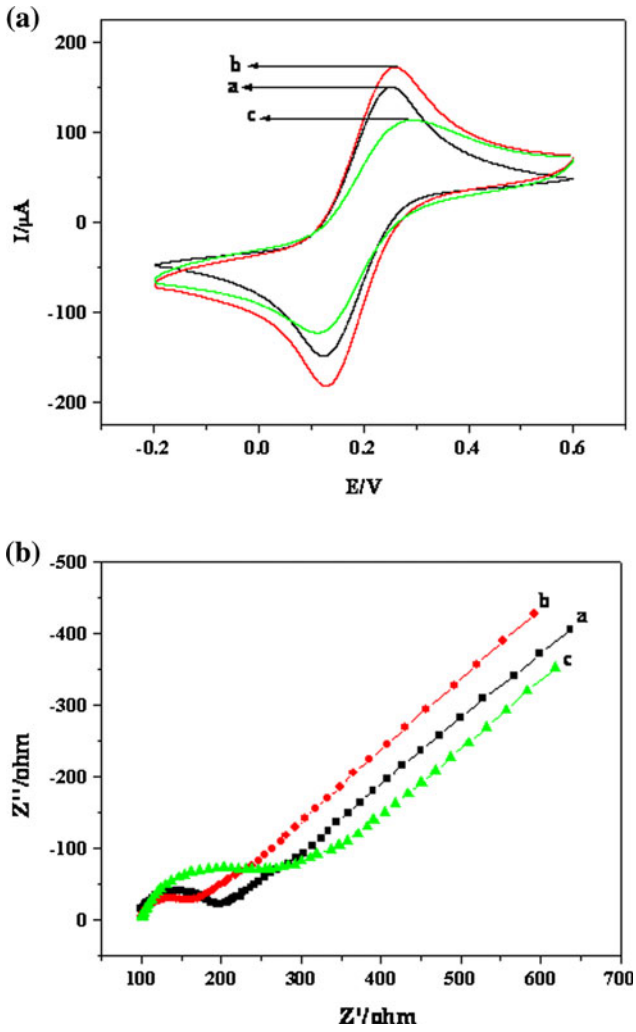

Fig. 3 **a** CV and **b** EIS obtained for different modified GCEs in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution. *a* bare GCE, *b* PtNPs-CS/GCE, *c* GOD/PtNPs-CS/GCE. The scan rate was 50 mV/s

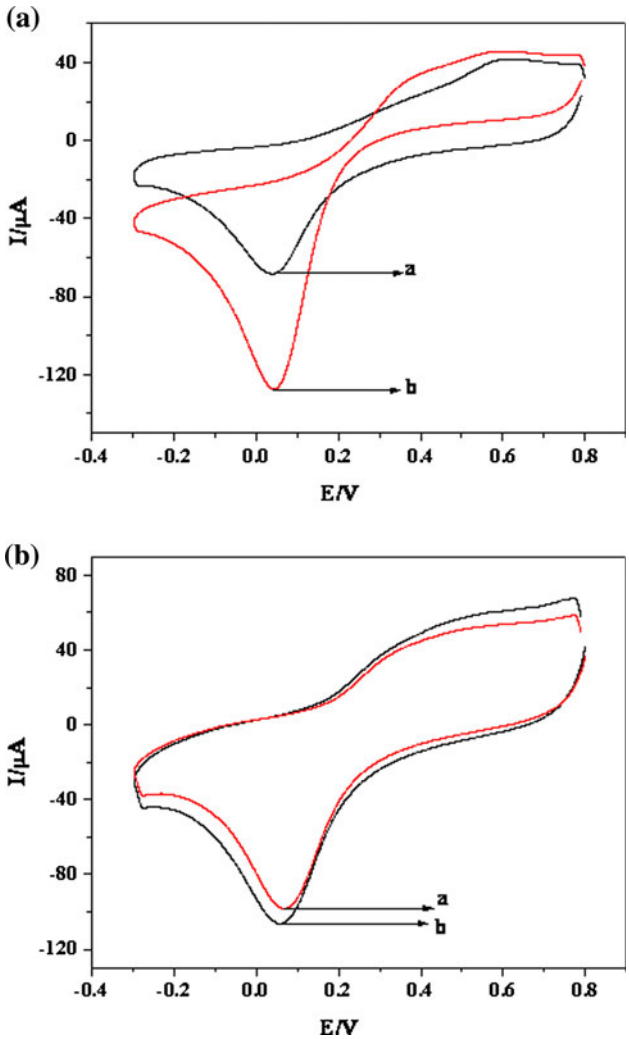

Fig. 4 CVs of **a**: GOD/PtNPs-CS/GCE in 0.1 M PBS (pH 7.0) without (*curve a*) and with (*curve b*) 0.35 mM H_2O_2. **b** GOD/PtNPs-CS/GCE in 0.1 M PBS (pH 7.0) without (*curve a*) and with (*curve b*) 0.3 mM glucose. Scan rate 50 mV/s

the response of GOD/PtNPs-CS/GCE were studied. The change of chronoamperometric current at various pHs ranged from 4.0 to 9.0 under constant glucose concentration (0.3 mM) was shown in Fig. 5a. As can be seen, the maximum response appears at pH 7.0. So the buffer solution of pH 7.0 was selected for amperometric measurements.

Figure 5b shows the dependence of the chronoamperometric current response to constant concentration (0.3 mM) glucose on the applied potential in the range from 0 to 0.6 V. From Fig. 5b, it can be seen that the response increases sharply from 0 to 0.6 V, and reached the maximum at 0.6 V. In order to minimize the risk for interfering reactions of the other electroactive species and get enough current responses, an applied potential of +0.35 V was chosen for amperometric measurements.

As shown in Fig. 5c, the effect of deposition time on the chronoamperometric current response was examined. It can be seen that the response current could reach the maximum at 60 s. In contrary, the response current decreases while decreasing or increasing the deposition time. The reason was that the decrease of the real surface area of the electrode resulting from the deposition of a large amount of PtNPs-CS on the electrode surface. Hence, a deposition time of 60 s was selected for the amperometric determination of glucose.

Amperometric response of glucose sensor

In order to check up the electrochemical responses of different modified sensors, a comparative study of the chronoamperometric responses for continuous addition of 0.3 mM glucose was carried out using three kinds of sensor which were developed as: (a) GOD/CS/GCE, (b) PtNPs-CS/GCE and (c) GOD/PtNPs-CS/GCE. As shown in Fig. 6, GOD/PtNPs-CS/GCE (curve c) showed the best response to

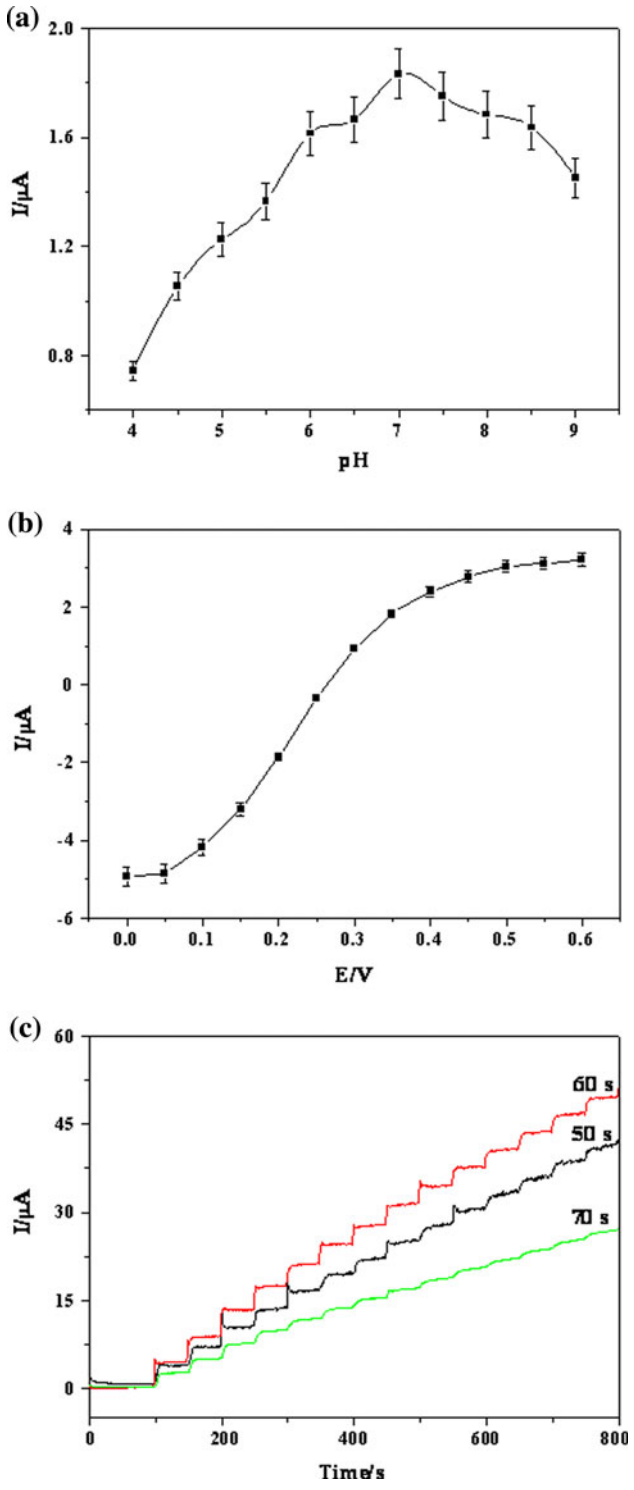

Fig. 5 Dependence of the current response of biosensor to 0.3 mM glucose on the pH of buffer solutions at an applied potential of 0.35 V (**a**) and on the applied potential in 0.1 M PBS (pH 6.0) (**b**), the influence of deposition time on the response of the biosensor with successive injection of 0.3 mM glucose into a stirred solution of 0.1 M PBS (pH 7.0) (**c**)

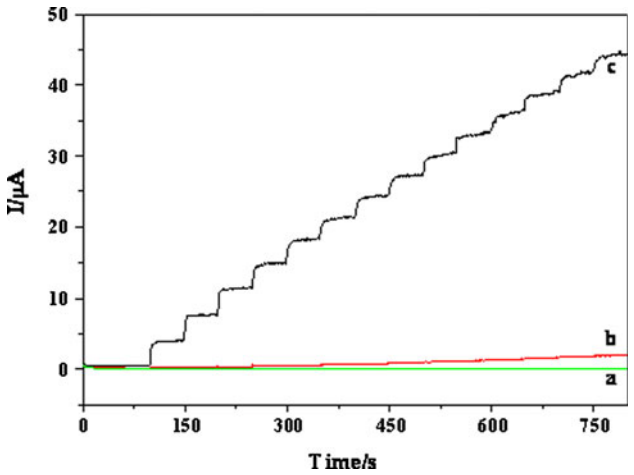

Fig. 6 The chronoamperometry response at applied potential of 0.35 V with successive injection of 0.3 mM glucose into a stirred solution of 0.1 M PBS (pH 7.0) for GOD/CS/GCE (*a*), PtNPs-CS/GCE (*b*) and GOD/PtNPs-CS/GCE (*c*), respectively

the addition of the same glucose concentration. As controlled experiment, the GOD/CS/GCE (curve a) exhibited less sensitive amperometric responses to glucose as the lack of PtNPs. The reason was that PtNPs-CS composite film could provide plenty of active sites for the immobilization of GOD compared to the CS. Without the GOD, the PtNPs-CS/GCE (curve b) also appeared certain electrochemical catalysis response to glucose, which can be attributed to the inherent catalysis of PtNPs.

The amperometric response of the glucose biosensor was investigated by successively adding glucose to a continuous stirring of PBS solution under the optimized conditions. The typical current–time curve and the linear calibration curve of the biosensor were shown in Fig. 7. In Fig. 7a, the proposed biosensor achieves 95% of the steady-state current in less than 5 s, indicating a fast response process. This may be ascribed to good electron transfer from the GOD molecules to the PtNPs-CS composite film. There is an excellent linear relation of the current with concentration of glucose from 1.2 μM to 4.0 mM, as shown in Fig. 7b. The linear regression equation is i (μA) = 1.6 + 1.03 [glucose] (mM) with a correlation coefficient of 0.995 (n = 18). From the slope of calibration curve, the detection limit of 0.4 μM is estimated at signal-to-noise ration of three. Compared with electrochemical glucose sensor [12], the prepared biosensor displayed good amperometric responses to glucose.

It is well known that a smaller Michaelis–Menten constant (K_M^{app}) implies a higher catalytic activity of the immobilized enzyme. According to the Lineweaver–Burk equation [20]:

$$1/I_{ss} = 1/I_{max} + K_M^{app}/I_{max}C.$$

where I_{ss} is the steady-state current after the addition of substrate; I_{max} is the maximum current measured under saturated substrate condition; C is the bulk concentration of the substrate. The value of K_M^{app} is determined by analyzing the slope and intercept of the plot of the reciprocals of the I_{ss} and C. Thus the K_M^{app} of the obtained biosensor was calculated to be 2.4 mM. The low value of K_M^{app} indicated that GOD immobilized in PtNPs-CS film retains its bioactivity and has a high biological affinity to glucose. The value was smaller than amperometric glucose biosensor in which GOD immobilized in platinum-multiwalled carbon nanotube-alumina-coated silica (Pt-MWCNT-ACS) nanocomposite modified glassy carbon electrode (K_M^{app} = 10.5 mM) [21]. The smaller value of the K_M^{app} showed that PtNPs-CS film had better retention of bioactivity of the immobilized GOD.

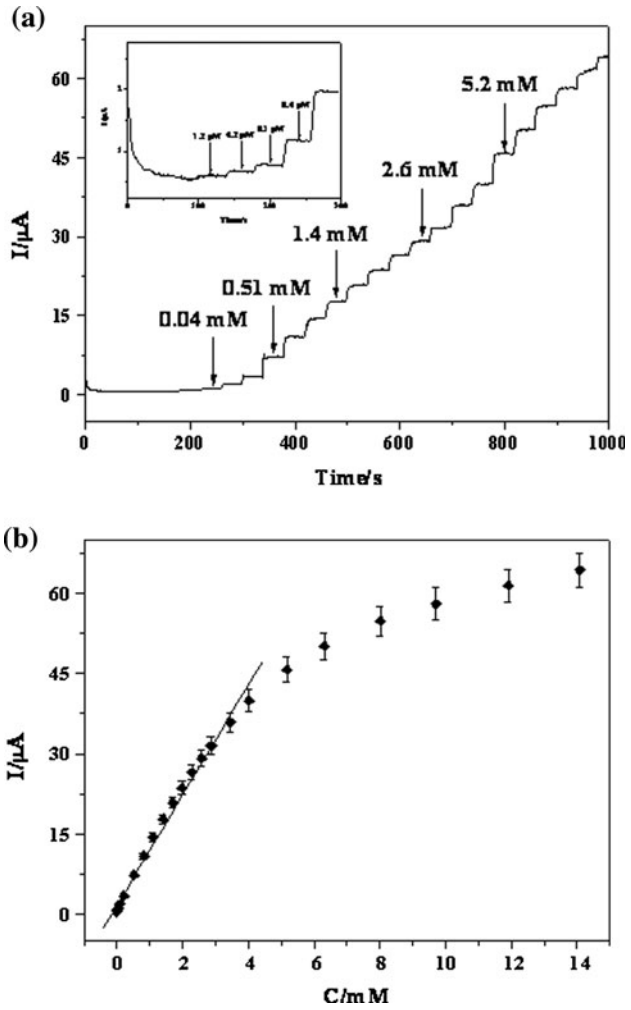

Fig. 7 **a** Amperometric response of the GOD/PtNPs-CS/GCE (at +0.35 V) to successive injections of different concentration of glucose into a stirred solution of 0.1 M PBS (pH 7.0) at an applied potential of 0.35 V in the time intervals of 40 s. *Upper inset* amplified response curve. **b** Calibration curve for the electrochemical responses of the proposed biosensor to glucose in PBS solution

Interference determination

Selectivity is also important in practical use of the proposed biosensors. To investigate the effect of some possible interfering substances on the biosensor, four common interfering species including ascorbic acid (AA), threonine, L-cysteine and uric acid (UA) were tested and the results were listed in Table 1. The current ratios were calculated by reading the current of the biosensor in the assay solution containing 0.3 mM interfering substance and 0.3 mM glucose, and comparing it with the current from the biosensor in the same assay solution containing 0.3 mM glucose. As can be seen from Table 1, four interfering species did not interfere significantly with the resulting biosensor, which was largely associated with the low working potential of 0.35 V used in the determination of glucose.

Stability and repeatability of the biosensor

The storage stability of the proposed biosensor was also examined. When not in use, the biosensor was stored dry at 4 °C and measured at every 3-day-interval, and it lost only 6.9% of the initial response after 3 weeks and maintained more than about 89.6% of the initial values after storage for 1 month. The decreased current may be ascribed to the enzymes which partly lose the bioactivity for long-storing time.

The fabrication reproducibility of five electrodes, showed an excellent reproducibility with a R.S.D. of 5.8% for determining the same glucose concentration of 0.3 mM. This result suggested that the glucose biosensor showed acceptable reproducibility.

Recovery experiment

In order to investigate its applicability, the biosensors were tested for detecting the recovery of glucose by a standard addition method. The results were showed in Table 2 and the recovery rate was between 96.7 and 101.3%. The satisfying results demonstrated a great potential for practical application in the analysis of glucose.

Conclusions

In this work, a novel and simple glucose biosensor was successfully developed by immobilizing the model enzyme, GOD, on the PtNPs-CS film which is prepared by a simple and controllable electrodeposition method. The fabricated PtNPs-CS nanocomposite film which had high

Table 1 Possible interference tested with the obtained biosensor

Potential interfering substance	Current ratio[a] (%)
Ascorbic acid	105.5 ± 2.2
Threonine	103.3 ± 1.6
L-cysteine	102.8 ± 1.3
Uric acid	104.2 ± 2.5

[a] Mean ± SD of three measurements

Table 2 Recovery measurements of glucose at the GOD/PtNPs-CS/GCE

Samples	Concentration (mM)	Found (mM)	RSD (%) ($n = 3$)	Recovery (%)
1	0.03	0.029	1.26	96.7
2	0.15	0.151	2.34	100.7
3	0.9	0.912	3.03	101.3

stability and good biocompatible ability was used to improve the characteristic of the biosensor. Moreover, the constructed biosensor had excellent performance on glucose detection. Finally, this electrodeposition method can be used in the preparation of other enzyme biosensors.

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