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A simple strategy for one-step construction of bienzyme biosensor by in-situ formation of biocomposite film through electrodeposition

Feng Li, Zhen Wang, Wei Chen, Shusheng Zhang*

Key Laboratory of Eco-Chemical Engineering, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China

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ABSTRACT

One-step construction of horseradish peroxidase (HRP) and glucose oxidase (GOD) bienzyme biosensor was established based on a simple and controllable electrodeposition approach by in-situ formation of biocomposite film on electrode. With hydrogen peroxide (H_2O_2) instantly generated by GOD-catalyzed oxidation of glucose, quantitative measurement of glucose could be achieved. In the proposed electrodeposition strategy, Concanavalin A (Con A) was applied as bifunctional cross-linker for in-situ incorporating of HRP–GOD in biocompatible chitosan (CS) matrix due to its biospecific affinity interaction with CS and glycoproteins. Scanning electron microscopy showed that the HRP–GOD/Con A/CS biocomposite film possessed highly porous surface. The developed bienzyme biosensor exhibited a fast amperometric response for the determination of glucose. The linear response of the developed biosensor for the determination of glucose ranged from 1.0×10^{-6} to 2.2×10^{-4} M with a detection limit of 6.7×10^{-7} M. The biosensor can be used to determine glucose in serum directly. The biosensor presented high stability owing to the design of the introduction of Con A and instant generation of H_2O_2 with bienzyme system. The one-step construction of biosensor based on non-manual technique in biocompatible environment was direct and facile.

1. Introduction

The determination of glucose is of significant importance due to the high demand for blood glucose monitoring. Owing to the procedural simplicity, intrinsic sensitivity and selectivity, electrochemical biosensors have gained ever-increasing attention (Jia et al., 2008; Xue et al., 2006; Zhu et al., 2007; Li et al., 2008a). Electrochemical biosensor based on horseradish peroxidase (HRP) and glucose oxidase (GOD) bienzyme biosensor is inherently attractive because H_2O_2 needed for the determination of glucose could be instantly formed by GOD-catalyzed oxidation of glucose. As a result, inhibit for the activity of HRP in the presence of high concentration of added H_2O_2 could be avoided (Bucur et al., 2004). To develop a reliable bienzyme biosensor, the technique used to immobilize enzymes is of great significance and biocompatible material that can improve the analytical performance of the biosensors is highly desired.

Recently, electrodeposition has been reported as a simple method for the preparation of biocomposite film (Xue et al., 2006; Song et al., 2006; Zou et al., 2008). Compared with traditionally manual process, electrodeposition method provided a facile way under moderate conditions and the thickness of the electrodeposited film could be controlled. As a result, the electrodeposition method is simple and controllable. In addition, functional substances such as gold nanoparticles, carbon nanotubes and ionic liquid could be efficiently incorporated in the electrodeposition process.

Chitosan (CS) was a biocompatible polymer and remained a focus of study as one of the most promising materials for enzyme immobilization due to the unique characteristics such as cheapness, hydrophilicity and remarkable biocompatibility (Kaushik et al., 2008; Li et al., 2008b; Li et al., 2006). In addition, CS was a pH-shift polymer because of the possessing of amino groups with a pK_a of about 6.3. Consequently, the solubility and the net charge of CS were pH-dependent. It has been reported that CS could be electrochemically deposited onto electrodes and the electrodeposited CS hydrogel can tightly attach to the electrode and retain its natural properties. Such electrodeposition of CS was pioneered by Payne group and Chen group (Wu et al., 2002, 2003, 2005; Xu et al., 2004; Xi et al., 2008; Luo et al., 2004). The mechanism was the formation of pH gradient near the cathode surface due to the reduction of water at reducing potentials. In brief, H^+ in the solution was reduced to H_2 at the cathode. Acidic side chains of CS were titrated by the locally generated H^+ gradient (Fernandes et al., 2003). The change of CS solubility resulted in the deposition of CS. Therefore, electrodeposition method could supply a facile and universal way to prepare

* Corresponding authors.
E-mail address: shushzhang@qust.edu.cn (S. Zhang).

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functional biocomposite film by incorporation functional enzymes in the biocompatible CS matrix under enzyme-friendly conditions.

This work attempts to establish the one-step construction of HRP and GOD bienzyme biosensor based on electrodeposition approach by in-situ formation of HRP–GOD/Concanavalin A (Con A)/CS biocomposite film on electrode. The proposed procedure was simple and controllable. Con A was applied as bifunctional cross-linker for in-situ incorporating of HRP–GOD in biocompatible CS material. Con A is a lectin protein found in Jack bean (Anzai and Kobayashi, 2000; Liu et al., 2007). Due to the biospecific affinity for glycoproteins, Con A could be used as a versatile platform to immobilize HRP and GOD. Con A molecule dispersed in the biocompatible CS chain through affinity with natural polysaccharide CS. Based on biospecific affinity between Con A, HRP and GOD, stable HRP–GOD/Con A/CS biocomposite film was subsequently formed through electrodeposition process. Glucose could be detected with H_2O_2 instantly generated by GOD-catalyzed oxidation of glucose. The biocompatible CS matrix, the introduction of Con A, the porous surface of the biocomposite film, and in-situ generation of H_2O_2 with bienzyme system endowed the as-prepared bienzyme biosensor with characteristics of fast response, high sensitivity and selectivity, and good stability.

2. Experimental

2.1. Reagents

HRP (EC 1.11.1.7, RZ > 3.0, 237 U mg^{-1}) and GOD (EC 1.1.3.4, 905 U mg^{-1}) were obtained from SERVA Electrophoresis GmbH. CS with 98% deacetylation and an average molecular weight of 6×10^4 g mol^{-1} was obtained from Yuhuan biomedical Corp., China. Con A (Shanghai sanjie biotechnology CO. LTD) from canavalin ensiformis (Jack Bean) was used in this study. All other chemicals were of analytical reagent grade and used without further purification. The 0.1 M phosphate buffer solutions (PBS) at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, and then adjusting the pH with 0.10 M H_3PO_4 or NaOH. Doubly-distilled water was used throughout this work.

2.2. Apparatus and instrumentations

Amperometric and cyclic voltammetric experiments were performed with a CHI 832B electrochemical analyzer (Shanghai CH Instrument Company, China). Electrochemical impedance spectroscopy (EIS) was performed on a CHI660C electrochemical analyzer (Shanghai CH Instrument Company, China). A conventional three-electrode system was used with bare gold electrode or modified gold electrode as the working electrode, Ag/AgCl electrode (saturated with KCl) as the reference electrode, and platinum wire as auxiliary electrode, respectively. Scanning electron microscopy (SEM) images were obtained at 5.0 kV on a SIRION (FEI, USA) field emission scanning electron microscope.

2.3. Preparation of bienzyme biosensor by in-situ formation of biocomposite film through one-step electrodeposition

Gold electrode was used as the base electrode for the fabrication of the bienzyme biosensor. Before use, bare gold electrode was polished with 1.0, 0.3, 0.05 μm α-Al_2O_3 slurry, respectively. The freshly polished electrodes were then pretreated by cleaning with piranha solution (7:3 mixture of concentrated sulfuric acid and 30% hydrogen peroxide). Afterwards, the Au electrode was electrochemically cleaned by potential cycling between −0.2 and 0.6 V versus Ag/AgCl in 0.1 M H_2SO_4. The obtained electrode was rinsed with doubly-distilled water followed by sonicating in acetone, ethanol and doubly-distilled water successively. Then, the electrode was dried at room temperature.

A homogenous mixture containing CS, Con A, HRP and GOD was firstly prepared as electrodeposition solution for one-step preparation of bienzyme biosensor. CS aqueous solution was prepared according to the previously reported procedure (Xu et al., 2004). Briefly, CS flakes were dissolved in dilute HCl solution and a solution with pH near 3.0 was obtained. After undissolved material was filtered, the pH was adjusted to 5.0 using 1.0 M NaOH. Con A stock solution was prepared by 0.1 M PBS (pH 7.0) containing 1.0 mM $CaCl_2$ and 1.0 mM $MnCl_2$. Typically, a homogeneous stock solution of Con A/CS solution was firstly formed by blending CS solutions (0.5 wt.%, pH 5.5) with Con A (0.3 mg ml^{-1}) and sonicated for 3 min. HRP–GOD/Con A/CS solution was obtained by blending such Con A/CS solution with 1.0 mg ml^{-1} HRP and 1.0 mg ml^{-1} GOD solution, and then sonicated for 2 min. The resulting HRP–GOD/Con A/CS biocomposite solution was stocked at 4 °C for 12 h before use as electrodeposition solution.

For electrodeposition, gold electrode was immersed in such HRP–GOD/Con A/CS biocomposite solution and used as the cathode by applying a voltage of −1.2 V for 8 min. In the electrodepositon, Con A, HRP, and GOD were in-situ entrapped in CS gel and the HRP–GOD/Con A/CS biocomposite film was formed. The obtained electrode (HRP–GOD/Con A/CS/Au) was washed with 0.1 M PBS and dried at room temperature for about 5 min, and then stocked at 4 °C in the dry state for 12 h before use (Zhao et al., 2006). For comparison, the HRP–GOD/CS/Au/Au, Con A/CS/Au, and CS/Au were independently prepared.

2.4. Characterization of the as-prepared bienzyme glucose biosensor

Cyclic voltammetric experiments were carried out in quiescent solutions with the scan rate of 50 mV s^{-1}. In steady-state amperometric experiments, the potential was set at 0.05 V under magnetic stirring and typical steady-state response of the biosensor to successive injection of glucose was recorded. All potentials are reported versus the Ag/AgCl reference electrode. Glucose stock solutions were allowed to mutarotate overnight at room temperature before use (Luo et al., 2004). EIS were performed in 1.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture containing 0.1 M KCl with the frequencies ranging from 10^4 to 10^{-1} Hz versus Ag/AgCl reference electrode. The morphology of the HRP–GOD/Con A/CS biocomposite film was examined by SEM observation.

3. Results and discussion

3.1. Fabrication of bienzyme biosensor by in-situ formation of HRP–GOD/Con A/CS biocomposite film through one-step electrodeposition

In present investigation, a versatile methodology was developed for preparing electrochemical sensing platform by integration of natural polysaccharide CS and biomolecules (Con A, HRP and GOD) through one-step electrodeposition. The biocompatible CS matrix, the introduction of Con A, the porous surface of the biocomposite film, and in-situ generation of H_2O_2 with bienzyme system were combined in the proposed method. Schematic illustration of the construction of HRP–GOD bienzyme biosensor based on in-situ formation of HRP–GOD/Con A/CS film by biospecific Con A – glycoconjugates (glycoproteins HRP, GOD and polysaccharide CS) interactions was given in Fig. 1.

As a pH-shift biopolymer, CS was ideally suited for electrodeposition because its net charge and solubility were pH dependent. The pK_a of CS was about 6.3 and CS chains had net positive charge

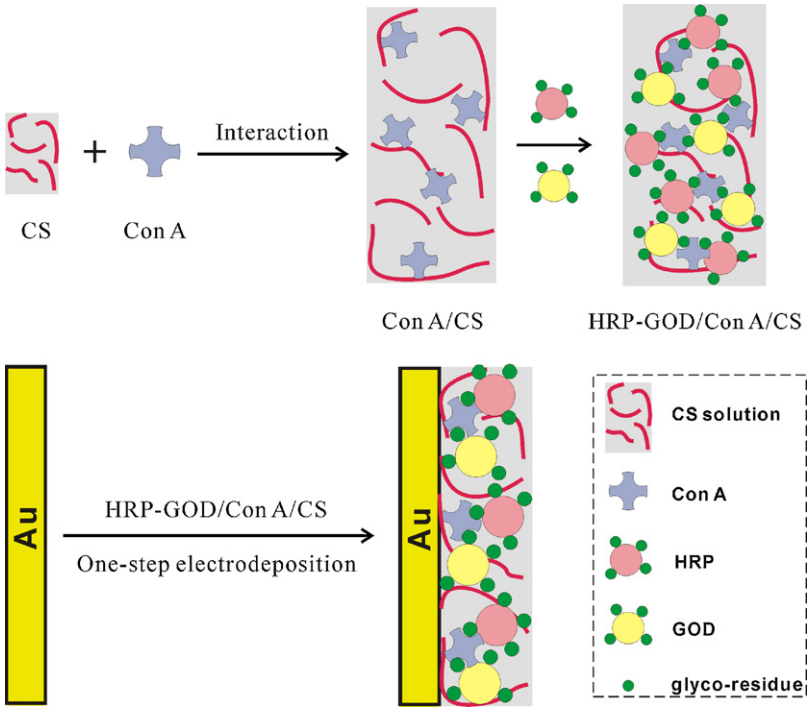

Fig. 1. Schematic illustration of electrodeposition construction of HRP–GOD bienzyme biosensor based on in-situ formation of HRP–GOD/Con A/CS film by Con A–glycoconjugates biospecific interactions.

in the electrodeposition solution. As a lectin protein found in Jack bean (Anzai and Kobayashi, 2000; Liu et al., 2007), Con A possessed biospecific affinity with glycoconjugates such as glycoproteins HRP, GOD and polysaccharide CS (Che et al., 2008). As a result, Con A molecule was dispersed in the biocompatible CS chain, as shown in Fig. 1. Through biospecific affinity between Con A and HRP and GOD, stable HRP–GOD/Con A/CS biocomposite was formed in the electrodeposition solution. Subsequently, in-situ incorporation of Con A, HRP and GOD into CS hydrogel was achieved through simple and convenient electrodeposition. Such non-manual electrodeposition approach was simple and controllable.

3.2. Morphology characterization of the HRP–GOD/Con A/CS biocomposite film

The surface morphology of the formed biocomposite film was investigated using SEM. Fig. 2 represented the SEM image of the formed HRP–GOD/Con A/CS biocomposite film. It could be found that the HRP–GOD/Con A/CS biocomposite film formed by one-step electrodeposition possessed highly porous surface. As a result, Con A, HRP and GOD was in-situ incorporated into the three-dimensional network of biocompatible CS. Such morphological structure might improve diffusion of the electroactive probes and provide good sensitivity and fast response of the biosensor.

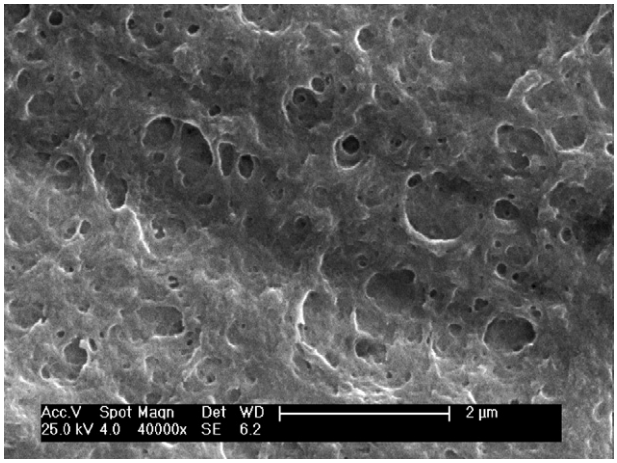

Fig. 2. SEM image of the HRP–GOD/Con A/CS biocomposite film.

3.3. Electrochemical characteristics of the developed bienzyme biosensor

The electrochemical activity of the $Fe(CN)_6^{3-/4-}$ redox probe at the as-prepared bienzyme biosensor was investigated using cyclic voltammetry (CV). CVs of bare Au electrode (a) and the HRP–GOD/Con A/CS/Au (b and c) in 0.1 M PBS (pH 7.0) containing 1.0 mM $K_4Fe(CN)_6$ at a scan rate of 50 mV s^{-1} were given in Fig. 3. The curve b was obtained without glucose and curve c was with 80 μM glucose. The peaks (Fig. 3b) represented characteristics of redox couple of $Fe(CN)_6^{3-/4-}$. With the addition of glucose, reduction peak current increased while oxidation peak current decreased (Fig. 3c), indicating that a catalytic reaction occurred on this biosensor. These results demonstrated that the response of the biosensor to glucose only resulted from the catalytic activity of bienzymes immobilized in the biocomposite film. The determination of glucose catalyzed by GOD and HRP and mediated by $K_4Fe(CN)_6$ can be described as follow:

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{GOD} \text{D-Glucono-1, 5-lactone} + H_2O_2 \quad (1)$$

$$H_2O_2 + 2Fe(CN)_6^{4-} + 2H^+ \xrightarrow{HRP} 2H_2O + 2Fe(CN)_6^{3-} \quad (2)$$

In brief, H_2O_2 was formed by GOD-catalyzed oxidation of glucose. The response of H_2O_2 catalyzed by HRP and mediated by $K_4Fe(CN)_6$ gave the electrochemical signal. CVs of the HRP–GOD/Con A/CS/Au in 0.1 M PBS (pH 7.0) containing 1.0 mM $K_4Fe(CN)_6$ at different scan rates were also investigated (see supplementary material). A linear relationship of peak current to the square root of the scan rate was revealed. For the reduction peak

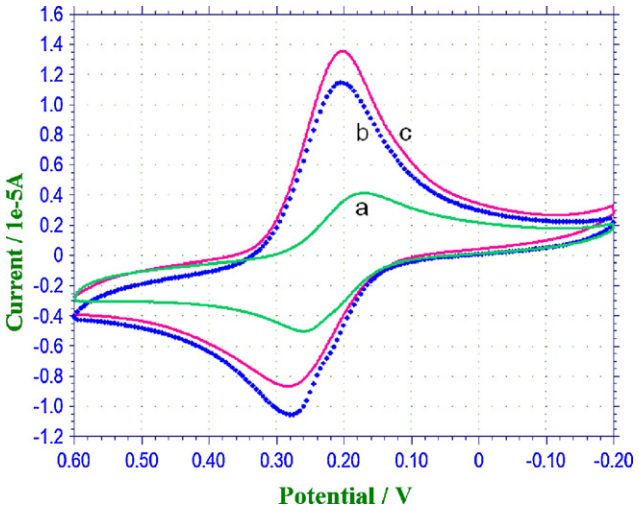

Fig. 3. CVs of bare Au electrode (a) and the HRP–GOD/Con A/CS/Au (b and c) in 0.1 M PBS (pH 7.0) containing 1.0 mM $K_4Fe(CN)_6$ at a scan rate of 50 mV s^{-1}. The curve b was obtained without glucose and curve c was with 80 μM glucose.

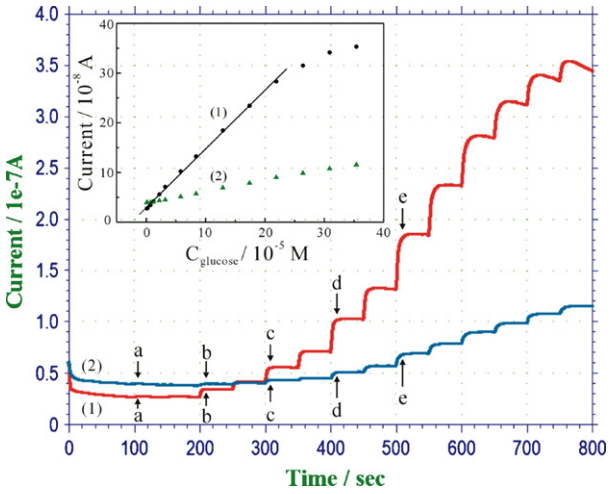

Fig. 5. Typical amperometric response of HRP–GOD/Con A/CS/Au (1) and HRP–GOD/CS/Au (2) to the successive addition of different concentrations of glucose: (a) 1 μM, (b) 5 μM, (c)10 μM, (d) 26 μM, (e) 45 μM into stirring 0.1 M PBS (pH 7.0) containing 1.0 mM $K_4Fe(CN)_6$ at the applied potential of 0.05 V versus Ag/AgCl.

current, a slope of 2.294 μA $s^{0.5}$ $mV^{-0.5}$ and a correlation coefficient of 0.9982 were revealed. For the oxidation peak current, a slope of −1.059 μA $s^{0.5}$ $mV^{-0.5}$ and a correlation coefficient of 0.9976 were obtained. The results indicated that the electrode reaction was mainly controlled by diffusion process.

EIS has been used to characterize the interface properties of the HRP–GOD/Con A/CS biocomposite modified electrodes. Fig. 4 showed the typical results of AC impedance spectra of bare Au (a), CS/Au (b), Con A/CS/Au (c), and HRP–GOD/Con A/CS/Au (d) in a solution of 0.1 M KCl containing 1.0 mM $Fe(CN)_6^{3-}$ and 1.0 mM $Fe(CN)_6^{4-}$ versus Ag/AgCl, respectively. Significant differences in the impedance spectra were observed. The electron transfer resistance (R_{et}) of bare Au (a) was estimated to be 516.4 Ω. For CS/Au, the value of R_{et} was found to be 36.4 Ω. The observation suggested that the formation of CS film effectively improved electron transfer between electrochemical probe and the electrode. The results agreed with those obtained in the CV experiments as shown in Fig. 3. The phenomenon might be ascribed to the tiny positively charged surface that electrostatically adsorbed $Fe(CN)_6^{3-/4-}$. On the other hand, the R_{et} of CS/Au (b) was apparently small, the results indicated that the real surface area of CS/Au electrode was significantly increased, which would favor the immobilization of

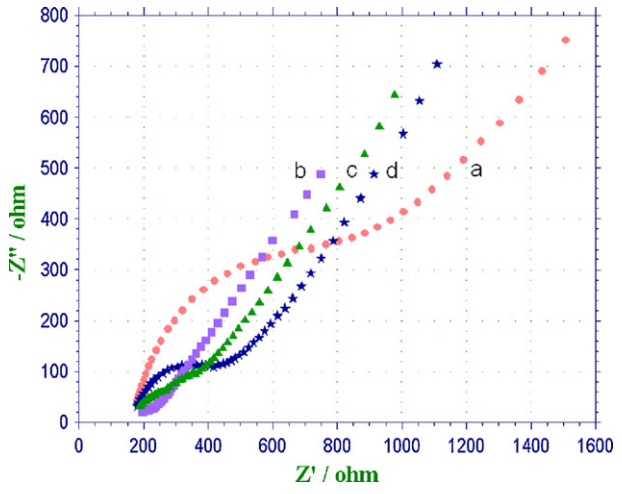

Fig. 4. EIS for bare Au (a), CS/Au (b), Con A/CS/Au (c), and HRP–GOD/Con A/CS/Au (d) in a solution of 0.1 M KCl containing 1.0 mM $Fe(CN)_6^{3-}$ and 1.0 mM $Fe(CN)_6^{4-}$.

HRP–GOD/Con A/CS on the electrode. The R_{et} for Con A/CS/Au (c) and HRP–GOD/Con A/CS/Au (d) were 112.9 and 208.0 Ω, respectively. Such increase of the R_{et} indicated the incorporation of the biomolecules.

3.4. Influence of pH and applied potential on response of the developed bienzyme biosensor

The dependence of the biosensor response on the pH of the measurement solution was investigated. A range of pH values between 4.0 and 9.0 was studied (see supplementary material). The current response reached the maximum at pH 7.0, which is close to the optimum pH observed for free GOD (Xue et al., 2006). Therefore, the suitable pH with the maximum performance of the biosensor was set at pH 7.0.

Studies to investigate the dependence of the biosensor response on the applied potential were also performed. The amperometric response of the HRP–GOD/Con A/CS/Au biosensor to glucose was investigated over the potential range of −0.10 to 0.25 V (see supplementary material). Little current was seen at 0.25 V, but current highly increased at 0.15 V. The current response arrived at a maximum value at 0.05 V and a flat step was observed from 0 to −0.10 V. As a result, the operating potential of 0.05 V was determined to give good sensitivity for further study.

3.5. Amperometric response of the developed bienzyme biosensor

Amperometric response of the HRP–GOD/Con A/CS/Au electrode to successive addition of glucose in 0.1 M PBS (pH 7.0) containing 1.0 mM $K_4Fe(CN)_6$ was given in Fig. 5. For comparison, the determination of glucose using HRP–GOD/CS/Au electrode prepared without the addition of Con A was also listed. At HRP–GOD/Con A/CS/Au electrode, as seen, rapid response and sharp increase were observed when glucose was added to the stirring PBS. The 95% of the steady-state current was obtained within 7 s. However, the HRP–GOD/CS/Au electrode, prepared without the bifunctional cross-linker, Con A, showed relatively low amperometric response. The inset displayed calibration curves of the two amperometric response of the biosensor to the concentration of glucose. Well-defined current proportional to the glucose concentration was observed for HRP–GOD/Con A/CS/Au electrode. The linear range of such biosensor for the determination of glucose was found to be 1.0×10^{-6} to 2.2×10^{-4} M with sensitivity of 1.18 μA mM^{-1} and a

Table 1
Determination of glucose in human serum samples.

Sample no.	Determined by biosensor (mM)[a]	Measured by hospital (mM)	Glucose added (mM)	Glucose found (mM)[a]	Recovery (%)
1	4.32	4.39	5.00	9.38	101
2	5.36	5.25	5.00	10.27	98
3	4.71	4.76	5.00	9.51	96

[a] Average of four determinations.

correlation coefficient of 0.9993. A detection limit of 6.7×10^{-7} M can be estimated using 3σ (where σ is the standard deviation of the blank solution, $n = 11$). It was a little higher than 0.5 μM based on nano-Au-PANI-GOD-GCE electrode (Xian et al., 2006), but a little lower than 1.5 μM at SiO_2-GOD-Pt electrode (Yang and Zhu, 2006), 2 μM at GOD-nano-FcDS (ferrocene-doped silica)-CS-GCE (Zhang et al., 2004) and much lower than 13 μM based on CS-nano-Au-GOD-GCE (Du et al., 2006) and 58 μM based on GOD-BISM (bamboo inner shell membrane)-OSOM (oxygen-sensitive optode membrane) (Yang et al., 2006). For HRP–GOD/CS/Au electrode, however, a significantly low sensitivity of 0.235 μA mM^{-1} was observed. The phenomenon indicated that the introduction of Con A for the affinity immobilization of HRP–GOD resulted in stable biocomposite film (Liu et al., 2007; Chen et al., 2008).

3.6. Reproducibility, stability and selectivity of the developed bienzyme biosensor

The reproducibility and storage stability of the proposed biosensor have also been studied. The relative standard deviation (RSD) of the biosensor response to 80 μM glucose was 2.9% for seven successive measurements. The fabrication reproducibility was investigated by preparing six biosensors independently. The reproducibility with the RSD of 3.8% for the detection of 80 μM glucose was obtained. The stability of the HRP–GOD/Con A/CS/Au bienzyme biosensor was evaluated by monitoring the response currents in the presence of 80 μM glucose, over a 5 week period. The electrode was stored at 4 °C in a refrigerator when not used and measured at intervals of 1 week. After 5 weeks, the biosensor retained about 89% of its original response. It was lower than that obtain at electrode based on GOD-BISM-OSOM (Yang et al., 2006), but higher than those obtained at SiO_2-GOD-Pt electrode (Yang and Zhu, 2006) and GOD-nano-FcDS-CS-GCE electrode (Zhang et al., 2004). The high stability might be ascribed to the biocompatible environment of CS, the strong biospecific affinity for assembly and the prevention of negative effect that high H_2O_2 concentration has on HRP activity (Bucur et al., 2004; Li et al., 2006; Tangkuaram et al., 2007).

The species usually appeared in biological and food samples were selected for interference studies to investigate the selectivity of the as-prepared biosensor. No change of the response was observed when 10 mM sucrose, fructose, lactose or maltose was individually added to 100 μM glucose. The phenomenon might result form the relative low applied potential of 0.05 V versus Ag/AgCl for the detection of glucose. Only high concentration of ascorbic acid (1.0 mM) interfered significantly. Ascorbic acid can reduce the hexacyanoferrate(III) produced in the peroxidase reaction and thus interfered the determination.

3.7. Glucose determination in serum samples

To demonstrate the practical usage of the glucose biosensor, human serum samples were assayed. A series of serum samples were analyzed. The sample was 100-fold diluted with 0.1 M PBS at pH 7. The diluted samples were then analyzed by the prepared biosensor at a potential of 0.05 V. The measured current was converted to the glucose concentration by extrapolating the value from the working curve. Results were summarized in Table 1. As can be seen, the values measured by the as-prepared bienzyme system are very close to results obtained by the clinic determination. These results indicate that the biosensor can be directly used to determine glucose in serum. The phenomenon might be ascribed to the tiny low working potential and the high stability of the biosensor.

4. Conclusion

An electrodeposition method for one-step construction of a HRP–GOD bienzyme biosensor by in-situ formation of HRP–GOD/Con A/CS biocomposite film was proposed. The biocompatible CS matrix, the introduction of Con A, the porous surface of the biocomposite film, and in-situ generation of H_2O_2 with bienzyme system were combined. The excellent characteristics and performance of the proposed biosensor, such as fast response, high sensitivity and selectivity, and good stability, make this methodological study and the application of such biocomposite film attractive in biosensor construction.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.03.017.

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