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Electrodeposition of chitosan–ionic liquid–glucose oxidase biocomposite onto nano-gold electrode for amperometric glucose sensing

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ABSTRACT

A sensitive glucose biosensor was fabricated by electrodepositing chitosan–ionic liquid–glucose oxidase biocomposite onto nano-gold electrode. First, nano-gold electrode was constructed by electrochemically depositing gold nanoparticles onto a flat gold electrode surface. Then the nano-gold electrode was immersed in the bath containing *p*-benzoquinone (BQ), chitosan (CS), glucose oxidase (GOD) and ionic liquid (IL) for electrodeposition of enzymatic electrode. The proton consumption during electroreduction of BQ increased the local solution pH near the electrode surface and led to the deposition of CS hydrogel on the electrode surface. Co-deposition of GOD and IL with the CS hydrogel was achieved. The proposed biosensor exhibited a fast amperometric response (<5 s) to glucose. Under the optimal conditions, the proposed biosensor exhibited a high current sensitivity (14.33 $\mu A\,mM^{-1}\,cm^{-2}$), which was 2.8 times of the biosensor prepared by electrodepositing CS–IL–GOD biocomposite on flat gold electrode. The detection limit for glucose was 1.5 μM, which was 20-fold lower compared to the biosensor prepared on flat gold electrode. The linear range for glucose detection was wide from 3.0 μM to 9.0 mM. Moreover, the proposed biosensor exhibited high reproducibility, long-time storage stability and satisfactory anti-interference ability. The applicability of the proposed biosensor to serum samples analysis was also evaluated.

1. Introduction

Diabetes mellitus has become a worldwide public health problem. It is one of the most prevalent and costly diseases in the world (Pei et al., 2004). There are nearly 40 million people suffering from diabetes in China (CDC Center, 2005). The metabolic disorder, named as hyperglycemia and hypoglycemia, may result in numerous complications such as heart disease, kidney failure and blindness. Therefore, monitoring of blood glucose levels becomes an indispensable test for the diagnosis and management of diabetes mellitus. Although innumerable methods have been developed, glucose biosensor has become the most popular means for its economical, simple and easy-to-use properties. Since the initial concept of glucose enzyme electrodes was proposed (Clark and Lyons, 1962), glucose biosensor has become a widely interested research field. Many studies on the development of glucose biosensors have been carried out (Wang, 2008). Many commercialized glucose biosensors for diabetes control have also been developed (Hu, 2009). For designing and fabricating biosensors, immobilization of enzymes is the key step. The development of simple and reliable procedures to immobilize and stabilize reactive enzymes on the electrode has received considerable attentions (Cosnier, 1999). The ideal immobilization process should be cheap, quick, and enzyme friendly, resulting in high sensitivity.

Ionic liquids (ILs), as molten salts with the melting point close to or below room temperature, consist of two asymmetrical ions of opposite charges (organic cations and various anions) that only loosely fit together. Due to its good properties such as non-volatility, non-flammability, high ionic conductivity, electrochemical and thermal stability and wide electrochemical window, ILs are attracting intensive interest in various areas of electrochemistry (Wei and Ivaska, 2008). On one side, ILs can be used as the supporting electrolytes. They are considered to replace the conventional organic solvents to reduce the environmental and economic cost. Recently, several authors have reported that increased stability and activity of enzymes were observed in ILs compared to it in aqueous solution or in some conventional organic solvents (Lozano et al., 2001; Laszlo and Compton, 2002; Persson and Bornscheuer, 2003; Lou et al., 2004; Machado and Saraiva, 2005). On the other hand, fabrication of biosensors incorporating ILs attracts more and more attentions. Many researches indicate that ILs can be entrapped in

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conventional matrices, such as chitosan (Lu et al., 2006a,b; Xi et al., 2008), cellulose (Turner et al., 2004), carbon materials (Zhao et al., 2004; Zhao et al., 2005), and sol–gel-based silica matrices (Liu et al., 2005). The resulted composites created a unique kind of materials for immobilization of enzyme. Persson and Bornscheuer assumed that the hydrogen bond and the electrostatic interaction between the IL and the enzyme resulted in a high kinetic barrier for unfolding of the enzyme. Therefore, the rigid structure of the enzyme can be protected from being destroyed (Persson and Bornscheuer, 2003). Accordingly, the stability and sensitivity of biosensors were improved effectively by the incorporating of ILs and conventional matrices.

Chitosan (CS) is a linear hydrophilic polysaccharide obtained by deacetylation of nature chitin. It is an attractive biocompatible, biodegradable, and nontoxic natural biopolymer that exhibits excellent film-forming ability. Due to its desirable properties, CS has been used as one of the most promising matrix for enzyme immobilization. CS–IL composite materials could find potential applications as electrochemical biosensing platforms. By entraping hemoglobin or horseradish peroxidase into CS–IL composite materials, direct electron transfer between enzyme and electrode was realized (Lu et al., 2006a,b). In addition, Wang et al. fabricated a β-nicotinamide adenine dinucleotide biosensor based on the CS–multi-walled carbon nanotubes (MWCNT)–IL composite (Wang et al., 2007). However, in these systems, the modification of electrodes with CS-based composite was achieved by casting or dip-coating method. In that way, the thickness of the resulting films was hardly uniform and controllable, and the biosensor fabrication was not so reproducible.

Electrochemical deposition has been reported recently as a simple method to obtain films on electrode. CS owns many primary amino groups, and has a pK_a value of about 6.3. At pH sufficiently below the pK_a, CS exists as a water-soluble cationic polyelectrolyte, since most of the amino groups are protonated. When the solution pH is raised near or above the pK_a, many of the amino groups are deprotonated, and CS becomes insoluble. Usually, electrochemical deposition of CS was performed via water reduction (Wu et al., 2002, 2003, 2005; Yi et al., 2004, 2005). At a reducing potential, H^+ in the solution was consumed at the cathode. Using the locally generated H^+ gradient, acidic side chains of CS were titrated. So the solubility of CS changed, eventually led to the controlled deposition of CS film. Meanwhile, other substances such as IL and even enzymes (Xi et al., 2008) could be effectively entrapped into CS film during the electrodeposition. Compared with the traditional process, this method is simple and it is suitable for selective deposition of films with controllable thickness. Zhou et al. reported that some deliberately added oxidants, e.g., *p*-benzoquinone (BQ) or H_2O_2, could be used as a "proton-consumer" instead of water. By this means, the electrochemically deposited CS can be carried out under much milder conditions and the resulted biosensor exhibited better performance (Zhou et al., 2007). However, the previous researches about electrochemical deposition of CS-based composite were all performed on the flat electrode. There are no works reported on the electrochemical deposition of CS-based composite on nano-gold electrode. Compared with the flat electrode, nanoparticle covered electrode exhibited many attractive characteristics such as large active surface areas, small charge-transfer resistance (Li et al., 2005; Szamocki et al., 2007). On the other hand, using nanomaterials will improved the electrical contact between the redox center of GOx and electrode supports (Wang, 2008). Thus the electrochemical signal would be increased on the nanoparticle covered electrode, which is significant for fabricating biosensors. In this paper, nano-gold electrode was prepared by electrochemical deposition of gold nanoparticle on a flat gold electrode. Then, biocomposite films of CS, IL and glucose oxidase were electrochemically deposited on the nano-gold electrode to construct a biosensor. Both biocompatibility of chitosan and inherent conductivity of IL enable the composite material to become an excellent biosensing platform. The effect of the biocomposite electrodeposition conditions on the biosensor response and performance of the resulting biosensor were investigated in detail.

2. Experimental

2.1. Reagents

CS from crab shells (90% deacetylated) and Nafion (5%) were purchased from Sigma. GOD (EC 1.1.3.4; type II from *Aspergillus niger*, activity ≈100 U mg^{-1}) and $HAuCl_4{\cdot}4H_2O$ (Au% > 48%) was purchased from Aldrich. 1-Butyl-3-methyl-imidazolium tetrafluoroborate ($BMIM{\cdot}BF_4$) was purchased from Shanghai Chengjie Chemical Co., Ltd. (Shanghai, China). Ionic liquid used in this paper is corresponding to $BMIM{\cdot}BF_4$. All other chemicals were of analytical grade and were used without further purification. CS solution was prepared by dissolving CS solid in 0.10 M acetic acid (HAc). After the undissolved material was filtered, the solution pH was adjusted to pH 5.0 via careful additions of concentrated NaOH under vigorous stirring.

2.2. Apparatus and instrumentations

All the electrochemical measurements were performed with a CHI 660A electrochemical workstation (Shanghai Chenhua Instrument Company, China). A conventional three electrode system was used with a modified gold electrode (2 mm in diameter) as working electrode, a platinum wire as counter electrode, and an Ag/AgCl (3 M KCl) electrode as reference electrode. All potentials were reported against Ag/AgCl. A magnetic stirrer and a stirring bar provided the convective transport during the amperometric studies. A 0.05 M, pH 7.0 phosphate buffer solution (PBS) was used as the electrolyte. All experiments were done at room temperature (~25 °C).

Electrochemical impedance spectroscopy (EIS) measurements were carried out at open circuit potentials and were performed in 5.0 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture containing 0.1 M KCl with the frequencies ranging from 100 kHz to 1 Hz.

The surface morphology of nano-gold electrode was analyzed by JSM-5600 LV (Japan) scanning electron microscopy (SEM).

The effective surface areas of the gold electrode and nano-gold electrode were determined according to the literature (Tremiliosi-Filho et al., 1997) by cyclic voltammetry between 0 and +1.5 V in 0.5 M H_2SO_4 by the integration of the cathodic peak during the reduction of the superficial gold oxide.

2.3. Preparation of the glucose biosensor

Nano-gold electrode was prepared by electrodeposition of gold nanoparticles onto the flat gold electrode (Li et al., 2005). Prior to electrodeposition, flat gold electrode (diameter 2 mm) was polished with 0.3 μm and 0.05 μm alumina, then ultrasonicated in ethanol and twice-distilled water, respectively. Then the electrode was electrochemically pre-treated by cyclic potential scanning between 0 and 1.5 V in 0.5 M H_2SO_4 until cyclic voltammogram became reproducible. After these pretreatments, the nano-gold deposition was carried out in 0.5 M H_2SO_4 solution containing 10 mM $HAuCl_4$ using chronoamperometry. The electrode potential is stepped from an initial potential E_i = 0.8 V, where no reaction occurs, to a overreduction potential E_r = 0.20 V, at which potential $AuCl^{4-}$ is fully reduced to Au nanocrystals. The deposition time was 10 s.

According to published procedures (Zhou et al., 2007), electrodeposion of CS–IL–GOD biocomposite on nano-gold electrode was triggered by reduction of BQ at −0.1 V for 300 s. The bath

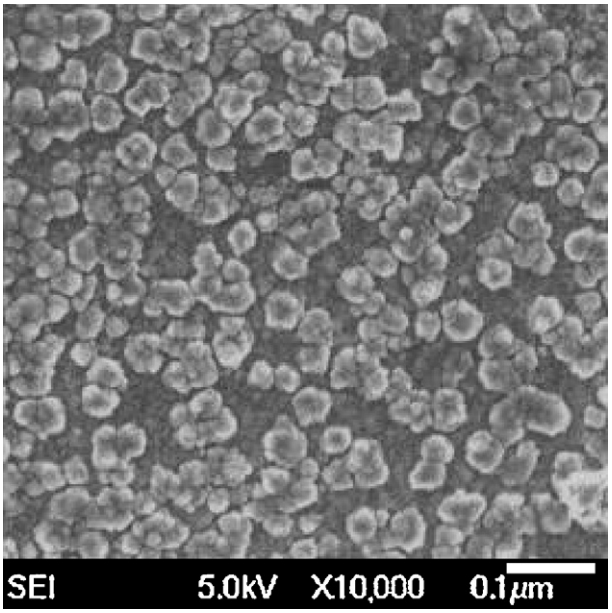


Fig. 1. SEM image of electrodeposited nano-gold electrode prepared at $E = 0.2$ V vs. Ag/AgCl with the deposition time of 10 s in 0.5 M H_2SO_4 solution containing 10 mM HAuCl4. The initial potential step at $t = 0$ s was started from 0.8 V.

for the electrodeposition of enzymatic electrode was 5.0 mL of a 0.5% CS solution containing 20 mM BQ, 5.0 mg mL^{-1} GOD and 1% (v/v) BMIM·BF_4, except where otherwise specified. After deposition, the modified electrode (CS–IL–GOD/nano–Au) was removed from the solution, rinsed with 0.05 M PBS (pH 7.0). To prevent interference, 5 μL 0.5% Nafion solution was cast on the surface of the prepared biosensor. For comparison, CS–IL–GOD/Au electrode was prepared by electrodeposion of CS–IL–GOD biocomposite on flat gold electrode using the same procedures above. When not in use, the prepared enzyme electrodes were stored in refrigerator (4 °C).

3. Results and discussion

3.1. Characterization of nano-gold electrode

The SEM image of electrodeposited nano-gold electrode is shown in Fig. 1. It was found that the Au nanoparticles deposited on the electrode surface had average particle sizes in the range of 20–40 nm.

The merit of the electrodeposition method for the fabrication of nano-gold electrode is that it is easy to control the size and density of gold nanoparticles by varying the concentration of the $HAuCl_4$ and the deposition time. In order to obtain a uniform nano-gold surface, 10 mM $HAuCl_4$ and a 10 s deposition time were chosen as the optimum deposition conditions. Longer deposition time or higher concentration of $HAuCl_4$ will lead to the formation of a continuous gold film. While when shorter deposition time or lower concentration of $HAuCl_4$ was adopted, only a small quantity of Au nanoparticles was obtained (Li et al., 2005).

The effective surface area of the gold electrode can be determined by accepting the charge of 0.386 mC cm^{-2} as the charge necessary to form a monolayer of electrosorbed O, in the form of AuO allowing for the double-layer charging (Tremiliosi-Filho et al., 1997). The effective surface areas of flat gold electrode was calculated to be 0.036 cm^2. Correspondingly, the effective surface area of nano-gold electrode reached 0.071 cm^2, which was almost twice as large as the flat gold electrode.

EIS is a powerful technique for studying the interface properties of electrode surfaces (Baranski et al., 1998). Fig. 2 shows the Nyquist plots of a flat gold electrode and nano-gold electrode, respectively. Significant differences in the impedance spectra were observed. The electron-transfer resistance (R_{et}), which equals the semicircle diameter at high frequencies in the Nyquist plots, of the nano-gold electrode surface was estimated to be 61 Ω, noticeably smaller than the flat gold electrode surface, 397 Ω. This result implied that the nano-gold array facilitated the electron transfer process on the electrode surface effectively.

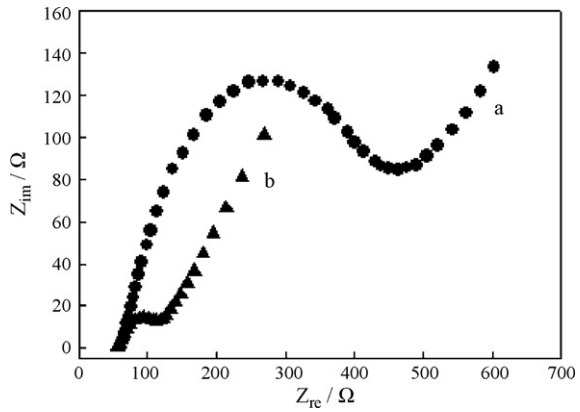


Fig. 2. Nyquist plots in 5 mM $Fe(CN)_6^{3-/4-}$ solution containing 0.1 M KCl at (a) bare gold electrode, (b) nano-gold electrode. The EIS measurements were carried out at open circuit potentials.

3.2. The mechanism of glucose determination

In this paper, amperometry technique was used to measure glucose. The proposed mechanism is shown in Scheme 1. The biocatalytical process for the oxidation of glucose in the presence of GOD can be summarized as following two processes:

First, the flavin group (FAD) in the enzyme is reduced of by reaction with glucose to the reduced form of the enzyme GOD($FADH_2$)

$$\text{GOD(FAD)} + \text{glucose} \rightarrow \text{GOD}(FADH_2) + \text{gluconolactone} \quad (1)$$

Then, the flavin is reoxidated by molecular oxygen to regenerate the oxidized form of the enzyme GOD(FAD)

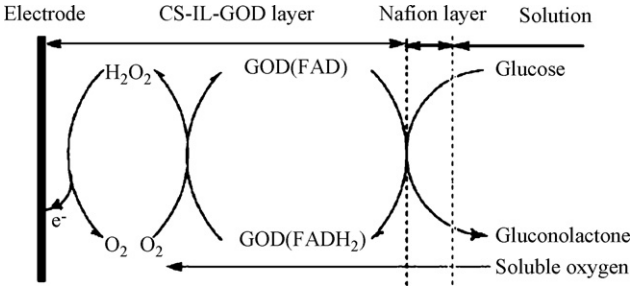
$$\text{GOD}(FADH_2) + O_2 \rightarrow \text{GOD(FAD)} + H_2O_2 \quad (2)$$

The quantification of glucose can be achieved via electrochemical detection of the enzymatically liberated H_2O_2:

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

3.3. Optimization of the electrodeposition conditions

The electrodeposition time plays an important role in the thickness of the deposited chitosan film (Wu et al., 2002). Thus, it



Scheme 1. The construction and sensing mechanism for the proposed glucose biosensor.

Table 1
Effects of possible interferents on the determination of glucose with CS–IL–GOD/nano–Au electrode.

Substrates[a]	Response current [b](μA)	Current ratio[c]
Glucose	2.28 ± 0.05	–
Glucose + ascorbic acid	2.35 ± 0.06	1.03
Glucose + uric acid	2.31 ± 0.05	1.01
Glucose + acetaminophen	2.40 ± 0.07	1.05

[a] The concentrations of substrates were at their physiological normal level (glucose: 5 mM; AA: 0.1 mM; UA: 0.5 mM; AP: 0.1 mM).
[b] Average of five determinations ± standard deviation.
[c] Current ratio = I_{G+I}/I_G. I_{G+I}, response current to glucose in the presence of interferents; I_G, response current to glucose.

would affect the performance of the biosensor. The response current of glucose increased evidently as the electrodeposition time increased from 100 s to 300 s. When the electrodeposition time was longer than 300 s, the response current began to level off (see supplemental Fig. S.1A). These would be caused by two reasons: first, the amount of GOD entrapped in the film increased as the film thickness increased at longer deposition time, and second, too thick film would affect the activity of GOD. On the other hand, longer deposition time resulted in longer response time. Therefore, the optimum electrodeposition time was chosen as 300 s.

Both the enzyme and IL contents in the deposition bath have significant effect on the performance of the biosensor. According to the previous procedures (Zhou et al., 2007; Luo et al., 2004), a glucose concentration of 0.5 mM was selected to study the effect of GOD and IL concentrations in the deposition bath. With the increase of the GOD concentration from 0 mg mL^{-1} to 5.0 mg mL^{-1}, the response current of glucose increased accordingly, which implies that higher GOD concentration resulted in higher sensitivity. When the concentration of GOD was higher than 5.0 mg mL^{-1}, no significant improvement of the response current was observed (see supplemental Fig. S.1B). This result agreed well with the previous researches (Zhou et al., 2007; Luo et al., 2004). The content of IL also had significant effect on the response of the biosensor. The best IL content was found to be 1% in the electrodeposition bath (see supplemental Fig. S.1B). Therefore a moderate GOD concentration of 5.0 mg mL^{-1} and an IL content of 1% in the deposition bath were selected, since they gave the highest current sensitivities at the minimum cost of reagents.

3.4. Optimization of applied potential

To improve the performance of the biosensor, the effect of applied potential on the response of proposed biosensor to glucose has been investigated in detail. The effect of applied potential on the response current was shown in supplemental Fig. S.2. With the increase of the applied potential from 0.2 V to 0.6 V, the response current increased accordingly. This means that the response of the enzyme electrode is resulted from the electrochemical oxidation of hydrogen peroxide. When the applied potential was higher than 0.6 V, the response current began to level off and the noise which was estimated as the fluctuating value of the steady state current increased obviously. Thus, a potential of 0.6 V was selected for glucose detection. At this potential, a higher sensitivity and a good signal/noise ratio could be offered.

3.5. Amperometric determination of glucose with the biosensor

Fig. 3A illustrates current–time plots for the CS–IL–GOD/nano–Au electrode under the optimized experimental conditions with successive additions of 1 mM glucose. For comparison, the response of glucose on the CS–IL–GOD/Au electrode is also shown.

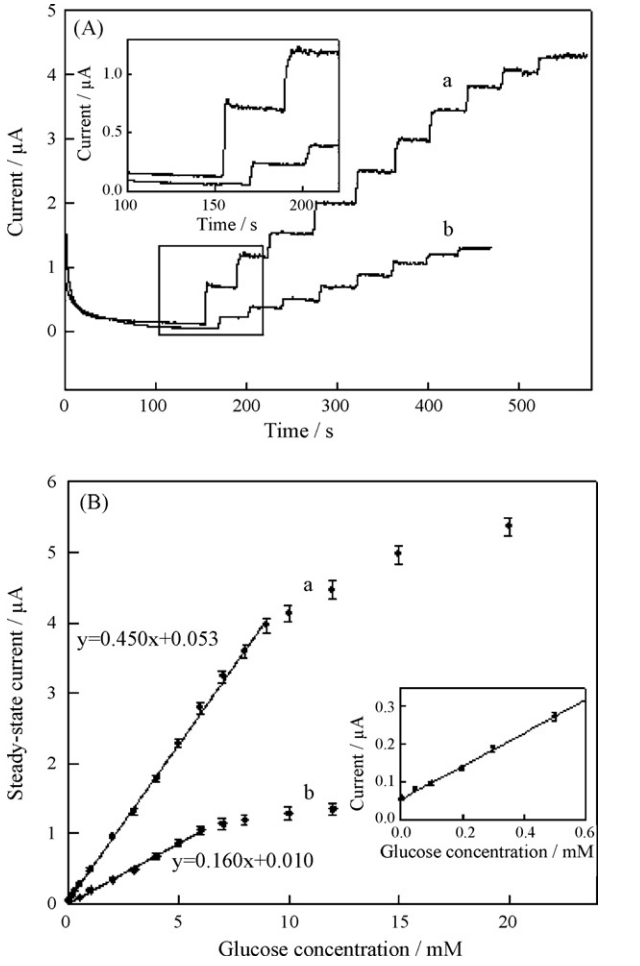


Fig. 3. (A) Typical amperometric response of (a) CS–IL–GOD/nano–Au electrode and (b) CS–IL–GOD/Au electrode to successive addition of 1 mM glucose in a stirred blank 0.05 M PBS (pH 7.0) containing no glucose. The applied potential was +0.6 V. (B) The calibration curves of (a) CS–IL–GOD/nano–Au electrode and (b) CS–IL–GOD/Au electrode. Insert: Enlarged drawing of calibration curve b in the range of lower glucose concentrations.

It is clear that rapid (less than 5 s) and sensitive response to glucose could be achieved for both electrodes. But interestingly, the amperometric response signal of CS–IL–GOD/nano–Au electrode is much greater than that of CS–IL–GOD/Au. The calibration curves of both electrodes are shown in Fig. 3B. As can be seen, the CS–IL–GOD/nano–Au electrode displays an expanded linear glucose concentration range from 3.0×10^{-6} to 9.0×10^{-3} M with a correlation coefficient of 0.997. The current sensitivity is 14.33 μA mM^{-1} cm^{-2} (0.45 μA mM^{-1}) to glucose. The amperometric response signals of gradually diluted glucose were measured for estimating the detection limit. At a signal-to-noise ratio of 3 (S/N = 3), the detection limit was found to be 1.5×10^{-6} M. Here, the noise was estimated as the fluctuating value of the steady state background current. The detection limit obtained with the proposed biosensor was lower than the values obtained with the GOD–nano gold–CS/glassy carbon electrode (13 μM) (Du et al., 2007) and the GOD–nano gold–CS multilayer film modified Pt electrode (7 μM) (Wu et al., 2007). However, the linear range at the CS–IL–GOD/Au electrode was only from 5.0×10^{-5} to 6.0×10^{-3} M with a detection limit of 3×10^{-5} M. The current sensitivity decreased to 5.12 μA mM^{-1} cm^{-2} (0.16 μA mM^{-1}), which means the electrodeposited nano-gold surface increased the glucose-detection sensitivity by a factor of 2.8. The higher sensitivity at the nano-gold electrode would be resulted from the large

Table 2
Results of the glucose assay and the recovery test for serum samples.

Serum sample	Glucose content provided by the hospital (mM)	Glucose added (mM)	Determined by the proposed biosensor (mM)[a]	Recovery (%)
1	6.18	0	6.05 ± 0.15	–
		5	11.15 ± 0.18	102
		10	15.74 ± 0.26	97
2	5.34	0	5.48 ± 0.09	–
		5	10.36 ± 0.24	98
		10	14.84 ± 0.40	94

[a] Average of five determinations ± standard deviation.

active surface areas and the excellent electron-transfer ability of gold nanoparticles.

The apparent Michaelis–Menten constant (K_m^{app}) can be calculated from the electrochemical version of the Linweaver–Burk equation (Kamin and Wilson, 1980):

$$1/i_{ss} = 1/i_{max} + K_m^{app}/(ci_{max})$$

where i_{ss} is the steady-state current after the addition of substrate, i_{max} is the maximum current under saturated substrate conditions, c is the concentration of substrate. The K_m^{app} value for the CS–IL–GOD/nano-Au electrode was found to be 7.8 mM, which was much smaller than the biosensor prepared by sol–gel CS film, 21 mM (Chen et al., 2003), and smaller than the biosensor prepared by CS–nano gold–GOD multilayer film modified Pt electrode, 10.5 mM (Wu et al., 2007). The smaller K_m^{app} value meant that GOD entrapped in the CS–IL biocomposite exhibited high enzymatic activity and affinity to glucose.

3.6. Reproducibility and stability of the biosensor

The reproducibility and storage stability of the proposed biosensor have also been studied. The relative standard deviation (RSD) of the current response of CS–IL–GOD/nano–Au electrode to 0.5 mM glucose was 3.2% for 10 successive measurements. For six electrodes modified identically, the R.S.D. was 4.5%. The stability of the CS–IL–GOD/nano–Au electrode under storage conditions (0.05 M PBS, pH 7.0, 4 °C) was investigated. The current response for 0.5 mM glucose was measured everyday up to 30 days. The current response did not change in a noticeable way during the 1st week, and only 5% of the current response was lost after 2 weeks. After 30 days, the current response still remained above 90% of its initial response. But, when the pH value of solution was lower than 6, the stability of the proposed biosensor became very poor. This disadvantage was induced by the detachment of CS film under acid condition.

3.7. The anti-interference ability and real sample analysis

Selectivity is important in practical use of the biosensors. The oxidizable compounds such as ascorbic acid (AA), uric acid (UA) and acetaminophen (AP) are usually co-existed with glucose in real samples. The current response obtained in the mixture of glucose and the interfering species at their physiological normal level (5 mM glucose, 0.1 mM AA, 0.5 mM UA and 0.1 mM AP) (Cass et al., 1984) was compared with the results obtained in the pure glucose solution. As shown in Table 1, all these interferences have littler influence on the glucose determination.

The satisfactory anti-interference ability would be resulted from the good properties of the outer Nafion film, which can effectively inhibit the penetration of negatively charged substrates towards the electrode surface (Shankaran et al., 2003; Mao et al., 2000). Thus, the proposed biosensor can be used in the physiological conditions without the significant interference from the substances present in the body fluids. Human serum samples were assayed to demonstrate the practical use of the proposed biosensor. The results of the glucose determination and the recovery of the serum samples are summarized in Table 2. As shown, the results obtained by the biosensor are in good agreement with those measured by the biochemical analyzer in the hospital and the recovery is satisfied.

4. Conclusion

In this paper, CS–IL–GOD biocomposite was electrodeposited onto nano-gold electrode for glucose sensing. The electrochemical deposition of gold nanoparticles on gold electrode surfaces led to a significant improvement in the detection sensitivity for assay of glucose. The proposed nano-gold electrode biosensor resulted in a detection limit of 1.5 μM for glucose, which was 20-fold lower compared to the biosensor prepared on flat gold electrode. The proposed glucose biosensor presented a number of other attractive features such as fast response, long-time storage stability, high reproducibility and satisfactory anti-interference ability.

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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.2009.02.027.

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