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Chitosan coated on the layers' glucose oxidase immobilized on cysteamine/Au electrode for use as glucose biosensor

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ABSTRACT

A glucose biosensor was developed via direct immobilization of glucose oxidase (GOD) by self-assembled cysteamine monolayer on Au electrode surface followed by coating chitosan on the surface of electrode. In this work, chitosan film was coated on the surface of GOD as a protection film to ensure the stability and biocompatibility of the constructed glucose biosensor. The different application ranges of sensors were fabricated by immobilizing varied layers of GOD. The modified surface film was characterized by a scanning electron microscope (SEM) and the fabrication process of the biosensor was confirmed through electrochemical impedance spectroscopy (EIS) of ferrocyanide. The performance of cyclic voltammetry (CV) in the absence and presence of 25 mM glucose and ferrocenemethanol showed a diffusion-controlled electrode process and reflected the different maximum currents between the different GOD layers. With the developed glucose biosensor, the detection limits of the two linear responses are 49.96 μM and 316.8 μM with the sensitivities of 8.91 μA mM^{-1} cm^{-2} and 2.93 μA mM^{-1} cm^{-2}, respectively. In addition, good stability (up to 30 days) of the developed biosensor was observed. The advantages of this new method for sensors construction was convenient and different width ranges of detection can be obtained by modified varied layers of GOD. The sensor with two layers of enzyme displayed two current linear responses of glucose. The present work provided a simplicity and novelty method for producing biosensors, which may help design enzyme reactors and biosensors in the future.

1. Introduction

Enzymes-modified electrode is a basic method employed for constructing biosensors and enzymatic bioreactors. In terms of the applicability of the biosensors, the enzymes should be immobilized on the electrode to avoid many complications linked to the solution systems. Therefore, suitable electrode immobilization methods of enzymes onto the electrode surface are of importance for obtaining their electrochemical reaction and keeping their bioactivities. If an enzyme immobilized on an electrode is available for direct electron transfer and keeping its bioactivity, it may be used in biosensors even without the addition of mediators. However, the accessibility of direct electron transfer between redox centers and electrode surface is limited by the three-dimensional structure of enzyme.

In recent years, considerable attention has been paid for the direct electron transfer of enzymes immobilized on the surface of an electrode and the majority of the developed methods rely on chemical modified (Degani and Heller, 1988; Ianniello et al., 1982; Narasimhan and Wingard Jr., 1986; Savitri and Mitra, 1998) or the usage of different materials related to the immobilization of enzymes, such as nanomaterials (Cai and Chen, 2004; Huang et al., 2005; Kang et al., 2009; Liu et al., 2004, 2005; Liu and Ju, 2003; Shan et al., 2009; Chen and Ma, 2014; Wu et al., 2009), porous materials (Coradin and Livage, 2003; Dai et al., 2007; Yu et al., 2014; Li et al., 2010), and biocomposite materials (Albareda-Sirvent et al., 2001; Bellezza et al., 2003; Chen and Gorski, 2001; Chen et al., 2002; Gole et al., 2000; Jia et al., 2007; Morales et al., 2000). Those applied materials reported demonstrate good performance but their costs are not economical. Meanwhile, the fabrication process with those materials may reduce the natural activities of enzymes. For the ideal method of enzyme immobilization should be enzyme benign and cost effective.

Au electrode has been used for constructing biosensors due to its good performance. Adsorptions of thiols and disulfides on Au electrode have been proved to form self-assembled monolayers (Folkers et al., 1992; Hostetler et al., 1996; Katz et al., 1996; Strong and Whitesides, 1988; Willner et al., 1996; Wirde et al., 1999). Cysteamine has been employed as bi-functional building blocks in which the sulfur atoms of the molecules are bound to the Au

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surface whereas the amino groups are attached to other groups from the layer (Doron et al., 1995; Gu et al., 2001; Willner et al., 1993; Willner and Riklin, 1994). However, it is obvious that the product via GOD immobilized on the Au electrode of self-assembled cysteamine (Sun et al., 2006) is unstable due to the weak attachment between GOD and the surface of electrode. Therefore, an important issue for successfully constructing stable biosensors is to prevent the active enzymes dropping off.

Chitosan is a unique physico-chemical biopolymer for enzyme immobilization with attractive properties. It has an excellent film-forming ability and a good adhesion, nontoxicity and biocompatibility (Kaplan., 1998; Luo et al., 2004), which provide friendly enzyme function. In addition, chitosan has primary amino groups resulting that the pKa value is about 6.3 (Ligler et al., 2001; Sorlier et al., 2001). At pH above the pKa, chitosan's amino groups will be deprotonated so that it becomes insoluble. In this study, chitosan coated on the modified electrode is used as the protection film, where chitosan's amino groups will be deprotonated in an electrolyte solution (pH 7.4) so that it becomes insoluble. As reported, chitosan hydrogel can also be deposited onto electrodes and the electrochemically deposited chitosan hydrogel can be tightly attached to the electrode and retain its natural properties (Fernandes et al., 2003). Thus, this assay proposed using chitosan, which not only retain its natural properties but also keep biocompatibility with GOD, to construct biosensor.

On the basis of EIS investigations, we demonstrated that GOD is successfully immobilized on the surface of Au electrode. In the present work, the main purpose is to develop and characterize an easy-making and novel method for glucose biosensor. The developed processes include the immobilization of GOD directly on the surface of chemically modified Au electrode and coating chitosan on the modified electrode as a protection film are convenient. The developed biosensors characterized by CV when ferrocenemethanol was used as a mediator demonstrated that we can obtain the wide signal ranges of sensors by developing multiple layers of enzyme. Double layers of GOD sensor appeared two linear current responses for glucose which made the single biosensor exceptional properties. The developed biosensor is characterized by SEM, EIS, CV, open circuit potential–time and current–time curve in detail. The proposed method for glucose biosensor is simple and the performance conditions are moderate, and the applied materials including cysteamine and chitosan are cost effective and enzyme benign.

2. Experimental section

2.1. Reagents

Glucose oxidase (EC1.1.3.4, from *Aspergillus niger*, CAS no. 9001-37-0) was purchased from MP Biomedicals of America. Cysteamine hydrochloride ($>98\%$) was obtained from Sangon Company of China. Ferrocenemethanol (98%) was purchased from Adamas reagent Co. Ltd. Phosphate buffer saline (0.01 M, pH 7.4) was purchased from Beijing Ding Guo Company of China. Chitosan of crab shells (80–95% deacetylated) was purchased from Sinopharm Chemical Reagent Co., Ltd. All other chemicals were of analytical grade and all solutions were prepared using ultrapure water.

2.2. Instrument and measurements

The scanning electron microscopic (SEM) images of Au/cysteamine/GOD, Au/cysteamine /GOD/chitosan were obtained with a S-4800 (Hitachi, Japan) scanning electron microscopy at an accelerating voltage of 5000 V.

Cyclic voltammetry, electrochemical impedance spectroscopy, open circuit potential–time and amperometric experiments were performed with a CHI 660A electrochemical workstation (Shanghai Chenhua Apparatus, China). All experiments were carried out using a conventional three-electrode cell. The working electrodes (WE) used were modified Au electrodes (Model CHI101, 2.0 mm diameter). A platinum plate electrode was used as the counter electrode (CE), and a saturated Ag/AgCl electrode was used as the reference electrode (RE). Phosphate buffer saline (0.01 M, pH 7.4) with or without 0.25 mM ferrocenemethanol was used as the supporting electrolytes.

Open circuit potential–time measurement was conducted in 3600 s under phosphate buffer saline (0.01 M, pH 7.4) at ambient laboratory temperature.

The XPS experiments were performed on a ESCALAB 250 Xi spectrometer (Thermo Scientific, America) using monochromatic AlKα radiation; the pass energy was kept at 40 eV. The Au 4f peak at 83.98 eV was used to check the binding energy scale of the instrument, and all spectra were also referenced to this peak. The S 2p and N 1s regions were recorded at each analysis position.

Amperometric measurements were conducted under phosphate buffer saline by applying the potential of 0.30 V at ambient laboratory temperature. Current–time data were recorded after a steady-state current had been achieved. Faradaic impedance measurements were performed in the presence of a 5×10^{-3} M $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1)-mixture as a redox probe, using an alternating current voltage of 5 mV. Impedance measurements were performed at a bias potential of 0.20 V in the frequency range from 0.1 to 1×10^5 Hz.

2.3. Preparation of the biosensor

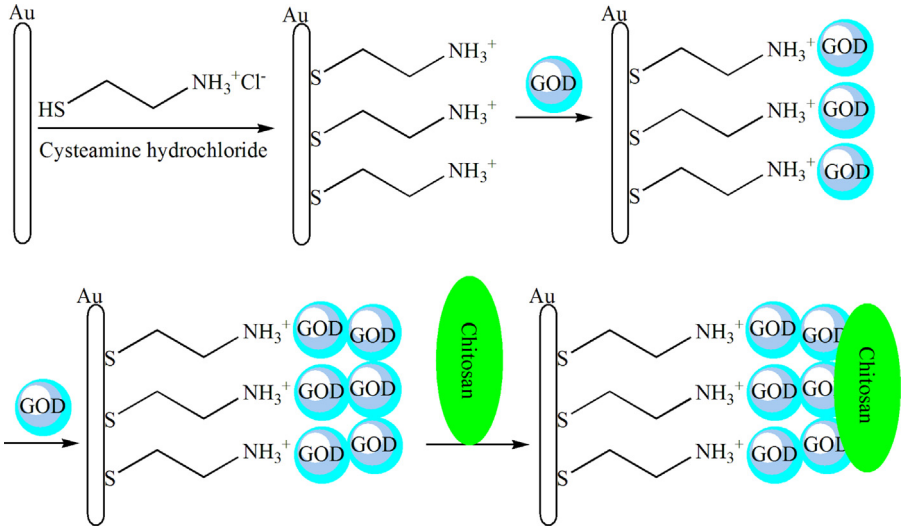
The Au electrode (2.0 mm in diameter) was polished sequentially with metallographic abrasive papers (No. 6) and slurries of 1.0, 0.3 and 0.05 μm gamma alumina powder to a mirror finish. After being rinsed with ultrapure water, it was sonicated with absolute ethanol and then with ultrapure water for 5 min, respectively. The cleaned Au electrode was first immersed in 20 mM cysteamine aqueous solution for 12 h at room temperature. The resulting self-assembled monolayer modified electrode was rinsed thoroughly with ultrapure water and soaked in water for a while to remove the physically adsorbed cysteamine. GOD solution (10 mg mL^{-1}) was poured on the surface of cysteamine modified Au electrode and dried at 4 °C and then this procedure was repeated several times. Chitosan solution (0.1%) was coated on the GOD film modified Au electrode and dried at 4 °C. Finally, the modified electrode was immersed in ultrapure water for a while and dried under flowing nitrogen. All resulting electrodes were stored at 4 °C in a refrigerator under dry conditions when not in use.

3. Results and discussion

3.1. Fabrication process of the biosensor

Scheme 1 shows the stepwise fabrication process of the two layers of GOD modified biosensor. The sulfur atoms of the cysteamine molecules are bound to the Au surface whereas the ammonium-terminates are attached to GOD mainly by noncovalent interaction: hydrogen bonding, electrostatic interaction and Vander Waals force. For example, hydrogen bonding between amino groups and Ala148, Asn168 at the surface of GOD, electrostatic interaction between NH_3^+ of cysteamine and COO^- of Asp181 and Glu221 at the surface of GOD. Finally, chitosan is coated on as a protection film.

Fig. 1 shows the surface image of the stepwise fabrication process of biosensor. The morphologies of GOD films and chitosan



Scheme 1. Stepwise assembly of cysteamine, two layers of GOD, chitosan on an Au electrode.

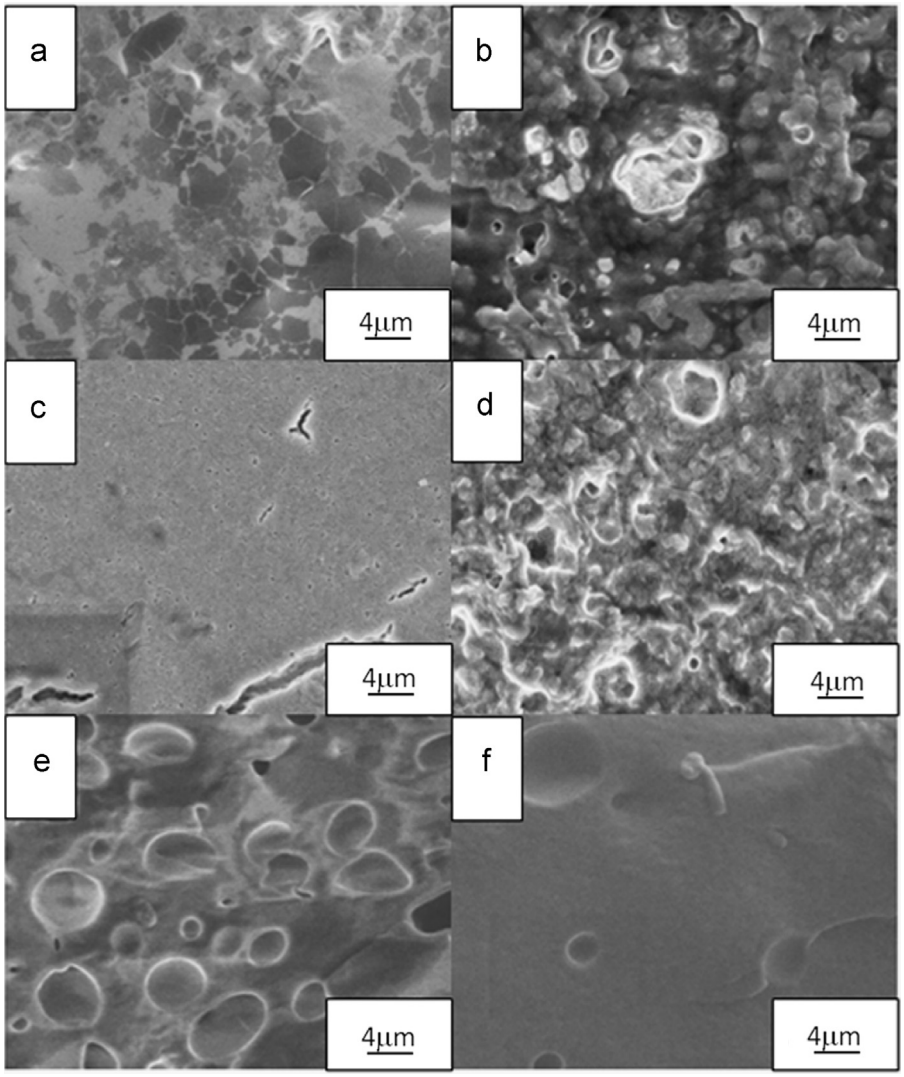


Fig. 1. SEM of the morphologies of (a) Au/cysteamine/GOD, (b) Au/cysteamine/GOD/chitosan, (c) Au/cysteamine/GOD/GOD, (d) Au/cysteamine/GOD/GOD/chitosan on the surface of Au film, SEM of the sectional view of (e) Au/cysteamine/GOD/chitosan and (f) Au/cysteamine/GOD/GOD/chitosan.

films which modified on gold film by cysteamine were characterized by scanning electron microscopy (SEM). GOD is easy to aggregate and rupture after drying as shown in Fig. 1a. When we increased the layers of GOD, the electrode's surface membrane is also easy to rupture (Fig. 1c). The SEM of chitosan film which is coated on one layer of GOD displays a porous structure (Fig. 1b).

Fig. 1d shows the SEM of the surface morphology of chitosan film coated on two layers of GOD. The porous structure on the surface of modified Au electrode is decreased when increased layers of GOD films. Besides, the porous structure is decreased when two layers GOD (Fig. 1f) instead of one layer of GOD (Fig. 1e), which corresponds to the sectional views of Fig. 1e and f, respectively. Thereby, the decrease in porous structure may account for the stability of the developed biosensor.

The electrochemistry of cysteamine was studied in a 10 mM aqueous phosphate buffer of pH 7.4 by CV in the potential region between +1.5 and −1.0 mV, using a 2 mm Au electrode and a 20 mM cysteamine solution. Compared to the CV responses of the Au electrode before and after immersing in 20 mM cysteamine aqueous solution for 12 h at room temperature (Fig. S1), the oxidation current in the gold oxide region (Fig. S1a) was significantly higher than the oxidation current (Fig. S1b), while the gold reduction peak areas were approximately equal. The latter indicates an irreversible oxidation of the self-assembled monolayer of cysteamine. The oxidation of the cysteamine layer probably involves an oxidation of the SH groups, formed upon the adsorption of cysteamine, to yield either SO_3^- ions (Walczak et al., 1995) or SO_2^- groups (Widrig et al., 1991), which desorbed from the gold surface. The present experimental results confirmed that cysteamine was oxidized in the gold oxide formation potential region, in agreement with previous findings (Owens and LaCourse, 1996).

The results from open circuit potential-time measurements (Fig. S2) also show that the cysteamine has been firmly immobilized on the Au electrode. The open circuit potential of cysteamine modified electrode is about 0.079 V (Fig. S2b), which is less than the clean Au electrode at 0.088 V (Fig. S2a).

The appearance of S 2p and N 1s of cysteamine in XPS (Fig. S3) also provides evidence that adsorbed layers are formed on gold. In the S 2p spectra (Fig. S3A), the normal S–Au bond binding energy is 162.1 eV; the other component is attributed to unbound sulfur (Castner et al., 1996) which is located at about 163–164 eV. The S–Au bond component is increasing obviously when compared to the 7 levels of deep analysis (Fig. S3A). The cysteamine N 1s spectrum (Fig. S3B) shows component at between 399.2 and 400.3 eV, and the component is decreasing in the deep analysis compared to all 7 curves.

In addition, the assembly process of the Au electrode was monitored by EIS experiments as EIS is an effective method for probing the features of surface-modified electrodes (Ehret et al., 1997; Patolsky et al., 1999). Fig. 2 shows the impedance features, presenting Nyquist plots (−Z'' vs. Z') of electrodes at different modification steps. During the process of modification, significant differences in the impedance spectra were observed. The Au/cysteamine electrode exhibited an almost straight line (Fig. 2a), which is characteristic of a diffusion limited electrochemical process. Insulating layers modified on the electrode surface functioned as a barrier to the interfacial electron transfer. This is reflected by the appearance of the semicircular part of the spectrum. Fig. 2b shows GOD immobilized on the Au/cysteamine electrode as a barrier, and Fig. 2c shows that chitosan layer is also an insulating film. The diameter of the respective semicircular element corresponds to the electron transfer resistance (Ret) at the electrode surface. The diameter increased when increasing layers of GOD on the electrode (Fig. 2c–f).

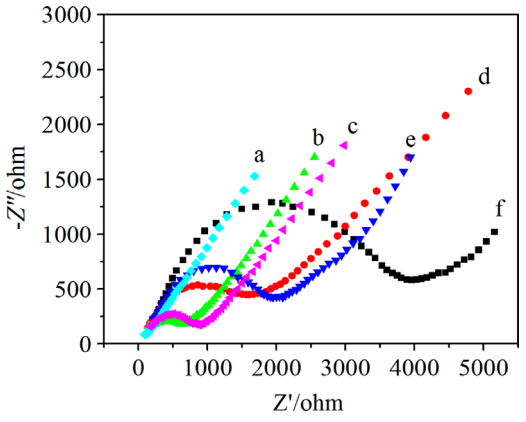


Fig. 2. EIS for the cysteamine modified Au electrode with different numbers of glucose oxidase in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (molar ratio is 1:1) solution. Au/cysteamine; (b) Au/cysteamine/GOD and Au/cysteamine/GOD/chitosan; (c)–(f) with different layers of GOD ((c) one layer; (d) two layers; (e) three layers; (f) four layers).

3.2. The electrocatalytic performance of GOD at the biosensor

Fig. 3A shows the cyclic voltammetric responses of the biosensor modified two layers of GOD coated with one layer of chitosan in 0.01 M phosphate buffer solution (pH 7.4) containing 0.25 mM ferrocenemethanol as the mediator. The mediator is used to observe and enhance the electron transfer. Fig. 3A shows the effect of scan rate. The pair of peaks is assigned to one-electron reversible redox reaction of Fc^+/Fc. The redox peak currents are proportional to the square root of scan rate $v^{1/2}$ (Fig. 3B), indicating a diffusion-controlled electron-transfer process (Brown and Anson, 1978).

In Fig. 3C, Curve a shows one-electron reversible redox reaction of Fc^+/Fc on modified electrode, one pair of waves with anodic peak potential (E_{pa}) at +0.212 V and cathodic peak potential (E_{pc}) at +0.141 V; the peak potential separation (ΔE_p) is about 71 mV. With the addition of glucose to the above solution, a well-defined sigmoidal catalytic wave was developed as a consequence of the GOD catalytic oxidation of glucose as shown in Curve c. The reaction can be described by the mechanism (Fig. 3D) as reported (Bourdillon et al., 1993; Zhang et al., 2005). On the other hand, without chitosan coated on, the modified Au/cysteamine/GOD (two layers of GOD) electrode demonstrates a tiny increase in redox current and a weak stability of catalytic oxidation of glucose as shown in Curve b (Fig. 3C). Therefore, it can be contributed to the coated chitosan film protecting the layers GOD from being lost, and enhanced the stability of the immobilized GOD.

3.3. Performance of the biosensor

The electrochemical response of GOD modified Au electrode is relevant to the concentration of glucose and the quantity of active GOD. Fig. 4 shows voltammetric responses of the different layers of GOD modified Au electrode in phosphate buffer solution (pH 7.4) containing 0.25 mM ferrocenemethanol and 25 mM glucose. Fig. 4a belongs to the bare Au, in the presence of 25 mM glucose and a pair of well-reversible redox wave with formal potential at 0.178 V (vs. Ag/AgCl) was observed. Fig. 4b–e shows voltammetric responses of the modification of one, two, three and four layers of GOD respectively. The anodic peak current increased with the modified layers of GOD. On the contrary, in Fig. 4b–e no change or slightly decrease in the cathodic peak current is observed. The difference between two peak currents results from the involved oxidation of glucose.

Fig. 5 shows one example of the current–time recordings of the biosensor on successive step changes of glucose concentration under operating potential of 0.3 V vs. saturated Ag/AgCl. The biosensor exhibited a rapid and sensitive response to changes in glucose concentration, indicating excellent electro-catalytic behavior of the biosensor. The sensor can reach 96% of the steady-state current within 9 s. The inset in Fig. 5 shows a calibration plot of the steady-state current vs. glucose concentration. The current responses of the sensor consist of two parts: one linear range between 1.5 and

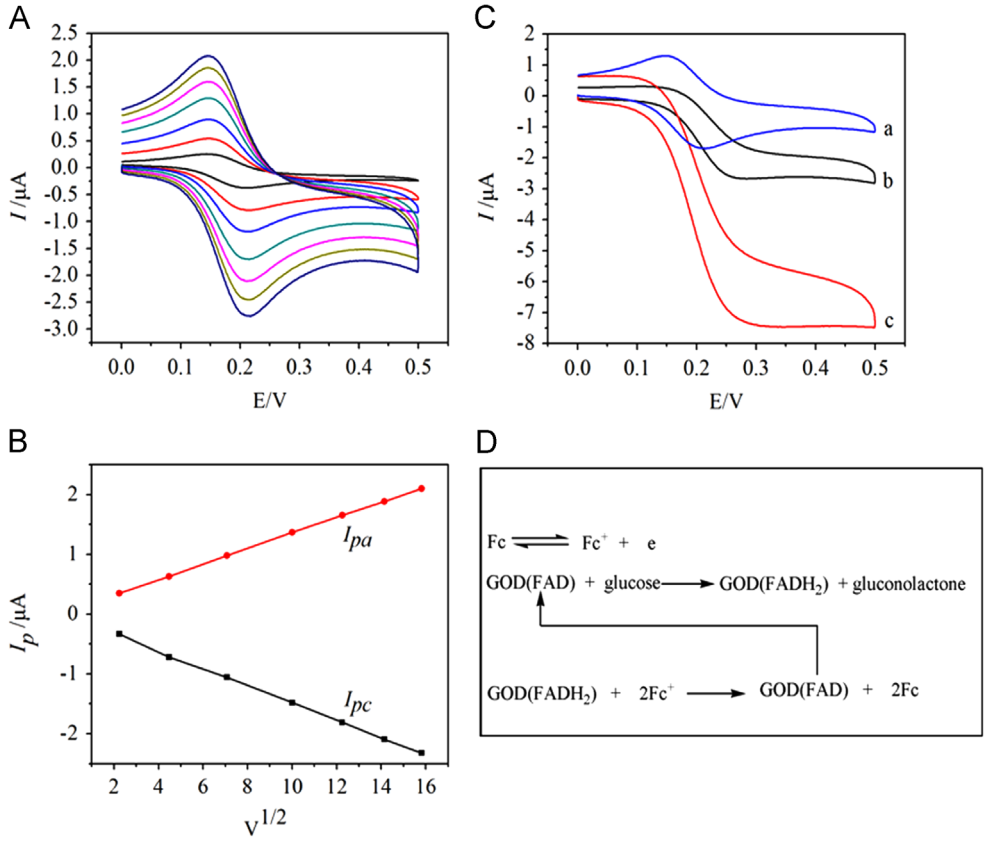


Fig. 3. (A) CV responses of two GOD layers modified Au/cysteamine/GOD/chitosan electrode in 0.01 M phosphate buffer solution (pH 7.4) containing 0.25 mM ferrocenemethanol at different scan rates and (B) the relationship of anodic and cathodic peak currents vs. the square root of scan rate. The scan rate (from inside to outside) is 5, 20, 50, 100, 150, 200, 250 mV/s, respectively. (C) CV responses of the Au/cysteamine/GOD/chitosan with two layers of GOD modified electrodes in 0.01 M phosphate buffer solution (pH 7.4) containing 0.25 mM ferrocenemethanol in the absence (a) and presence (c) of 25 mM glucose. Curve b is bioelectrocatalytic responses of the Au/cysteamine/GOD with two layers of GOD modified electrodes in 0.01 M phosphate buffer solution (pH 7.4) containing 0.25 mM ferrocenemethanol and 25 mM glucose. Scan rate: 100 mV/s. (D) The reaction described the mechanism of GOD catalytic oxidation of glucose (GOD ($FADH_2$) and GOD (FAD) are the reduced and oxidized forms of GOD, Fc and Fc^+ are the reduced and oxidized forms of ferrocenemethanol, respectively).

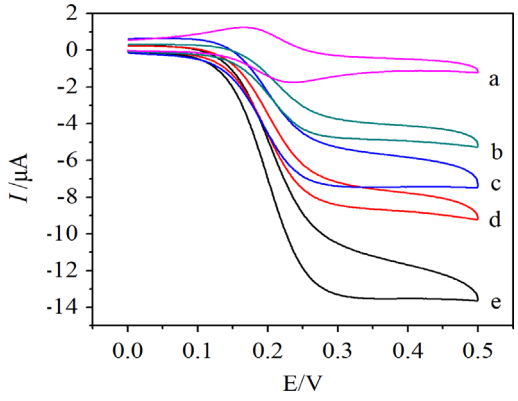


Fig. 4. CV responses of the modified electrodes in 0.01 M phosphate buffer solution (pH 7.4) containing 0.25 mM ferrocenemethanol and 25 mM glucose. Scan rate: 100 mV/s. (a) bare Au; (b)–(e) Au/cysteamine/GOD/chitosan with different layers of GOD; (b) one layer; (c) two layers; (d) three layers; (e) four layers.

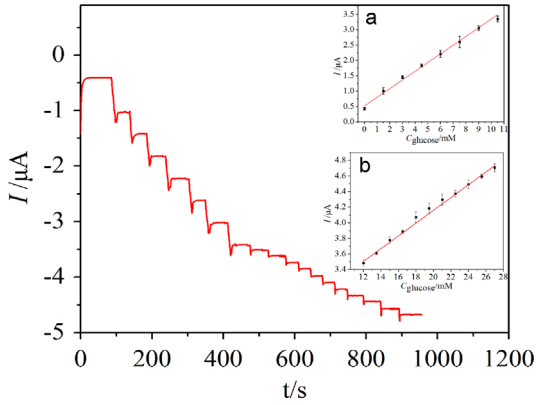


Fig. 5. Current–time curve obtained at the modified two layers of glucose oxidase (GOD) electrode for successive addition of 3 μmol glucose to 15 mL phosphate buffer solution (pH 7.4) containing 0.25 mM ferrocenemethanol; applied potential, 0.3 V vs. saturated Ag/AgCl. Inset: calibration plot of the steady-state current vs. glucose concentration with (a) range between 1.5 and 10.5 mM; (b) range from 10.5 to 27 mM.

10.5 mM with the sensitivity of 8.91 μA mM^{-1} cm^{-2} and the detection limit of 49.96 μM at a signal-to-noise ratio of 3 (inset, Fig. 5a; linear regression equations: $I = 0.516 + 0.284C_{glucose}$, $R^2 = 0.9917$, RSD is in range from 0.65% to 9.6%); the other linear is in range from 10.5 to 27 mM with the sensitivity of 2.93 μA mM^{-1} cm^{-2} and the detection limit of 316.8 μM at a signal-to-noise ratio of 3 (inset, Fig. 5b; linear regression equations: $I = 2.517 + 0.082C_{glucose}$, $R^2 = 0.9965$, RSD is in range from 0.052% to 2.47%).

3.4. Stability of the biosensor

The reproducibility of the four layers GOD biosensor is poor according to the response from cyclic voltammetry. The stability of the two-layer GOD biosensor during continuous operation was investigated by measuring the changes in voltammetric current during potential cycling. This two-layer GOD biosensor showed no

obvious changes in I_{cat} when subjected to 15 potential cycles from 0 to 0.5 V at a scan rate of 100 mV/s. The storage stability of this type of biosensor was checked by CV test every 2–3 days. The sensor was stored at 4 °C in 0.01 M phosphate buffer solution (pH 7.4). The current response of the sensor to glucose remained almost constant during the first 30 days with the RSD range from 1.3% to 7.2% (Fig. S4). Thus, the chitosan coated on electrode is quite efficient for retaining the activity of GOD. Good storage stability observed may be attributed to the good biocompatibility of interaction between cysteamine and GOD, GOD and chitosan.

4. Conclusions

In summary, we have developed an easy but novel process to fabricate a glucose biosensor by covalently and noncovalently binding GOD to a cysteamine monolayer modified Au electrode. Cysteamine employed as bi-functional create a self-assemble monolayer in building blocks with the sulfur atom of the molecule bound to the Au surface whereas the ammonium-terminates are attached to GOD layer. The presence of chitosan protection film enhances the stability of loaded GOD without the sacrifice of GOD activity. SEM, EIS and CV were used successfully to characterize the developed biosensor. The developed biosensor demonstrated distinct values including material affordability, high sensitivity (8.91 μA mM^{-1} cm^{-2}) and fast response time (< 10 s), easy way to produce (coating and drying), good stability (up to 30 days) and versatility by changing the modified enzyme layer. The use of chitosan in the developed process not only provide an avenue to overcome the weak attachment between GOD and the surface of electrode, but also may lead to other enzymes-modified biosensors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.04.035.

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