Biosensors and Bioelectronics 60 (2014) 271–276

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

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

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ARTICLE INFO

Article history:
Received 21 January 2014
Received in revised form
5 April 2014
Accepted 17 April 2014
Available online 30 April 2014

Keywords:
Cysteamine
Glucose oxidase
Au electrode
Chitosan
Immobilization

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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http://dx.doi.org/10.1016/j.bios.2014.04.035
0956-5663/

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