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Glucose oxidase/colloidal gold nanoparticles immobilized in Nafion film on glassy carbon electrode: Direct electron transfer and electrocatalysis

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Abstract

The direct electron transfer of glucose oxidase (GOD) was achieved based on the immobilization of GOD/colloidal gold nanoparticles on a glassy carbon electrode by a Nafion film. The immobilized GOD displayed a pair of well-defined and nearly reversible redox peaks with a formal potential ($E^{\circ\prime}$) of −0.434 V in 0.1 M pH 7.0 phosphate buffer solution and the response showed a surface-controlled electrode process. The dependence of $E^{\circ\prime}$ on solution pH indicated that the direct electron transfer reaction of GOD was a two-electron-transfer coupled with a two-proton-transfer reaction process. The experimental results also demonstrated that the immobilized GOD retained its electrocatalytic activity for the oxidation of glucose. So the resulting modified electrode can be used as a biosensor for detecting glucose.

Keywords: Direct electron transfer; Glucose oxidase; Colloidal gold nanoparticles; Electrocatalysis; Glucose

1. Introduction

One of the enduring challenges in electrochemical research is achieving direct electron transfer of enzymes or proteins with electrode. The quest to achieve this goal is because direct electron transfer will firstly offer an electrochemical basis for the investigation of the structure of enzymes (proteins), mechanisms of redox transformations of enzymes (proteins), and metabolic process involving redox transformations and secondly establish a foundation for fabricating biosensors, catalytic bioreactors, and biomedical devices. It is difficult for an enzyme (protein) to exchange electrons directly with bare solid electrodes due to several factors. First, it would be adsorbed on the electrode surface, resulting in large changes in its conformation and loss of its electrochemical activity and bioactivity. Second, its redox center is deeply buried in its electrochemically "insulated" peptide backbone, resulting in the inaccessibility of the redox center, so that promoters and mediators are needed to obtain the electrochemical reactions of enzymes (proteins) [1–3]. Extensive research has been done over the last twenty years to study the direct electron transfer of small redox proteins, such as cytochrome *c*, hemoglobin or myoglobin [4–6]. However, direct electron transfer of large redox enzymes has proved to be less successful.

Glucose oxidase (GOD) is a homodimer with a molecular weight of about 150–180 kDa containing two tightly bound flavine adenine dinucleotide (FAD) cofactors [7] and catalyzes the electron transfer from glucose to oxygen accompanying the production of gluconic acid and hydrogen peroxide. The most important application of GOD is in biosensors for the quantitative determination of glucose in body fluids, foodstuffs, beverages and fermentation liquor. A great amount of work has been devoted to fabrication of various kinds of biosensors based on GOD [8–11]. But only a few examples of quasi-reversible voltammograms for direct electron transfer between the GOD active site and the electrode surface are reported. Godet et al. [12] reported the direct electron transfer of GOD by fixed in the bulk of a graphite paste electrode in the absence of additives or modifiers. The direct electron transfer of GOD, immobilized onto aligned nanotube electrode arrays formed by self-assembly, was studied by Liu et al. [13]. Ianniello et al. [14], using differential pulse voltammetry, studied the direct electron transfer of adsorbed GOD at a graphite electrode and a cyanuric

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chloride-modified graphite electrode. So developing novel and convenient method to fulfill the direct electron transfer of GOD has great significance.

Nafion (perfluorinated sulphonate ionomer), due to its easy fabrication, good electrical conductivity, high chemical stability and good biocompatibility, has been widely used in biosensor making as a protective and selective coating material and as a support for enzyme immobilization. In addition, Nafion film has negative charge, so foreign species such as ascorbic acid, paracetamol, uric acid, etc. are readily repelled [15–17]. The number of potential foreign species is then restricted by molecular size, permeation and/or (bio)chemical reaction. As a consequence, the analytical sensitivity and system versatility are obtained.

Recently, there is an increasing interest in using all kinds of nanoparticles to construct electrochemical biosensors [18–21] since they have high specific surface area, good biocompatibility, and good conductivity. It was reported that gold nanoparticles (GNPs) could adsorb redox enzymes (proteins) without loss of their biological activities [22]. In addition, GNPs can act as tiny conduction centers and can facilitate the transfer of electrons. So it has been used for study of the direct electron transfer of proteins [23,24].

In this work, we study the direct electron transfer of GOD immobilized on GNPs by a Nafion film, in which the GNPs is used to retain the bioactivity of GOD and facilitate the direct electron transfer between GOD and electrode. The biological and electrochemical activities of GOD were characterized with UV–Vis spectroscopy and cyclic voltammetry. The immobilized GOD exhibits a fast direct electron transfer and retains its electrocatalytic behavior to glucose oxidation.

2. Experimental

2.1. Reagents

Glucose oxidase (EC 1.1.3.4, from *Aspergillus niger*, 100 U/mg) was purchased from Amersco. Hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4 \cdot 3H_2O$), Nafion (5 wt.%) solution in a mixture of lower aliphatic alcohols and water and ferrocenemethanol were obtained from Aldrich. 0.1 M phosphate buffer solutions (PBS) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4 and adjusting the pH values with 0.1 M H_3PO_4 or NaOH solutions. All other chemicals were of analytical grade. All solutions were made up with doubly distilled water.

2.2. Apparatus

UV–Vis spectra were recorded on a GBC Cintra 10_e UV–Visible spectrometer. The electrochemical experiments were carried out with a CHI 660A electrochemical workstation (CH Instruments Co., USA) with a conventional three-electrode cell. The modified or unmodified glassy carbon electrode was used as the working electrode. The Pt wire and the Ag/AgCl (3.0 M KCl) electrode were used as the counter and reference electrodes, respectively. All solutions were purged with high-purity nitrogen for at least 20 min prior to experiments and a nitrogen environment was then kept over the solution in the cell. All experiments were performed at a temperature of 25 ± 1 °C. Amperometric experiments were carried out in a stirred system by applying a potential step of 0.27 V to the working electrode. Aliquots of glucose standard solution were added successively to the solution. Current-time data were recorded after a steady-state current had been achieved.

2.3. Preparation of GNPs

All glassware used in the procedures was firstly washed with freshly prepared HNO_3/HCl (1 : 3, v/v), then rinsed thoroughly with doubly distilled water and dried before use. Spherical GNPs were prepared according to the literature [25] by adding sodium citrate solution to a boiling $HAuCl_4$ aqueous solution. The solution was stored in brown glass bottles at 4 °C. The average nanoparticle diameter is 12 nm as measured by transmission electron microscopy (not shown here).

2.4. Preparation of Nafion/GOD–GNPs/GC electrode

Glassy carbon electrodes (3 mm-diameter) were polished first with 1.0-, 0.3- and 0.05-μm alumina slurry. After rinsing thoroughly with doubly distilled water, they were sonicated in absolute ethanol and doubly distilled water for about 1 min, respectively. GOD solution was obtained by dissolving 10.0 mg of GOD in 1 mL of 0.1 M pH 6.82 PBS, and the GNPs were used as prepared. Then a 10 μL mixture of GOD and GNPs (v/v = 1 : 1) was dropped onto the surface of a cleaned glassy carbon electrode with a microsyringe and allowed to dry at ambient temperature. Finally, 1 μL of Nafion (5 wt.%) was casted and used as a net to hold the GOD–GNPs on the electrode surface stably. The solvent was allowed to evaporate before use. The final electrode is taken as the Nafion/GOD–GNPs/GC electrode. The similar procedures were employed to fabricate the Nafion/GOD/GC and Nafion/GNPs/GC electrode. All resulting electrodes were stored at 4 °C when not in use.

3. Results and discussions

3.1. UV studies of the interaction between GOD and GNPs

UV–Vis absorption spectra have been carried out to study the effects of GNPs on the microstructures of GOD. UV–Vis is sensitive to the possible changes inside the microenvironment of GOD. Fig. 1 shows the UV–Vis absorption spectra of (a) GOD in 0.1 M pH 6.82 PBS, (b) GNPs, and (c) the mixture of GOD and GNPs. As can be seen, in the case of GOD solution (native state) an intense band appeared at 280 nm and two well-defined peaks of light absorption at 375 and 450 nm could be distinguished. The above peaks could also be observed in the mixture of GOD and GNPs and nearly have no difference in the site or shape, indicating the GOD keeps its natural structure in the mixture. In addition, the GNPs solution exhibited a distinct surface plasmon absorption band at 519 nm. When mixed with GOD, there is nearly no change in the site. The result shows that

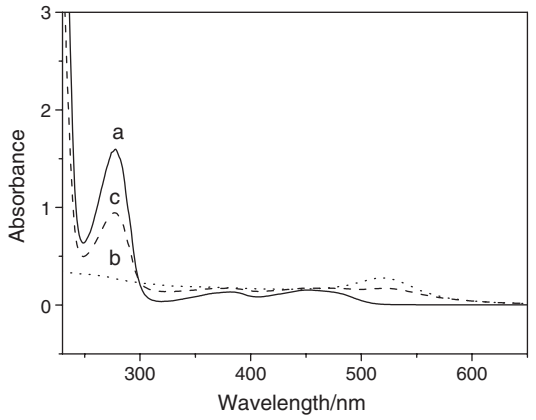

Fig. 1. UV–Vis absorption spectra of (a) GOD solution; (b) GNPs solution; (c) the mixed solution of GOD and GNPs (v/v=1:1).

GNPs are uniformly dispersed in the mixture and no obvious aggregation of GNPs occurred.

3.2. Direct electron transfer of GOD

The cyclic voltammograms of bare glassy carbon electrode in PBS in the presence or in the absence of GOD in solution showed no response. However, a pair of well-defined and nearly reversible redox peaks for the direct electron transfer of GOD could be observed on Nafion/GOD–GNPs/GC electrode in 0.1 M pH 7.0 PBS, as shown in Fig. 2a. The anodic peak potential (E_{pa}) and cathodic peak potential (E_{pc}) are located at −0.423 and −0.444 V, respectively, at scan rate of 50 mV/s. The separation of peak potentials, ΔE_p, is 21 mV. The formal potential ($E^{\circ\prime}$), calculated from the average value of the anodic and cathodic peak potentials, is −0.434 V. In contrast, no peak was observable at Nafion/GNPs/GC electrode (Fig. 2c). When only GOD was entrapped in the Nafion film without the presence of GNPs, the cyclic voltammogram showed a small response of GOD (Fig. 2b). However, the response was much smaller than that of Nafion/GOD–GNPs/GC electrode. Thus, the GNPs played an important role in facilitating the electron exchange between the GOD and electrode. It is most likely that the GNPs have relatively larger specific surfaces to integrate enzyme and to aid in the orientation of the enzyme absorption as well. Simultaneously, GNPs act as electron transfer tunnels which may favor the electron transfer between the enzyme and electrode.

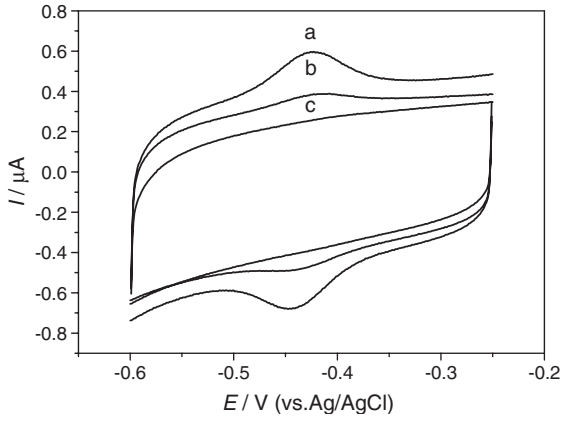

Fig. 2. Cyclic voltammograms of (a) Nafion/GOD–GNPs/GC electrode; (b) Nafion/GOD/GC electrode; (c) Nafion/GNPs/GC electrode; in 0.1 M pH 7.0 PBS at scan rate of 50 mV/s.

It was reported that the direct electron transfer of GOD immobilized on the heterogeneous surface originated from the conversion of GOD(FAD) and GOD ($FADH_2$) [14]. In GOD, FAD is deeply seated in a cavity and therefore not easily accessible for conduction of electrons to the electrode surface. The formal potential of FAD/$FADH_2$ redox couple at pH 7.0 is −0.434 V, which is close to the standard electrode potential of −0.46 V (vs. SCE) for FAD/$FADH_2$ at pH 7.0 (25 °C) [26], indicating that most GOD molecules preserved their native structure after immobilized on the gold nanoparticles.

Fig. 3A shows the cyclic voltammograms of Nafion/GOD–GNPs/GC electrode in pH 7.0 PBS at different scan rates. Both the cathodic and anodic peaks currents are linearly proportional to the scan rate in the range from 20 to 250 mV/s (Linear regression equations: P_a: $y=0.00844+0.0094x$, $r=0.999$; P_c: $y=-0.09728-0.00928x$, $r=-0.999$), which indicates a surface-controlled electrode process.

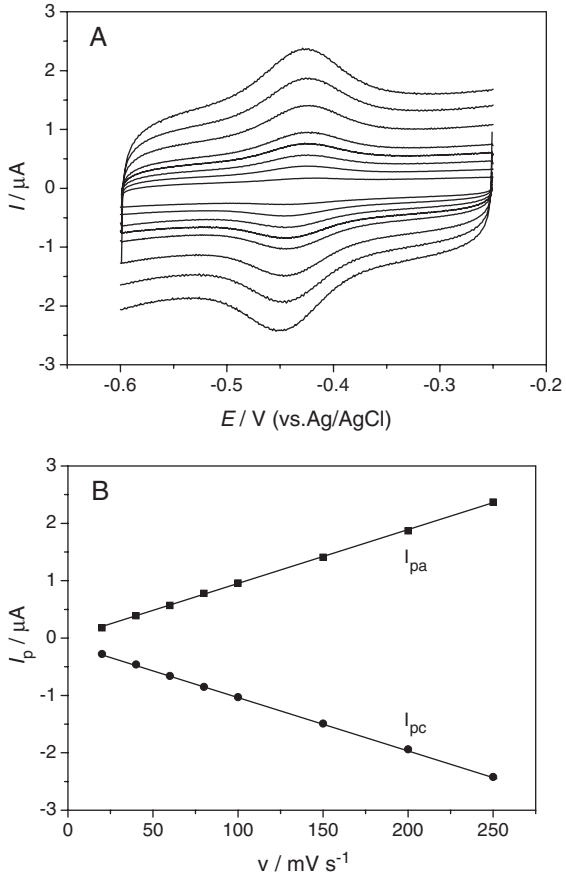

Fig. 3. (A) Cyclic voltammograms of the Nafion/GOD–GNPs/GC electrode in 0.1 M pH 7.0 PBS at various scan rates. The scan rate is 20, 40, 60, 80, 100, 150, 200 and 250 mV/s (from inner to outer). (B) Relationship between scan rate and the cathodic and anodic peak current.

An estimation of the electron transfer rate constant (k_s) has been made from the peak potential separation value using the model of Laviron [27] for a surface-controlled electrode process. Taking a charge transfer coefficient α of 0.5, and a scan rate of 50 mV/s, ΔE_p=21 mV, and then the electron transfer rate constant was 1.30 s^{-1}.

Cyclic voltammograms of Nafion/GOD–GNPs/GC electrode showed a strong dependence on pH of external solutions (Fig. 4). An increase of the solution pH leads to a negative shift in potential for both anodic and cathodic peaks. $E^{\circ\prime}$ has a linear relationship with pH from pH 5.0 to 8.0 with a slope of −58.5 mV/pH, which is nearly the same as the theoretical value of −58.6 mV/pH at 22 °C for a reversible, two-proton coupled with two-electron redox reaction process.

The direct electron transfer of GOD is stable. When the Nafion/GOD–GNPs/GC electrode was scanned continuously in 0.1 M pH 7.0 PBS, the voltammetric response decreased slowly with the increase of the cycles. The peak current almost remained 90% of the initial response after 150 cycles (Fig. 5). The storage stability of the Nafion/GOD–GNPs/GC electrode was investigated by keeping the electrode at 4 °C when not in use; every few days the cyclic voltammogram was recorded and using the same electrode. The peak current decreased with the increase of storage time. However, the electrode can still retain about 70% of the initial response after 20 days storage. The above results indicate that the Nafion/GOD–GNPs/GC electrode has a good stability.

3.3. Electrocatalytic oxidation of glucose at the Nafion/GOD–GNPs/GC electrode

A review [28] on fundamentals and analytical applications of enzyme-catalysed direct electron transfer related that a number of enzymes were found to be capable of interacting directly with an electrode while catalyzing the corresponding enzymatic reaction, whereas controversial results were reported on the direct electrocatalysis of GOD. So in amperometric biosensors based on GOD, $FADH_2$ is usually reoxidized by means of either the natural electron acceptor O_2 [29] or artificial redox mediators [3].

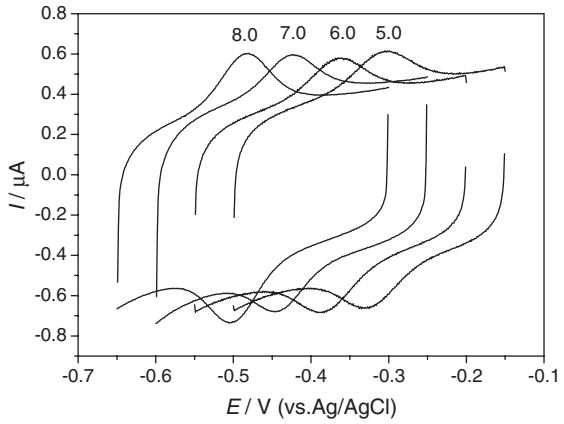

Fig. 4. Cyclic voltammograms of Nafion/GOD–GNPs/GC electrode in 0.1 M PBS at different pH. The scan rate is 50 mV/s.

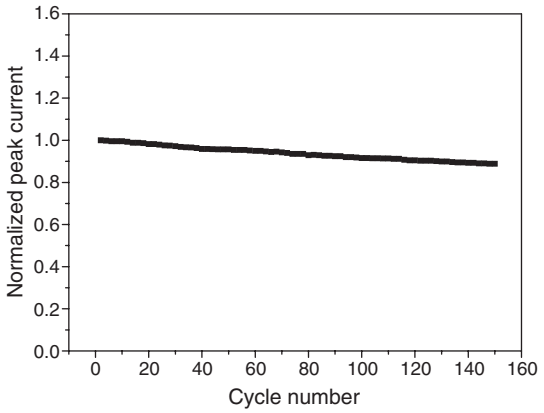

Fig. 5. Stability of the Nafion/GOD–GNPs/GC electrode on continuous scanning. The normalized peak current was calculated by comparing the response of the electrode with that of the first cycle.

Cyclic voltammetric experiments demonstrated that the immobilized GOD still remained its electrocatalytic activity for the oxidation of glucose, as showed in Fig. 6. Curves *a* and *b* are the cyclic voltammetric responses of the Nafion/GOD–GNPs/GC electrode in 0.1 M pH 6.82 PBS in the absence and presence of 0.25 mM ferrocenemethanol. No electrochemical response is observed in the absence of ferrocenemethanol (curve *a*). In the presence of ferrocenemethanol, a pair of well-reversible redox waves with formal potential at 0.19 V (vs. Ag/AgCl) was observed (curve *b*), which was assigned to one-electron reversible redox reaction of Fc^+/Fc. The anodic peak current was almost the same as the corresponding cathodic peak current, and increased linearly with the square root of scan rate from 5 to 150 mV/s, as expected for a diffusion-controlled electron transfer process. Adding glucose to above solution, a well-defined sigmoidal catalytic wave was developed as a consequence of the GOD catalytic oxidation of glucose, and the electrocatalytic current increases with the increase of the concentration of glucose in buffer. The above results indicate

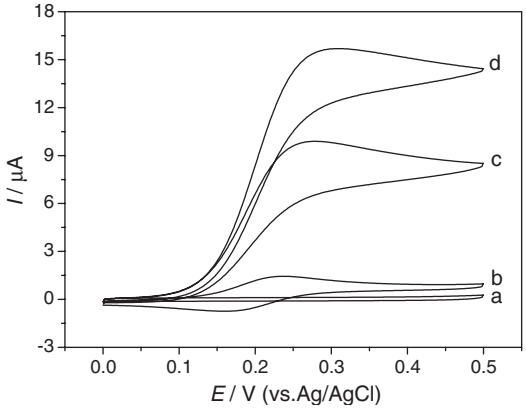

Fig. 6. Cyclic voltammograms of the Nafion/GOD–GNPs/GC electrode in 0.1 M pH 6.82 PBS (a) in the absence (b) and presence of 0.25 mM ferrocenemethanol. Curves *c* and *d* are electrocatalytic response of the Nafion/GOD–GNPs/GC electrode to the oxidation of glucose in 0.1 M pH 6.82 PBS containing 10.0 (c) and 20.0 mM (d) glucose, respectively, in the presence of 0.25 mM ferrocenemethanol. The scan rate is 10 mV/s.

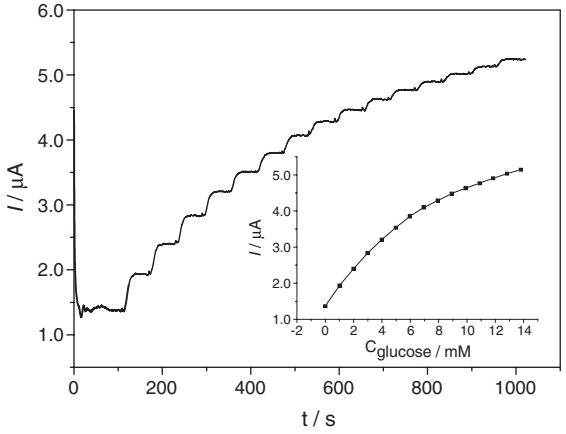

Fig. 7. Current-time curves obtained at the Nafion/GOD–GNPs/GC electrode for successive addition of 1 mM glucose. Conditions: 0.1 M pH 6.82 PBS containing 0.25 mM ferrocenemethanol; applied potential, 0.27 V (vs. Ag/AgCl). Inset: calibration curve of the sensor as a function of glucose concentrations.

that the immobilized GOD remains its electrocatalytic activity for the oxidation of glucose.

Fig. 7 shows a typical current-time plot of the Nafion/GOD–GNPs/GC electrode on successive step changes of glucose concentration. The Nafion/GOD–GNPs/GC electrode reached 95% of the steady-state current within 15 s. The current response of the enzyme electrode increased along with glucose concentration. Fig. 7 (inset) shows a calibration plot of the steady-state current versus glucose concentration. The linear response range of the Nafion/GOD–GNPs/GC electrode to the concentration of glucose can be extended at least to 6 mM. In the linear range, the Nafion/GOD–GNPs/GC electrode has a high sensitivity of 6.5 μA mM^{-1} cm^{-2}. The detection limit of the sensor was determined to be 3.4×10^{-5} M at a signal to noise ratio of 3. When glucose concentration is high, a plateau current was observed, showing the characteristics of the Michaelis–Menten kinetics. The apparent Michaelis–Menten constant (K_m^{app}), an important parameter to reveal enzyme–substrate reaction kinetics, can be calculated by the electrochemical version of the Lineweaver–Burk plot [30].

$$1/I_{ss} = 1/I_{max} + K_m^{app}/I_{max} \times C$$

where I_{ss} is the steady-state current after the addition of the substrate, C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturate condition. K_m^{app} for the enzyme electrode was estimated to be 4.6 mM, which is much smaller than that reported in the literature [31], indicating the present electrode exhibits higher affinity to glucose.

The storage stability of the Nafion/GOD–GNPs/GC electrode towards the catalytic oxidation of glucose has been examined as well. After being stored at 4 °C for 2 weeks, signals decrease by about 10%, indicating the good stability of this enzyme electrode. The reproducibility of the Nafion/GOD–GNPs/GC electrode was examined at a glucose concentration of 20 mM (0.25 mM ferrocenemethanol as mediator). The relative standard deviation is 2.5% for eight successive assays. The fabrication reproducibility of five enzyme electrodes, made at the same electrode independently, was also examined in above solutions. An acceptable reproducibility with a relative standard deviation of 4.6% is obtained. From the above results, it can be deduced that the enzyme electrode could be used as an amperometric biosensor in the determination of glucose.

4. Conclusions

In this paper, we realized the direct electron transfer of GOD by entrapping GOD/ GNPs in a Nafion film on GC electrode. Cyclic voltammetric results showed a pair of well-defined and nearly reversible redox peaks, which originate from the direct electron transfer of GOD. The dependence of the formal potential on solution pH indicated that the direct electron transfer reaction of GOD was a two-proton coupled with two-electron redox reaction process. The experimental results further confirmed that the immobilized GOD remained its electrocatalytic activity for the oxidation of glucose. So the enzyme electrode can be used as an amperometric biosensor for the determination of glucose. In addition, the biosensor also possesses high sensitivity and good chemical and mechanical stability.

Acknowledgements

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