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Direct electrochemistry of glucose oxidase in a colloid Au–dihexadecylphosphate composite film and its application to develop a glucose biosensor

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Abstract

Colloid Au (Au_{nano}) with a diameter of about 10 nm was prepared and used in combination with dihexadecylphosphate (DHP) to immobilize glucose oxidase (GOD) onto the surface of a graphite electrode (GE). The direct electrochemistry of GOD confined in the composite film was investigated. The immobilized GOD displayed a pair of redox peaks with a formal potential of −0.475 mV in pH 7.0 O_2-free phosphate buffers at scan rate of 150 mV s^{-1}. The GOD in the composite film retained its bioactivity and could catalyze the reduction of dissolved oxygen. Upon the addition of glucose, the reduction peak current of dissolved oxygen decreased, which could be developed for glucose determination. A calibration linear range of glucose was 0.5–9.3 mM with a detection limit of 0.1 mM and a sensitivity of 1.14 μA mM^{-1}. The glucose biosensor showed good reproducibility and stability. The general interferences that coexisted in human serum sample such as ascorbic acid and uric acid did not affect glucose determination.

Keywords: Glucose; Glucose oxidase; Colloid Au; Dihexadecylphosphate; Biosensor

1. Introduction

Glucose is a major component of animal and plant carbohydrates. Quantitative determination of glucose is very important in biochemistry, clinical chemistry, and food analysis. Electrochemical sensors have been widely used for daily glucose monitoring due to their high accuracy, low cost, rapidity, simplicity and better detection limits. Classical electrochemical glucose biosensors were based on monitoring either the consumption of oxygen or the production of hydrogen peroxide [1,2] (first-generation biosensors). Unfortunately, the amperometric determination of hydrogen peroxide requires high anodic potential [3]. To overcome such a shortcoming, redox mediators such as ferrocene [4], or osmium complex [5], etc, were often used to shuttle the electrons between the redox centers of GOD and the electrodes (second-generation biosensors). However, the redox mediators used in conjunction with redox proteins are not selective but rather general, facilitating not only the electron transfer between electrode and protein but also various interfering reactions. Therefore, the mediator-free biosensors (third-generation biosensors) have received more and more interest recently [6].

Since the flavin adenine dinucleotide (FAD) moiety is deeply embedded within a protective protein shell, well-defined direct electrochemical behavior of GOD is rather difficult. In literature, only a few examples of quasi-reversible voltammograms from GOD active site were reported. Ianniello et al. [7] studied the direct electron transfer of adsorbed GOD at a graphite electrode and a cyanuric chloride-modified graphite electrode using differential pulse voltammetry. The direct electrochemistry of GOD, immobilized at a self-assembled monolayer of

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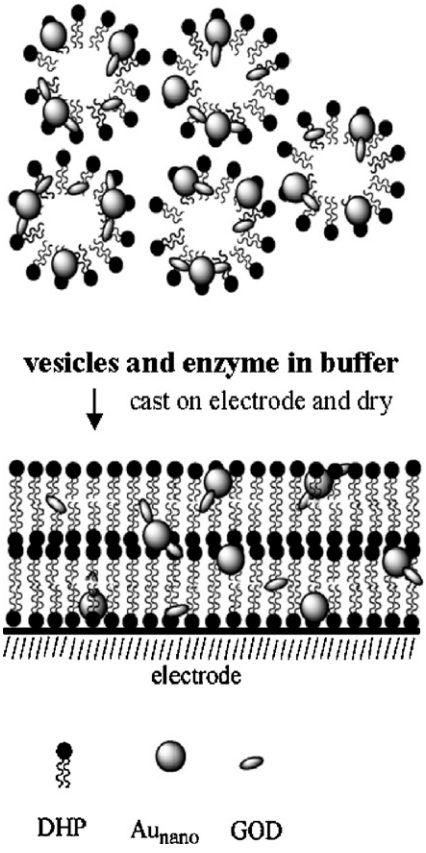

Scheme 1. Representation of the process of GOD immobilization.

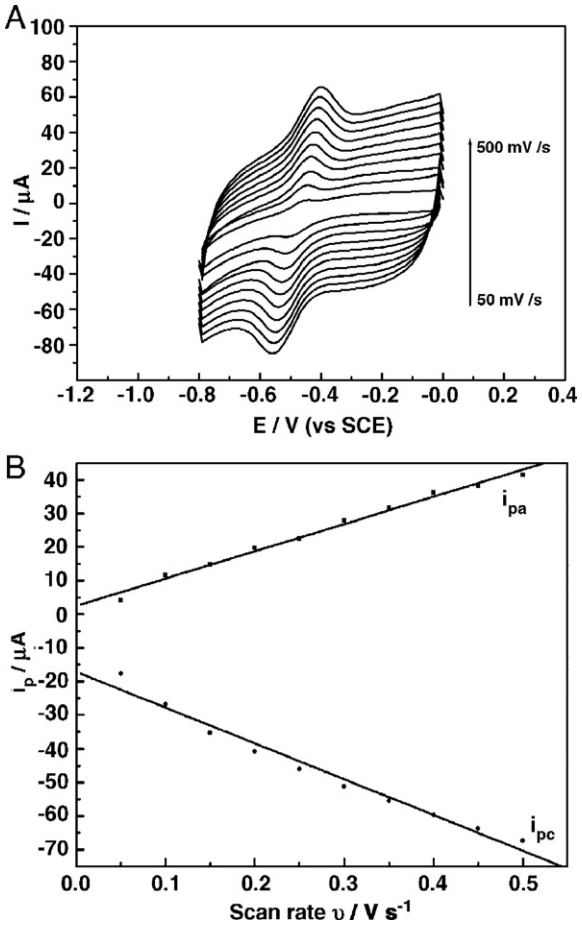

Fig. 2. CVs of GOD–Au$_{nano}$–DHP/GE at various scan rates (A). Plots of peak currents vs scan rate (B).

3,3-dithiobis-sulfosuccinimidyl propionate, was reported by Jiang et al. [8]. Direct electron transfer of GOD realized by carbon nanotubes was also studied by various groups in recent years [9–11].

Dihexadecylphosphate, a surfactant with a negative charged headgroup and two nonpolar tails, is insoluble in water and does not form micelles. Stable films can be cast from its aqueous vesicle dispersions. Evaporation of the solvent leaves self-assembled multi-bilayer films, similar to stacks of biomembranes. Thus, DHP have been used to immobilize redox proteins onto the electrode surface to promote their electron transfer [12–14]. Au colloids possess biocompatibility and proteins bound with Au colloids electrostatically can retain biological activity [15]. Some groups have demonstrated that several enzymes could maintain their enzymatic and electrochemical activity when immobilized on colloid Au [16,17]. The immobilization of a redox protein on Au$_{nano}$ can help the protein to keep a favored orientation or to make possible conducting channels between the prosthetic groups and the electrode surface, and they will both reduce the effective electron transfer distance, thereby facilitating electron transfer between electrode and enzyme [18].

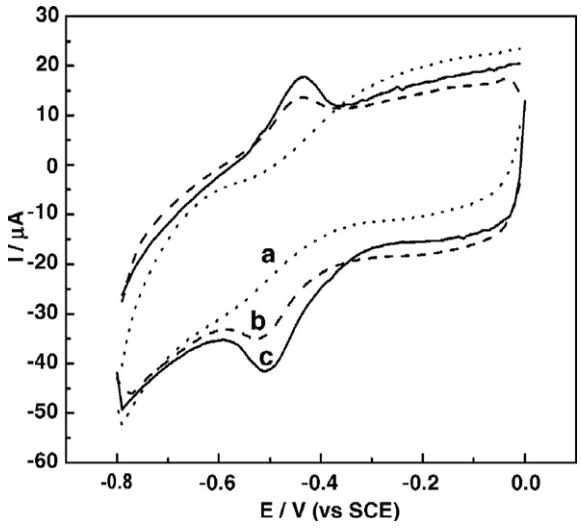

Fig. 1. CVs of a Au$_{nano}$–DHP/GE (a); GOD–DHP/GE (b); GOD–Au$_{nano}$–DHP/GE (c) in 0.06 M air-free phosphate buffers (pH 7.0) at a scan rate of 150 mV s^{-1}.

In this work, we combined the advantageous features of colloidal Au and dihexadecylphosphate to prepare a composite film for the immobilization of glucose oxidase onto a graphite electrode surface. GOD confined in Au$_{nano}$–DHP composite film have realized its direct electrochemistry. The immobilized GOD can catalyze the reduction of dissolved oxygen, and glucose determination is developed based on the decrease of reduction peak current of oxygen with the addition of glucose. The applied

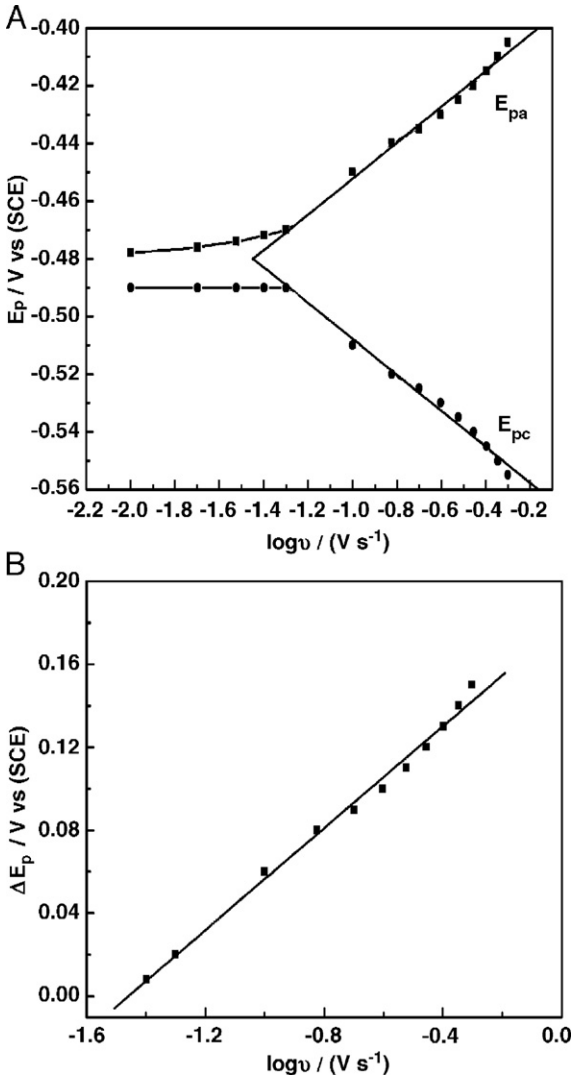

Fig. 3. Plots of the anodic peak potential, E_{pa}, and cathodic peak potential, E_{pc} vs the logarithm of the scan rate (A). Plot of ΔE_p vs log$v$ (B).

potential of the biosensor is −0.5 V, therefore, it prevents the interference from electrochemically oxidizable compounds, such as ascorbic acid and uric acid.

2. Experimental

2.1. Reagents

Glucose oxidase (GOD, EC 1.1.3.4., from *Aspergillus niger*, 191 units mg^{-1}) and dihexadecylphosphate were purchased from Fluka. Glucose was purchased from Shanghai Boao bioscientific Ltd. Co. (Shanghai, China). All other chemicals were of analytical grade and were used without further purification. Doubly-distilled water was used throughout and the supporting electrolyte was usually phosphate buffer containing 0.06 M Na_2HPO_4 and NaH_2PO_4.

Colloid Au was prepared as described by Hillier et al. [19]. In brief, $HAuCl_4$ aqueous solution (4 ml of 0.4 mg ml^{-1}) was boiled, then 1 ml sodium citrate (1% aqueous solution) was added to the boiled solution above. The resulting solution was maintained at 100 °C for several minutes until the color of the solution was not changed.

2.2. Apparatus

All the voltammetric determinations were performed with a computer controlled Model CHI 830A electrochemical analyzer (ChengHua Instrument Co., ShangHai, China). The working electrode is a modified graphite electrode with the geometric area of 0.071 cm^2. The saturated calomel electrode (SCE) is used as reference electrode, and a Pt wire used as counter electrode. All the potentials were reported versus SCE.

2.3. Film preparation and electrode modification

1 mg DHP was added into 1 ml colloid Au aqueous solution or into 1 ml redistilled water. A homogeneous dispersion of Au_{nano}–DHP or DHP aqueous solution was achieved with the aid of ultrasonication agitation. 10 μl of 3 mg ml^{-1} GOD in pH 7.0 phosphate buffers was mixed with 10 μl of Au_{nano}–DHP dispersion or DHP aqueous solution. Before modification, a graphite electrode was polished with 0.05 μm aluminum slurry (CH Instruments, USA), then rinsed thoroughly with redistilled water,

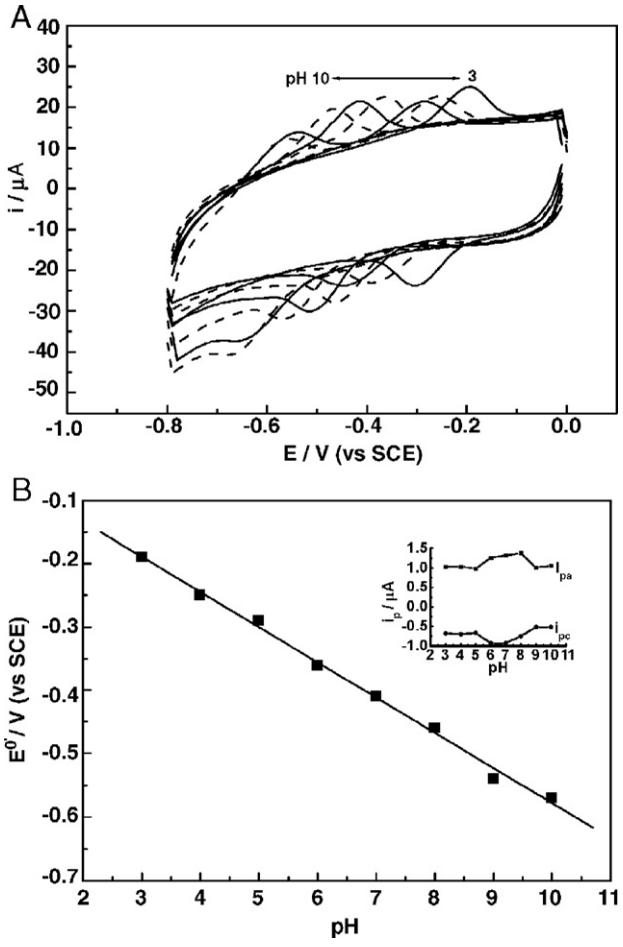

Fig. 4. CVs of GOD–Au_{nano}–DHP/GE in different pH values with scan rate of 150 mV s^{-1}. (A). Plot of peak potential vs pH value (B). Inset of B: plots of peak currents vs pH value.

and finally sonicated successively in 1:1 HNO_3–H_2O (v/v) and redistilled water, each for 1 min. After that, 10 μl of the above mixed GOD–Au_{nano}–DHP solution or GOD–DHP solution was pipetted onto the surface of GE and the solvent was evaporated at room temperature. By this means, GOD was immobilized onto the GE surface. The modified electrodes were denoted as GOD–Au_{nano}–DHP/GE and GOD–DHP/GE, respectively.

The process of GOD immobilization is illustrated in Scheme 1. The association of GOD to the Au colloid surface is due to the interaction between cysteine or NH_3^+-lysine residues of GOD and the Au colloid surface.

3. Results and discussion

3.1. Electrochemical behavior of GOD confined in the Au_{nano}–DHP composite film

Curves a, and b in Fig. 1 are the cyclic voltammograms (CVs) of an Au_{nano}–DHP/GE electrode and a GOD–DHP/GE electrode in pH 7.0 phosphate buffer at scan rate of 150 mV s^{-1}, respectively. No any observable peak was obtained at the Au_{nano}–DHP/GE electrode, but a pair of well-defined and stable peaks was observed at the GOD–DHP/GE electrode, corresponding to the reaction of GOD–FAD+2e+2H$^+$ ↔ GOD–$FADH_2$. It suggests that DHP can immobilize GOD and provides a favorable microenvironment to facilitate its electron communication. However, when using Au_{nano}–DHP to immobilize the same amount of GOD, the peak currents increase obviously (curve c in Fig. 1), indicating Au_{nano} acts as an improved active interface for the direct electron transfer of GOD. The formal potential ($E^{0'}$) of GOD in Au_{nano}–DHP film electrode is around −0.475 V and the peak potential separation (ΔE_p) is 50 mV at scan rate of 150 mV s^{-1}, close to the literature value in which GOD was immobilized on carbon nanotubes [10].

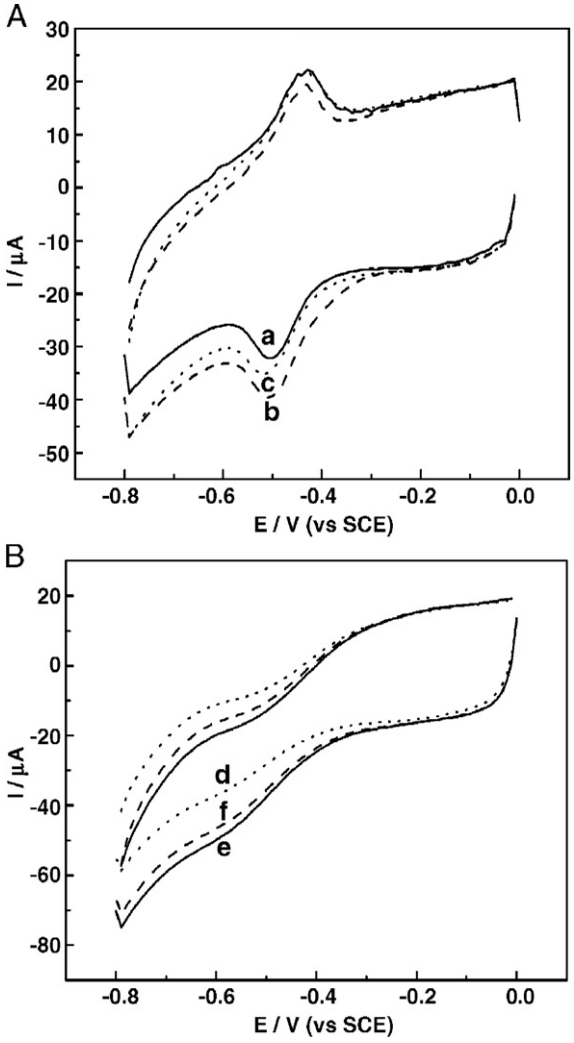

Fig. 5. CVs of GOD–Au_{nano}–DHP/GE (A) and Au_{nano}–DHP/GE (B) in air-free pH 7.0 phosphate buffer (a and d), air-saturated pH 7.0 phosphate buffer (b and e) and air-saturated pH 7.0 phosphate buffer with addition of 8 mM glucose (c and f) at scan rate of 150 mV s^{-1}.

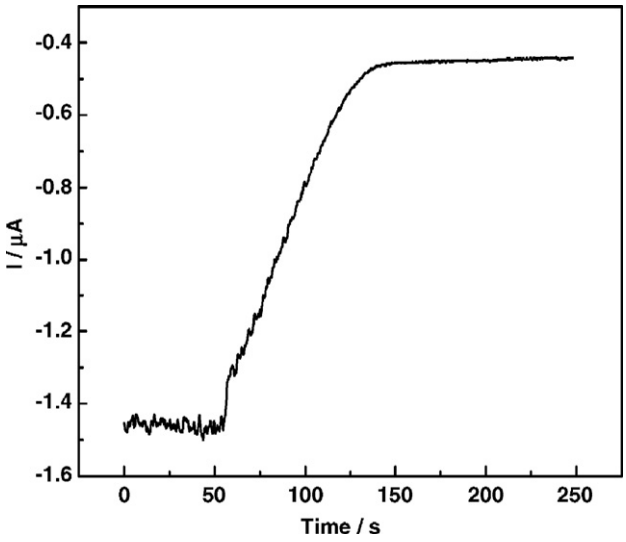

Fig. 6. An amperometric i–t curve of GOD–Au_{nano}–DHP/GE in air-saturated pH 7.0 phosphate buffer at a constant potential of −0.45 V while glucose was added at 65 s with the concentration of 15 mM.

The surface coverage (Γ) of GOD on the GE surface was estimated from integration of the reduction peak in the CVs according to $\Gamma = Q/nFA$, where Q is the charge involved in the reaction, n is the number of electron transferred, F is Farady constant, and A is the electrode area. The value of $\Gamma = 7.82 \times 10^{-12}$ mol cm^{-2} was calculated. It indicated that a monolayer of GOD molecules takes the electrode reaction [20].

The effect of scan rate on the voltammetric response of GOD is shown in Fig. 2A. With the increasing scan rate, both peak currents and peak-to-peak separation increase. The anodic and cathodic peak currents are linearly proportional to the scan rate ranging from 50 to 500 mV s^{-1} (Fig. 2B), indicating the redox reaction is a surface process [21].

For a redox monolayer modified electrode, the peak potentials can be represented by Laviron [22]:

$$E_{pc} = E^{0'} - \frac{2.3RT}{\alpha nF}\left\{\log\frac{\alpha Fn}{RT} + \log[v/(\ \mathrm{Vs^{-1}})] - \log[k/(\mathrm{s^{-1}})]\right\} \quad (1)$$

$$E_{pa} = E^{0'} - \frac{2.3RT}{(1-\alpha)nF}\left\{\log\frac{(1-\alpha)Fn}{RT} + \log[v/(\mathrm{Vs}^{-1})] - \log[k/(\mathrm{s}^{-1})]\right\} \quad (2)$$

$$\Delta E_p = \frac{2.3RT}{(1-\alpha)\alpha nF}\left\{\alpha\log(1-\alpha) + (1-\alpha)\log\alpha - (1-2\alpha)\log\frac{RT}{nF} - (1-2\alpha)\log[k/(\mathrm{s}^{-1})]\right\} + \frac{2.3RT(1-2\alpha)}{(1-\alpha)\alpha nF}\log[v/(\mathrm{Vs}^{-1})] \quad (3)$$

where E_{pa}, and E_{pc} are anodic peak potential, and cathodic peak potential, respectively, $\Delta E_p = E_{pa} - E_{pc}$, α is the transfer coefficient, v is the scan rate (V s^{-1}), k is the heterogeneous electron transfer rate constant (s^{-1}). According to the slopes of anodic process and the cathodic process in Fig. 3A, it was calculated that $\alpha = 0.44$. Based on $\Delta E_p \sim \log v$ plot (Fig. 3B), the value of $k = 1.713$ s^{-1} was calculated, close to the literature value in which GOD was immobilized on carbon nanotubes [10].

The influence of buffers pH on the voltammetric response of GOD was examined. An increase in pH value from 3.0 to 10.0 caused a negative shift in both anodic and cathodic peak potentials of GOD (Fig. 4A), indicating hydrogen ion is involved in the electrode reaction of GOD. The slope of the $E^{0'}$ vs pH line was 55.6 mV pH^{-1} (Fig. 4B), which is close to the theoretical value (59.0 mV pH^{-1}) for a two-proton coupled with two-electron redox reaction process. The pH value does also affect the redox peak currents of GOD (inset in the Fig. 4B). Obviously, the maximum peak currents are observed at an optimal pH range between 6.0 and 7.0. It may be due to the immobilized GOD that maintains its higher bioactivity at such a pH range.

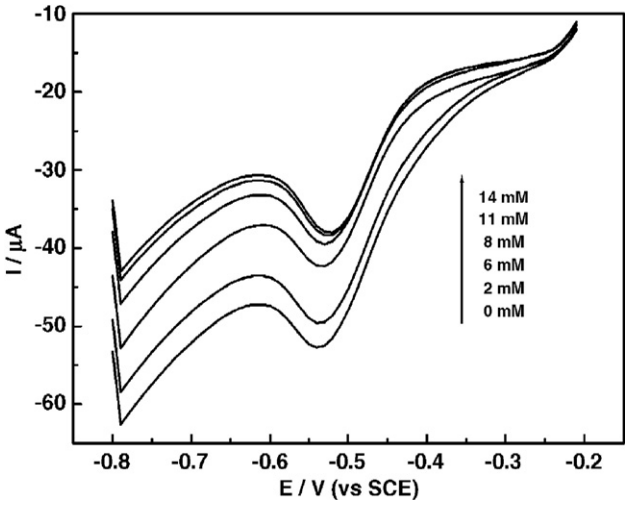

Fig. 7. LSVs of GOD–Au_{nano}–DHP/GE in a pH 7.0 air-saturated phosphate buffer with addition of 0, 2.0, 5.0, 8.0, 11.0, and 14.0 mM glucose (from bottom to top) at scan rate of 200 mV s^{-1}.

Table 1
Determination of glucose in human serum sample by the GOD biosensor utilizing standard addition method ($n = 7$)

Sample no.	$C_{glucose}$/mM				Recovery/ %
	Found before adding	Added	Expected	Found after adding	
1	5.50	2.0	7.50	7.62	101.6
2	5.76	2.0	7.76	7.68	98.9
3	6.07	2.0	8.07	8.12	100.6

3.2. Bioactivity of GOD confined in the Au_{nano}–DHP composite film

It is well known that GOD catalyzes the oxidation of glucose to glucono-1,5-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. Whether GOD immobilized in the Au_{nano}–DHP composite film retains its bioactivity is important to be investigated. Curves a, and b in Fig. 5A show the CVs of GOD–Au_{nano}–DHP/GE in air-free and air-saturated buffers, respectively. The reduction peak of GOD significantly increases in the presence of oxygen, indicating that GOD can catalyze the reduction of oxygen. It follows the reaction as:

$$\mathrm{GOD{-}FADH_2} + \mathrm{O_2} \rightarrow \mathrm{GOD{-}FAD} + \mathrm{H_2O_2} \quad (4)$$

Curve c in Fig. 5A shows the CV of GOD–Au_{nano}–DHP/GE in air-saturated buffers with addition of 8 mM glucose. The reduction peak current decreases with the addition of glucose. This result demonstrates that molecular oxygen was consumed at the electrode interface in the presence of glucose because the dissolved oxygen mediates the enzymatic oxidation of the glucose by GOD. It follows the reaction as:

$$\mathrm{Glucose} + \mathrm{O_2} \rightarrow \mathrm{Gluconolactone} + \mathrm{H_2O_2} \quad (5)$$

In order to demonstrate that the reduction of oxygen at −0.5 V is catalyzed by GOD, Au_{nano}–DHP/GE was investigated to compare. In the presence of dissolved oxygen, no peak current was observed at Au_{nano}–DHP/GE (curve e in Fig. 5B) at −0.5 V, though there is an increase in cathodic current at the potential that is more negative than −0.5 V.

An amperometric i–t experiment on GOD–Au_{nano}–DHP/GE at a constant potential of −0.45 V was carried out under constant stirring. Upon the addition of glucose, the monitored current was dramatically increased as shown in Fig. 6. All these experiment results demonstrated that the immobilized GOD retained its bioactivity.

Table 2
The comparison between the GOD biosensor and YSI blood sugar analyzer (average of 7 determinations)

Sample no.	$C_{glucose}$/mM		Bias/ mM
	Determined by biosensor	Determined by YSI blood sugar analyzer	
1	5.50	5.58	−0.08
2	5.76	5.69	+0.07
3	6.07	6.18	−0.11

3.3. Determination of glucose by linear sweep voltammetry

The decrease of the reduction current of oxygen with the addition of glucose is employed to determine the glucose concentration. The linear sweep voltammograms of GOD–Au_{nano}–DHP/GE with successive addition of glucose to air-saturated buffers (pH 7.0) are shown in Fig. 7. With increasing glucose concentration, the reduction current decreased. When the concentration of glucose was above 11 mM, the reduction peak did not decrease any more, indicating that it arrived to the GOD's substrate saturated concentration. The relationships between the decrease of the reduction peak current of oxygen and the concentration of glucose were examined. The decrease of the reduction peak current is linear with the concentration of glucose from 0.5 mM to 9.3 mM. The linear regression equation is ΔI_p (μA)=2.20+1.14C (mM) with the correlation coefficients of 0.992. The detection limit is 0.1 mM based on a signal-to-noise ratio of 3. The sensitivity of GOD–Au_{nano}–DHP/GE to glucose was found to be 1.14 μA mM^{-1}.

3.4. Stability and reproducibility of the glucose biosensor

The stability tests were carried out at room temperature by measuring the response from day to day. The GOD–Au_{nano}–DHP/GE and GOD–DHP/GE were kept at 4 °C. It was shown that the GOD–Au_{nano}–DHP/GE and GOD–DHP/GE maintain their 90% and 80% initial activity after 15 days, respectively. The biosensor shows a good stability due to the stable film formation of DHP and its prevention of the leakage of colloid Au and GOD from the electrode surface. The repeatability was estimated from two series of 15 repetitive measurements of 0.8 mM and 8 mM glucose solution, and the relative standard deviations (RSD) were 4.2% and 3.3%, respectively. The fabrication of the GOD–Au_{nano}–DHP/GE composite film onto the same GE surface for seven times independently showed an acceptable reproducibility with a relative stand deviation of 6.7% for 1 mM glucose. These results suggest that the GOD biosensor has a good repeatability for the determination of glucose.

3.5. Determination of glucose in human serum sample

The determination of glucose in human serum sample was performed by the glucose biosensor utilizing standard addition method. After the current response was determined in 10.0 ml of pH 7.0 buffers containing the sample of 5.0 m1, 20 μl of 1 M glucose solution was added to the above system for standard addition determination. The results are listed in Table 1. Comparative determinations of the concentration of glucose in the sample were carried out by a yellow springs blood sugar analyzer (YSI Model 2300). The results are shown in Table 2 with a good agreement.

4. Conclusions

The colloid Au and dihexadecylphosphate composite film was used to immobilize glucose oxidase. GOD in the composite film is electroactive and can catalyze the reduction of dissolved oxygen. Upon the addition of glucose, the reduction peak current of dissolved oxygen decreases, which has been developed for glucose biosensor. The biosensor showed long stability and excellent reproducibility due to advantageous features of colloidal Au and dihexadecylphosphate, and it was applied to determine glucose in human serum sample successfully.

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