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Zinc oxide nanoparticles/glucose oxidase photoelectrochemical system for the fabrication of biosensor

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ABSTRACT

Nanosized semiconductor crystals can increase efficiency of photochemical reactions and greatly improve the catalytic activity of enzymes to generate novel photoelectrochemical systems. In this work, glucose oxidase (GOx)/zinc oxide (ZnO) is selected as a model system to assess the photovoltaic effect of semiconductor nanoparticles on the enzyme electrode. UV-spectrum and circular dichroism (CD) results show that the structure of GOx is preserved after conjugation with ZnO nanoparticles. The current response of the enzyme electrode containing ZnO nanoparticles increases from 0.82 to 21 $\mu A\,cm^{-2}$ in the solution of 10 mM β-D-glucose. Furthermore, after irradiating the enzyme electrode with UV light for 2 h, the current response can be increased nearly 30% and the detection limit can be lowered about two orders compared with the catalytic reactions in the dark, which indicate that a technique to fabricate a novel photocontrolled enzyme-based biosensor may be developed.

1. Introduction

Biosensors are reported to offer the possibility of carrying out analytical and diagnostic procedures simply and rapidly. Among biosensors, enzymatic biosensors have been the subject of much intensive research owing to their high selectivity and quick response to specific substrates. In order to increase the sensitivity of enzyme electrodes, many advanced materials have been used in biosensor fabrication [1–5]. However, the efficient electrical communication between enzyme's redox center and solid electrode surfaces still forms a barrier [6], due to the fact that the enzyme active site is deeply buried in the proteins shell. Therefore, it is important to find a feasible way to further enhance the efficiency of the electrical communication.

Semiconductor colloids have been used in a range of optoelectronic applications because of their unique electrical and optical properties [7]. Specifically, the photophysical features of semiconductor nanoparticles are employed to develop sensor and biosensor systems [6,8–12]. Among these materials applied in biosensor, zinc oxide (ZnO) is an advisable one, which is a large band gap (3.3–3.6 eV) *n*-type semiconductor [13] that shows both photoconductivity and photocatalytic activity. Some researches have revealed that ZnO is suitable for enzyme immobilization, because of its biocompatibility [14] and high electron communication features [15]. Furthermore, ZnO nanoparticles have large excitation binding energy of 60 meV at room temperature. When exposed to sunlight or ultraviolet radiation, they can release electron–hole pairs, which can assist the catalytic reactions of enzyme electrodes. So they can provide a possible method to tune the current response of enzyme electrodes for development of photocontrolled enzyme-based biosensors. Recently, few articles [6,16] have reported use of the photovoltaic effect of semiconductor nanoparticles (TiO_2 or ZnO) to prepare highly sensitive biosensor. However, all of them dealt with small heme containing proteins. To our best knowledge, no paper has reported the effect of semiconductive nanoparticles linked to an enzyme with complex structure, such as GOx, whose active site is deeply buried.

In this report, we describe the fabrication, characterization and analytical performance of a glucose biosensor based on ZnO nanoparticles. The secondary structure of pure GOx, GOx/ZnO is examined using the circular dichroism (CD) spectroscopic technique. Our experiment shows that these ZnO nanoparticles can significantly enhance the current sensitivity of GOx enzyme electrode. In the photoelectrochemical system, the photovoltaic effect of the zinc oxide nanoparticles can play an interesting role in the catalytic ability of GOx and further improve the sensitivity of glucose biosensor. This fabrication method of glucose biosensor is simple and effective, which can be potentially applied in other enzyme/semiconductor system.

2. Materials and methods

2.1. Chemicals and reagents

Glucose oxidase (GOx) (E.C.1.1.3.4, from *Aspergillus niger*, 130 U/mg) was purchased from Fluka. β-D (+)-Glucose was supplied by

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Sigma Chemical Company. Hydroxypropyl cellulose (HPC) was obtained from Tokyo Chemical Industry Ltd. (TCI, Chuo-Ku, Tokyo, Japan). Polyvinylbutyral (PVB) and gluctaraldehyde were obtained from Chinese Medicine Company and Chinese Beitong Chemical Company, respectively. All reagents were used without further purification. All solutions were prepared with redistilled water.

2.2. Preparation of ZnO nanoparticles

ZnO nanoparticles were synthesized by precipitation from 2-propanol [17]. For a typical synthesis, 1.2 mmol of $ZnCl_2$ was dissolved in 25 mL 2-propanol under constant stirring at 60 °C. Thereafter, 18 mL of KOH solution (0.03 M, in 2-propanol) was added in to the $ZnCl_2$ solution with the temperature maintained at 60 °C. Eighty milligram of HPC was added before the injection of KOH, which used as protective agent. The mixture was allowed to stir for 2 h. Then the white precipitate was centrifugated, washed with 2-propanol and diluted to 200 mL with 2-propanol.

2.3. Preparation of enzyme electrodes

The platinum electrode with a diameter of 1 mm were first boiled in nitric acid for 5 min and washed in redistilled water. 12 U GOx was added to different concentrations of ZnO suspensions to form mixture in a 5 mL beaker. Two milliliters of PVB 2-propanol solution ($w = 2\%$) used as auxiliary membrane matrix and 100 μL gluctaraldehyde aqueous solution ($w = 1\%$) used as crosslinking agent were added to the beaker successively. The contents were stirred evenly by the platinum electrode. And then the platinum electrode was dipped into the mixture to a depth of 1 cm for 8 min and then taken out. These electrodes were stored at 4 °C for 24 h before measurement.

2.4. Apparatus and measurements

Scanning electron microscope (SEM) of ZnO nanoparticles was obtained with a HITACHI S-4300 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at an acceleration voltage of 10 kV. Selected area electron diffraction (SAED) pattern were taken with JEOL-200CX electron microscope, operating at 160 kV. V-570 UV/vis/NIR spectrophotometer (Jasco Japan) was used to measure the UV-spectrum of samples.

The circular dichroism (CD) spectra were taken using a Jasco J-810 spectropolarimeter. Quartz cells of 1-mm optical path length were used for all CD measurements of GOx (GOx concentration = 6.5×10^{-7} M) and GOx/ZnO (GOx/ZnO = 1:1 weight ratio) solutions. The phosphate buffer (Na_2HPO_4–NaH_2PO_4, pH 6.8) was used to prepare these solutions. The spectra were analyzed for the content of secondary structure by using the software accompanying the spectropolarimeter [18].

Cyclic voltammetry studies used a CHI604B electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China). All experiments were carried out using a three-electrode cells equipped with a Pt-wire as counter electrode and Ag/AgCl electrode as reference electrode. The applied scan rate was 50 mV/s.

Amperometric measurements were carried out using a three-electrode cell consisting of an enzyme working electrode, a counter electrode of platinum wire, and a reference electrode of Ag/AgCl. Measurements were conducted in a 5 mL phosphate buffer (Na_2HPO_4–KH_2PO_4–KCl, pH 6.8) cell at 35 °C. A fixed potential of 0.4 V was applied to this electronic cell. Firstly, working electrode and reference electrode were put into a phosphate solution at 35 °C. When background current reached a constant value, different concentrations (from 1.4 to 22 mM) of β-D-glucose solution were added. Then response current was noted down, and background current was deducted, and the correlation between response currents and different concentrations of glucose solution was obtained.

The surface of the modified electrode was irradiated with UV–visible light for 2 h by facing the surface to a UV lamp (8 W) in a distance of 10 cm.

3. Results and discussion

3.1. Preparation of ZnO nanoparticles

Fig. 1A shows the typical scanning electron microscope (SEM) of the ZnO nanoparticles, which indicates that the sample is composed of a large quantity of well-dispersed spherical nanoparticles. The average size of these particles estimated from the SEM image is about 140 nm. The surface of every particle is rough and with many smaller particles. The high-magnification SEM image in Fig. 1B also reveals that particles are built up of numerous small nanoparticles. It appears that these primary small nanoparticles interconnected with one another to form larger secondary particles with recognizable boundaries or voids between the component subunits. The selected area electron diffraction (SAED) pattern of the same single nanoarchitecture in the inset of Fig. 1B can be indexed to the hexagonal structure of ZnO with phase purity. The ZnO particles with such structure would have larger ratio of surface to volume and are more suitable for the immobilization of the biomolecule.

3.2. Characterization of GOx/ZnO bioconjugates

CD is one of the most sensitive physical techniques for determining structures and monitoring structural changes of biomolecules. It can directly interpret the changes of protein secondary structure. In this present work, CD spectra are used to characterize the secondary structure of GOx before and after bioconjugate with ZnO nanoparticles. Fig. 1C shows the CD spectra of GOx and GOx/ZnO bioconjugates. It can be seen that the CD spectrum of GOx/ZnO bioconjugates looks similar to pure GOx, but the peak has slightly less intensity. It is indicated that GOx have been adsorbed on the surface of ZnO nanoparticles. Table 1 shows the secondary structure estimated from the CD data. After the conjugation, the percentage of α-helix increases from 26.2% to 30.5%, and the ratio of α-helix to β-sheet increases from 0.51 to 0.63. It is indicated that there is no significant change in the secondary structure of the enzyme [19].

Fig. 1D showed the absorption spectrums of GOx, ZnO and bioconjugation of GOx with ZnO separately. Pure GOx had absorption in the visible region with maximum values at 380 and 452 nm (curve a), which were consistent with previous report [20]. ZnO nanoparticles had its own strong absorption at 378 nm (curve b). After bioconjugation of GOx with ZnO (curve c), the absorption value at 452 nm kept stable, while the absorption value at 380 nm shifted between 380 and 378 nm, which was the due to conjugation between GOx (380 nm) and ZnO nanoparticles (378 nm). The results indicated that GOx was firmly immobilized on ZnO nanoparticles.

3.3. Electrochemical characterization

Cyclic voltammetry was performed to assess the effect of ZnO nanoparticles on the electric conductivity of the enzyme membrane. The cyclic voltammograms for the electrode in the glucose were shown in Fig. 2. Compared to the CV of bare Pt electrode, which exhibited high peaks (curve a), the CV of PVB modified Pt electrode (curve b) exhibited a markedly decrease in the current response due to the insulation of PVB membrane, indicating that

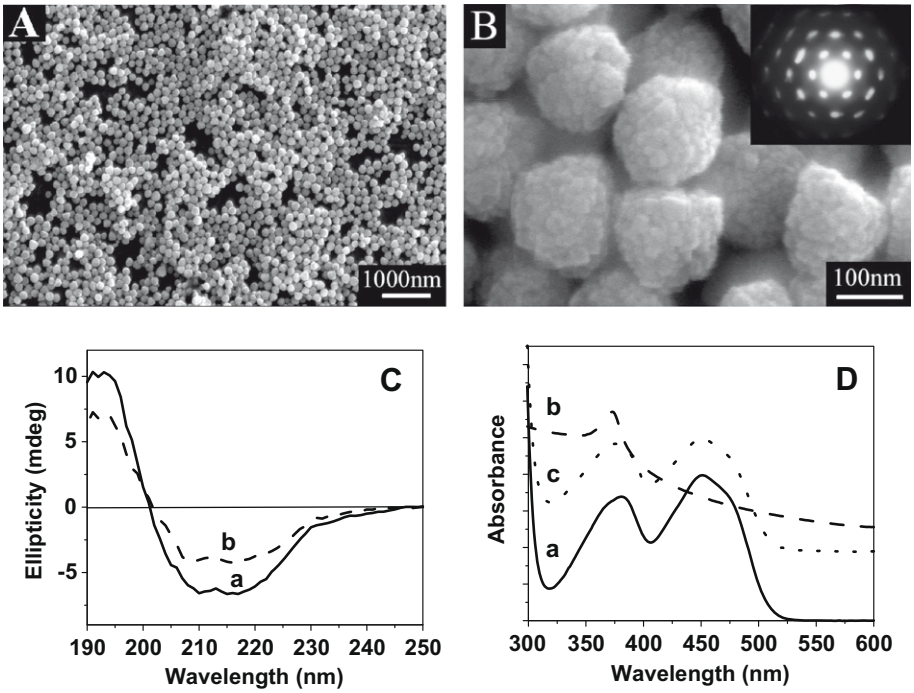

Fig. 1. (A) Typical SEM image of the ZnO nanoparticles; (B) high-magnification SEM image of the ZnO nanoparticles. The inset shows the selected area electron diffraction (SAED) pattern obtained by focusing the electron beam on a ZnO nanoparticle; (C) CD spectra of pure GOx (a) and GOx/ZnO bioconjugates (b); (D) UV–vis spectra of (a) GOx (b) ZnO and (c) GOx/ZnO bioconjugates.

Table 1
Enzyme conformation of pure GOx and GOx/ZnO bioconjugates.

	α-Helix (%)	β-Sheet (%)	α/β
GOx	26.2	51.6	0.51
GOx/ZnO	30.5	48.0	0.63

PVB membrane had been formed on the surface of bare Pt electrode. With further introduction of GOx/ZnO bioconjugates (curve c), the peaks appeared again. It is evident from the figure that ZnO nanoparticles have a marked influence on the barrier property of PVB-modified electrodes. They can provide a conductive path from the enzyme to the electrode, and then improve the electric conductivity of the enzyme electrode.

3.4. The current response curves of the immobilized GOx electrode with ZnO nanoparticles

To study the effect of the ZnO nanoparticles on the sensitivities of the glucose biosensor, enzyme electrodes containing ZnO nanoparticles were tested by amperometric measurements. The current response curves of GOx electrodes without and with ZnO nanoparticles were shown in Fig. 3(a and b). When glucose concentration was 10 mM, the current response of the electrode without nanoparticles was 0.82 μA cm^{-2}, and that of the electrode with ZnO particles was 21 μA cm^{-2}. It could be seen that nanoparticles could significantly enhance the current response of the electrodes. It was mainly due to the large surface area of ZnO nanoparticles, which could make GOx inevitably adsorbed on the surface of nanoparticles. Our previous research had given the proof [21] that the

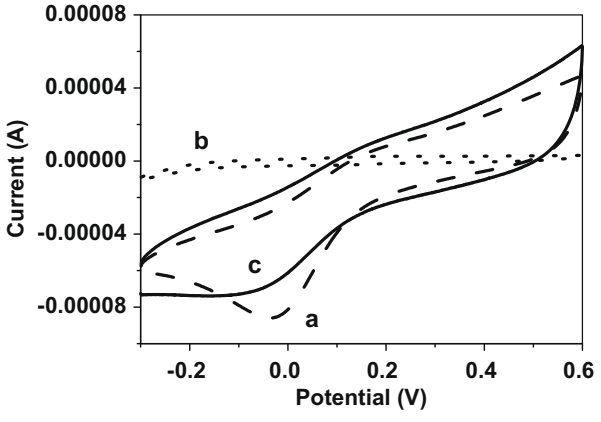

Fig. 2. Cyclic voltammograms of the electrodes immersed in 22 mmol/L glucose solution; (a) bare Pt electrode; (b) the electrode with PVB film; (c) the electrode with PVB film added GOx and ZnO nanoparticles; scan rate is 50 mV/s.

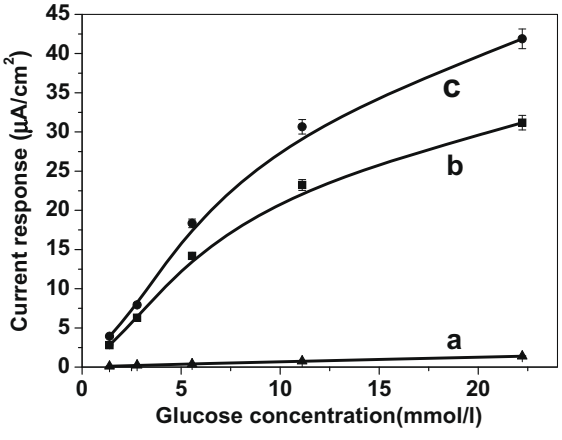

Fig. 3. Effect of ZnO nanoparticles on the GOx enzyme electrode current response (a) without ZnO nanoparticles; (b) with ZnO nanoparticles; (c) with ZnO nanoparticles under illumination.

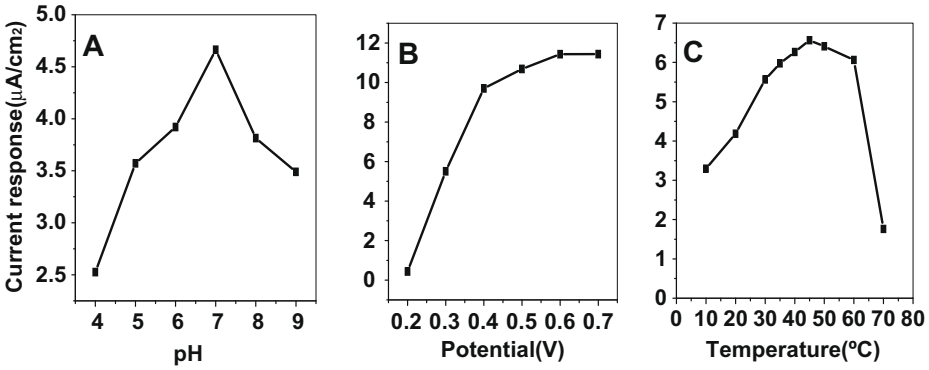

Fig. 4. (A) Effect of pH on the response of the biosensor to 2.8 mmol/L glucose. (B) Effect of working potential on the response of the biosensor to 2.8 mmol/L glucose. (C) Effect of temperature on the response of biosensor to 2.8 mmol/L glucose.

surface of nanoparticles facilitates the immobilization enzyme, and improves the activity and stability of the enzyme. On the other hand, from cyclic voltammograms we know that ZnO nanoparticles can provide a conductive path from GOx to the platinum electrode, so it can accelerate the electron transfer rate between GOx and the platinum wire and make the current response improved markedly.

3.5. Optimization of the biosensor

3.5.1. Effect of pH, working potential and temperature on the biosensor

Fig. 4A showed the effect of pH of the buffer solution on the performance of the biosensor. The pH dependence of the sensor response was evaluated at 2.8 mmol/L glucose over the pH range from 4.0 to 9.0. The optimum response was achieved at the pH 6.8, which is close to the optimum pH for free GOx. So pH 6.8 (phosphate buffers) was selected to ensure higher sensitivity and stability of the biosensor.

The results of the effect of working potential on biosensor had been presented in Fig. 4B. The working potential was stepped from 0.2 to 0.7 V in 2.8 mmol/L glucose. The steady-state current response increased with the working potential increases from 0.2 to 0.4 V, and then reached a plateau from 0.4 to 0.7 V. Therefore, 0.4 V was chosen as the working potential for amperometric detection of glucose concentration.

The effect of temperature on the biosensor had been examined between 10 and 70 °C in the 2.8 mmol/L concentration of glucose solution. Fig. 4C showed that the current response of the biosensor increased with increasing temperature and reached a maximum at approximately 45 °C, and then went down as the temperature turned higher, which indicated that the enzyme was denatured. In addition, the current response kept correspondingly steady between 30 and 60 °C, indicating enzyme bioconjugate with ZnO nanoparticles had good thermodynamic stability and life span. In order to keep consistent with the temperature of human body, 35 °C was selected for this work.

3.5.2. Stability of the enzyme electrode

The stability of the biosensor was investigated by amperometric measurements in the presence of 1.4 mmol/L glucose. Stored at 4 °C, the current response of biosensor was retained about 70% of its original response after one month.

3.6. Photovoltaic effect of ZnO nanoparticles on the current response of glucose biosensor

Further studies reveal an interesting result that the current response of the modified enzyme electrode further increased under illumination compared to the value obtained in room light using the same electrode [as shown in Fig. 3(c)] and reached 27 μA cm^{-2} when glucose concentration was 10 mM.

In order to investigate the photovoltaic effect of ZnO nanoparticles on the biosensor, the current response of GOx electrodes was measured in different conditions. All experiments were carried out in 1.4 mmol/L glucose solution. As the curves indicated (see Fig. 5A), the current response of enzyme electrode under illumination (curve b) increased about 30% compared with that obtained in the dark (curve a) using the same electrode.

The effect of the time of the ultraviolet irradiation on the response of the biosensor was shown in Fig. 5B. It was found that the current response of the biosensor reached a maximum after 2 h UV irradiation. When the time of irradiation was prolonged, the current declined. It may be due to the denaturation of the enzyme.

The detection limits of enzyme electrode containing ZnO nanoparticles in the dark and under illumination were also investigated. The result showed that the detection limit decreased to 5.6×10^{-6} M under illumination while the detection limit was 1.1×10^{-4} M in the dark, which made it possible to fabricate a more sensitive glucose biosensor.

It is not very clear how the photovoltaic effect of ZnO nanoparticles works. A preliminary explanation can be addressed as follow. As is known, nanosemiconductors are photosensitive materials. When ZnO nanoparticles are exposed under solar or ultraviolet, they may generate electrons and holes, respectively, in the conduction and valence bands, which can be used for the substrate reduction (oxidation) through electron accepter (donor) [22]. For instance, the hole can oxidize a water molecule to yield $OH^{\cdot}$ [23], and the electron can reduce dissolved oxygen (O_2) to give the superoxide anion (O_2^-) or H_2O_2 [24]. These highly reactive molecules may have effect on the oxidation–reduction center of enzyme and finally enhance the catalytic activity of GOx. Synchronously, when the rate of O_2 reduction is not sufficiently fast to match the rate of hole reaction, an excess of electrons will accumulate on the ZnO nanoparticles. Electrochemical experiments have shown that electrons accumulated on the surface can be collected at an electrode to produce current [25]. So in the glucose biosensor system, the photovoltaic effect from ZnO nanoparticles can improve the catalytic ability of GOx and promote the current response of enzyme electrode.

As a comparison, the current response of electrodes containing ZnO nanoparticles without enzyme [see Fig. 5C(a and b)] and containing GOx without nanoparticles [see Fig. 5C(c and d)] were tested in dark and under illumination, respectively. The results showed the light less effected on these electrodes, which indicated the enhancement of the current response resulted from the cooperation of ZnO nanoparticles and enzyme. In our system, the photovoltaic effect of ZnO nanoparticles played an assistant function in

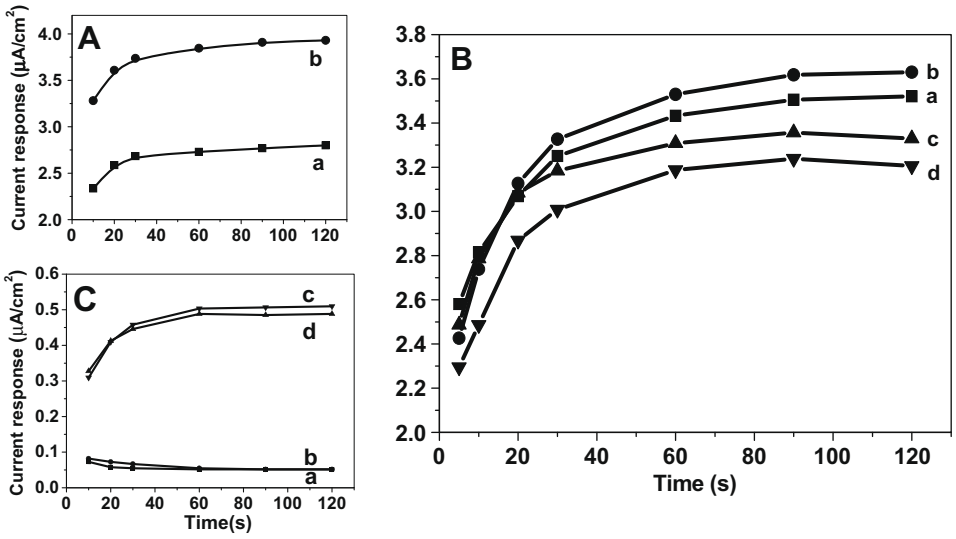

Fig. 5. (A) Photovoltaic effect on the calibration curves of the electrodes containing ZnO nanoparticles (a) in the dark and (b) under illumination. (B) Effect of the time of the ultraviolet irradiation on the response of the biosensor (a) 1 h, (b) 2 h, (c) 3 h and (d) 4 h. (C) Photovoltaic effect on the calibration curves of the electrodes containing (a and b) only ZnO nanoparticles and (c and d) only GOx. The concentration of β-D-glucose solution is 1.4 mmol/L.

enzymatic reaction, making the catalysis of enzyme more efficiently and contributing to the higher sensitivity and reactivity.

4. Summary

We have demonstrated a simple and effective method for the fabrication of enzyme biosensor based on ZnO nanoparticles. The UV-spectrum and CD results show that the structure of GOx can be maintained after bioconjugation with ZnO. The experimental results indicate the enzyme electrode containing ZnO nanoparticles significantly enhances the response current compared with the electrodes containing no nanoparticles. Moreover, it has been proved that illumination plays an important role in biosensor's performance. The photovoltaic effect of ZnO nanoparticles can greatly enhance the catalytic activity of GOx and then improve the performance of enzyme electrode. This method provides a new feasible way to further enhance and tune the current response of biosensor, which is favorable for miniaturization of biosensor. We believe this photoelectrochemical system is suitable for other amperometric biosensor system.

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