



Immobilization and direct electrochemistry of glucose oxidase on a tetragonal pyramid-shaped porous ZnO nanostructure for a glucose biosensor

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ABSTRACT

A tetragonal pyramid-shaped porous ZnO (TPSP-ZnO) nanostructure is used for the immobilization, direct electrochemistry and biosensing of proteins. The prepared ZnO has a large surface area and good biocompatibility. Using glucose oxidase (GOD) as a model, this shaped ZnO is tested for immobilization of proteins and the construction of electrochemical biosensors with good electrochemical performances. The interaction between GOD and TPSP-ZnO is examined by using AFM, N₂ adsorption isotherms and electrochemical methods. The immobilized GOD at a TPSP-ZnO-modified glassy carbon electrode shows a good direct electrochemical behavior, which depends on the properties of the TPSP-ZnO. Based on a decrease of the electrocatalytic response of the reduced form of GOD to dissolved oxygen, the proposed biosensor exhibits a linear response to glucose concentrations ranging from 0.05 to 8.2 mM with a detection limit of 0.01 mM at an applied potential of −0.50 V which has better biosensing properties than those from other morphological ZnO nanoparticles. The biosensor shows good stability, reproducibility, low interferences and can diagnose diabetes very fast and sensitively. Such the TPSP-ZnO nanostructure provides a good matrix for protein immobilization and biosensor preparation.

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1. Introduction

Over the last two decades considerable attention has been paid to the development of new biocompatible materials with high porosity and large surface area for protein immobilization (Volodkin et al., 2004; Pierre et al., 2006). A series of porous materials such as clay (Carrado et al., 2004), montmorillonite (Lin et al., 2007), porous alumina (Dai et al., 2006) and sol–gel matrix (Brennan et al., 2003) have been used and proven to be promising as the immobilization matrices because of their high mechanical, thermal, and chemical stability as well as good adsorption and penetrability. The incorporation of proteins into pores could provide an active biomaterial (Xiao et al., 2003).

Zinc oxide (ZnO) is a typical semiconductor material with a wide band gap ($E_g = 3.37$ eV) and a large exciton binding ability (60 meV) (Wong and Searson, 1999). In the area of bioscience, the special properties of nano-ZnO have also attracted much attention gradually (Krishnamoorthy et al., 2006; Dorfman et al., 2006; Wang et al., 2006; Wei et al., 2006; Corso et al., 2007; Zhu et al., 2007). Its nice biocompatibility and fast electron transfer between the enzyme's active sites and the electrode have made the material

be favor for functioning as the biomimic membrane to immobilize and modify proteins. Nano-ZnO also deserves further investigation as an important promising candidate for the supporting material in the fabrication of biosensors (Chen et al., 2007, 2008). To date, although various ZnO nanostructures, such as nanorods (Cheng and Samulski, 2004), nanowires (Rout et al., 2007), nanobelts (Height et al., 2006), nanoring (Wang et al., 2004), nanosheet (Park et al., 2004), tetrapod (Yan et al., 2003), hexagonal pyramid and cylinder (Joo et al., 2005), and radial nanowire array (Yang et al., 2005) have been prepared and ZnO nanorod-modified electrodes have been reported (Zhang et al., 2004, 2007; Wei et al., 2006), to the best of our knowledge, there are fewer reports on ZnO porous nanostructures (Polarz et al., 2007) and their application in biosensing.

Diabetes mellitus is a worldwide public health problem. The metabolic disorder results from insulin deficiency and hyperglycemia and is reflected by blood glucose concentrations higher or lower than the normal range of 4.4–6.6 mM (Wang, 2008). The determination of glucose concentration is very important in clinic for diagnosing diabetics (Yu et al., 2003). In this work, a tetragonal pyramid-shaped porous ZnO (TPSP-ZnO) nanostructure was synthesized and for the first time used for the immobilization of GOD by physical adsorption. The high isoelectric point (IP) of 9.5 of ZnO provides a friendly microenvironment for the negatively charged GOD (IP: 4.2) to retain its activity and ZnO promotes the direct electron transfer between the GOD and the electrode to a large extent.

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The immobilized GOD shows fast direct electrochemistry corresponding to its FAD/FADH₂ (FAD: flavin adenine dinucleotide) redox couple. The reduced form of GOD can electrocatalyze the reduction of dissolved oxygen. In the presence of glucose the electrocatalytic reaction is restrained due to the enzyme-catalyzed reaction between the oxidized form of GOD and glucose, which results in a decrease of electrocatalytic response. Based on the decrease, a new method for glucose determination is proposed. This competitive assay-like method is different from the conventional detection method based on the measurement of oxygen consumed, which is usually influenced by the concentration of the dissolved oxygen. The limitation of the solubility of the dissolved oxygen will influence the detection limit. Furthermore, the reduction of oxygen is irreversible. The peak caused by the reduction of oxygen is broad. The constructed sensor has the linear response range of 0.05–8.2 mM with a low detection limit to glucose (0.01 mM), and can operate under air without the exclusion of the dissolved O₂. It has better biosensing properties than those from other morphological ZnO nanoparticles, which might result from the larger specific surface area and the specific shape. Thus TPSP-ZnO provides an efficient strategy and a new promising platform for the further study of direct electron transfer of protein and the development of biosensors.

2. Experimental

2.1. Reagents

GOD (EC 1.1.3.4, 35.3 units mg⁻¹. Type II from *Aspergillus niger*) and β-D-(+)-glucose were purchased from Sigma and used as received. Nafion (10% in methanol with equivalent weight of about 1100) was obtained from Aldrich and was diluted to 5% with H₂O before use. All other chemicals were of analytical grade and were used without further purification. All solutions were made up with doubly distilled water. Phosphate buffer (PB, 0.1 M) solutions with various pH values were prepared by mixing stock standard solutions of K₂HPO₄ and KH₂PO₄ and adjusting the pH with H₃PO₄ or NaOH.

2.2. Electrode modification

TPSP-ZnO was prepared following a recipe similar to that reported by our previous work (Dai et al., 2008). The spherical solid ZnO particles with diameters of 30–50 nm were prepared according to the literature (Yuan et al., 2003).

10 mg of TPSP-ZnO was dispersed into 10 mL doubly distilled water to obtain a suspension of 1 mg mL⁻¹ TPSP-ZnO. 2 μL of TPSP-ZnO suspensions was mixed with 2 μL of GOD (2 mg mL⁻¹ in PB)

thoroughly. Then 2 μL of the mixture was dropped onto the surface of a glassy carbon electrode (GCE) and allowed to dry at ambient temperature to obtain the GOD/ZnO-modified electrode. Finally, 2 μL of Nafion (5%) was cast on the GOD/ZnO-modified electrode surface and the GOD/TPSP-ZnO/Nafion-modified electrode was obtained. The solvent was allowed to evaporate before use. The modified electrodes were rinsed with doubly distilled water for twice or thrice to get rid of the non-firmly adsorbed GOD. They were then immersed into the blank 0.1 M pH 7.0 PB until a stable electrochemical response of GOD was observed. The obtained modified electrodes were stored in 0.1 M pH 7.0 PB at 4 °C in a refrigerator when not in use. The same procedure was employed to fabricate other modified electrodes.

2.3. Apparatus and measurements

The phase characterization was performed by means of X-ray diffraction (XRD) using a D/Max-RA diffractometer with Cu Kα radiation. The morphologies and particle sizes of the samples were characterized by JEM-200CX transmission electron microscopy (TEM) working at 200 kV. The X-ray photoelectron spectra (XPS) were recorded on an ESCALAB MK II X-ray photoelectron spectrometer, using Mg K-X-ray as the excitation source. Atomic force microscopic (AFM) experiments were performed with Agilent series 5100. Nitrogen adsorption isotherms were obtained using an ASAP 2000 instrument. Spectrophotometric measurements were carried out using a Hitachi (model U-2001) spectrophotometer. Cyclic voltammetric and amperometric measurements were performed on CHI 660 electrochemical workstation. A three-electrode system comprising a platinum wire as auxiliary, a saturated calomel electrode as reference and the modified electrode as working electrodes were used for all electrochemical experiments. The electrochemical behavior of GOD was performed by deoxygenating with highly pure nitrogen for 15 min, and then a nitrogen atmosphere was kept over the solutions during measurements. The detection of glucose was carried out in air-saturated solution. All experiments were carried out at laboratory temperature.

3. Results and discussion

3.1. Characterizations of the prepared ZnO

The TEM image of the prepared ZnO particles is shown in Fig. 1A. It can be seen that ZnO particles display the flat base and have the tetragonal pyramid-shaped structure (marked with arrows). The lengths of the side edge and basal edge mainly range from 70 to 90 nm and from 40 to 60 nm, respectively. The shape of some particles to be nonpyramid is attributed to their different orientations on

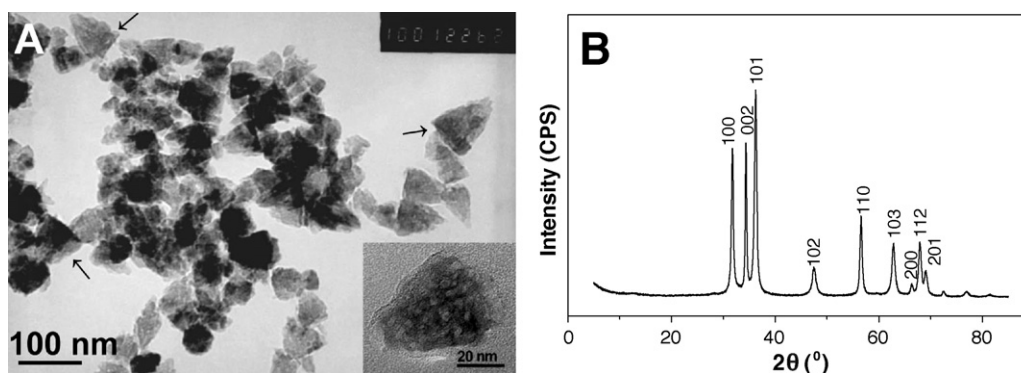


Fig. 1. TEM images at lower (A) and higher (inset in A) magnification and XRD pattern (B) of ZnO particles prepared by the reaction of ZnSO₄ with KOH in an aqueous solution of polyglycol.

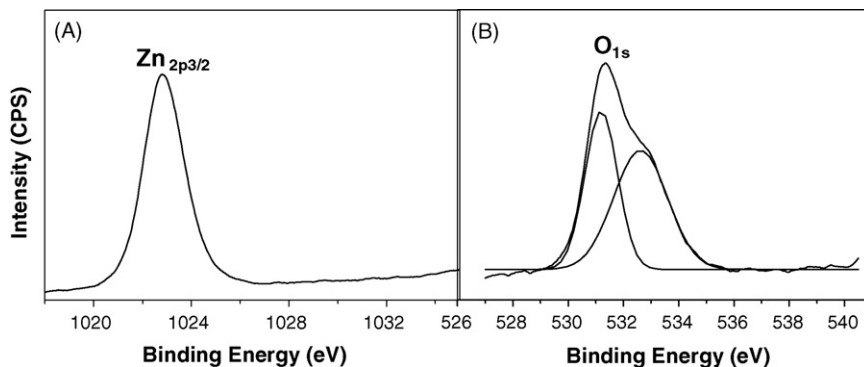


Fig. 2. XPS spectra of the TPSP-ZnO particles: (A) Zn_{2p} and (B) O_{1s} .

a solid support. From inset in Fig. 1A, there exist many pores in the ZnO particles and the diameter of the pores is about 4 nm. Fig. 1B shows the XRD pattern of TPSP-ZnO. All the diffraction peaks in the range $5^\circ < 2\theta < 85^\circ$ can be indexed as the hexagonal ZnO with lattice constants $a = 3.251 \text{ \AA}$ and $c = 5.215 \text{ \AA}$, which are in good accordance with the values on the standard card (JCPDS 36-1451).

XPS spectra taken from the Zn and O regions of the sample are shown in Fig. 2A and B, respectively. The peak at about 1022.8 eV (Fig. 2A) is attributed to $\text{Zn}_{2p_{3/2}}$, which agrees well with the data for Zn_{2p} in ZnO. From Fig. 2B, O_{1s} XPS is asymmetric and can be fitted by two peaks centered at 531.1 and 532.6 eV. The high binding energy component can be attributed to the presence of loosely bound oxygen on the surface of ZnO particles. The low binding energy component can be attributed to O^{2-} ions on the wurtzite structure of the hexagonal Zn^{2+} ions array, surrounded by Zn. The atom ratio of Zn with O is calculated by using the integrated peak area and sensitivity factors, which is also about 1:1, suggesting ZnO is formed.

3.2. Interaction between TPSP-ZnO and GOD

Brunauer–Emmett–Teller (BET) surface area of the TPSP-ZnO calculated from N_2 adsorption isotherm is about $41 \text{ m}^2 \text{ g}^{-1}$, which is larger than the value of about $30.2 \text{ m}^2 \text{ g}^{-1}$ calculated for the surface of spherical solid ZnO particles with the diameter in the range of 30–50 nm (Wang and Caruso, 2004). It is reported that

protein can be fixed in the pores of porous materials by simply immersing the porous material in the protein solution (Diaz and Balkus, 1996). To verify the effect of GOD on TPSP-ZnO, N_2 adsorption isotherms before and after GOD loading are investigated. The surface area of ZnO decreases upon the immobilization treatment. The surface area of ZnO is $24 \text{ m}^2 \text{ g}^{-1}$ after GOD loading and the pore volume of TPSP-ZnO is 60% of that before GOD loading, indicating GOD intercalates into the pores of ZnO (Takahashi et al., 2001).

Fig. 3 demonstrated AFM images of the TPSP-ZnO before (A) and after (B) GOD loading, respectively. The parameter of the base was about 40–60 nm and the porous structure also could be observed which was consistent with the observation of the TEM analysis. After GOD was loaded, the pores on the surface of TPSP-ZnO cannot be observed and the diameter of the ZnO slightly increased, indicating that GOD molecules were immobilized on the surface of TPSP-ZnO. From the results of N_2 adsorption isotherm and AFM, GOD not only adsorbed on the surface of TPSP-ZnO, but also intercalated into the pores of ZnO, which indicated TPSP-ZnO was a good matrix for GOD immobilization.

3.3. Direct electron transfer of GOD/ZnO/Nafion-modified electrode

FAD, a part of the GOD molecule, is known to undergo redox reaction where two protons and two electrons are released or

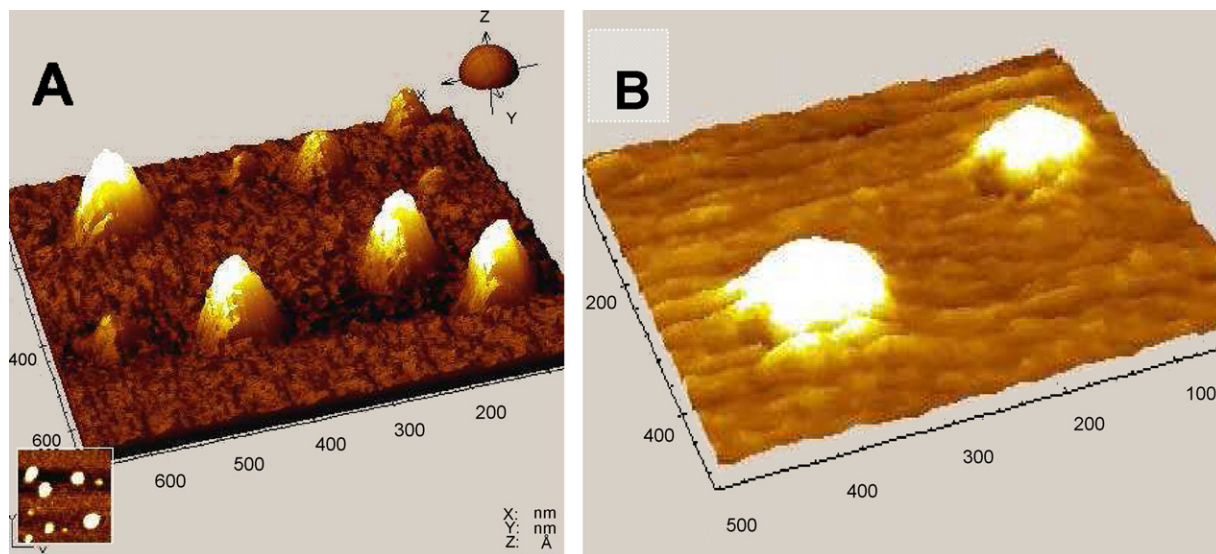


Fig. 3. AFM images of TPSP-ZnO before (A) and after (B) GOD loading, respectively.

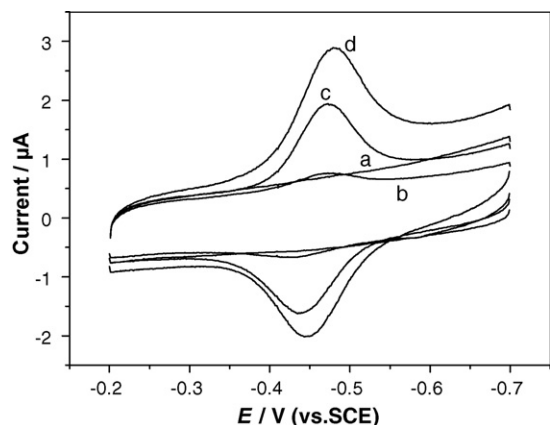


Fig. 4. Cyclic voltammograms of TPSP-ZnO/Nafion (a), GOD/Nafion (b), GOD/spherical ZnO/Nafion (c) and GOD/TPSP-ZnO/Nafion (d) modified GCEs in 0.1 M pH 7.0 PB at 0.1 V s^{-1} .

taken up (Ianniello et al., 1982; Savitri and Mitra, 1998). According to the conclusion of Ianniello et al. (1982), the electrochemistry response of GOD immobilized on the heterogeneous surface is due to the redox of FAD. Fig. 4 shows the cyclic voltammograms of different electrodes in 0.1 M pH 7.0 PB at 0.1 V s^{-1} . No redox peak is observed at TPSP-ZnO/Nafion-modified GCE, which indicates TPSP-ZnO is electroinactive in the potential window. GOD/Nafion-modified electrode also shows slight redox peaks because FAD is deeply seated in a cavity and therefore not easily accessible for conduction of electrons to the electrode surface.

The GOD/TPSP-ZnO/Nafion-modified electrode exhibits a couple of stable redox peaks that are attributed to the redox of immobilized GOD, indicating TPSP-ZnO can facilitate the electron transfer between the electroactive center of GOD and electrode. It might result from the high isoelectric point of ZnO (9.5) and the low isoelectric point of GOD (4.2). ZnO can provide a friendly microenvironment for the negatively charged GOD to retain its activity. For comparison, GOD/spherical ZnO/Nafion-modified electrode has been carried out (curve c in Fig. 4). The GOD/spherical ZnO/Nafion-modified electrode also exhibits a couple of stable redox peaks with which the same formal potential of GOD/TPSP-ZnO/Nafion-modified electrode and the peak current is 1.7 times smaller than that of GOD/TPSP-ZnO/Nafion-modified electrode. It results from the larger specific surface area of TPSP-ZnO, which results in a large response.

The ratio of anodic to cathodic peak current is close to 1, which indicates the electrode process is reversible. The anodic and cathodic peak potentials of the immobilized GOD are at -480 and -448 mV , respectively. The formal potential of -464 mV is very close to the standard electrode potential of -460 mV (vs. SCE) for FAD/FADH_2 at pH 7.0 (25.8°C) (Tinoco et al., 1978), suggesting that most GOD molecules preserve their native structure after the adsorption process (Liu and Ju, 2002).

The effect of scan rate on direct electron transfer of the immobilized GOD was evaluated in Fig. 5. With an increasing scan rate, the anodic peak potential of GOD shifted to a more positive value and the cathodic peak potential shifted in a negative direction. The redox peak currents were proportional to the scan rate, indicating a surface-controlled behavior. The average k_s value of $7.5 \pm 0.4 \text{ s}^{-1}$ was larger than 2.76 s^{-1} of GOD immobilized on the carbon nanotube wrapped by polyelectrolyte (Wen et al., 2007) and 3.1 s^{-1} of GOD immobilized on carbon nanotubes/chitosan matrix (Liu et al., 2005).

Cyclic voltammogram of GOD/ZnO/Nafion-modified electrode showed a strong dependence on solution pH. An increase in solu-

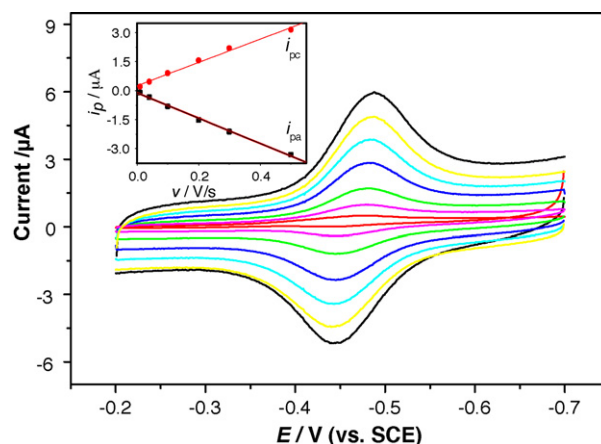


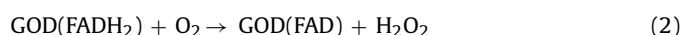
Fig. 5. Cyclic voltammograms of GOD/TPSP-ZnO/Nafion/GCE in pH 7.0 PB at 0.01, 0.04, 0.08, 0.1, 0.2, 0.3 and 0.5 V s^{-1} (from inner to outer). Inset: Plots of peak currents vs. v .

tion pH caused a negative shift in both cathodic and anodic peak potentials and the maximum peak currents of GOD occurred at pH 7.0. Plot of the formal potentials vs. pH (from 5 to 8.0, $P=0.9973$) produced a line with the slope of -50.94 mV/pH which was close to the expected value of -59.0 mV/pH , indicating two protons and two electrons were involved in the electron transfer process.

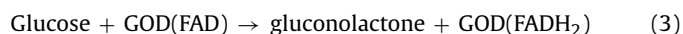
3.4. The determination of glucose

GOD catalyzes the oxidation of glucose by oxygen to produce gluconolactone and hydrogen peroxide, and oxygen is used as the electron acceptor.

The electrocatalytic process is expressed as follows (Liu and Ju, 2003):



Upon the addition of glucose, the electrocatalytic reaction is restrained to the enzyme-catalyzed reaction between the oxidized form of GOD, GOD(FAD), and glucose.



In the presence of dissolved oxygen, no peak was observed at TPSP-ZnO/Nafion electrode (curve b in Fig. 6A), though an increase in cathodic current at the potentials more negative than -0.4 V . Curves d and e in Fig. 6B show the cyclic voltammograms of GOD/TPSP-ZnO-modified electrode in oxygen-free and oxygen-saturated buffers, respectively. The reduction peak of GOD significantly increases in the presence of oxygen, indicating that its reduced form, GOD(FADH₂), can electrocatalyze the reduction of dissolved oxygen following Eqs. (1) and (2).

Upon addition of glucose to air-saturated PBS, the reduction current response of GOD/TPSP-ZnO/Nafion electrode decreased (curve f in Fig. 6B) which resulted from the electrocatalytic reaction restrained to the enzyme-catalyzed reaction between the oxidized form of GOD, GOD(FAD), and glucose, following Eq. (3), while no obvious change was observed at TPSP-ZnO/Nafion electrode (curve c in Fig. 6A). As well known, glucose is the substrate of GOD, its presence will result in an enzyme-catalyzed reaction according to Eq. (3) and decrease the concentration of the oxidized form of GOD on electrode surface. Thus, the addition of glucose restrained the electrocatalytic reaction and led to the decrease of reduction current.

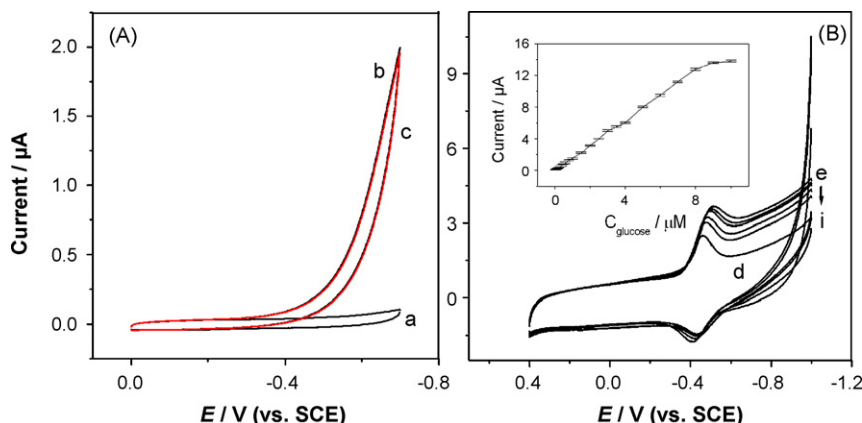


Fig. 6. Cyclic voltammograms of the TPSP-ZnO/Nafion (A) and GOD/TPSP-ZnO/Nafion (B) modified electrodes in 0.1 M pH 7.0 PB in the absence (a and d) and presence (b and e) of dissolved oxygen and 0.1 M pH 7.0 PB + 0.1 mM (f), 0.2 mM (g), 0.4 mM (h) and 0.5 mM (c and i) glucose in the presence of dissolved oxygen at 0.1 V s⁻¹. Inset in B: plot of the electrocatalytic current of O₂ vs. glucose concentration at -0.50 V.

With increasing β -D-(+)-glucose concentration the reduction current decreased. The calibration curve of the sensor under the optimized experimental conditions detected at the applied potential at -0.5 V was shown as an inset in Fig. 6B. The calibration range of β -D-(+)-glucose concentration was from 0.05 to 10.0 mM. The linear response range of the sensor to β -D-(+)-glucose concentration was from 0.05 to 8.2 mM (correlation coefficient: 0.9992) with a detection limit was 0.01 mM at a signal-to-noise ratio of 3 (inset B in Fig. 6). The average relative standard deviation (R.S.D.) of the plots was 4.1%. It is a little low stability, which might result from a little loss of the activity of the enzyme during the reaction. It is well known that the diabetic glucose concentration is above 7.0 mM. Compared with the linear range of 0.01–3.45 mM for GOD immobilized on ZnO nanorod (Wei et al., 2006) and 0.001–0.76 mM for GOD immobilized on ZnO nanowires (Polarz et al., 2007), the linear range of our prepared sensor was much wider and more suitable for diagnosing diabetics. The detection limit was 0.01 mM, which was the same as GOD immobilized on ZnO nanorod (Wei et al., 2006) and smaller than 0.05 mM for GOD immobilized on carbon nanotube materials (Yao and Shiu, 2007).

As a comparison, the amperometric response of the GOD/spherical ZnO/Nafion-modified GCE was evaluated. Under optimal conditions, the linear range of the GOD/spherical ZnO/Nafion-modified GCE was from 0.1 to 4.2 mM with a correlation coefficient of 0.9990. The detection limit was estimated to be 0.07 mM at a signal-to-noise of 3. So the constructed biosensor based on immobilized GOD on TPSP-ZnO had better biosensing properties than those from spherical ZnO which might result from the larger specific surface area of TPSP-ZnO causing more GOD molecules loading and the TPSP nanostructure having high fraction of surface atoms located on the corners and edges causing the better catalytic activity (Narayanan and El-Sayed, 2005).

The effect of applied potential on the responses of the glucose sensor showed the electrocatalytic reduction of oxygen could be observed at around -0.45 V. Upon a decreasing applied potential from -0.45 to -0.70 V, the steady-state current increased due to the increased driving force for the fast reduction of glucose at the lower potentials. Here we select -0.50 V as the working potential for determinations.

3.5. Interference

The influences of foreign species were investigated by analyzing a standard solution of 0.20 mM glucose to which interfering species were added. 0.10 mM uric acid and 0.10 mM *p*-acetaminophenol did

not cause any observable interference to the response to glucose, and only ascorbic acid at the concentration of 0.10 mM reduced the reduction current by 2.1%. This was probably due to the smaller size of ascorbic acid, which could diffuse through the porous film to the electrode surface and be oxidized. Thus, the response was not interfered by cooxidizable substances such as ascorbic acid, uric acid and *p*-acetaminophen.

3.6. Stability and reproducibility of the GOD/TPSP-ZnO/Nafion-modified electrode sensor

The direct electrochemistry of the GOD/TPSP-ZnO/Nafion-modified electrode could retain the constant current values upon the continuous cyclic sweep. After more than 300 successive measurements the immobilized GOD/ZnO/Nafion-modified electrode only lost 12.3% of its initial activity. Thus, TPSP-ZnO particles were very efficient for retaining the electrocatalytic activity of GOD and preventing it from leaking out of the sensor. After a storage period of one month in 0.1 M pH 7.0 PB the biosensor showed a 10% loss of activity.

The fabrication reproducibility of ten electrodes, made independently, showed an acceptable reproducibility with a R.S.D. of 5.3% for the current determined at a glucose concentration of 0.1 mM. With one sensor, the mean steady-state current was 0.16 μ A with a R.S.D. of 4.2% for six determinations at a glucose concentration of 0.1 mM.

3.7. Determination of glucose in practical serum sample

Five parallel determinations were carried out. The glucose level was determined to be 8.15 close to the 8.4 mM determined by spectrophotometry, showing a good accuracy. The recoveries for the assays of 0.10–0.80 mM glucose were between 95 and 103% for eight measurements, indicating good practicability of the biosensor in clinic laboratory.

4. Conclusions

In this work, a TPSP-ZnO nanostructure has been prepared with a larger specific surface area and can be applied in protein immobilization and biosensing. TPSP-ZnO exhibits favorable biocompatibility for facilitating the electron transfer between protein and electrode surface. The immobilized GOD preserves its natural structure and bioactivity and displays better responses to glucose than those from other morphological ZnO nanoparticles. The

constructed biosensor shows a good reproducibility and stability. TPSP-ZnO provides an efficient matrix for promoting the direct electron transfer of proteins and developing biosensors.

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