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A novel hydrogen peroxide biosensor based on the immobilization of hemoglobin on three-dimensionally ordered macroporous (3DOM) gold-nanoparticle-doped titanium dioxide (GTD) film

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ABSTRACT

The three-dimensionally ordered macroporous gold-nanoparticle-doped titanium dioxide (3DOM GTD) film was modified on the indium-tin oxide (ITO) electrode surface. Hemoglobin (Hb) has been successfully immobilized on the 3DOM GTD film and the fabrication process was characterized by Raman and UV–vis spectra. The results indicated that the Hb immobilized on the film retained its biological activity and the secondary structure of Hb was not destroyed. The direct electrochemistry and electrocatalysis of Hb immobilized on this film have been investigated. The Hb/3DOM GTD/ITO electrode exhibited two couples of redox peaks corresponding to the Hb intercalated in the mesopores and adsorbed on the external surface of the film with the formal potential of −0.20 and −0.48 V in 0.1 M PBS (pH7.0), respectively. The Hb/3DOM GTD/ITO electrode exhibits an excellent eletrocatalytic activity, a wide linear range for H_2O_2 from 5.0 μM to 1.0 mM with a limit of detection of 0.6 μM, high sensitivity (144.5 μA mM^{-1}), good stability and reproducibility. Compared with the TiO_2 nanoneedles modified electrode, the GTD modified electrode has higher sensitivity and response peak current. The 3DOM GTD provided a good matrix for bioactive molecules immobilization, suggesting it has the potential use in the fields of H_2O_2 biosensors.

1. Introduction

The sensitive and accurate detection of hydrogen peroxide (H_2O_2) is becoming of practical importance in the pharmaceutical, environmental, biological, clinical and industrial settings fields (Bartlett et al., 1998; Chen et al., 2006; Yu and Ju, 2003). Electrochemical methods for H_2O_2 detection have been demonstrated to be simple, rapid, and inexpensive methods and extensively employed for the design of H_2O_2 biosensors. However, the miniaturization trend of electrochemical biosensors makes the design and utilization of them have been of high interest.

The direct electrochemistry of enzymes and proteins has attracted considerable attention because it provides fundamental knowledge of redox behavior of enzymes or proteins in a biological system and is a more effective way of fabricating biosensors, bioreactors, and biomedical devices than using mediators (Armstrong and Wilson, 2000; Hamachi et al., 1997; Armstrong et al., 1988; Szpakowska et al., 2009). Although enzymes or proteins electron-transfer are quite fast in biological systems, they exhibit rather slow rate of heterogeneous electron-transfer at conventional electrodes because of the deep burying of the electroactive center of enzymes or proteins, the adsorptive denaturation and the unfavorable orientations on the electrodes. Therefore, it is still a challenge to achieve direct electrochemistry of enzymes and proteins.

In order to improve the electrochemical properties of enzymes or proteins and retain their bioactivities, multiporous structure materials have been used to prepare the modified electrode for their high active surface area and increase significantly detection signals (Alwarappan et al., 2010; Teng et al., 2009). Among the multiporous structures, three-dimensionally ordered macroporous (3DOM) (>50 nm) modified electrodes materials have recently received extensive attention (Lu and Eychmueller, 2008; Song et al., 2005; Szamocki et al., 2006, 2007). These 3DOM modified electrodes can ensure the accessibility of reactants to the active surface sites of electrodes, increasing both the mass transport of reactant and mass-normalized activity. These open, interconnected and periodic 3DOM structures provide a high stability, an increase of up to 2 orders of magnitude active surface area, more than one order of magnitude signal compared to that on a flat electrode. Therefore, the electrochemical detection with the 3DOM modi-

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fied multiporous electrodes have become apparent and fascinating in the electrochemistry field. Among the modified electrodes, the 3DOM TiO_2 film is of particular interest for its optical transparency, good biocompatibility, environmental safety and relatively good electrical conductivity. Since the first report claiming the synthesis of 3DOM TiO_2 photonic crystals, 3DOM TiO_2 films have been used in rechargeable lithium ion batteries (Bing et al., 2006), solar cells (Huisman et al., 2005), photocatalysts (Environ, 2007) and electrochemical biosensor (Zhang et al., 2004). Recently, 3DOM TiO_2 film modified electrodes have been reported for immobilization of HRP (Zhu et al., 2009) and glucose oxidase (Cao et al., 2008a,b). In our previous work, we fabricated a new 3DOM gold-nanoparticle-doped titanium dioxide (GTD) photonic crystals modified electrode by the colloidal crystal template technique and developed a new HRP-based H_2O_2 biosensor using the 3DOM GTD/ITO electrode (Li et al., 2009a,b). 3DOM gold nanoparticles/TiO_2 composite architecture exhibited to promote the direct electron transfer of the immobilized enzymes and further enhance the sensitivity of the designed H_2O_2 electrochemical biosensor.

Hemoglobin (Hb) is a desirable model molecule for the study of electron transfer reactions of heme enzymes (Ma et al., 2007; Tang et al., 2008; Wei et al., 2009; Wu and Hu, 2007) and can be used as a substitute of horseradish peroxidase (HRP) to catalyze the reduction of H_2O_2 (Feng et al., 2005; Li et al., 2009a,b; You et al., 2009). In this work, Hb was successfully immobilized on the 3DOM GTD film and Hb-based H_2O_2 electrochemical biosensor was fabricated. The fabrication process was characterized by Raman spectroscopy, UV–vis spectroscopy. This is the first report on the 3DOM GTD/ITO electrode for biosensor for direct electrochemistry of Hb. The results indicated that the secondary structure of Hb immobilized on the film was not destroyed and retained its biological activity. The amperometric response of the immobilized Hb had a good linear relation with the concentration of H_2O_2 and the quantitative relationship between the responsive current intensity and H_2O_2 concentration was established. The electrode modified with Hb/3DOM GTD film has higher stability and eletrocatalytic activity toward the reduction of H_2O_2 compared with the electrode modified with the gold-nanoparticle-doped titanium dioxide nanoneedles (GTDNs) film. The developed biosensor provides a new matrix for study of direct electrochemistry of enzymes and proteins and detection of H_2O_2 on low conductivity electrode biosensor.

2. Experimental

2.1. Materials

Bovine hemoglobin (Hb, MW 64,500, EC 1.11.17, RZ > 3.0, A > 300 units/mg) purchased from Sigma Chemical Co. was used without further purification. Titanium (IV) butoxide, H_2O_2 (30%) and ethanol absolute were purchased from Chemical Reagents Co., Ltd. of China. Triethanolamine (TEA), $HAuCl_4$ were purchased from the Nanjing Sunshine Biotechnology Ltd., China. TiO_2 nanoneedles were synthesized in Southeast University, China. The water was Mili-Q water at 18' MΩ. All other chemicals were of analytical grade and were used without further purification. ITO glasses (<20 Ω/cm^2) were purchased from Wuxi Kang Li Electron Industry Co., Ltd of China. The diameter 290 ± 15 nm polystyrene (PS) microspheres were synthesized in our laboratory.

2.2. Apparatus

The UV–vis spectra were recorded using a Lambda 17 UV spectrophotometer (Perkin–Elmer, USA) with a cuvette of 1 cm path length. The Raman spectra were obtained on a Labram 800 UV Raman spectrometer at ambient temperatures using 514.5 nm excitation and a spectral slit width of 600g cm^{-1}. The scanning electron microscopy (SEM) images were obtained with a Quanta-200 microscope (FEI, Holand).

Cyclic voltammetric (CV) and amperometric response measurements were carried out on a CHI760 electrochemical workstation (CHI Instruments, USA) employing a conventional three-electrode cell. The saturated calomel electrode (SCE) and a platinum electrode were used as the reference electrode and counter electrode, respectively. A Hb/3DOM GTD/ITO electrode was used as the working electrode.

2.3. Substrate preparation

Gold colloids were prepared according to the method reported by Doron et al. (1995). The products were stored in brown glass bottles at 4 °C. GTD sol–gel solution was synthesized according to our previous work (Li et al., 2009a,b). The preparation of 3DOM GTD photonic crystals was performed by the colloidal crystal template method. The surface morphology of the 3DOM GTD photonic crystals was characterized by SEM shown as Fig. 1A. The SEM image shows that the film is face-centered cubic ordered hexagonal network structure and the gold nanoparticles are introduced within the framework. The hexagonal networks stack layer-by-layer forming a 3DOM periodical structure with the interpenetrated pores.

Prior to modification, ITO glasses were cleaned ultrasonically 3 times with deionized water. The cleaned ITO substrates were immersed into 0.2% (v/v_0) colloidal suspension of PS microspheres for 48 h at the room temperature. The fix of PS template on ITO substrates, the infiltration of GTD colloids solution into the interspaces of PS opal template and the removing of the template were performed (Li et al., 2009a,b). The ITO substrates modified with GTD photonic crystals were cut at 1 cm × 1 cm area.

The TiO_2 nanoneedles were dispersed in deionized water with the aid of ultrasonic agitation for 2 h. Then TiO_2 nanoneedles were mixed with 1% Au colloid solution and ultrasonic agitation for 1 h. The suspension was adjust to pH = 6 and centrifugated at 1.1×10^4 *g* for 15 min. During the process, the gold-nanoparticles would be absorbed on the surface of the TiO_2 nanoneedles forming gold-nanoparticle-doped TiO_2 nanoneedles adducts (GTDNs). The surface morphology of the GTDNs adducts was shown as Fig. 1B. The GTDNs/ITO electrode was fabricated by casting a sol of GTDNs on the surface of treated ITO electrode through the dip-coating method. Then the GTDNs/ITO electrode was sinteringed in the oven at 500 °C for 1 h. The ITO substrates modified with the GTDNs film were also cut at 1 cm × 1 cm area.

2.4. Hb immobilization

For preparation of the Hb electrode, the 3DOM GTD/ITO electrode was cleaned 2 times with ethanol absolute and immersed in 0.1 M pH 7.0 phosphate buffer solution (PBS) containing 5 mg/mL Hb at 4 °C for 24 h. Before electrochemical experiments, the electrode was rinsed thoroughly with doubly distilled water to remove unbound Hb and kept in PBS at 4 °C when not in use.

2.5. Electrochemical detection of H_2O_2

Cyclic voltammetric (CV) and amperometric response measurements were performed in a 10 mL electrochemical cell by a conventional three-electrode cell. A Hb/3DOM GTD/ITO electrode, SCE and platinum electrode were used as the working electrode, reference electrode and counter electrode, respectively. Prior to experiments, high-purity nitrogen was purged into solution for at least 15 min, and a continuous flow of the gas was kept over the solution during the experiments to protect the solution from