A Tyrosinase Biosensor Based on ZnO Nanorod Clusters/ Nanocrystalline Diamond Electrodes for Biosensing of Phenolic Compounds

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An amperometric biosensor was constructed by using ZnO nanorod clusters as platforms for immobilizing tyrosinase on the nanocrystalline diamond (NCD) electrodes. The results showed that ZnO nanorod clusters provided an advantageous microenvironment due to their favorable isoelectric point (IEP) for tyrosinase loading; immobilized tyrosinase generally retained its activity. The tyrosinase/ZnO/NCD electrode showed a linear response range of 1–210 and sensitivity of 179.9 $\mu\text{A mmol}^{-1} \text{cm}^{-2}$ for *p*-cresol. The corresponding values were 1–190 and 90.2 for phenol, and 1–250 and 121.3 for 4-chlorophenol. The low detection limits were estimated to be 0.2 μM for *p*-cresol, 0.5 μM for phenol, and 0.4 μM for 4-chlorophenol ($S/N = 3$). The prepared enzyme electrode could keep 85% of its original activity after intermittent use for 4 weeks when stored in a dry state at 277 K. Therefore, the ZnO nanorod cluster thin films have potential applications as platforms to immobilize other enzymes and bioactive molecules in biosensors.

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Introduction

Recently, much effort has been devoted to the design of functional electrode surfaces for immobilization of enzyme molecules for sensitive electrochemical analytical applications and specific biological recognition. Boron-doped nanocrystalline diamond (NCD) thin film is regarded as the ideal biosensor support and an attractive new electrode material because of its excellent biocompatibility. It features good electrochemical properties, including a wide electrochemical potential window, very low double-layer capacitance, extreme electrochemical stability and high resistance to deactivation by fouling.¹ Thus, the NCD electrodes are of interest for a variety of electrically based chemical and biological sensing applications.² However, the as-deposited diamond electrode surface is hydrogen-terminated and biomolecules can not be directly immobilized on it. For the realization of biosensors from diamond film, bonded linker molecular layers are required. Recent studies have shown that the functionalization of diamond film using various chemical methods to link molecular layers to the diamond surface can serve as a starting point for preparing biomolecular interfaces exhibiting high stability and excellent biomolecular recognition properties.^{3–8} However, these methods are time-consuming, and diamond electrodes can not be reused.

ZnO is a key functional material with versatile properties; those ZnO nanostructures such as nanowires, nanotips and

nanorods have received more and more attention in biosensor applications due to their distinguished performances.^{9–19} Firstly, ZnO nanomaterial is a good biocompatible material with a high isoelectric point (IEP) of about 9.5, which makes it suitable for absorption of proteins with low IEPs, as the protein immobilization is primarily driven by electrostatic interaction. Secondly, ZnO nanostructures have unique advantages including the high specific surface area, nontoxicity, chemical stability, electrochemical activity, and high electron communication features. Thirdly, they can be easily prepared through chemical solution processes including sol-gel, hydrothermal synthesis and electrochemical deposition. Hence, ZnO nanomaterials have been widely used for different biosensors,^{10–12,20–26} such as glucose sensor, tyrosinase sensor, uric acid biosensor, horseradish peroxidase biosensor, and DNA biosensor. There have been a few reports about depositing ZnO films on diamond surfaces including radio frequency magnetron sputtering, chemical vapor deposition and electrochemical deposition. Such films have been widely used for field emission displays, ultraviolet photodetectors and surface acoustic waves (SAW). However, the fabrication of ZnO nanostructures onto the diamond film substrates and the construction of enzyme biosensors have not been reported.

Here we describe the construction of an amperometric biosensor based on tyrosinase/ZnO nanorod clusters/NCD electrode. The response dependences and the amperometric characteristics of the prepared enzyme electrode in the detection of phenolic compounds were investigated. The developed biosensor exhibited good performances.

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Fig. 1 Schematic illustration of the preparation process of tyrosinase electrode.

Experimental

Reagents and chemicals

Tyrosinase (EC 1.14.18.1, 3216 U mg⁻¹ from mushroom) was purchased from Fluka (packed in Switzerland). Phenol and *p*-cresol were obtained from Wako Chemical Co. (Japan). 4-Chlorophenol was purchased from Vas Lab Supplies Co. (analytical grade, China). Nafion in ethanol/water was obtained from Sigma (5 wt%). All other chemicals used were of analytical grade and all the solutions were prepared in deionized water (produced by a Millipore Milli Q system) in the experiment.

Apparatus

Voltammetric and amperometric measurements were carried out with a potentiostat/galvanostat (Princeton 263 A) in a three-electrode cell system. The NCD film was deposited on *n*-Si (111) wafers using a hot filament chemical vapor deposition (HFCVD) apparatus (made by Shanghai Jiaotong University, P. R. China). An NCD electrode was used as the working electrode. A saturated calomel reference electrode (SCE) and a Pt stick counter electrode were also employed. Cyclic voltammetric and amperometric measurements were carried out in a 50-mL electrochemical cell. Cyclic voltammetry (CV) was typically performed at a scan rate of 25 mV s⁻¹. Unless otherwise stated, all experiments were carried out at room temperature in phosphate buffer solution (PBS) (0.1 M, pH 6.5). Phosphate buffer solution was prepared using disodium hydrogen phosphate and potassium dihydrogen phosphate in Milli-Q water. The scanning electron microscope (SEM) images were taken using a Hitachi Ultra-high-Resolution S-4800 scanning electron microscope. The crystalline phase of the ZnO nanorods was determined on a Rigaku D/max 2550 V X-ray diffractometer (XRD) with high-intensity Cu K α radiation and a graphite monochromator. The Raman spectra were recorded under ambient conditions using a Renishaw 1000 Raman spectrometer (Renishaw Ltd., UK).

Fabrication of tyrosinase modification electrodes

NCD electrodes were sonicated successively in 2-propanol and Milli-Q water, and then allowed to dry in a flow of nitrogen gas. The fabrication process of the enzyme electrode is schematically illustrated in Fig. 1. Each NCD electrode was immersed in a solution containing an equimolar (0.025 M) aqueous solution of zinc nitrate hexahydrate and hexamethylenetetramine, and reacted at 338 K for 1 h; rinsing with Milli-Q water followed. For preparation of a tyrosinase-modified electrode, 10 μ L of 20 mg L⁻¹ tyrosinase solution was dropped onto the surface of a ZnO nanorod cluster-NCD electrode and this electrode was stored overnight at 277 K so that the tyrosinase could be sufficiently immobilized onto the surface of the ZnO nanorod clusters. After the electrode dried, a 10- μ L 1 wt% Nafion solution was dropped on the top to

Fig. 2 The SEM image and Raman spectrum of as-prepared NCD film. (a) SEM image and (b) Raman spectrum.

form a covered film. After drying, the electrode was immersed in PBS and then rinsed with PBS to wash away the non-immobilized tyrosinase. Finally, the electrode was ready for use.

Results and Discussion

Characterization of NCD film

Figure 2 showed the SEM image and the Raman spectra of as-prepared NCD film. As shown in Fig. 2a, the grain size of NCD obtained by HFCVD was about 20–30 nm. The intense wide peak at 1330 cm⁻¹ is assigned to the crystalline sp³-hybridized carbon Raman peak (Fig. 2b). Broadening of the diamond band is a result of decreasing the grain size to the nanometer scale.²⁷ The presence of a scattering peak at 1545 cm⁻¹ is due to increasing graphite-like sp² bonded components at the grain boundaries in films, and the two scattering peaks at 1126 and 1440 cm⁻¹ are attributed to NCD Raman peaks.²⁸

The characteristics and the formation mechanism of ZnO nanorod clusters

The morphologies of ZnO nanorod clusters were characterized by SEM. Figure 3a shows the results; a large quantity of ZnO nanorod clusters can be observed. Every cluster is composed of closely packed nanorods with a diameter of 40–60 nm and a length of 700–1000 nm. Figure 4a represents the XRD pattern of ZnO nanorod clusters. All the diffraction peaks are in good accordance with the values of the hexagonal ZnO on the standard card (JCPDS 36-1451). The sharpness of the peaks indicates that the product is well crystallized. The EDS (Fig. 4b) analysis for the sample shows that it is composed of Zn and O with an

Fig. 3 SEM images of ZnO nanorod clusters before (a) and after (b) immobilization tyrosinase (the inset shows the higher magnification SEM image).

atom ratio of about 1:1, indicating that ZnO was formed. The formation mechanism of ZnO nanorod clusters was also investigated. The overall reaction for the growth and dissolution of ZnO crystals can be described as follows:

During the heating process, hexamethylenetetramine hydrolyzes and releases OH^- into solution by Eq. (1), and then OH^- reacts with Zn^{2+} to form ZnO_2^{2-} ; finally, ZnO crystals are formed *via* homogeneous precipitation under this mild condition.

In general, the morphology of crystal principally depends on the relative growth rates of various crystal faces, *i.e.* the more rapid the growth rate, the quicker the disappearance of the plane.^{29,30} It is well known that the hexagonal ZnO crystal has both polar and nonpolar faces: a basal polar oxygen plane, top tetrahedron corner-exposed polar zinc plane, and low index faces (parallel to the *c* axis) consisting of a nonpolar face. Polar faces with surface dipoles are thermodynamically less stable than nonpolar faces; such polar surfaces usually undergo rearrangement to reduce their surface energy, and tend to grow more rapidly than nonpolar faces. At the early stage of the reaction system, ZnO_2^{2-} ions, the growth units in the solution

Fig. 4 XRD (a) and EDS (b) of ZnO nanorod clusters on the NCD electrode.

near the surface of ZnO nuclei, are likely adsorbed on the positive polar face of the (0001) surface, resulting in faster growth along the [0001] direction, and ZnO nanorods were obtained. Additionally, as the precursor concentration is higher, a large amount of ZnO nuclei are generated. These selectively aggregate and grow into closely packed ZnO nanorods. With the reaction time increasing, both the widths and the lengths of the packed ZnO nanorods increase and those ZnO nanorod clusters are formed.

Fabrication of ZnO nanorod cluster tyrosinase biosensor

The fabrication process of the tyrosinase biosensor was described above. ZnO nanorod clusters were firstly fabricated on the NCD electrode surface by the homogeneous precipitation method, and then tyrosinase was immobilized onto the ZnO nanorod cluster surfaces by the electrostatic force. As is shown in Fig. 3b, after immobilization of tyrosinase, the SEM image of ZnO nanorod clusters was changed; it became blurry because of adsorption of the non-conductive enzyme molecules. Such results also demonstrated that there are the strong forces between ZnO nanorods and tyrosinase. Subsequently, the Nafion solution was dropped onto the electrode surface and the tyrosinase biosensor was successfully constructed.

CV curves for phenolic compounds at the tyrosinase/ZnO/NCD electrode

Tyrosinase is also known as polyphenol oxidase; it can catalyze the oxidation of phenol and various phenol derivatives to *o*-quinone derivatives *via* catechol derivatives.⁷ Thus, the generated *o*-quinones can be reduced electrochemically at low potential without any mediator. Figure 5 shows the CV curves of the tyrosinase/ZnO/NCD electrode before and after addition of *p*-cresol in 0.1 M PBS (pH 6.5). In the absence of *p*-cresol, only a low background current was observed; after adding *p*-cresol to the PBS, the CV curves gave a well-defined peak located at -0.2 V, which was due to the reduction of *o*-quinone

Fig. 5 Typical CV curves of *p*-cresol on tyrosinase/ZnO/NCD electrode. (a) Blank, (b) 5 μM , (c) 50 μM , (d) 100 μM *p*-cresol, respectively (the supporting electrolyte is 0.1 M PBS solution, and the scan rate is 25 mV s^{-1}).

species liberated from the enzymatic reaction on the electrode surface; the reduction currents also increased with increasing the concentration of *p*-cresol.

Optimization of the experimental conditions

An enzyme is a biological catalyst, usually speeding up the rate at which chemical reactions occur under favorable conditions. Their performance is affected by a variety of factors; the pH value, oxygen concentration and other factors. The most favorable pH value, where the enzyme is most active, is known as the optimum pH, so we investigated the effect of the pH value of the solution. The effect of pH on the tyrosinase/ZnO/NCD electrode in 0.1 M PBS containing 50 μM *p*-cresol is shown in Fig. 6a. The reduction current increases as the pH changes from 4.5 to 6.5, and then decreases gradually from 6.5 to 9.5. The pH value of 6.5, at which the current reaches the maximum, is in accordance with the activity values over the pH range ($4.5 < \text{pH} < 9.5$) for the free tyrosinase.⁸ To obtain the maximum response, a pH of 6.5 for the PBS was selected for the following experiments. The oxygen effects on the reaction were also investigated. The results showed that the current signals have no obvious changes under the air-saturated and oxygen-saturated solutions. Therefore, the following experiments were done in the air-saturated solution.

Furthermore, the effect of potential applied to the tyrosinase/ZnO/NCD electrode on the amperometric signal and background current is shown in Fig. 6b. It can be seen that the current increased rapidly with decreasing the potential from 0 to -0.2 V. When the applied potential was less than -0.2 V (vs. SCE), a higher signal current was achieved, but background current increased more rapidly because of the reduction of dissolved oxygen. Therefore, the potential of -0.2 V (vs. SCE) was used as the optimum potential for the amperometric measurements.

Amperometric response of the tyrosinase/ZnO/NCD electrode

The performance of the biosensor was evaluated by testing phenolic compounds with increased concentrations. Figure 7 shows a typical amperometric response for the tyrosinase/ZnO/NCD electrode at -0.2 V (vs. SCE) after the addition of successive aliquots of *p*-cresol to the 0.1 M PBS (pH 6.5) under constant stirring. A well-defined reduction current proportional to the concentration of *p*-cresol was observed. The tyrosinase/ZnO/NCD electrode achieves about 95% of

Fig. 6 The response dependences of the tyrosinase/ZnO/NCD electrode: (a) the effect of pH value in 0.1 M PBS containing 50 μM *p*-cresol. Applied potential: -0.2 V (vs. SCE). (b) Current-potential curves vs. SCE in air-saturated 0.1 M PBS (pH 6.5) without (1) and with (2) 50 μM of *p*-cresol under stirring.

steady-state current in less than 3 s, which is similar to the results reported by Zhou *et al.*⁷ The response rate is much faster than that of other tyrosinase electrodes.^{31,32} Such fast response is attributed to a rapid electron transfer between the enzymatically-produced quinones and the prepared electrode. The inset in Fig. 7 showed the typical calibration curve of the tyrosinase/ZnO/NCD electrode to *p*-cresol. The characteristics of the tyrosinase/ZnO/NCD electrode to *p*-cresol, phenol and 4-chlorophenol, including sensitivity, linear range and detection limit, are listed in Table 1. As were shown in Table 1, the sensitivity of the tyrosinase/ZnO/NCD electrode is higher than those of other tyrosinase biosensors;^{7,33-35} the differences can be attributed to the biocompatible microenvironment for the enzyme provided by the ZnO/NCD electrode and the high loading of enzyme in the present method. As tyrosinase has different affinity towards its substrates, each phenolic compound has different sensitivity. Sensitivities follow the order: *p*-cresol > 4-chlorophenol > phenol, which is consistent with the previous reports.⁷ The detection limits were also calculated according to the formula 3 *S/N* criteria. The detection limits were 0.2, 0.5 and 0.4 μM for *p*-cresol, phenol and 4-chlorophenol, respectively, under present experimental condition; such values are slightly higher than other reports.⁷ Furthermore, the developed enzyme electrode shows good reproducibility. The relative standard deviation (RSD) estimated by 15 successive measurements of 50 μM *p*-cresol solution at -0.2 V (vs. SCE) was about 2.1%. For different batches of enzyme electrodes, prepared under the same condition, the acceptable reproducibility was obtained with a coefficient of 4.6% for the current response to the same sample at -0.2 V (vs. SCE). Furthermore, compared with the photochemical method and the diazonium method as

Fig. 7 Typical amperometric response of the tyrosinase/ZnO/NCD electrode to *p*-cresol in 0.1 M PBS solution (pH 6.5) (operational potential is -0.2 V vs. SCE).

Table 1 Response characteristics of the tyrosinase/ZnO/NCD electrode to phenolic compounds

Analyte	Sensitivity/ $\mu\text{A mmol}^{-1} \text{cm}^{-2}$	Linear range/ μM	Limit of detection/ μM	<i>R</i>
<i>p</i> -Cresol	179.9	1 – 210	0.2	0.994
Phenol	90.2	1 – 190	0.5	0.995
4-Chlorophenol	121.3	1 – 250	0.4	0.997

R represents as the correlation coefficient of the linear range.

described in Refs. 2–7, the proposed method has another distinct advantage, *i.e.*, the NCD electrode can be reused repeatedly.

Stability of the tyrosinase/ZnO/NCD electrode

The stability of the tyrosinase/ZnO/NCD electrode was also studied. The biosensors were stored dry at 277 K and their response to 50 μM *p*-cresol was measured in 0.1 M PBS (pH 6.5) at -0.2 V (vs. SCE) every other day. The best sensitivity of biosensor was found after a day or two of storage, and the value during the first week was quite stable. After that, the developed enzyme electrode retained about 85% of its initial activity after 4 weeks and the response decreased to about 70% of the initial value after 6 weeks. The decrease in sensitivity may be due to the leakage of enzyme during the electrochemical measurement; at the same time, the activity of the enzyme may also be decreasing with time.

Conclusions

We have fabricated ZnO nanorod clusters on the NCD electrode surface by the homogeneous precipitation method, and developed the tyrosinase biosensor based on the immobilization of tyrosinase onto the ZnO nanorod clusters-modified NCD electrode. The resulted enzyme electrode exhibited fast response, high sensitivity and good stability for the amperometric detection of phenolic compounds because of the high loading of tyrosinase and the rapid electron transfer between the enzymatically-produced quinones and the prepared electrode. As the ZnO nanorod clusters were a favorable matrix for immobilizing the low IEP enzymes, they could be extended to other enzymes and other bioactive molecules. Possible

applications of this kind of biosensor include environmental and industrial monitoring.

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