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A Novel Biomolecular Immobilization Matrix Based on Nanoporous ZnO/Chitosan Composite Film for Amperometric Hydrogen Peroxide Biosensor

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Abstract: A novel ZnO/Chitosan composite matrix was developed to fabricate the H_2O_2 biosensor. This material combined the advantages of inorganic species, ZnO, and organic polymer, chitosan. Horseradish peroxidase immobilized in the material maintained its activity well as the usage of glutaraldehyde was avoided. The activity of enzyme was 7.9 times greater than the cross-linked enzyme. The parameters affecting the fabrication and experimental conditions of biosensors were optimized. With the aid of hydroquinone mediator, the biosensor had a fast response of less than 10 s. The linear range was 5.0×10^{-6} to 2.0×10^{-3} mol/L with a sensitivity of 43.8 μA L/mmol. This matrix can also be used to immobilize other biomolecule.

Keywords: Nano ZnO, chitosan, composite film, biosensors, horseradish peroxidase.

The performance of an enzyme electrode often depends on the method of enzyme immobilization when fabricating biosensors[1]. Therefore, the technique used to immobilize the enzyme is one of the key factors in developing a reliable biosensor. Organic-inorganic composite (or hybrid) materials have emerged in recent years. It combines the physicochemical attributes of components and improves their features. Organic components benefit the formation of defect-free inorganic membranes and make it less brittle. Organic membranes can have their chemical and thermal stability improved by an inorganic phase[2-4]. Because chitosan has excellent film-forming and adhesion ability, together with nontoxicity and biocompatibility, it has gained growing interest in using it to immobilize biomolecules in recent years[5].

In this paper, we presented for the first time a novel enzyme biosensor based on nanoporous ZnO/chitosan inorganic–organic composite film as immobilization matrix, with good stability. This material combined the advantage of inorganic species, ZnO, and organic polymer, chitosan, which is used as a dispersant of ZnO. The immobilization of enzyme is based on the absorption of nanoporous ZnO; therefore, the

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usage of glutaraldehyde can be avoided. Well-studied horseradish peroxidase (HRP) was chosen as a model enzyme. The results obtained from scanning electrode microscopy indicated that ZnO/chitosan film is porous and highly homogeneous. HRP can be effectively entrapped in the film with well-retained bioactivity comparing to that of cross-linking HRP by glutaraldehyde.

Experimental

Chitosan (CHIT, MW~1×10^6 ,75-85% deacetylation) was supplied by Sigma (St. Louis, Mo, USA). Nanoporous ZnO was produced by Nano Material Application Engineering Technology Center (Zhejiang, China). All other chemicals used were of analytical-reagent grade and used as received without further purification. All solutions were prepared with doubly distilled water.

Cyclic voltammetric experiments were performed using a VMP2 Multichannel potentiostat (Advanced Measurement Technology Inc., USA). The three-electrode system consisted of enzyme electrode as the working electrode, a SCE as the reference electrode and a Pt foil as the counter electrode.

Appropriate amount ZnO nanoporous were dispersed in 0.5 % of chitosan (0.05 mol/L acetic acid), the mass ratio of ZnO: chitosan was 1:100. The mixture was sonicated for 15 min after stirring 1 h. Finally, a highly dispersed colloidal solution was formed. A solution of HRP was prepared in 0.067 mol/L phosphate buffer at pH 6.9. A casting solution was obtained by mixing 20 μL of ZnO/chitosan composite solution and 20 μL of enzyme solution. This resulting casting solution of 10 μL was pippted onto the surface of a glass carbon (GC) electrode (0.3 mm diameter), which was polished before each experiment with 0.05 μm α-alumina powder, and rinsed thoroughly with absolute alcohol and distilled water in ultrasonic bath and dried in air. The casting solution was allowed to dry at 4 °C overnight. Finally, the enzyme electrode was rinsed with 0.067mol/L phosphate buffer (pH 6.9) to wash out the unimmobilized enzyme. When not in use, the electrode was stored dry at 4°C in a refrigerator.

Results and Discussion

To investigate the microstructure of ZnO/CHIT matrix, the morphology of ZnO/CHIT film was examined by SEM and shown in **Figure 1**. The result shows a uniform porous structure. This provided a significantly enhanced effective electrode surface for high enzyme loading.

Figure 2 displays the cyclic voltammograms of GC/ZnO/CHIT electrode (a) and bare glass carbon electrode (b) in PB (pH 6.9) containing 1.0 mmol/L hydroquinone and 5 mmol/L H_2O_2. After ZnO/CHIT film was plated, the current signal decreased compared with bare glass carbon electrode due to the barrier effect of ZnO/CHIT film. **Figure 2** (c, d) were obtained with the GC/ZnO/CHIT/HRP electrode in unstirred 0.067 mol/L PB solution (pH 6.9) containing 1.0 mmol/L hydroquinone without H_2O_2 (c) and with 5 mmol/L H_2O_2 added (d). In the presence of 5 mmol/L H_2O_2 (**Figure 2** (d)), the cathode peak current increased and anodic peak current decreased compared with (a, b, c). This confirmed that the current response recorded in **Figure 2** (d) was catalyzed by HRP. Obviously this current is also related to the activity of HRP enzyme.

Figure 1 SEM of ZnO/CHIT film

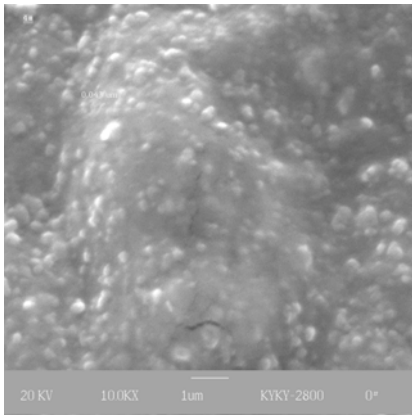


Figure 2 The electrode cyclic voltammograms

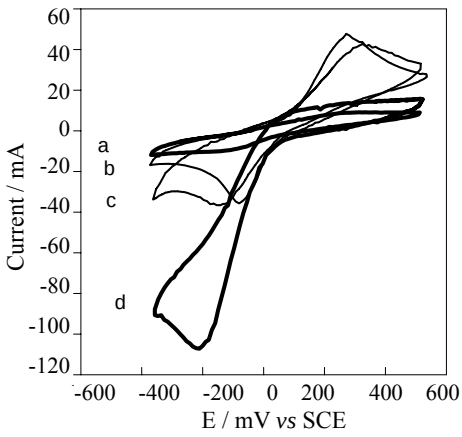


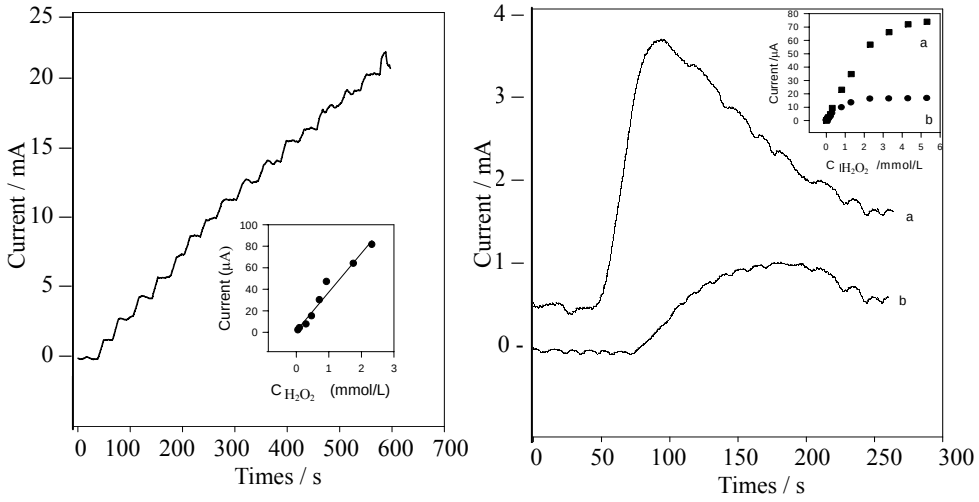
Figure 3 displays typical chronoamperometric results of the biosensor for successive additions of the same amounts of H_2O_2 under optimized experimental conditions. The current reaches a saturation at high H_2O_2 concentration which suggests that the active sites of HRP units are saturated at those H_2O_2 level. The response time is less than 10s. The calibration curve is shown in **Figure 3** (inset). Over a concentration range 5.0×10^{-6} to 2.0×10^{-3}mol/L, the electrode provided a linear response to H_2O_2 with a sensitivity of 43.8 μA L/mmol. The regression equation is $I = 43.8\times C + 2.78$ with a detection limit of 2.5×10^{-6} mol/L, and the correlation coefficient is 0.99.

The sensor retained 81% of its initial current response after 40 days. Thus, the ZnO/CHIT film immobilization was quite efficient in retaining the enzyme activity. This could be due to the good biocompatibility and the negligible swelling of the ZnO/CHIT film in aqueous solutions. Moreover, the nanoporous structure of ZnO greatly enhances the active surface available for enzyme binding over the geometrical area [6]. On one hand, ZnO has general affinity for the binding of proteins because the amine and carboxyl groups on the surface of enzyme can act as ligands to ZnO. On the other hand, a large amount of hydrogen bonds in the composite film is favorable to maintaining the configuration of enzyme molecule [7].

To investigate the activity of enzyme immobilized on the electrode surface without and with glutaraldehyde, the static state response and steady state current of GC/ZnO/ CHIT/HRP electrode (a) were compared with those obtained from GC/CHIT/GLU/HRP electrode (b). The static state response of both electrodes to 0.2mmol/L H_2O_2 in unstirred PB were studied and shown in **Figure 4**. The slopes of current-time curve (rising part), namely $\Delta i / \Delta t$ can be represented the activity of enzyme because the same amount of enzyme (10 mg/ml) was used to immobilized onto the surface of the electrodes. The $\Delta i / \Delta t$ values were 0.079 for the former and 0.01 for the later. These results clearly indicated the activity of enzyme immobilized by ZnO/chitosan was 7.9 times greater than the enzyme immobilized through cross-linking by glutaraldehyde. **Figure 4** (inset) displays the calibration curve of both GC/ZnO/CHIT/HRP(a) and GC/CHIT/GLU/HRP (b) electrode in stirring solution. As is apparent from it, the

sensitivity and response current of the former was 1.4 times that those of latter. In addition, the sensitivity of obtained GC/ZnO/CHIT/HRP electrode (43.8 μA L/mmol) was also 2.3 times great than the value 18.7 μA L/mmol reported in the previous work [5] in which HRP was immobilized by chitosan film crosslinked with glutaraldehyde.

Figure 3 Dynamic response of the electrode **Figure 4** The comparison of activity of enzyme



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