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Electrocatalytic activity of horseradish peroxidase/chitosan/carbon microsphere microbiocomposites to hydrogen peroxide

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ABSTRACT

Colloidal carbon microspheres (CMS) are dispersed in chitosan (CHIT) solution to form an organic–inorganic hybrid with excellent micro-environment for the immobilization of biomolecules. A novel amperometric biosensor for the determination of hydrogen peroxide (H_2O_2) has been constructed by entrapping horseradish peroxidase (HRP) in as-synthesized CMS/CHIT hybrid. The modification of glassy carbon electrode is made by a simple solution-evaporation method. The electrochemical properties of the biosensor are characterized in electrochemical methods. The proposed biosensor shows high sensitive determination and fast response to H_2O_2 at −0.15 V. The constructed HRP/CHIT/CMS/GC electrode also exhibits a fine linear correlation with H_2O_2 concentration. The calculated value of the apparent Michaelis–Menten constant, 2.33 mM, suggests that the HRP in CMS/CHIT hybrid keeps its native bioactivity and has high affinity for H_2O_2.

1. Introduction

In recent years, amperometric biosensors based on horseradish peroxidase (HRP) have been considered as the most effective measure for the determination of hydrogen peroxide (H_2O_2), which is of practical importance in many fields, including chemistry, biology [1,2], industry, clinical control and environmental protection, etc. [3–5]. Conventional H_2O_2 detection methods, such as titrimetry [6], chemiluminescence [7–10], fluorimetry [11–13], and spectrometry [14], are generally time-consuming and cumbrous for operation. Now electrochemical sensors offer an attractive route because of their simplicity, high sensitivity, and selectivity.

Carbon-based materials, such as glassy carbon, graphite, and diamond, have been extensively used in electrochemistry due to their low background response, and good conductivity [15–17]. As one of the most interesting carbon nanostructures, carbon nanotubes (CNTs) have been extensively used in electrocatalysis and biosensing [18–22]. The insolubility of CNTs in most solvents, however, limits their application in designing CNTs-based biosensing devices. Oxidation with acids or ozone is used to be a conventional modification of carbon surface. The oxygenated functionalities such as carboxylic acid, esters are generated on the carbon surface. The subsequent reaction of thionyl chloride with the carboxylic groups makes it possible to graft and further elaborate the surface properties. However, the modification technique has a deadly disadvantage in the low degree of functionalization and the corrosion of the carbon surface during the oxidative treatment [23].

New kinds of biocompatible materials have attracted much interest to improve the behavior of biosensors. The carbon microspheres (CMS, colloidal carbon spheres [24]) are synthesized through a facile hydrothermal synthetic process. In contrast to other materials, two advantages become apparent: (1) The good biocompatibility and relatively active surface, which contains abundance of functional groups such as —C=O, —OH, is easily obtained by only one-step process without further modification. Partially dehydrated functional groups on the carbon frameworks improve the hydrophilicity and reactivity of the microspheres in aqueous systems. These functional groups, which provide a crucial chemical environment, make the microspheres that are easily solubilized in aqueous solution of a biopolymer, chitosan (CHIT), because of van der Waals force. At the same time, the effective attachment of the enzyme is occurred because of covalently binding through an amide bond between the CHIT and enzyme. (2) The preparation of the carbon microsphere is an absolutely environment friendly and low-cost approach. The hydrothermal reaction procedure involves none

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of the organic solvents or surfactants which are commonly used for preparation of polymer micro- or nanospheres. These advantages ensure that the microsphere is nontoxic, which enlarges their application in biosensor.

Herein we successfully utilize as-synthesized CMS as a new kind of biomaterial to construct a biosensor for the first time. CMS act as efficient conductor for electrons transfer, CHIT is an electrochemical promoting polymeric binder as backbone, and HRP is a biological catalyst that facilitates the transfer of substrate into product by lowering the activation energy. In contrast to previous sensors, the CMS-based H_2O_2 sensor has shown the advantages of low applied potential, high affinity, low background current and excellent sensitivity.

2. Experimental

2.1. Reagents

HRP (EC.1.11.1.7, 250 U/mg, from Horseradish) and CHIT were purchased from Sigma and used without further purification. H_2O_2 (30% v/v aqueous solution), glucose was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). All other reagents were of analytical grade. All solutions were prepared with double distilled water, which was purified with a Milli-Q purification system. Phosphate buffer solution (PBS, 1/15 M, pH 6.98) was used as supporting electrolyte in all measurement.

2.2. Apparatus

Cyclic voltammetry was performed in 1/15 M PBS (pH 6.98) with CHI760B electrochemical workstation (Shanghai, China). The modified glassy carbon (GC) electrode was used as the working electrode, a Pt foil as the counter electrode and a saturated calomel electrode (SCE) as the reference electrode in a three-electrode cell (10 mL). Fourier transform infrared spectra (FT-IR) measured by KBr pellets was obtained on a WQF-410 Fourier transform infrared spectrophotometer (Beijing Secondary Optical Instruments, China). Transmission electron microscope (TEM) image was taken with a FEI Tecnai G^2 20 TEM (FEI, America).

2.3. Preparation of CMS

The carbon microspheres (CMS) were prepared through the polycondensation reaction of glucose under hydrothermal condition according to previously literature [25]. Briefly, 8 g of glucose was dissolved in 40 mL of deionized water. The mixture solution was then put into a Teflon-lined stainless steel autoclave with a capacity of 40 mL and maintained at 180 °C for over 8 h. The black product was precipitated with ethanol and distilled water followed by centrifugation. Finally the uniform CMS were obtained.

2.4. Preparation of HRP/CHIT/CMS/GC biosensor

Prior to modification, the GC electrodes (3 mm diameter) were polished with emery paper and 0.05 μm alumina powder, cleaned in nitric acid (dilute with distilled water to 1:1% v/v), ethanol and distilled water for 5 min, successively. Then thoroughly rinsed with distilled water and dried at room temperature. 125 mg of CHIT flakes were dissolved into 50 mL of 0.05 M acetic acid and the mixture was vigorously stirred for 2 h at room temperature. A viscous and translucent CHIT (0.25 wt %) solution was formed. 5 mg of CMS were ultrasonically dispersed into 20 mL of CHIT solution by ultrosonication for 2 h. Then equal volume of CMS/CHIT composite solution and the enzyme solution (5 μL of CMS/CHIT and 5 μL of 5 mg/mL HRP) were mixed adequately. The electrode

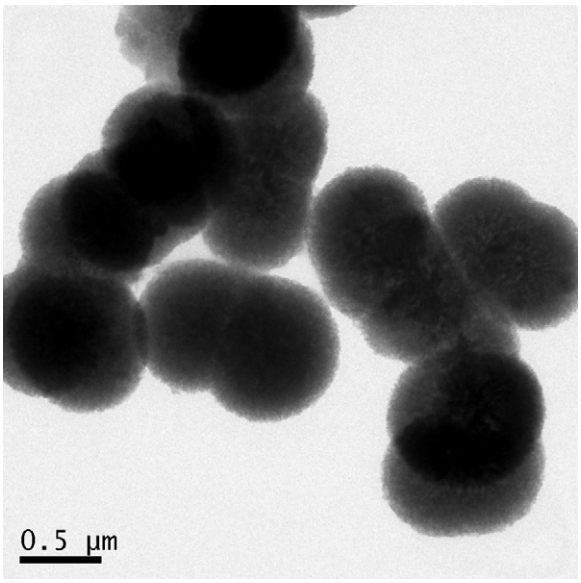

Fig. 1. TEM image of CMS of 500 nm prepared a 180 °C, 8 h.

modified by horseradish peroxidase/chitosan/carbon microspheres (HRP/CHIT/CMS) was prepared by casting 8 μL of the mixture solution onto the surface of a GC electrode. When the solution was dried at 4 °C overnight, a CHIT thin film contained CMS and HRP was prepared. As a comparison, the electrodes modified by HRP/CHIT and CMS/CHIT were also prepared by the similar manner.

3. Results and discussion

3.1. Characterization of the carbon microspheres (CMS)

Fig. 1 shows the typical TEM image of the sample prepared with reaction time of 8 h under the hydrothermal condition at 180 °C. The diameter of the particles is around 500 nm, with some large ones reaching about 800 nm. It can be seen that it is very coarse on the surface which contained some distributed uniformly nanosized pores of the obtained CMS [25]. The coarse surface can not only increase the surface area, but also assist adsorption of reactive species. The diameters of the CMS were affected by temperature, concentration of the glucose and reaction time.

Fig. 2 shows the FT-IR spectra of the carbon spheres. The sample displays feature peaks for hydroxyl at 3383 cm^{-1} (O—H stretching),

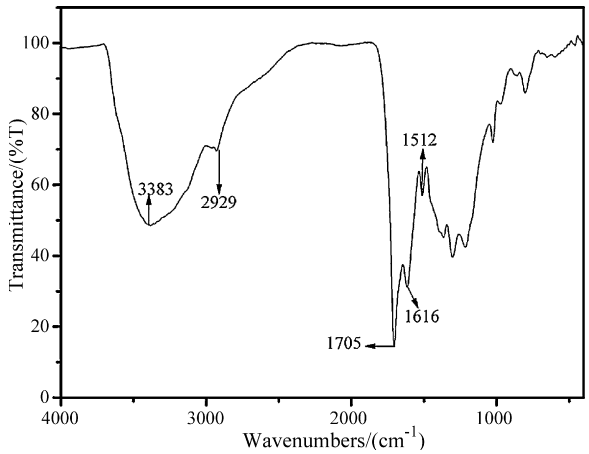

Fig. 2. FTIR spectra of carbon spheres.

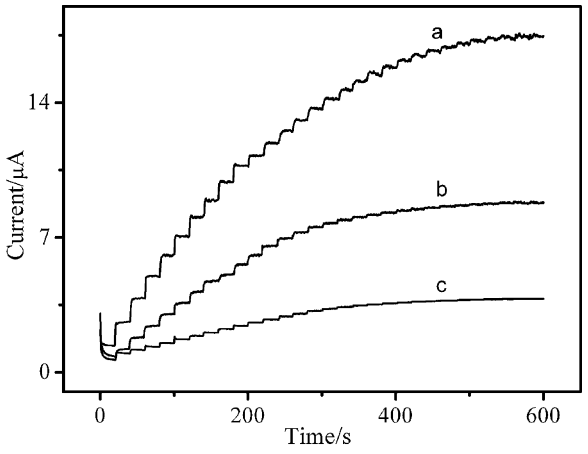

Fig. 3. Current–time responses for successive addition of 1 mM hydrogen peroxide at the (a) HRP/CHIT/CMS/GC, (b) CHIT/CMS/GC, (c) HRP/CHIT/GC electrodes in 1/15 M PBS (pH 6.98) containing 0.1 M hydroquinone. Operating potential, −0.15 V.

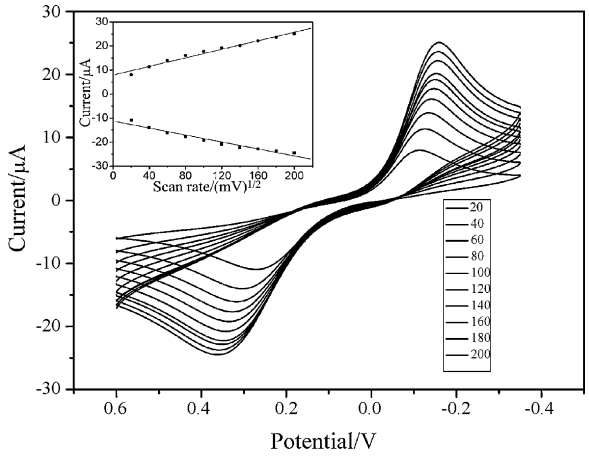

Fig. 4. Voltammetric responses of the GC electrode modified by HRP/CHIT/CMS films to 1 mM hydroquinone at different scan rates (inner to outer): 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 mV s^{-1}. The inset shows the anodic and cathodic peak currents plotted against the square root of the scan rate.

carbonyl at 1705 cm^{-1} (C═O stretching), and stretching peaks at 1616 and 1512 cm^{-1} (aromatic C═C). The bands in the range from 1000 to 1300 cm^{-1}, which include the C—OH stretching and —OH bending vibrations, imply the existence of large numbers of residual hydroxy groups. After dehydration, partial residues in which reductive —OH or —CHO groups are covalently bonded to the carbon frameworks improve the hydrophilicity and stability of the microspheres in aqueous systems. These residue groups improve the hydrophilicity and stability of the microspheres in aqueous systems.

3.2. Electrochemical behavior of the HRP/CHIT/CMS/GC/GC electrode

To distinguish the role of individual components and possible synergy between them, the electrochemical properties of HRP/CHIT/CMS/GC, CHIT/CMS/GC and HRP/CHIT/GC were monitored. The advantages of the HRP/CHIT/CMS/GC electrode are illustrated in connection with the detection of H_2O_2. Fig. 3 compares the response to increasing levels of H_2O_2 in 0.1 mM steps at the HRP/CHIT/CMS/GC electrode (Fig. 3a), CHIT/HRP/GC electrode (Fig. 3b) and CMS/CHIT/GC electrode (Fig. 3c) at potential of −0.15 mV. As expected from the curve, the CHIT/CMS/GC electrode gives a slight response signal to the addition of H_2O_2. Higher increase of the response signal is observed for the HRP/CHIT/GC electrode. In contrast, the HRP/CHIT/CMS/GC electrode responds rapidly to the changes of the concentration, reaching a steady-state signal within 5 s. The difference between these electrodes mainly attributes to the excellent properties of HRP immobilized on the CMS/CHIT hybrid. The micro-environment of the hybrid is advantageous to the incremental adsorption of HRP. The functional groups and the coarse surface of the carbon frameworks aid the large amount of distributing of CHIT in the hybrid through van der Waals between CMS and CHIT. HRP could be covalently bound to the functional surface mainly due to amide bonds between CHIT and HRP.

For the fabricated biosensor, the cyclic voltammograms (CVs) corresponding to various scan rates were also investigated. Both the anodic and cathodic peak currents clearly increase with increasing potential scan rate. As shown in Fig. 4, well-characterized redox peaks could be observed through the scan rate in a range from 20 to 200 mV s^{-1}. Moreover, it is found from Fig. 4 (inset) that the peak currents are proportional to the square roots of the scan rates, which suggest a typical the semi-infinite linear diffusion-controlled electrochemical behavior feature of the redox process. The catalytic process of HRP immobilized onto CHIT/CMS electrode surface can be expressed as follows [26]:

$$\text{HRP}(Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

$$\text{Compound I} + H_2Q \rightarrow \text{compound II} + BQ \quad (2)$$

$$\text{Compound II} + H_2Q \rightarrow \text{HRP}(Fe^{3+}) + BQ + H_2O \quad (3)$$

$$\text{The net reaction is : } BQ + 2e^- + 2H^+ \rightarrow H_2Q$$

where compound I (oxidation state + 5) and II (oxidation state + 4) represent the enzyme intermediates in the reaction H_2Q and BQ represent hydroquinone and benzoquinone, respectively. In the first two electrons transfer step, H_2O_2 is reduced to water and the HRP is oxidized to compound I. Compound I is then reduced in a one electron step to form compound II, and then compound II is reduced to the original form of HRP by the redox mediator (H_2Q). The BQ is subsequently reduced to H_2Q by a rapid reaction involving the consumption of two electrons from the electrode. The resulting cathodic currents are therefore correlated with the concentration of H_2O_2.

In order to observe the activity of HRP on the CMS/CHIT modified glassy carbon electrode, its response to the reduction of H_2O_2 was studied. Fig. 5 is the CVs of the HRP/CHIT/CMS/GC electrode in 1/15 M PBS (pH 6.98) in the absence (curve a) and in presence of 4 mM H_2O_2 (curve b) between −0.35 and 0.6 V at the scan rate of 100 mV s^{-1}. In blank PBS, the biosensor only exhibited the electrochemical behavior of hydroquinone, a pair of anodic and cathodic waves (curve a). With the addition of H_2O_2, an obvious increase of the cathodic and anodic current is observed. The reduction peak current increases greatly (from 18.67 to 73.66 μA) with slightly negative shift of peak potential. Meanwhile the oxidation peak current decreased markedly, indicating an obvious electrocatalytic process to the enzymatic reduction of H_2O_2 by the immobilized HRP. The inset shows the process of addition of successive aliquots of 1 mM H_2O_2 in PBS. The peak current enhances with the increase of H_2O_2 concentration. The excellent electrocatalytic activity of the electrode toward H_2O_2, which is a common product of a great many of oxidase-based enzyme reactions, means that the electrode can provide a signal transduction in the fabrication of biosensors.

The dependence of the biosensor response on the applied potential for determination of 0.1 mM H_2O_2 is shown in Fig. 6. The foot of the electrocatalytic reduction of H_2O_2 was observed around 0 V

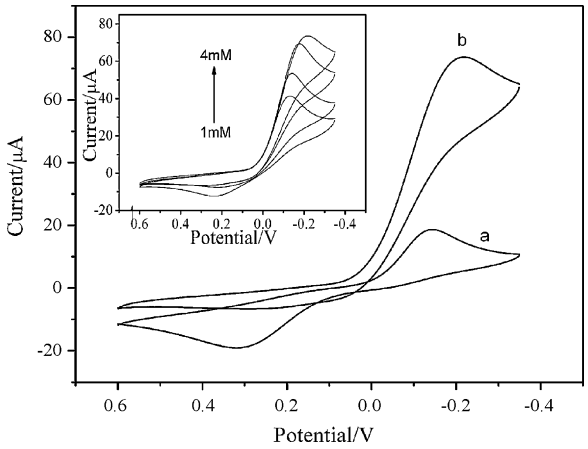

Fig. 5. CVs of the HRP/CHIT/CMS/GC electrode in 1/15 M PBS (pH 6.98) in the absence (a) and presence of 4 mM H_2O_2 (b). Inset: CVs of addition of successive aliquots of 1 mM H_2O_2 in PBS (pH 6.98).

and the maximum amperometric response value was at −0.15 V. As is well known, a lower potential can avoid or decrease the interference caused by some electroactive species and make the background current and noise levels reach their lowest value [27]. −0.15 V was chosen as the applied potential for the amperometric measurement. Fig. 7 displays a typical amperometric response of the CMS biosensor after addition of successive aliquots of 0.1 mM H_2O_2 in PBS (1/15 M, pH 6.98) under stirring. It can be seen that HRP/CHIT/CMS/GC electrode has a fast and sensitive response to H_2O_2 over a wide range of concentrations. The plot of current vs. H_2O_2 concentration is shown in inset A of Fig. 7. The equation of the fitting curve was:

$$I = 8.14C_{H_2O_2}(\text{mM}) + 0.9146(\mu\text{A}) \quad (R = 0.997, n = 13)$$

The calibration curve was linear with H_2O_2 concentration from 0.1 to 1.6 mM, the sensitivity was 120.17 $\mu A\,cm^{-2}\,mM^{-1}$ and the detection limit was calculated as 0.93 μM. It can be seen from the plot that the biosensor showed a rapid and sensitive response to the change of H_2O_2 concentration, indicating a good electrocatalytic property of the modified electrode. The sensor retained about 90% of its initial current response after intermittent use after 25 days. The response time for the electrode is less than 5 s. Fast responses may be due to the easy diffusion of H_2O_2 in the ultrathin CMS/CHIT hybrid film. The apparent Michaelis–Menten constant, K_M^{app}, which

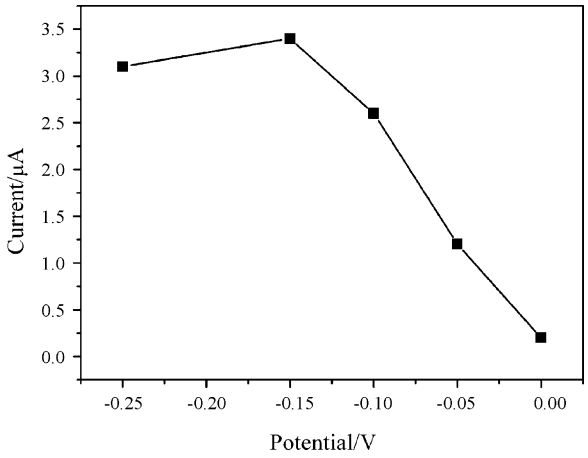

Fig. 6. Influence of applied potential on the amperometric response of the sensor to 0.1 mM H_2O_2.

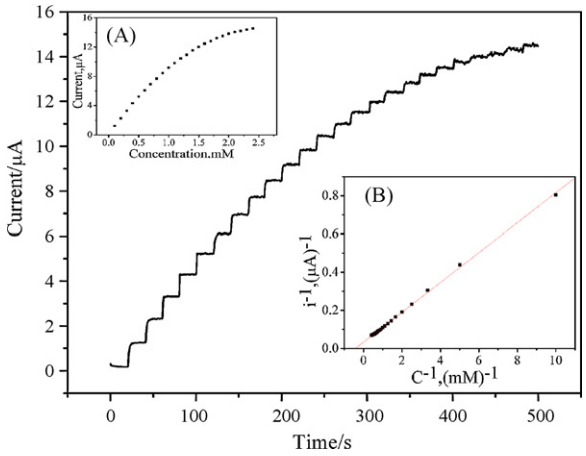

Fig. 7. Amperometric response of successive addition of 0.1 mM H_2O_2 at HRP/CHIT/CMS/GC electrode in PBS (pH 6.98, 1/15 M) containing 1 mM hydroquinone, −0.15 V constant potential, insets: (A) plot of chronoamperometric current vs. H_2O_2 concentration; (B) linear calibration curve for determination of K_M^{app}.

gives an indication of the enzyme-substrate kinetics and a reflection of the enzymatic affinity, could be calculated from the electrochemical version of the Lineweaver–Burk equation [28]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}C}$$

where I_{ss} is the steady-state current after the addition of substrate, C is the bulk concentration of the substrate, and I_{max} is the maximum current measured under saturated substrate condition. The K_M^{app} is determined by analyzing the slope and intercept for the plot of the reciprocals of the current vs. H_2O_2 concentration. As is well known the smaller K_M^{app} shows the higher catalytic ability. The Michaelis–Menten constant of the system (K_M^{app}) in this work was found to be 2.33 mM, which was similar to that of 2.198 mM at polysaccharide–silica-modified electrode [29] and much smaller than the reported 23.85 mM based on the direct electrochemistry of HRP in sol–gel derived ceramic–CNT nanocomposite film [8]. The result implied that the HRP/CHIT/CMS/GC electrode exhibited a higher affinity for hydrogen peroxide.

4. Conclusions

In summary, a biosensor with excellent properties was successfully demonstrated based on the combination of CMS, CHIT, and HRP. Inorganic–organic hybrid, CMS/CHIT, allowed the necessary efficient electron tunneling and large amount of adsorption of HRP. With the help of CMS, nice electrochemical response of HRP was obtained. The H_2O_2 biosensors exhibited a variety of good electrochemical characteristics including high sensitivity, low applied potential, high affinity.

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