Short communication

Electrochemical study of a new methylene blue/silicon oxide nanocomposition mediator and its application for stable biosensor of hydrogen peroxide

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Abstract

A novel organic–inorganic nanocomposite of methylene blue (MB) and silicon oxide was synthesized and characterized by TEM, FTIR, and UV–vis. The as-prepared material was able to transfer the electron of the MB to electrode and was different from other SiO₂ spheres structurally. It can be used as mediator to construct a biosensor with horseradish peroxidase (HRP) coimmobilized in the gelatine matrix and cross-linked with formaldehyde. The resulting biosensor exhibited fast amperometric response and good stability to hydrogen peroxide (H₂O₂). The linear range for H₂O₂ determination was from 1 × 10⁻⁵ to 1.2 × 10⁻³ M, with a detection limit of 4 × 10⁻⁶ M based on S/N = 3. Moreover, the lifetime is more than 3 months under dry conditions at 4 °C.

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1. Introduction

In the last few years, numerous investigations have been carried out on electrochemical biosensors, due to their possibility to couple the rapidity, selectivity, sensitivity and low cost of chemical analysis (Kurzawa et al., 2002; Kong et al., 2003; Khoo and Chen, 2002). The simplest biosensor format consists of a thin layer of protein (such as enzyme, antibody, DNA), which can be used for direct measurement. However, in addition to the leaching of the protein, various factors can prohibit direct electron transfer between electrodes and proteins, including the deep burial of the electroactive cofactors in the protein structure, denaturation of the protein at the electrode surface, and unfavorable orientation of the protein. Much effort has been focused on facilitating electron transfer between proteins and electrodes, including using electron transfer mediators, where immobilization of the mediator is as important as that of the enzyme (Chaubey and Malhotra, 2002).

A series of organic dyes have been used as electrode surface modifiers, such as methylene blue (Arvand et al., 2003), methylene green (Munteanu et al., 2001), prussian blue (Karyakin et al., 2004), phenazines (Pessoa et al., 1997), and thionin (John and Ramaraj, 2004), all displaying excellent mediating ability in bioelectrocatalytic reduction of hydrogen peroxide. Methylene blue (MB), a cationic dye whose electrochemical properties are well known in the solution phase, has been used as a redox indicator since its formal potential, E⁰, is between 0.08 and −0.25 V (versus SCE) in solution with pH 2–8. This redox potential is close to that of most biomolecular redox potentials. A modified carbon electrode based on using this dye as an electron mediator system may be of great interest (Dias et al., 2002). However, such low molecular weight soluble mediator is disadvantageous as it can leach out of the electrode, which may lead to a significant signal loss and affect the stability of biosensor. In order to overcome these shortcomings, we have developed a new...
kind of organic–inorganic nanocomposite material composed of methylene blue and SiO$_2$ as mediator. Nanoparticles frequently display unusual physical (structural, electronic, magnetic, and optical) and chemical (catalytic) properties. All these properties make nanoparticles suitable for application in different fields of analytical chemistry, such as optoelectronics and chemical- and bio-sensing. Furthermore, because of the large specific surface area and high surface free energy, nanoparticles can also be used in constructing electrochemical biosensors. For example, metal nanoparticles such as gold or silver nanoparticles can be used to promote electron transfer between proteins and electrodes (Su et al., 2001; Cai et al., 2002). Willner and co-workers applied semiconductive nanoparticles to organize the bioelectrochemical devices (Katz et al., 2004). These nanoparticles were often fabricated on the surface of electrode by self-assembling method. However, the above fabrication process of generation of biosensors is relatively complicated. Recently, silica based solid support has been used effectively for the immobilization of various biomolecules such as enzymes, proteins, and DNA (Fang et al., 1999; Yakovleva et al., 2003; Cox et al., 2003). Although the sol–gel technology can provide an efficient means to prepare a three-dimensional network suited for the encapsulation of a variety of biomolecules, the silica can not transfer electron from biomolecules to electrodes. Organic–inorganic nanocomposites have become attractive for many new electronic, optical or magnetic applications in catalysis (Mecking and Thomann, 2000), chromatography (Bottoli et al., 2002), and optics (Amaity et al., 2001). However, to the best of our knowledge, there are few reports about organic–inorganic nanocomposite as mediator on their application of electroanalysis.

In this paper, a novel organic–inorganic nanocomposite combining the efficient electrocatalytic properties of MB with the physical properties of the SiO$_2$ was synthesized and used for immobilization of enzyme to construct a hydrogen peroxide (H$_2$O$_2$) biosensor. A natural polymer, gelatin, was used as a structural material for designing functional layers. Gelatin was selected here because of its excellent membrane-forming ability, good adhesion, biocompatibility, and non-toxicity (Segtnan et al., 2003). The optimized conditions of the enzyme electrode were studied, and the analytical performance of the biosensor was evaluated.

2. Experimental

2.1. Reagents

Horseradish peroxidase (HRP, BE 1841) was obtained from Sigma (USA). Tetraethyl orthosilicate (TEOS) was obtained from Tianjin Reagent Factory (Tianjin, China). Methylene blue (MB, not purified before use) was purchased from Third Chemical Factory of Shanghai (Shanghai, China). H$_2$O$_2$ (30% w/v solution) was purchased from Shanghai Chemical Reagent Company (Shanghai, China). The concentration of the diluted hydrogen peroxide solutions prepared was determined by titration with cerium(IV) to a ferroin endpoint. All other reagents were of analytical grade. All the solutions were prepared with doubly distilled water.

2.2. Apparatus and measurements

All electrochemical experiments were carried out in a three-electrode cell controlled by CHI 660 electrochemical workstation (CH Instruments, USA). A H$_2$O$_2$ biosensor was used as the working electrode. Reference and counter electrodes were a SCE and platinum wire, respectively. Fourier transform infrared (FTIR) spectra by KBr pellets were obtained in the range of 500–4000 cm$^{-1}$ on a Bruker 22 FTIR spectrometer at room temperature. UV–vis absorption spectra were obtained with UV-3100 spectrophotometer (SHIMADZU) at room temperature. The images for transmission electron micrographs (TEM) were obtained with a JEOLEMD JEM 200CX electron microscope.

2.3. Preparation of silica/MB composites

An amount of 0.1 g of MB was dissolved in a mixed solution of 2 ml TEOS and 10 ml ethanol in a 50 ml beaker. Then 5 ml ammonia and distilled water were added till the total volume rose to 50 ml. This solution was irradiated with a high-intensity ultrasonic horn (Sonics, Model VCX750, 1.25 cm Ti-horn, 20kHz, 100W/cm$^2$) under ambient air for 30 min and a blue colloid solution was obtained. After cooled to room temperature, the colloid were centrifuged and washed by ethanol and distilled water in sequence for two times, respectively.

2.4. Construction of the SiO$_2$–MB/gelatine modified HRP electrode

Gelatine solution (1%) was prepared by dissolving gelatine flake in hot doubly distilled water. Glassy carbon working electrodes (GCE, 3 mm diameter) were polished successively with 1.0, 0.3, and 0.05 µm alumina powder on chamois leather, and rinsed thoroughly with doubly distilled water between each polishing step. Next, the polished electrode was sonicated in 1:1 nitric acid, acetone and doubly distilled water and then allowed to dry at room temperature.

An amount of 0.2 mg of HRP and the same amount of SiO$_2$–MB composite were dissolved in 0.5 ml of gelatine solution (1%), and then 10 µl of formaldehyde solution was added into the enzyme solution. The mixture was hand-mixed completely. The GCE was coated with a drop of 10 µl of the resulting mixture and then was left for at least 24 h at 4°C. Thus, HRP and SiO$_2$–MB composite were entrapped in the membrane. The enzyme biosensors were stored at 4°C in a refrigerator when not in use.
3. Results and discussion

3.1. Characteristics of the material

The TEM images (Fig. 1) exhibited the structure of the SiO$_2$–MB composite nanoparticles. The composites were uniform spheres with the average size of ca. 120 nm. Compared with the smooth SiO$_2$ spheres prepared in the same condition in the absence of MB, the composite spheres were much rougher on surface. As shown in the TEM image (Fig. 1b), a single sphere was congregated with many smaller colloid particles. What is more, many dispersed small colloid nanoparticles were also found during the reaction, and the percentage and size of spheres increased with time. It proved that the TEOS would first decompose and combine to small particles and the spheres were congregated by these initial particles gradually. For this growth mechanism, we can also control the size of the spheres from dozens to hundreds of nanometer. Because MB can dissolve in the TEOS/EtOH solution, once the TEOS decomposed and combined to SiO$_2$ nuclei and particles, MB would be adsorbed on their surface or combine with SiO$_2$ continuously. At last, the homogeneous SiO$_2$–MB composites were formed after the congregation. The structure of the composite we prepared was quite different from the general SiO$_2$ sphere with MB adsorbed on the surface, and the simple core-shell structure in which the MB was enwrapped by a SiO$_2$ shell. For the general SiO$_2$ sphere, the quantity of MB was low and the MB molecules were easy to desorbing. For the simple core-shell structural MB/SiO$_2$ the electron transfer capability between the MB and electrode was low due to the poor conductivity of SiO$_2$ shell. The as-prepared product was much more stable and contained the MB molecules in a high concentration. On the other hand, in the case of the composite sphere, the MB molecules homogeneously presented inside the inner and easily transferred electron through the nanoparticles directly. Furthermore, the formed rough surface was also more effective in adsorption of the protein molecule.
of MB–SiO2 composites (Fig. 2A, curve c), the absorption peaks at 664 nm disappeared, with the reaction of the SiO2, whereas the peak at around 614 nm shifted slightly towards lower wavelength (by about 10 nm). This was normally attributed to the dimeric form of the dye (Jockusch and Turro, 1995).

Fig. 2B showed the FTIR spectra of the MB, SiO2, and their composites. The characteristic peaks of SiO2 at ~3430, 1103, ~965, and ~801 cm⁻¹ and of MB at ~1600, ~1398, ~1351, ~1338, and ~882 cm⁻¹ appeared in the MB–SiO2 nanocomposites. However, there were some differences between the IR spectra of MB–SiO2 and the spectra of MB and SiO2. For MB (Fig. 2B, curve a), the adsorption band at 1600 cm⁻¹ corresponding to the vibration of the aromatic ring shifted to 1605 cm⁻¹ in the IR adsorption of the composites. For SiO2 (Fig. 2B, curve b), the adsorption band at 1103 cm⁻¹ attributed to Si–O–Si stretching vibration shifted to 1096 cm⁻¹. Thus, it can be concluded that the MB formed a composite with the SiO2 by a bonding interaction between the Si–O groups of SiO2 and the nitrogen of the aromatic ring of MB (Watanabe et al., 1999).

3.2. Electrochemical studies

3.2.1. Electrochemical characteristics of the coimmobilization of HRP and MB–SiO2 nanocomposite in gelatine membrane modified GC electrode

Typical cyclic voltammograms of MB–SiO2/HRP in 0.1 M phosphate buffer solution (pH 7.0) at different scan rates were shown in Fig. 3. It was clear that the peak potential was independent of the scan rate in the range between 25 and 300 mV s⁻¹. From this result, we confirmed that MB–SiO2 composites have good electrochemical reversibility. Both the anodic peak current and the cathodic peak current were proportional to υ at the above scan rate range, suggesting that peak currents were surface-confined. This suggested that the mediator was immobilized in the surface of the electrode successfully (Xu et al., 2003).

3.2.2. Electrochemical response to hydrogen peroxide

Fig. 4 showed the cyclic voltammetric behavior of the enzyme electrode. In blank phosphate buffer, the enzyme electrode only gave the electrochemical behavior of methylene blue. There was a pair of quasi-reversible anodic and cathodic waves. When 2.1 mM H2O2 was added into the solution, cathodic peak current increased significantly. With an increase of reduction peak current, the oxidation peak current decreased. A small peak-potential shift towards negative direction with the increase of H2O2 concentration was observed. The reaction mechanism of the sensor was summarized as follows (Aoki and Kaneko, 1988):

HRP reduced hydrogen peroxide to water:

\[
\text{H}_2\text{O}_2 + \text{HRP} \rightarrow \text{H}_2\text{O} + \text{HRP-I}
\]

HRP can be regenerated by using a mediator through two separate one-electron steps:

\[
\text{HRP-I} + (\text{MB–SiO}_2)_{\text{red}} \rightarrow \text{HRP-II} + (\text{MB–SiO}_2)_{\text{ox}}
\]

\[
\text{HRP-II} + (\text{MB–SiO}_2)_{\text{red}} \rightarrow \text{HRP} + (\text{MB–SiO}_2)_{\text{ox}}
\]

\[
(\text{MB–SiO}_2)_{\text{ox}} + 2e \rightarrow (\text{MB–SiO}_2)_{\text{red}}
\]

MB can then be recycled at the electrode as the mediator leading to an increase of its reduction current. This indicated that methylene blue incorporated in this matrix could effectively shuttle electrons between the base GCE and the bioactive center of HRP in the membrane.
3.2.3. Optimization of hydrogen peroxide monitoring

In order to determine the optimal working potential for H$_2$O$_2$ sensing, a plot of chronoamperometric current versus working potential was made. It was observed that the steady-state current changed with an increase of applied potential from −0.2 to −0.5 V; the potential reached −0.3 V, the electrocatalytic effect of H$_2$O$_2$ reached a plateau. Hence, the potential of −0.3 V was selected as the optimized monitoring potential.

The effect of pH on the enzyme electrode was investigated in the pH range 5.0–9.0 in the presence of 0.05 mM H$_2$O$_2$ at a working potential of −0.30 V. It was observed that the current response of the electrode was suitable in the pH range 5.0–7.5; however, it dropped quickly in the pH range 7.5–9. Therefore, we chose pH 7.0 phosphate buffer solution throughout this study, which is close to the optimum pH 7.0 observed for soluble peroxidase (Maehly, 1995).

The effect of temperature on the biosensor was examined between 15 and 55 °C. It was observed that an increase of temperature enhanced the sensitivity of the electrode to hydrogen peroxide, which reached a maximum value at 30 °C. However, the further increasing temperature led to a decrease of the enzyme possibly, because of the partial denaturation of the enzyme and the possible dissolution of the gelatine film (Segnian et al., 2003).

3.2.4. Steady-state amperometric response to hydrogen peroxide

Fig. 5 displayed the dynamic response of the electrode under the optimal experimental conditions with successive injections of H$_2$O$_2$ to the phosphate buffer solution under stirring. The trace clearly demonstrated the fast response and high sensitivity of the electrode to H$_2$O$_2$. The time required to reach 95% of the maximum steady-state current was less than 20 s. The response to H$_2$O$_2$ was linear in the range from 0.01 to 2 mM. The detection limit was 4 μM when the signal to noise ratio is 3.

The apparent Michaelis–Menten constant $K_{\text{app}}$ was generally used to evaluate the biological activity of immobilized enzyme and it could be calculated according to the Michaelis–Menten equation:

$$i = i_{\text{max}} - \frac{K_{\text{app}}}{C}$$

where $i$ is the steady-state catalytic current, $i_{\text{max}}$ the maximum current measured under saturated substrate conditions, $C$ referred to the H$_2$O$_2$ concentration and $K_{\text{app}}$ stands for the apparent Michaelis–Menten constant of the system. $K_{\text{app}}$, in this work, was evaluated as 0.9 mM (Kamin and Wilson, 1980), which revealed that the whole system was controlled by the catalytic kinetic process of the enzyme. The stability of the biosensor was investigated by amperometric measurements in the presence of 0.05 mM H$_2$O$_2$ periodically. It was found that the biosensor retained its original response after 1 month of testing. When not in use, it was stored under dry conditions at 4 °C in a refrigerator. The response of the sensor was maintained about 70% of the initial values after storage for more than 3 months. The good long-term stability can be attributed to the great stability of the mediator and the excellent biocompatibility and the stabilizing microenvironment around the enzyme provided by the organically modified gelatine composite matrix.

4. Conclusions

We have developed a novel organic–inorganic nanocomposite as mediator, which is composed of MB and SiO$_2$. The organic–inorganic nanocomposites and HRP have been successfully immobilized in the gelatine cross-linked matrix on the electrode. The biosensor that was studied exhibited fast response, good reproducibility, and long-term stability.

In this paper, several merits of utilizing the organic–inorganic nanoparticles are highlighted. First, the as-prepared MB–SiO$_2$ nanoparticles were congregated with many smaller homogeneous MB–SiO$_2$ composites and quite different from the simple core-shell MB–SiO$_2$ in structure. Therefore, these nanoparticles can act as tiny conduction centers and facilitate effectively the transfer of electrons from the base electrode to the biocatalytic center of HRP in the membrane. Second, the nanocomposite can provide a three-dimensional interface to absorb the enzyme, thus, it can increase the enzyme loading. Third, the nanocomposite is about 120 nm that can be successfully entrapped into gelatine cross-linked matrix. Thus it can avoid the mediator leaching from the surface of electrode under the operation conditions. The resulting biosensor exhibits high sensitivity and good stability implying that this nanocomposite can provide a suitable microenvironment for enzyme. In addition to the use of this nanocomposite as a mediator, other applications are being developed in our laboratory.
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