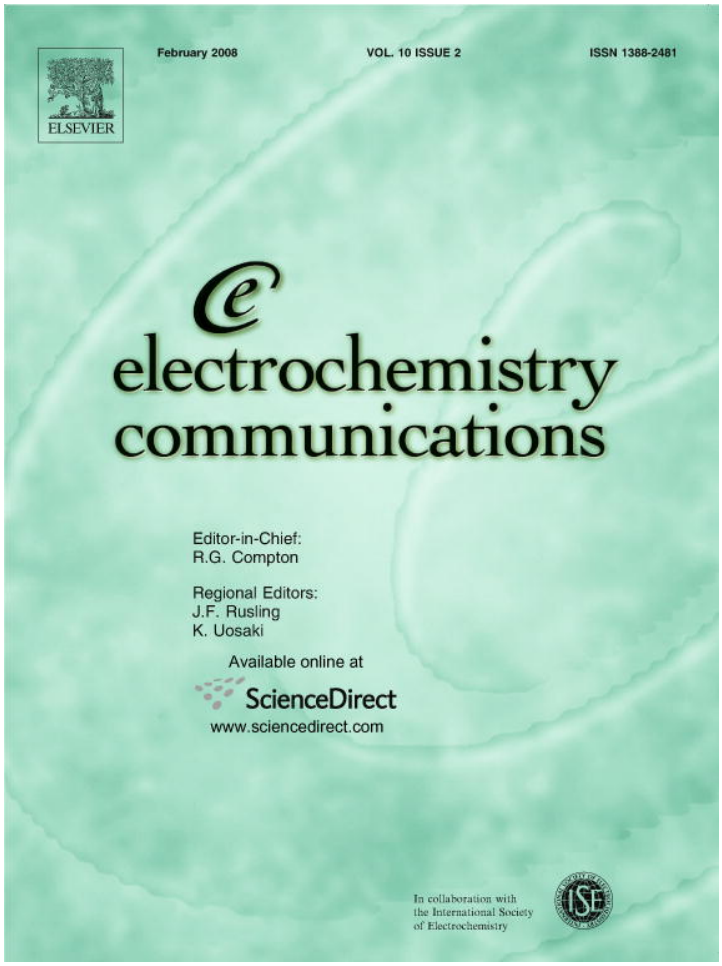

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Direct electron transfer of horseradish peroxidase and its electrocatalysis based on carbon nanotube/thionine/gold composites

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Abstract

Horseradish peroxidase (HRP) was incorporated into multiwalled carbon nanotube/thionine/Au (MTAu) composite film by electrostatic interactions between positively charged HRP and negatively charged MTAu composite. The results of electrochemical impedance spectroscopy (EIS) confirmed adsorption of HRP on the surface of MTAu modified GC electrode. Moreover, the electrochemical results showed that HRP retained its bioactivity and bioelectrocatalytical activity, and also showed good direct electron transfer behavior on such a composite film.

Keywords: Horseradish peroxidase; Direct electrochemistry; Multiwalled carbon nanotubes; Gold nanoparticles

1. Introduction

Direct electron transfer between the electrode and the redox enzyme is very important for fundamental studies and construction of biosensors [1–3]. However, the direct electron transfer between the enzyme and unmodified electrode is usually prohibited due to shielding of the redox active sites by the protein shells [4,5]. Therefore, several studies have been made to enhance the electron transfer. Mediators are widely used to access the redox center of an enzyme and then to act as the charge carriers. Mediators can minimize the effects of interferences, lower the operating potential of the electrodes, and improve the linear response range and sensitivity of the sensor [6]. Use of carbon nanotubes (CNTs) as mediators has attracted increasing attention in recent years. Comparing with traditional carbon electrodes, CNTs show unique properties, such as good conductivity, high chemical stability, and catalytic activities towards many electrochemical reactions [5,7,2,8,9]. More importantly, it is possible to bring the nanotubes close to the redox centers of the proteins [10,11].

As a member of the large class of peroxidases, horseradish peroxidase (HRP) has long been a representative enzyme to explore the structure, dynamic and thermodynamic properties of peroxidases, especially in understanding the biological behavior of catalyzed oxidation of the substrates H_2O_2. But, generally, direct adsorption of HRP onto bare electrode surface may result in denaturation and loss of bioactivity of the enzyme. Nanometer-sized colloidal gold particles have been reported to adsorb redox enzymes and proteins without any loss of their biological activity [12–14]. In addition, colloidal gold nanoparticles are widely used as a model system because of their easy synthesis and surface modification [15], good

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biocompatibility [13], as well as their ability to act as tiny conduction centers which facilitate the electron transfer [16].

In addition to denaturation of the protein, leak of small mediator molecules into the bulk solution is another problem. Therefore, development of a simple and stable immobilization method is of great importance. The isoelectric point of HRP is at pH 8.9, therefore it has positive surface charge at pH 7.0. Thus, electrostatic interactions between positively charged HRP and negatively charged gold nanoparticles will hold HRP well attached to the CNT/Au composite films. In addition, the redox peak potential of thionine (Th) is pH dependent, and the reduction process of Th involves two electrons and one proton at neutral pH [17,18].

Moreover, Th molecules contain aurophilic S and N atoms, both of which bind strongly to Au-nanoparticles [19]. Therefore, Th acts not only as a cross-linker to construct the multiwalled carbon nanotube/thionine/Au (MTAu) composite films [20], but also as functional component to enrich the properties of the composite films [21]. Moreover, Th, as reported early, can speed up electron communication between the enzyme and the electrode [22].

In this work, HRP is incorporated into the MTAu film and the electrochemical properties of the HRP–MTAu composite are characterized as well as the electrochemical effect on reduction of hydrogen peroxide and dissolve oxygen.

2. Experimental procedure

2.1. Reagents and apparatus

Thionin acetate (85%) and $HAuCl_4 \cdot 3H_2O$ (99.999%) were purchased from Aldrich. HRP (EC 1.11.1.7, $A \sim 150$ U/mg) was obtained from Fluka. Trisodium citrate and sodium borohydride ($NaBH_4$, 96%) were obtained from Beijing Chemical Reagents Company. MWNTs prepared by chemical vapor deposition (CVD) were purchased from Shenzhen Nanotech Port Ltd. Co. (China). All solvents were of analytical grade. Ultrapure water from a Milli-Q plus system (Millipore Co., >18 MΩ cm) was used in all aqueous solutions and rinsing procedures.

Zeta potentials (effective surface charge) were measured by dynamic light scattering (Zetasizer 3000, Malvern Instruments, France). Electrochemical impedance spectroscopy (EIS) measurements were carried out with Solartron 1255B Frequency Response Analyzer (Solartron Inc., UK). Cyclic voltammetry (CV) scans were recorded using a CHI660 electrochemical workstation (CHI, USA) with a conventional three-electrode electrochemical cell using a glassy carbon (GC) electrode (3 mm diameter), KCl-saturated silver–silver chloride (Ag|AgCl) and a platinum wire as the working, reference and counter electrodes, respectively. All potentials reported here refer to the Ag|AgCl (sat. KCl) reference electrode.

2.2. Preparation of HRP–MTAu films

The scheme for preparation of HRP–MTAu films is shown in Fig. 1. Preparation of MTAu composite is based on our previous report [20]. The MTAu ethanol suspension was drop casted onto the glassy carbon (GC) electrodes. After ethanol was evaporated, 10 μL of 0.2 mM HRP solution was dropped on the as-prepared GC electrode. This such-prepared GC electrode was then dried for ca. 24 h in air.

3. Results and discussion

3.1. TEM measurement

Fig. 2 shows the TEM image of MWCNT/thionine (MT) (A) and MWCNT/thionine/Au (MTAu) (B). The result of TEM (Fig. 2A) showed that thionine molecules were successfully attached onto the sidewalls of the individual MWCNTs. This attachment may result from a π–π stacking force between two kinds of conjugated frames [23]. TEM image (Fig. 2B) confirmed that gold nanoparticles were typically bound on MWCNT walls and ends with fairly even distribution, although a few aggregates were observed.

3.2. EIS characterization

EIS measurements give information on the impedance changes of the electrode surface. The high-frequency region contains information of kinetics of the faradaic process, while the low-frequency region gives information concerning the diffusion of species to electrode surface. The semicircle diameter of well conducting substrates equals the electron transfer resistance, R_{ct}. If the substrate is covered by a film with some ohmic resistance, R_f, the diameter of the semicircle will also be dependent on that resistance. In our system, however, the HRP–MTAu film has good conductivity and therefore, the contribution of R_f to the diameter of the semicircle may be negligible. Therefore, the larger semicircle in the high-frequency region of HRP–MTAu (Fig. 3) represents slower electron-transfer kinetics and more blocking behavior for the redox couple [24]. These results show that presence of HRP obstructed the electron transfer of the electrochemical probe to the electrode substrate. Therefore, we can conclude that HRP has successfully been adsorbed on the surface of MTAu modified GC electrode by electrostatic interactions.

3.3. Cyclic voltammetry

Fig. 4 shows the cyclic voltammograms of GC electrodes modified with HRP–MTAu (solid line), MTAu (dotted), and HRP (dashed) film in 0.1 M PBS buffer (pH 7.0) saturated with N_2. A pair of stable and well-defined redox peaks of HRP for the HRP(Fe(III))/HRP(Fe(II)) redox couple transformation [25] can be seen. This redox couple

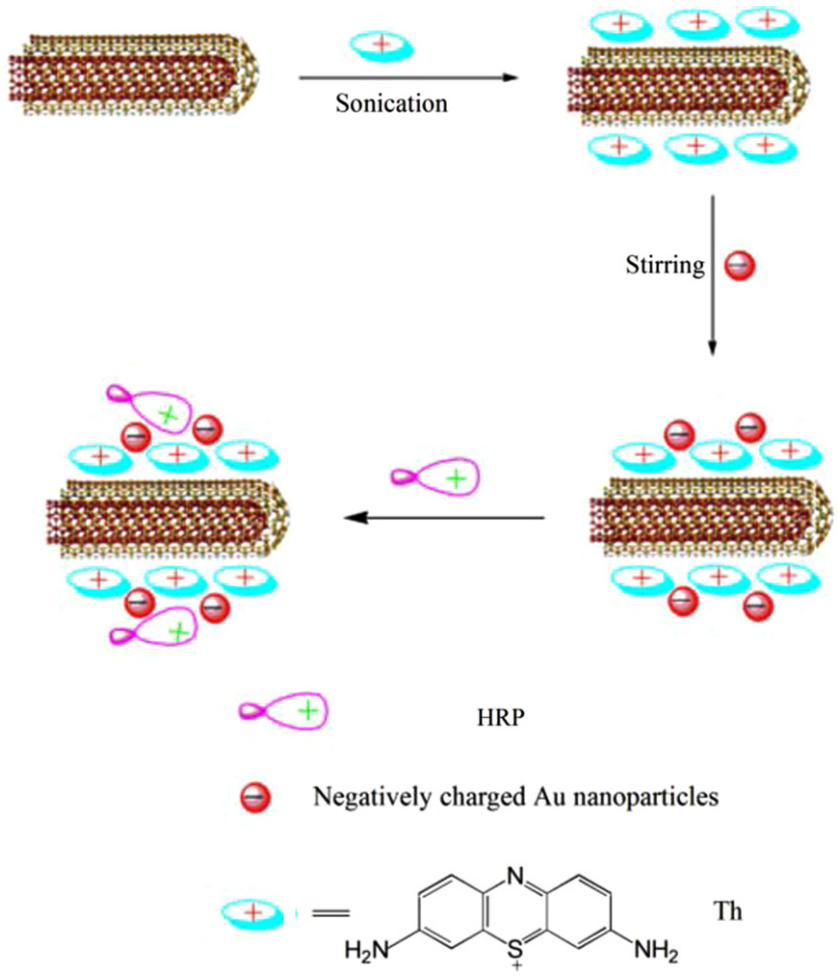

Fig. 1. Schematic illustration of the preparation procedure.

can be ascribed to the direct electron transfer between the HRP and the underlying electrode substrate, and was observed only at the HRP–MTAu modified GC electrode. The anodic peak potential (E_{pa}) and the cathodic peak potential (E_{pc}) are located at −0.17 V and −0.19 V, respectively. However, no redox peak can be observed in the voltammogram at the HRP-modified GC electrode. This shows that the MTAu composite can facilitate the direct electron transfer of HRP. Au nanoparticles might play an important role due to their high ratio of surface to volume [26].

Fig. 5 shows the cyclic voltammograms of MWCNTs (dashed) film-modified GC electrode and GC electrode (solid) in 0.1 M PBS (pH 7.0) buffer with saturated N_2. The MWCNT-coated electrode exhibits reduction peak at −0.53 V in the presence of H_2O_2. In contrast, no redox activity can be clearly observed at the naked GC surface over most of the potential range. Moreover, there is not any peak at MWCNT-modified electrodes (dotted, Fig. 5) in the absence of H_2O_2. Therefore, MWCNTs also showed electrochemical activity toward H_2O_2. It is believed that CNTs have two distinct possible reactive sites: basal plane sites which occur on the side walls of the CNTs and edge-plane-like sites/defects which occur at the ends of the tubes and, in the case of MWCNTs, along the tube axis [27]. Therefore, the electrochemical activity of MWCNTs resulted from either edge plane like-sites/defects [28–31] or metallic impurities [27,32–34] should be depended much on the electrochemical systems we explored. On the other hand, HRP attached to the gold colloid surface has more spatial freedom in its orientation, which makes it much easier for the electroactive center of HRP to unfold [12]. The cyclic voltammogram of MTAu modified GC electrode shows a reduction but no oxidation current. This can not be found in the HRP–MTAu modified GC electrode, which is still unclear for us.

3.4. Reduction of H_2O_2 at the HRP–MTAu modified GC electrode

Reduction of H_2O_2 is studied at both HRP–MTAu and MTAu modified GC electrodes by cyclic voltammetry. The voltammogram in the presence of 0.5 mM H_2O_2 in 0.1 M PBS (pH 7.0) are shown in Fig. 6 together with the voltammograms of the background electrolyte. Reduction of H_2O_2 can be observed at both electrodes. But the HRP–

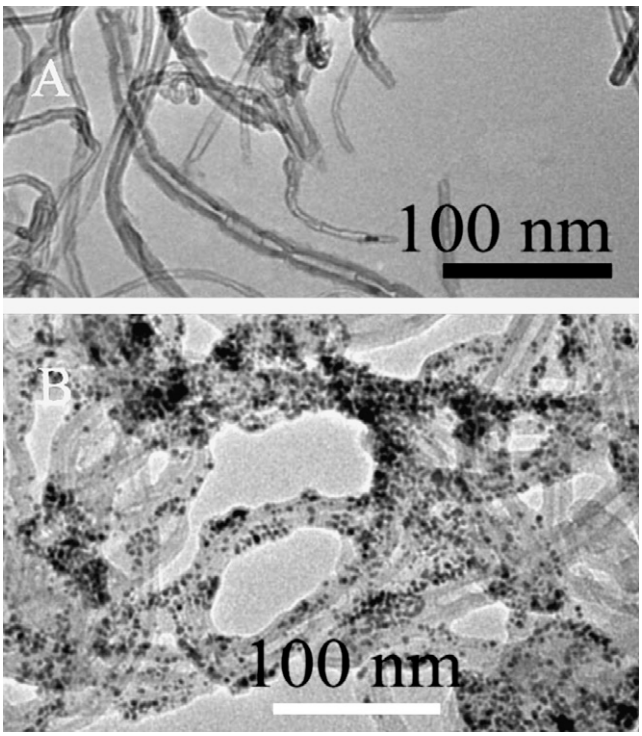

Fig. 2. TEM images of MT (A) and MTAu (B).

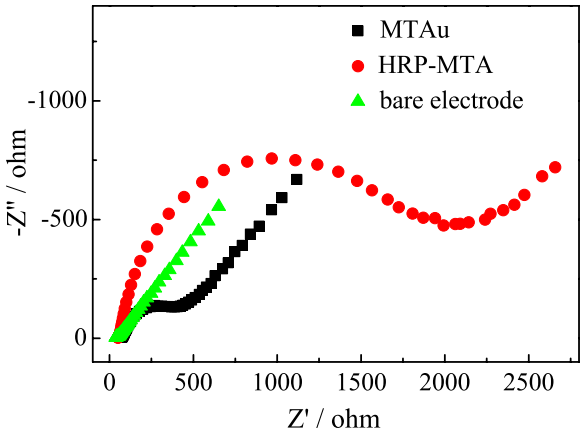

Fig. 3. Nyquist plots of bare GC electrode (▲), MTAu composite modified GC electrode (■), and HRP–MTAu film modified GC electrode (●) in 5 mM $Fe(CN)_6^{3-/4-}$ (in 1:1 ratio) in 0.1 M KCl solution, respectively. The frequency range is 10^5 Hz to 0.3 Hz, and the perturbation signal is 5 mV. The measurements are done at the open circuit potential.

MTAu modified GC electrode was at less negative potential (−0.28 V) compared with the peak of MTAu modified GC electrode (−0.35 V). This indicates that the presence of HRP in the film has catalytic activity to reduction of H_2O_2. Also, these results definitely showed a synergistic effect between HRP and MTAu. However, in nitrogen-saturated systems (dotted, Fig. 6), current peaks are not readily discernible.

The electrocatalytical reduction of H_2O_2 at HRP–MTAu modified GC electrode was also examined by

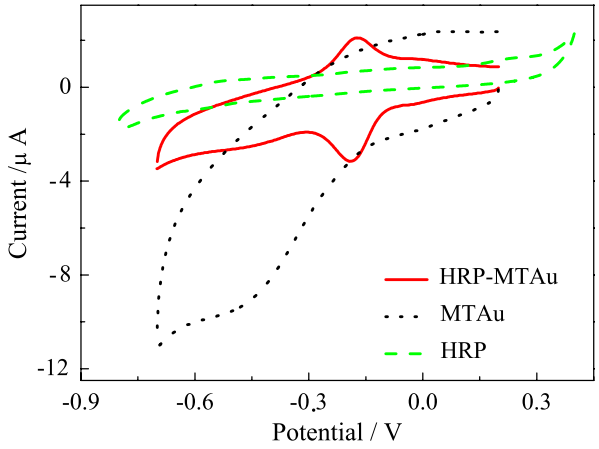

Fig. 4. Cyclic voltammograms of the HRP–MTAu film modified GC electrode (solid), MTAu composite modified GC electrode (dotted), and HRP modified GC electrode (dashed) in 0.1 M PBS (pH 7.0) saturated with N_2. Scan rate: 0.05 V s^{-1}.

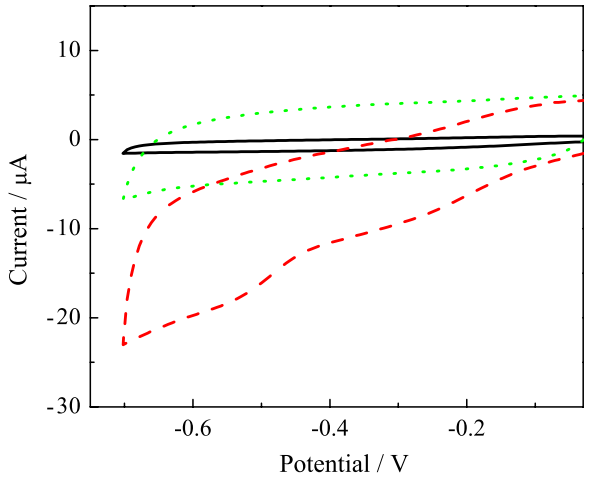

Fig. 5. Cyclic voltammograms of MWCNTs film-modified GC electrodes (dashed) and GC electrode (solid) in 0.1 M PBS (pH 7.0) with 0.5 mM H_2O_2 saturated with N_2 and cyclic voltammograms of MWCNTs film-modified GC electrode (dotted) in 0.1 M PBS buffer (pH 7.0) saturated with N_2. Scan rate: 0.05 V s^{-1}.

amperometry, which is one of the most commonly-used techniques in electrochemical biosensors. Fig. 7 shows the steady-state current response to successive additions of 0.64 mM H_2O_2 (in 0.1 M PBS (pH 7.0)) at the HRP–MTAu modified-GC electrode at 0 V. As can be seen in the Fig. 7, the response was linear and the linear regression equation was I (nA) = 1.81 + 4.86 × 10^3[H_2O_2] (M), with the correlation coefficient of 0.9995. In separate experiment, the detection limit was determined to be 10^{-7} M with the signal to noise ratio of three. The linear range between the electrocatalytic current and the concentration of H_2O_2 is extended to 7 mM (inset of Fig. 7) when 0 V was used as the working potential.

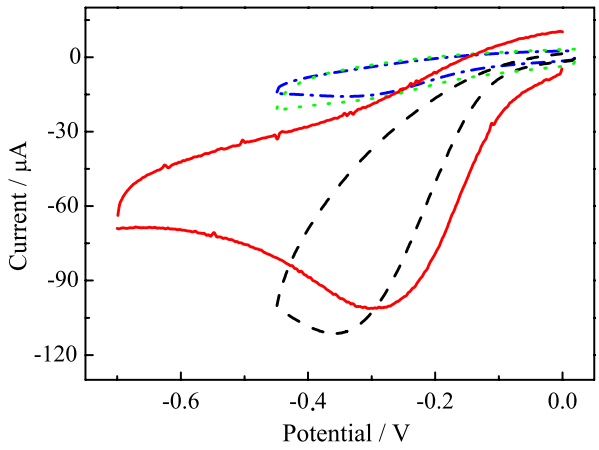

Fig. 6. Cyclic volgammograms of HRP–MTAu (solid), MTAu (dashed) film-modified GC electrodes in 0.1 M PBS (pH 7.0) with 0.5 mM H_2O_2, HRP–MTAu (dotted), MTAu (dash-dot) film-modified GC electrodes in 0.1 M PBS (pH 7.0) saturated with N_2. Scan rate: 0.05 V s^{-1}.

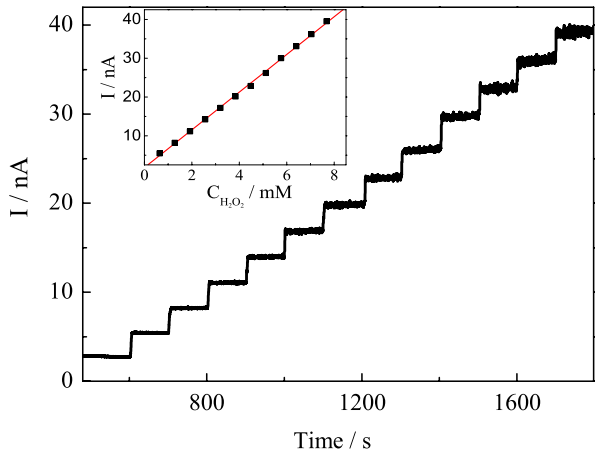

Fig. 7. Amperometric response of the HRP–MTAu composite with successive additions of 6.4×10^{-4} M H_2O_2 at an operating potential of 0 V.

4. Conclusions

HRP has successfully been incorporated into MTAu composite by electrostatic interactions between positively charged HRP and negatively charged MTAu composite. HRP retains its bioactivity and bioelectrocatalytical activity, and also exhibits good direct electron transfer between the redox center of the enzyme and the electrode. The presence of MWNT, Au nanoparticles and Th components plays key roles in accelerating the electron transfer between active sites in enzyme and electrode substrate.

Acknowledgements

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