

Direct Electrochemistry and Electrocatalytic Behavior of Horseradish Peroxidase on Attapulgitte Clay Modified Electrode

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A novel third-generation hydrogen peroxide (H₂O₂) biosensor was developed by immobilizing horseradish peroxidase (HRP) on a biocompatible attapulgitte (ATP) modified glassy carbon (GC) electrode. The ATP could provide a biocompatible microenvironment for enzyme molecules, greatly amplify the coverage of HRP molecules on the electrode surface, and most importantly facilitate the direct electron transfer between HRP and the electrode. The biosensor construction process was followed by atomic force microscopy (AFM). Cyclic voltammetry was employed to characterize the properties of the biosensor. A linear calibration plot of the enzyme electrode was obtained over the range of 5 μM to 0.3 mM for H₂O₂ with a detection limit of 5 μM. Furthermore, the biosensor showed high sensitivity, good reproducibility, and fine long-term stability.

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Introduction

The feasibility of heterogeneous direct electron transfer (DET) reactions between redox proteins and the electrode surface has attracted considerable interest; an understanding of these reactions fundamentally can establish a desirable model for studying the redox behavior of the proteins in biological systems.¹ Among numerous redox proteins, horseradish peroxidase (HRP) has commonly been chosen to construct H₂O₂ biosensors because of easy availability in high purity and low cost. An electrochemical technique based on a simple and low-cost enzyme electrode has been extensively employed for the determination of H₂O₂ for its simplicity, high selectivity and intrinsic sensitivity.²

However, the direct electron-transfer rate of redox proteins is rather slow at conventional electrodes. This slow electron exchange may be caused by an unsuitable orientation of proteins on the electrode surface, which increases the distance between the electroactive center of the protein and the electrode surface. Moreover, the adsorption of impurities and denatured protein onto an electrode surface could block electron communication between heme and the electrode.³ Therefore, one of the main challenges is to select a host matrix to provide a suitable microenvironment for the proteins, and to enhance direct electron transfer between the proteins and the underlying electrodes.⁴ Until now, many materials, such as polymer films,⁵ nano-particles,⁶⁻⁸ biomembranes,⁹ surfactants¹⁰ and clay¹¹⁻¹³ have been used to promote the electron transfer between redox

proteins and electrodes. Composite enzyme-clay films have been confirmed to improve the analytic performances, including adhesion, mechanical strength and enzymatic activity.¹⁴⁻¹⁶

Attapulgitte (ATP), as one kind of clay, has unique physical and chemical properties, and is receiving increasing attention in many areas, such as inorganic-organic polymer hybrids, fertilizer suspensions, drilling fluids, oil refining, catalysts, catalyst supports, the pharmaceutical industry and others.¹⁷⁻²⁰ Many national pharmacopoeias have even accepted ATP. In addition, ATP has fine biological affinity, which makes it friendly to enzymes.^{21,22} In a word, there are many reasons why we may choose ATP as the matrix for enzyme immobilization: low cost, high chemical stability, high ion-exchange properties, unique aluminosilicate-layered structure with appreciable surface area, and especially fine biological affinity.^{23,24}

In this paper, ATP was used as a matrix for HRP immobilization on the surface of a glassy carbon (GC) electrode, and the direct electron exchange between HRP and the electrode was studied. Meanwhile, HRP retains its bioactivity and displays a high affinity for H₂O₂, producing a novel reagentless sensor for the measurement of H₂O₂. Compared with conventional second generation sensors, this DET-based sensor has some practical advantages, including operation simplicity, low expense of fabrication and suitability for real-time detection.

Experimental

Reagents

HRP was purchased from Sigma (USA) and used as received. ATP was obtained from Jiangsu Junda AT Material Co., Ltd. (Jiangsu, China). Additions of H₂O₂ were made from a 100 mM

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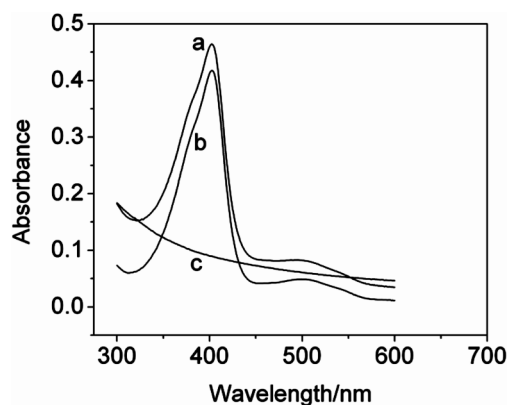


Fig. 1 UV-Vis absorption spectra of samples in 0.1 M pH 7.0 PBS: (a) HRP, (b) HRP/ATP composite, (c) ATP.

stock solution, prepared by dilution from a solution obtained from Beijing Chemical Reagent (Beijing, China) (30% w/w in water, 99% purity). The stock solution was prepared daily so as to avoid excessive decomposition of the H_2O_2 to water and oxygen. Phosphate buffer solutions (PBS) were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4 . Other chemicals were of analytical reagent grade. All solutions were prepared with pure water obtained by means of a Milli-Q water purification set.

Characterizations and electrochemical measurements

The UV-Vis spectra of the HRP solution, ATP suspension and HRP/ATP composite suspension were measured with a Cary 50 Scan UV-Vis spectrophotometer (Varian, USA). The morphology of ATP and HRP/ATP was recorded using an SPI3800N microscope (Seiko Instruments, Japan) in the tapping mode. All electrochemical experiments were performed by a CHI 660 (USA) electrochemical workstation using a conventional three-electrode system consisting of a modified GC electrode as a working electrode, a saturated calomel electrode (SCE) as a reference electrode and a platinum wire as an auxiliary electrode. The electrolyte was deoxygenated by purging pure nitrogen into the solution for about 30 min, and nitrogen gas was kept flowing over the solution during electrochemical experiments.

Preparation of the biosensor

A GC electrode (3 mm in diameter) was polished to a mirror-like surface with fine emery papers and 1.0, 0.3 and 0.05 μm alumina slurry, followed by thoroughly rinsing with Milli-Q water. The electrode was then successively sonicated in alcohol and Milli-Q water, and allowed to dry at room temperature. Then, 5 μl of a 1 mg/ml purified ATP sample prepared as previously described¹⁸ was directly dropped onto a pretreated GC electrode and dried at room temperature for 3 h to make an ATP modified GC electrode. A 5- μl aliquot of HRP (5 mg/ml, in pH 7.0 PBS) was dropped onto the surface of the ATP modified GC electrode, and then the electrode was dried at room temperature and rinsed with Milli-Q water twice to remove any non-firmly adsorbed HRP. The obtained HRP/ATP/GC electrode was stored at 4°C in a refrigerator when not in use.

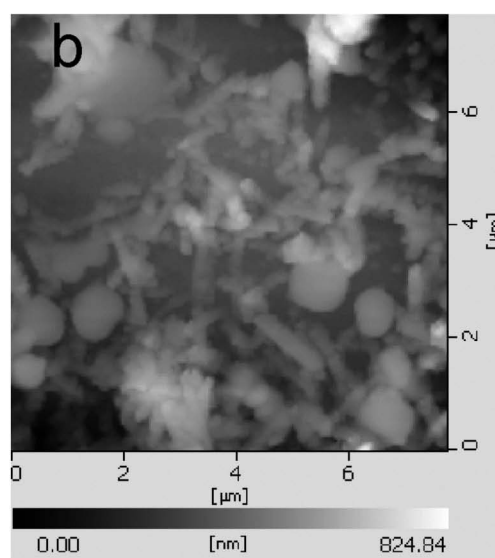
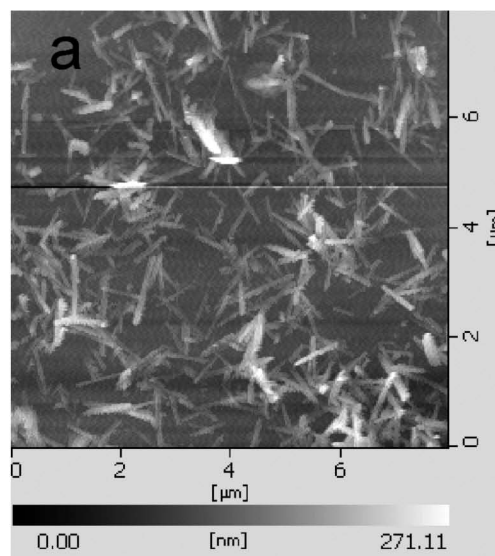


Fig. 2 AFM images of (a) ATP, (b) ATP with adsorbed HRP molecules.

Results and Discussion

UV-Vis absorption spectroscopic characterization

UV-Vis spectroscopy is a useful tool for monitoring any possible change of the Soret absorption band of HRP.²⁵ Figure 1 shows the UV-Vis spectra of a HRP and HRP/ATP composite in pH 7.0 PBS. The native HRP gave a heme band at 402 nm (curve a). For the HRP/ATP composite, the absorption band was also at 402 nm (curve b). This indicates that ATP does not destroy the proteins' structure, or change the fundamental microenvironment. It can also be concluded that as a matrix for the immobilization of protein, ATP will undoubtedly maintain the properties and reactivity of the protein.

AFM characterization of the biosensor construction

We used AFM to study the interaction between HRP and ATP. AFM images confirmed that the HRP molecules had been immobilized on ATP. Figure 2a shows the ATP without HRP molecules and Fig. 2b shows that with adsorbed HRP molecules.

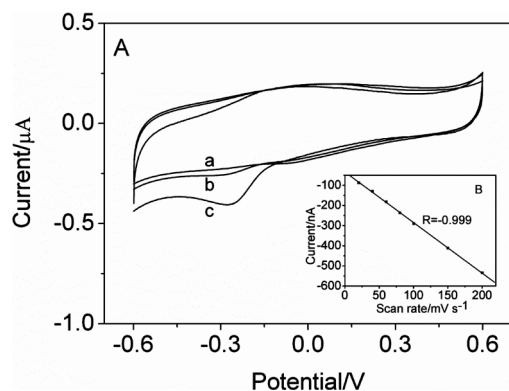


Fig. 3 (A) Cyclic voltammograms of electrodes in 0.1 M PBS (pH 6.0): (a) ATP/GC electrode, (b) HRP/GC electrode, (c) HRP/ATP/GC electrode. Scan rate: 50 mV/s. (B) Dependence of the cathodic peak current of the HRP/ATP/GC electrode on the scan rate. Scan rates: 20, 40, 60, 80, 100, 150 and 200 mV/s.

Comparing the two images, it can be directly observed that HRP molecules are mostly adsorbed onto the surface of ATP. To obtain the sample of Fig. 2b, HRP in pH 7.0 PBS was directly dropped onto the ATP and dried at room temperature, and then rinsed with Milli-Q water twice to remove any non-firmly adsorbed HRP. At pH 7.0, HRP (isoelectric point: 8.9) has a net positive surface charge,²⁶ and ATP (isoelectric point: 3.0) has a net negative surface charge.²⁷ Therefore, electrostatic interactions between the positively charged HRP and the negatively charged ATP make HRP to be adsorbed on the ATP. However, we also believe that some HRP could be adsorbed in the inner space of ATP because the ATP rod is porous,²⁷ with a large specific surface area, and possibly able to entrap HRP. These results indicate that the ATP can be used as a support for HRP.

The electrochemical characteristics of the different GC electrodes

Figure 3A shows cyclic voltammograms of the ATP/GC electrode, HRP/GC electrode (HRP dropped onto the pretreated GC electrode and dried at room temperature for 3 h) and HRP/ATP/GC electrode in 0.1 M pH 6.0 PBS at 50 mV/s. No distinct peak was observed at either the ATP/GC electrode (curve a) or HRP/GC electrode (curve b). However, a pair of cathodic-anodic peaks, E_{pa} and E_{pc} values at -0.05 and -0.27 V, were observed at the HRP/ATP/GC electrode (curve c). This pair of peaks are attributed to direct electron transfer between the heme group of HRP immobilized in the ATP films and the GC electrode. To further investigate the characteristics of the HRP/ATP/GC electrode surface, the effect of the scan rate on the response of immobilized HRP is shown in detail. It can be seen from Fig. 3B that the reduction peak currents increase linearly with scan rates, indicating that the redox process is a surface-controlled electrode process. In this work the electron transfer of HRP immobilized in the ATP film may be enhanced. This was possibly because the heme group of HRP in ATP films achieves a favorable orientation, which makes the heme group more easily exposed to the surface of the electrode.^{28,29} Therefore, the electron-transfer process between the protein and the electrode could be promoted by an electron-hopping mechanism.³⁰

Electrocatalytic behavior of HRP/ATP/GC electrode to the reduction of H_2O_2

Figure 4 shows cyclic voltammograms of the HRP/ATP/GC electrode in 0.1 M pH 6.0 PBS in both the presence and the

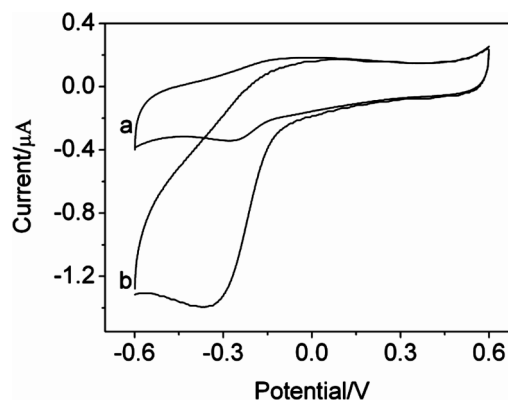
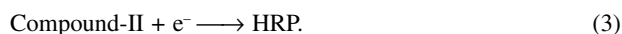
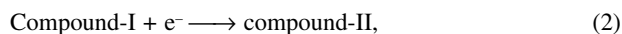
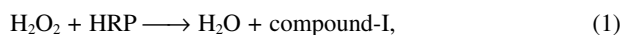


Fig. 4 Cyclic voltammograms of HRP/ATP/GC electrode in 0.1 M PBS (pH 6.0) containing: (a) 0 M H_2O_2 , (b) 50 μ M H_2O_2 . Scan rate: 50 mV/s.

absence of H_2O_2 . When H_2O_2 was added, compared with the system with no H_2O_2 present, the cathodic peak current of the electrode increased dramatically, and the anodic peak almost disappeared. This catalytic reduction behavior for hydrogen peroxide shows the similarity with that of hemoglobin on the ATP.²¹ Thus, the catalytic mechanism of HRP immobilized in the ATP films to H_2O_2 reduction may be explained by the following process:³¹



In the first 2 e^- step, hydrogen peroxide is reduced to water and the bound cofactor is oxidized. This oxidized form of peroxidase is usually denoted as compound-I. Compound-I is then reduced in the first 1 e^- step to form compound-II. Compound-II is reduced in the second 1 e^- step back to HRP. According to studies,³² compound-II shows a higher stability than compound-I, and thus the reduction process of compound-II to native HRP is slow, so the third step can be detected by cyclic voltammetry. These results indicate that HRP incorporated into the ATP films can catalyze the reduction of H_2O_2 efficiently and still retain its biological activity.

Amperometric response to H_2O_2

To evaluate the dependence of the electrocatalytic current on the concentration of H_2O_2 , the characteristics of the HRP/ATP/GC electrode were investigated by a chronoamperometric measurement at pH 6.0. The results presented in Fig. 5 indicate that the electrocatalytic current increases with increasing concentration of H_2O_2 . The linear range between the electrocatalytic current and the concentration of H_2O_2 can extend to 0.3 mM. The correlation coefficient, R is 0.997, and the detection limit value is 5 μ M, which imply that the HRP/ATP/GC electrode exhibits a higher affinity for H_2O_2 .

Reproducibility and stability

The repeatability of the current response of the same enzyme electrode was examined in the presence of 50 μ M H_2O_2 in 0.1 M PBS. The relative standard deviation (RSD) was 3.6% for seven successive assays. The electrode-to-electrode reproducibility was determined in the presence of 50 μ M H_2O_2

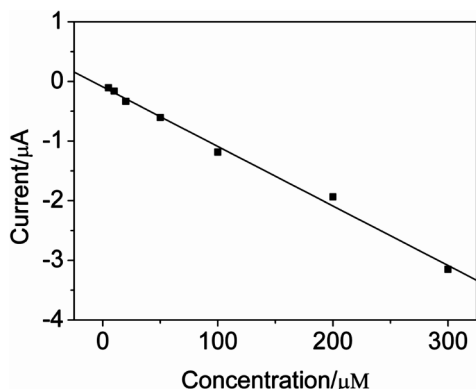


Fig. 5 Dependence of the electrocatalytic current on the concentration of H_2O_2 .

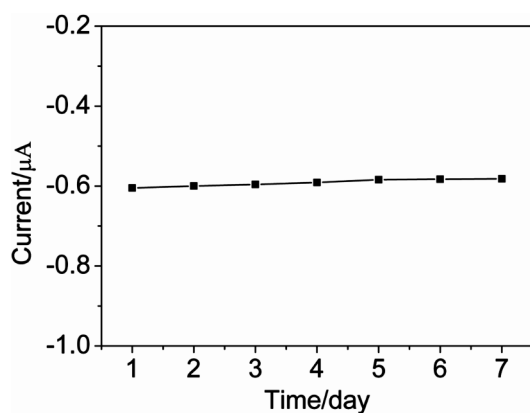


Fig. 6 Storage stability of the sensor: daily current response of the HRP/ATP/GC electrode to $50 \mu\text{M}$ H_2O_2 in 0.1 M phosphate buffer (pH 6.0) during a 7-day period. Scan rate: 50 mV/s .

in 0.1 M PBS with seven different enzyme electrodes. They yielded a 5.1% RSD. When the biosensor was stored in the refrigerator at 4°C and measured every day during a 7-day period, there was no obvious change of current in the response to $50 \mu\text{M}$ H_2O_2 , as shown in Fig. 6. The good reproducibility and good stability might be due to the fact that HRP molecules were firmly immobilized in the ATP, which provided a biocompatible microenvironment on the electrode surface.

Conclusion

ATP was used as a matrix to modify the GC electrode. HRP could be effectively immobilized on the matrix to produce a fast direct electron transfer and to retain its bioactivity and native structure. The resulted enzyme electrode showed high sensitivity, good reproducibility and fine long-term stability. This approach provides a simple method to develop a new kind of electrochemical H_2O_2 biosensor.

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