



A novel approach to construct a horseradish peroxidase|hydrophilic ionic liquids|Au nanoparticles dotted titanate nanotubes biosensor for amperometric sensing of hydrogen peroxide

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ABSTRACT

The direct electrochemistry of horseradish peroxidase (HRP) on a novel sensing platform modified glassy carbon electrode (GCE) has been achieved. This sensing platform consists of Nafion, hydrophilic room-temperature ionic liquid (RTIL) and Au nanoparticles dotted titanate nanotubes (GNPs-TNTs). The composite of RTIL and GNPs-TNTs was immobilized on the electrode surface through the gelation of a small amount of HRP aqueous solution. The composite was characterized by transmission electron microscopy (TEM), powder X-ray diffraction (XRD) and infrared spectroscopy (IR). UV–Vis and IR spectroscopy demonstrated that HRP in the composite could retain its native secondary structure and biochemical activity. The HRP-immobilized electrode was investigated by cyclic voltammetry and chronoamperometry. The results from both techniques showed that the direct electron transfer between the nanocomposite modified electrodes and heme in HRP could be realized. The biosensor responded to H_2O_2 in the linear range from 5×10^{-6} to $1 \times 10^{-3} \text{ mol L}^{-1}$ with a detection limit of $2.1 \times 10^{-6} \text{ mol L}^{-1}$ (based on the $S/N = 3$).

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1. Introduction

Direct electron transfer process between the redox proteins and electrodes have been studied in detail in the last few decades for the better understanding of kinetics and thermodynamics of biological redox processes (Armstrong et al., 1988; Zhao et al., 2008) and for the development of highly selective bioelectrocatalyst and biosensors (Zeng et al., 2009). As an important heme-containing enzyme, horseradish peroxidase (HRP) is considered to be an ideal model molecule for such study owing to its well known structure, high stability and bioactivity (Sun et al., 2010).

Due to the special characteristics such as excellent conductivity, biocompatibility, wide electrochemical windows and high chemical stability, ionic liquids have been widely applied as an electrode film material in the construction of electrochemical biosensors (Anderson et al., 2006; Musameh and Wang, 2008). Especially some long chain ionic liquids like 1-alkyl-3-methylimidazolium (abbreviated $[\text{Cn-mim}^+]\text{Br}^-$) ($n \geq 10$) salts have a very particular feature that they can realize an immediate conversion from a viscous liquid

to a stable, homogeneous gel when a certain amount of water is added into them (5 to 40%, w/w) (Firestone et al., 2002; Firestone et al., 2004). This special feature of ionic liquids provides us a new strategy to immobilize redox proteins onto the electrode surface.

As an electrode material with many excellent properties like biocompatibility, chemical inertness, photocatalytic capability and strong immobilization ability, TiO_2 nanotubes have been successfully used in the electrochemistry and photoelectrochemistry for many years (Han et al., 2010; Paramasivam et al., 2008). To improve conductivity and biocompatibility of TiO_2 nanotubes, gold-nanoparticles were modified on TiO_2 nanotubes to produce a nano-composite GNPs-TNTs (Kafi et al., 2008; Paramasivam et al., 2008). A combination between GNPs-TNTs and long chain ionic liquids is believed to be capable of providing a biocompatible microenvironment to retain the native structure and the bioactivity of redox proteins. Moreover, this biocompatible microenvironment can facilitate the direct electron transfer between redox centers of proteins and electrode surface (Zhao et al., 2004).

In the present work, aqueous solution of HRP was employed as a gelator to immobilize a composite scaffold consisting of RTIL, HRP and GNPs-TNTs on the electrode surface for the first time. The composite scaffold provided a biocompatible microenvironment for HRP to keep its bioactivity and obtain direct electrochemistry. GNPs-TNTs was also acting as support matrix to strengthen the immobilization layer and increase the affinity between GC

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electrode and protein composite film, which can minimize the loss of RTIL and HRP during the detection process. Finally, Nafion was applied on the composite scaffold to further enhance stability of the biosensor. The electrocatalytic detection of H_2O_2 at HRP|RTIL|GNPs-TNTs|Nafion electrodes was conducted in the absence of electron mediators.

2. Experimental

2.1. Reagents and instruments

Titanate nanotube ($\text{Na}_2\text{Ti}_2\text{O}_4(\text{OH})_2$) was prepared based on previous publication (Yang et al., 2003). Titanate nanotubes were successfully coated with Au nanoparticles by reduction of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ in a dispersion solution of titanate nanotubes, in

which sodium borohydride acts as a reducing reagent and sodium citrate as a stabilizing reagent (Bai et al., 2011). Horseradish peroxidase and $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ were purchased from Sigma–Aldrich. The chemical composition and morphology of the nanocomposite were examined using transmission electron microscopy (JEM-2010, Japan), X-ray diffractometry (X-PertPro, Netherlands, Cu $\text{K}\alpha$ radiation $\lambda = 1.5406 \text{ nm}$) and FT-IR spectrophotometer (Nicolet 170, USA). UV–vis absorbance spectra were recorded on a UV–vis spectrophotometer (UV-1750, SHIMADZU, Japan).

2.2. Preparation of the HRP electrodes

Glassy carbon electrodes (GCE, 3 mm in diameter) were polished to a mirror-like with 1.0, 0.3, and $0.05 \mu\text{m}$ alumina slurry and cleaned. HRP aqueous solution was employed to gelate the

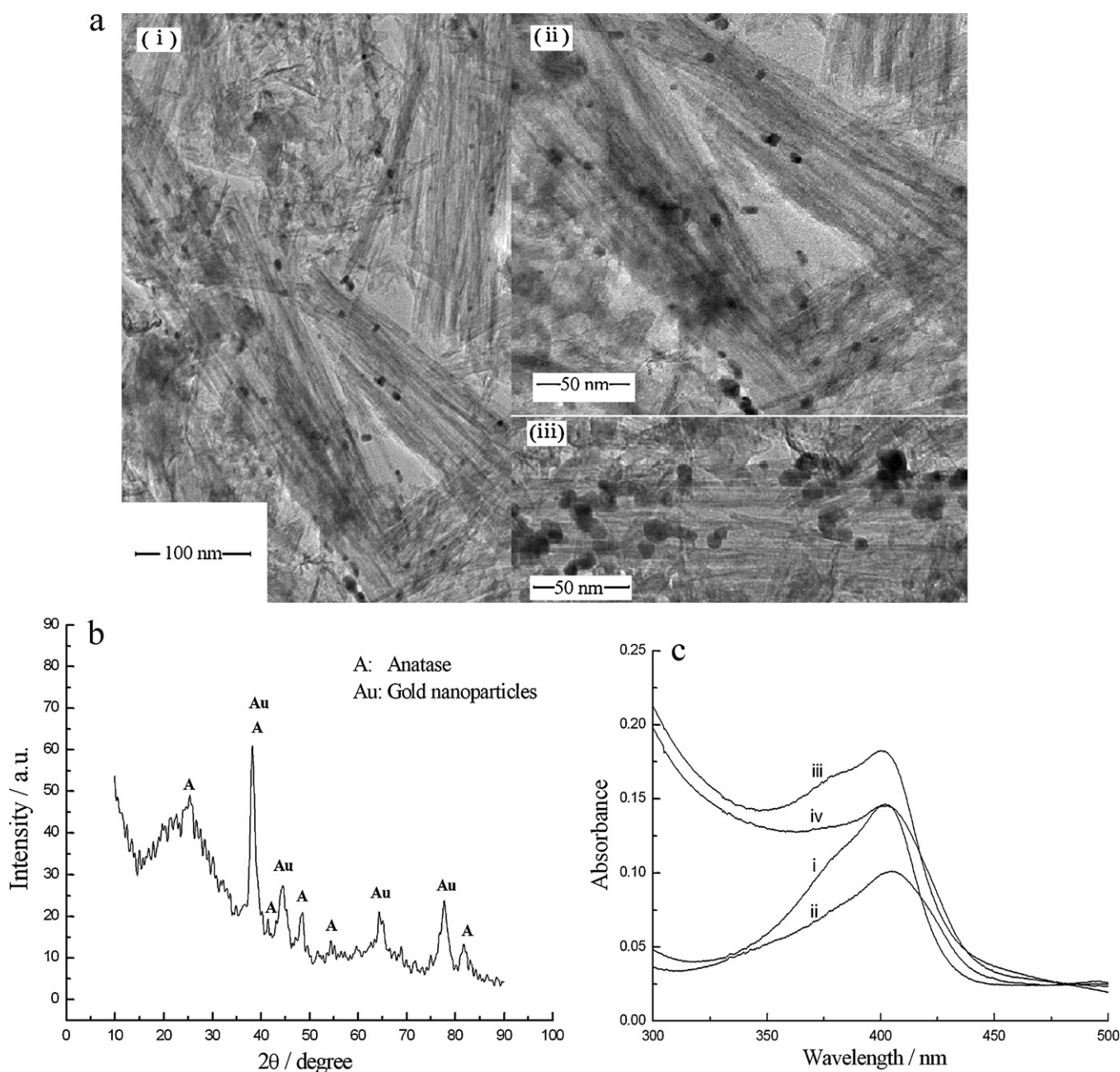


Fig. 1. (a) TEM images of GNPs-TNTs hybrid structures: (i) nanotubes in GNPs-TNTs hybrid structures; (ii) magnified image of nanotubes of GNPs-TNTs hybrid structures; (iii) a single nanotube of GNPs-TNTs hybrid structures. (b) XRD pattern of the GNPs-TNTs. (c) UV–vis absorption spectra of (i) HRP, (ii) HRP|RTIL, (iii) HRP|GNPs-TNTs and (iv) HRP|RTIL|GNPs-TNTs.

mixture of RTIL and GNPs-TNTs on the GC electrode surface. In brief, 150 μL HRP aqueous solution (2 mg/ml) was added in 850 μg RTIL and GNPs-TNTs composite (9:1, w/w). The obtained composite was sonicated in a water bath for 30 s and 10 μL suspension was taken out from the composite and dropped onto the glassy carbon electrodes immediately. Finally, 10 μL Nafion (1%) was casted onto the modified electrodes and dried in refrigerator at 4 °C. Water|RTIL|GNPs-TNTs|Nafion electrode was fabricated based on the similar procedure except that water replaced HRP solution to gelate the mixture of RTIL and GNPs-TNTs.

2.3. Electrochemical measurements

Electrochemical measurements were performed on a CHI 630C electrochemistry workstation (CH Instruments, Shanghai, China) with a conventional three-electrode system consisting of a nanocomposite-HRP modified electrode as working electrode, a platinum wire as counter electrode and an Ag/AgCl (3.0 M KCl) electrode as reference electrode. All the electrochemical measurements were performed under deoxygenating with nitrogen gas for 15 min.

3. Results and discussion

3.1. TEM, XRD and FT-IR characterization of GNPs-TNTs

The GNPs-TNTs was characterized by TEM and XRD as shown in Fig. 1a and b. It can be seen that well-dispersed, spherical gold particles were coated on the surface of TNTs (Fig. 1a) and the average particle size of GNPs is about 5.3 nm. The crystal structure of as-prepared GNPs-TNTs was characterized by XRD (Fig. 1b). The strong diffraction peaks at 2θ angles of 25.0°, 37.4° and 48.5° correspond to the spacing of (1 0 1), (0 0 4) and (2 0 0) of the anatase (tetragonal) phase (Han et al., 2010). And the peaks at 37.8°, 45.0°, 65.0° and 78.0° can be assigned to (1 1 0), (2 0 0), (3 1 1) reflection of GNPs (Paramasivam et al., 2008; Song et al., 2010). Furthermore, the average size of the Au particles was calculated to be 5.4 nm by the Scherrer formula (Park et al., 2002; Yang et al., 2005), which is almost same as the value estimated by TEM. The FT-IR characterization of GNPs-TNTs and TNTs was also conducted and the spectra were displayed in Supplementary Materials as Fig. S.1.

3.2. UV-Vis and FT-IR spectroscopic analysis of HRP|RTIL|GNPs-TNTs

UV-Vis spectrometry is considered to be an effective technique to probe the characteristic structure of proteins (Sun et al., 2010). Because the Soret band of protein UV-Vis spectrometry is sensitive to the variation of the microenvironment around the heme group, the band shift may provide useful information about the denaturation in heme protein (Arnstein and Neuberger, 1953; Zhao et al., 2008). Fig. 1c shows the UV-Vis spectra of HRP and its mixture with the composite materials. As we can see that the Soret band absorption for HRP, HRP|GNPs-TNTs, HRP|RTIL and HRP|RTIL|GNPs-TNTs (curves i, ii, iii, iv) is located at nearly the same wavelength (about 402 nm), indicating that HRP remained its native structure and no significant denaturation occurred to protein after mixing with the composite materials. FT-IR spectroscopy is another powerful tool to investigate the secondary structure of proteins (Sun et al., 2010; Zhu et al., 2010). The IR spectra of HRP and HRP|RTIL|GNPs-TNTs composite are shown in Supplementary Materials as Fig. S.2. As displayed in this figure, there is only little difference between these two spectra, therefore, we can confirm that the native structure of HRP is retained in the composite material. Both UV-Vis and FT-IR spectroscopic results demonstrated the composite materials could

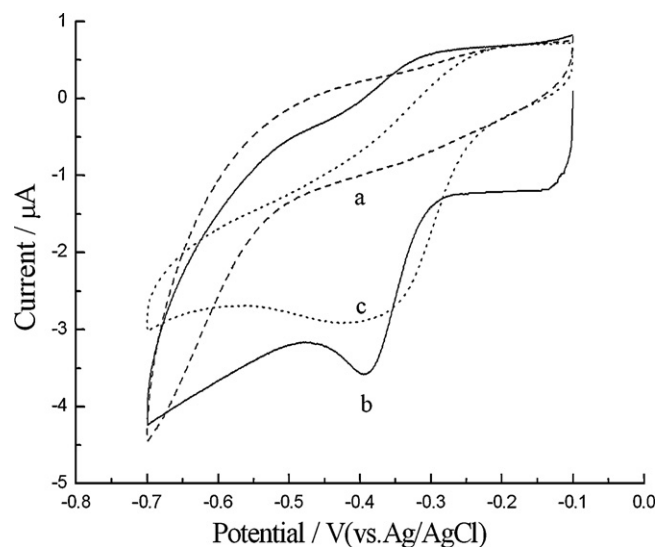


Fig. 2. Cyclic voltammograms obtained at different modified electrodes: (a) Water|RTIL|GNPs-TNTs|Nafion electrode (dash line), (b) HRP|RTIL|GNPs-TNTs|Nafion electrode (solid line) and (c) HRP|RTIL|TNTs|Nafion electrode (dot line) in 0.05 mol L⁻¹ PBS (pH 7.0) at a scan rate of 0.05 V s⁻¹.

provide excellent biocompatibility to keep the native structure and bioactivity of HRP.

3.3. Electrochemical characterization of HRP|RTIL|GNPs-TNTs electrode

Fig. 2 shows the cyclic voltammograms (CVs) of different modified electrodes in N₂-saturated PBS at a scan rate of 0.05 V s⁻¹. In Fig. 2 trace a, no peaks could be observed at the Water|RTIL|GNPs-TNTs|Nafion electrode. In contrast, a couple of stable redox peaks with a peak separation of 80 mV appeared at the HRP|RTIL|GNPs-TNTs|Nafion electrode as shown in trace b, indicating that the direct electron transfer of HRP has been achieved at this modified electrode. Moreover, compared with those of the HRP|RTIL|TNTs|Nafion electrode (trace c), the oxidation and reduction currents of the HRP|RTIL|GNPs-TNTs|Nafion electrode were increased 25.5% and 28.6% respectively. This can be attributed to the improved catalytic effect and electron transfer ability of the composite film in the presence of GNPs.

Electrocatalytic reduction of hydrogen peroxide at the HRP|RTIL|GNPs-TNTs|Nafion electrode in 0.05 mol L⁻¹ PBS (pH 7.0) is displayed in Fig. 3a. When increasing concentration of H₂O₂ was added, an obvious increase in the reduction peak was observed at about -0.39 V with decrease of the oxidation peak of HRP. These results indicated the strong catalytic effect of the biosensor to H₂O₂.

To further illustrate the relationship between the electrocatalytic reduction current and the concentration of H₂O₂, the chronoamperometry was performed and the results are shown in Fig. 3b. In Fig. 3b, the amperometric response of the HRP|RTIL|GNPs-TNTs|Nafion biosensor (trace II) is much bigger than that of the HRP|RTIL|TNTs electrode (trace I), demonstrating the strong catalytic effect of GNPs. In the presence of GNPs, the calibration plot can be represented by the following linear relationship:

$$\text{Peak current}/\mu\text{A} = (8.13 \pm 0.069) \times 10^{-4}/\mu\text{A } \mu\text{M}^{-1} [\text{H}_2\text{O}_2] + (1.20 \pm 0.0033)$$

where the errors denote 95% confidence intervals and a correlation coefficient of 0.997 could be obtained. The corresponding calibration plot (with a correlation coefficient of 0.992) obtained in

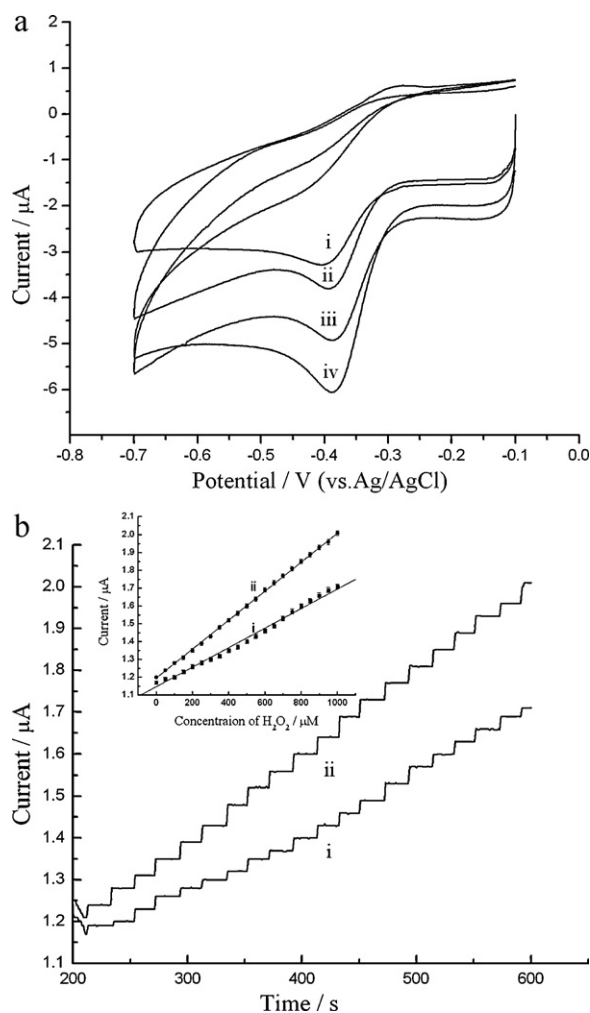


Fig. 3. (a) Cyclic voltammograms of HRP|RTIL|GNPs-TNTs|Nafion modified electrode at 0.05 V s^{-1} in 0.05 mol L^{-1} N_2 -saturated phosphate buffer solution (pH 7.0) in the presence of 1.0×10^{-4} , 3.0×10^{-4} , $1.0 \times 10^{-3} \text{ mol L}^{-1}$ H_2O_2 (from trace i to iv). (b) Amperometric response of (i) HRP|RTIL|TNTs|Nafion, (ii) HRP|RTIL|GNPs-TNTs|Nafion modified electrode in a stirred N_2 saturation PBS (pH 7.0) at -0.40 V to successive additions of $50 \mu\text{L}$ H_2O_2 . Insert: relationship between the catalytic current and the concentration of H_2O_2 .

the absence of GNPs is represented by

$$\text{Peak current}/\mu\text{A} = (5.48 \pm 0.082) \times 10^{-4}/\mu\text{A} \mu\text{M}^{-1} [\text{H}_2\text{O}_2] + (1.15 \pm 0.0043)$$

From the two linear relationships above, we can clearly see that GNPs significantly increased the sensitivity of the HRP biosensor. The response time for both biosensors is about 4 s, proving that the reduction of H_2O_2 is a fast kinetic process at the two biosensors. The HRP|RTIL|GNPs-TNTs|Nafion biosensor shows a superior linear response range of 5×10^{-6} to $1 \times 10^{-3} \text{ mol L}^{-1} \text{ M}$ ($R = 0.997$, $n = 20$) and a detection limit of $2.1 \times 10^{-6} \text{ mol L}^{-1}$ (based on the $S/N = 3$) over some reported HRP biosensors, as tabulated in Table S.1 in Supplementary Materials.

3.4. Stability and precision study of the HRP|RTIL|GNPs-TNTs|Nafion biosensor

The long-term stability of the biosensor was investigated over a 15-day period at successive intervals of 3 days. The biosensor

retained about 95% of its original sensitivity when it was stored at 4°C . The reproducibility of the biosensor was investigated by determining $0.1 \text{ mmol L}^{-1} \text{ H}_2\text{O}_2$ in PBS (pH 7.0) and a relative standard deviation (R.S.D.) of 5.3% was obtained for 10 successive assays. The fabrication repeatability for five electrodes from the same manufactured batch gave an R.S.D. of 4.2% for the determination of $0.1 \text{ mmol L}^{-1} \text{ H}_2\text{O}_2$. The control experiments were also performed to test the stability of HRP|RTIL biosensor in the absence of GNPs-TNTs and Nafion. The results showed that HRP|RTIL biosensor lost its 90% sensitivity after 2 tests at intervals of 3 days.

4. Conclusions

A novel immobilization method has been developed to construct a HRP biosensor. In this method, HRP aqueous solution was used for the first time as a gelation reagent to immobilize the mixture of RTIL and GNPs-TNTs nanocomposite onto the electrode surface. The RTIL-nanocomposite film provided a suitable microenvironment to facilitate the direct electrochemistry of HRP and keep the bioactivity of HRP to catalyze the reduction of H_2O_2 . This study is believed to have a great potential of developing a nonpoisonous and environmental friendly sensing platform in the construction of a third-generation biosensor for in vivo determination of the environmental pollutants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.09.045.

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