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A reagentless hydrogen peroxide sensor based on incorporation of horseradish peroxidase in poly(thionine) film on a monolayer modified electrode

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Abstract

A novel reagentless hydrogen peroxide sensor fabricated by the entrapment of horseradish peroxidase (HRP) into poly(thionine) film on a thionine self-assembled monolayer modified gold electrode is described. The direct electron transfer between the heme site of HRP and the electrode is mediated by thionine units incorporated in the polymer film. The sensor displays an excellent electrocatalytic response to the reduction of H_2O_2. The chronoamperometric current is proportional to the H_2O_2 concentration in the range of 6.2 µM to 9.4 mM with a correlation coefficient of 0.999. Moreover, such a modified electrode exhibits a good stability and reproducibility within the linear range of H_2O_2, the variation coefficients are 3.8% and 4.5% for 10 successive assays at a H_2O_2 concentration of 9.3 µM and 2.8 mM, respectively.

Keywords: Hydrogen peroxide sensor; Poly(thionine) film; Coimmobilization; Horseradish peroxidase; Thionine monolayer; Gold electrode

1. Introduction

The entrapment of enzymes in electropolymerized films has become an increasingly important technique for the immobilization of biomolecules in the fields of biosensors and molecular electronics [1,2]. A polymer film can be formed on the electrode surface by the electrochemical oxidation of a suitable monomer from an aqueous solution. Many biological components, ranging in size from whole cell [3] to protein fragments [4], have been incorporated into these polymer films. However, some biosensors built up of conducting and semi-conducting polymer films possess low electron transfer efficiency, giving rise to extremely low catalytic currents for the enzymatic reaction [5,6]. To improve the sensitivity of sensors based on non-conducting polymer films, mediators, such as ions, ligands or some molecules covalently attached to the monomer prior to electropolymerization, have been incorporated into the polymer film to transfer electrons between the active center of enzyme and the electrode [7,8]. A more simple and efficient approach is the preparation of a semi-conducting film by electropolymerization of a monomer which is itself a redox mediator.

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The detection of hydrogen peroxide plays an important role in many fields including industry, environmental protection and clinical control [9,10]. HRP is the most commonly used enzyme for the construction of H_2O_2 biosensors. It has been trapped into electropolymerized poly(pyrrole) [11] or poly(*o*-phenylenediamine) [12] to obtain the direct electron transfer between HRP and the units of the polymer film. The oxidized pyrrole oligomers in the PPy membrane could mediate the HRP–PPy electron transfer [11]. However, the sensitivity of poly(pyrrole) modified electrode to H_2O_2 was relatively low which was considered to be caused by the limited diffusion of H_2O_2 through the polymer layer [13,14]. The H_2O_2 sensors constituted by a hydrophilic, permeable film of HRP covalently bound to a 3-dimensional epoxy network having polyvinyl pyridine (PVP)-complexed $[Os(bpy)_2Cl]^{3+/2+}$ redox centers have also been fabricated [15]. A sensitivity of $1\ A\ cm^{-2}\ M^{-1}$ and a dynamic range of 1×10^{-7}–2×10^{-4} M was obtained at 0.0 V (vs. SCE). However, the response was restricted because slow electron flow (an electron diffusion coefficient below $10^{-8}\ cm^2\ s^{-1}$) within the redox hydrogels did not match the turnover rate of peroxide mass transport at optimal (10^{-4} M) substrate concentration. [15].

Here we present a H_2O_2 sensor based on the incorporation of HRP in a poly(thionine) film on a thionine monolayer modified gold electrode. Schlereth and Karyakin [16] confirmed that poly(thionine) film formed on a bare gold electrode was very unstable. The instability could be due to a weaker adsorption of the monomer on the gold electrode surface before the electropolymerization reaction. In this study, the gold electrode is firstly self-assembled by a thionine monolayer before the coimmobilization of HRP and poly(thionine). The stability of the poly(thionine) film is greatly improved, which is ascribed to the chemical bonding between the polymer film and the electrode surface. The poly(thionine) film shows an excellent efficiency of electron transfer between HRP and the electrode for the reduction of H_2O_2. The linear calibration of H_2O_2 is obtained in the range of 6.2 μM to 9.4 mM with a detection limit of 3 μM. The HRP/poly(thionine) electrode displays a good stability and reproducibility, and can be used to the determination H_2O_2 in practical samples.

2. Experimental

2.1. Apparatus

All electrochemical measurements were performed with a BAS-100B electrochemical analyzer (Bioanalytical System, USA) and a three-electrode system comprising a HRP/poly(thionine) sensor as working electrode, a saturated calomel electrode (SCE) as reference electrode, against which all potentials were referred to in our results, and a platinum wire as auxiliary electrode. Cyclic voltammetric experiments were carried out in a steady solution containing 0.1 M phosphate buffer solution (PB) at $37.0 \pm 0.2°C$ in a Faraday cage. Amperometric experiments were done in a thermostatic, stirred electrochemical cell holding 5 ml of 0.1 M PB (pH 7.0) at $37.0 \pm 0.2°C$. A potential step of -100 mV was applied to the working electrode, the current-time response was recorded following successive additions of stock H_2O_2 solution after a constant current had been established. All experimental solutions were deaerated with highly pure nitrogen for 10 min, and maintained in nitrogen atmosphere.

2.2. Chemicals

Horseradish peroxidase (HRP, E.C. 1.11.1.7, RZ > 3.0, 250 U/mg) and cysteamine were purchased from the Sigma. Thionine (> 95%) and hydrogen peroxide (30%) were obtained from Shanghai Biochemical Reagent (China). The concentration of more diluted hydrogen peroxide solutions was determined by titration with cerium(IV) to a ferroin end point [17]. 0.1 M phosphate buffers with various pHs were prepared by mixing the stock solutions of K_2HPO_4 and KH_2PO_4. Terephthaloyl chloride (TC, from Shanghai Reagent Factory) was of chemical grade and used without further purification. All solutions were made up with twice distilled water. All other reagents were of analytical grade.

2.3. Construction of H_2O_2 biosensor

Gold electrodes were prepared by sealing polycrystalline gold wires (> 99.99%) in soft glass tubes. The gold electrodes were abraded with successively finer grades of sandpapers and polished to a ‘‘mirror-like’’ surface with 0.3 μm and 0.05 μm alumina slurry on microcloth pads (Buehler), followed by rinsing with water and ethanol, and briefly sonicating in twice distilled water. The geometric area of $(1.3 \pm 0.1) \times 10^{-3}$ cm^2 was determined by using cyclic voltammetry in 0.1 M KNO_3 solution containing 1.01 mM $K_3[Fe(CN)_6]$. A roughness factor of 1.4 ± 0.2 was obtained from the ratio of the real to the geometric area. The real area of electrode was calculated with the charge evolved during oxidation of the superficial gold in 0.1 M H_2SO_4 [18].

The cysteamine functional monolayer was firstly prepared by soaking a clean gold electrode in 13 mM cysteamine aqueous solution for 4 h at room temperature. After the cysteamine modified electrode was rinsed with pH 7.0 PB and water, and dried in nitrogen, it was amido-derivatized by incubating in a saturated toluene solution of TC for 2 h. Then, the electrode was thoroughly rinsed in toluene and treated by electrochemical cyclic scanning between (600 mV in 0.1 M PB (pH 8.0) containing 0.1 mM thionine until a stable voltammogram was observed [19–21], producing a thionine monolayer modified electrode.

Electropolymerization was achieved by using cyclic voltammetry, which yielded films with more uniform thickness than using the potentiostatic mode. The HRP/poly(thionine) modified electrode was prepared by cyclic scanning from -500 mV to $+1100$ mV in 0.1 M PB (pH 8.0) containing 0.1 mM thionine and 3 mg ml^{-1} HRP at a thionine monolayer modified gold electrode at 20 mV s^{-1} for 2 h. The coimmobilization process of HRP and poly(thionine) was carried out in nitrogen atmosphere. After the polymerization procedure, the H_2O_2 sensor was rinsed with pH 7.0 PB and kept in the same PB at 4°C. The poly(thionine) electrode without the incorporation of HRP was fabricated in a similar way.

3. Results and discussion

3.1. Incorporation of HRP into poly(thionine) film on a thionine monolayer modified gold electrode

The isoelectric point of HRP is at pH 7.2. When pH > 7.2, the enzyme is negatively charged. Since the thionine is deposited as a polycation, electrostatic interaction between the polymer film and the enzyme plays an important role in the incorporation of the enzyme into the growing film. Experimental results indicated that the property of HRP/poly(thionine) modified electrodes formed from solutions with various pHs was quite different. The H_2O_2 sensor obtained from an acidic solution (pH 4.0) possessed good electroactivity, but exhibited no significant electrocatalytic response to the reduction of H_2O_2. However, the HRP/poly(thionine) modified electrode prepared in a weakly basic solution (pH 8.0) showed excellent catalytic behavior to H_2O_2.

The cyclic voltammograms of the coimmobilization of HRP and poly(thionine) on a thionine monolayer modified electrode with successive sweeps are shown in Fig. 1. During the electropolymerization process, a well-defined shoulder-peak appears at 1.0 V, which is attributed to the formation of a radical cation by the one-electron oxidation of thionine monomer [16,22–24]. With the potential cycling, the radical cation peak augments and more radicals are formed. These radicals are produced from both the monomeric dye in solution and the thionine units in the monolayer on the electrode surface [21,22]. Delocalization of unpaired electrons in the radical cation produces the electrophilic attacking site with high probability on either amine group or at its *ortho* position [25]. Radical dimerization can occur via the carbon–nitrogen coupling route to form a mixture of linkage species, as proposed by Bauldreay and Archer [22] for the electropolymerization mechanism of thionine. The increase of the broad cathodic peak at $+600$ mV indicates an increase in the amount of electroactive species on the electrode surface. In the meantime, the negatively charged HRP is entrapped in the growing poly(thionine) film due to electrostatic interaction.

The poly(thionine) film is electroactive because each thionine unit retains its electroactive heterocyclic nitrogen atom [25–27]. The HRP/poly(thionine) modified electrode showed a cathodic peak at -191 mV and a poorly-defined anodic peak at -156 mV in pH 7.0 PB (inset in Fig. 1). The broadness of the peaks may result from the complexity of the polymer or the interactions among molecules existing in the poly(thionine) film. The plot of cathodic current vs. the square root of scan rate showed a good linear relation (Fig. 2), which was not a surface-controlled electrode process, because a proton gradient was involved across the solution and/or the film in the electrode processes [28].

Fig. 3 shows the dependence of the ratio of i_{cat}/i_d on pH at a HRP/poly(thionine) modified electrode (where

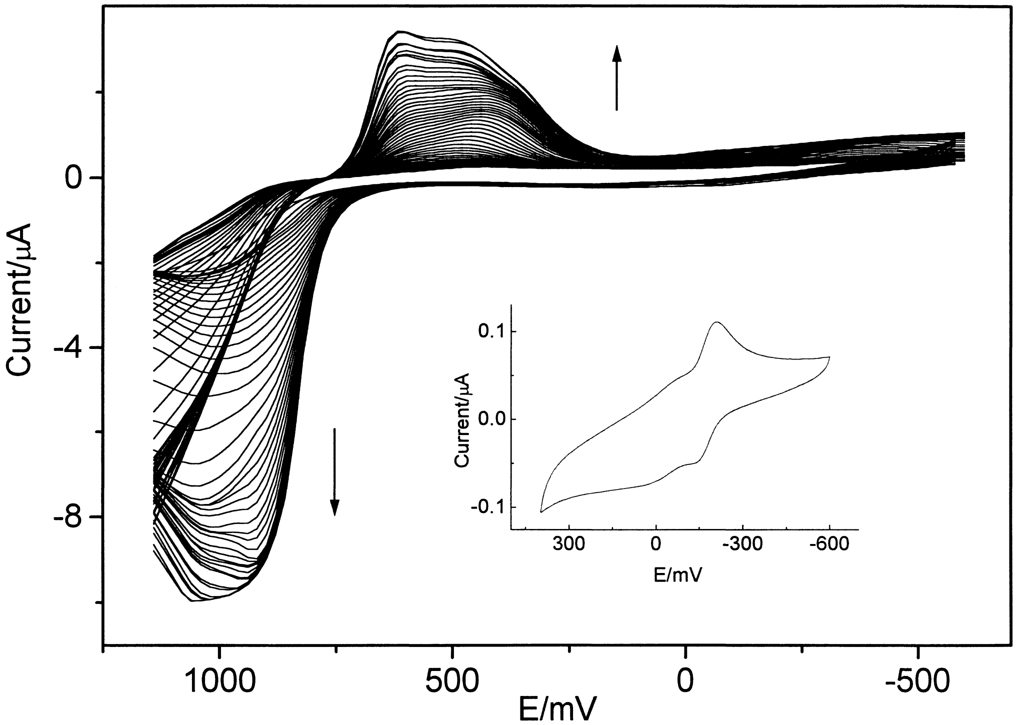

Fig. 1. The continuous cyclic voltammogram for coimmobilization of HRP and poly(thionine) on a thionine monolayer modified gold electrode in 0.1 M PB (pH 8.0) containing 0.1 mM thionine and 3 mg ml^{-1} HRP at 20 mV s^{-1}. Inset: cyclic voltammogram of the H_2O_2 sensor after the electropolymerization process in pH 7.0 PB at 50 mV s^{-1}.

i_d and i_{cat} are the cathodic peak currents before and after adding 0.194 mM H_2O_2, respectively. i_{cat}/i_d is used to quantify the electrocatalytic efficiency of the H_2O_2 reduction.). The ratio of i_{cat}/i_d exhibits a maximum value at pH 7.0, which is in agreement with the result observed for soluble peroxidase [29]. Thus, the poly(thionine) film does not alter the optimum pH value for the catalytic behavior of HRP. At the HRP/poly(thionine) modified electrode, there was a linear relation between cathodic potential and pH in the range of 4.0–8.6 with a slope of 53.3 mV/pH. Supposing the electron transfer number of each thionine unit in polymer film was 2, the numbers of H$^+$ ions participating in the electrode process was 2.

3.2. Amperometric response of H_2O_2 at HRP/poly(thionine) modified electrode

HRP molecules entrapped into a poly(thionine) film still retain their bioactivity. With the addition of H_2O_2 to PB, an obvious increase of the cathodic peak current and a decrease of the anodic peak current were observed (Fig. 4), indicating that poly(thionine) film could efficiently shuttle the electrons between the heme site of HRP and the gold electrode. In the present stage, the direct electron transfer between the gold electrode and the redox site of HRP was excluded due to the blocking of the "molecular-bridges" formed by the self-assembly of the thionine monolayer. We supposed that thionine units exited in the poly(thionine) film could be candidates for the mediators; in that case, HRP catalyzed the H_2O_2 reduction according to the following schemes [30,31]:

$$\mathrm{HRP(Fe^{3+})} + \mathrm{H_2O_2} \rightarrow \text{Compound I} + \mathrm{H_2O}$$

$$\text{Compound I} + \text{poly(thionine)(Red)} \rightarrow \text{Compound II} + \text{poly(thionine)(Ox)}^*$$

$$\text{Compound II} + \text{poly(thionine)(Ox)}^* \rightarrow \mathrm{HRP(Fe^{3+})} + \text{poly(thionine)(Ox)}$$

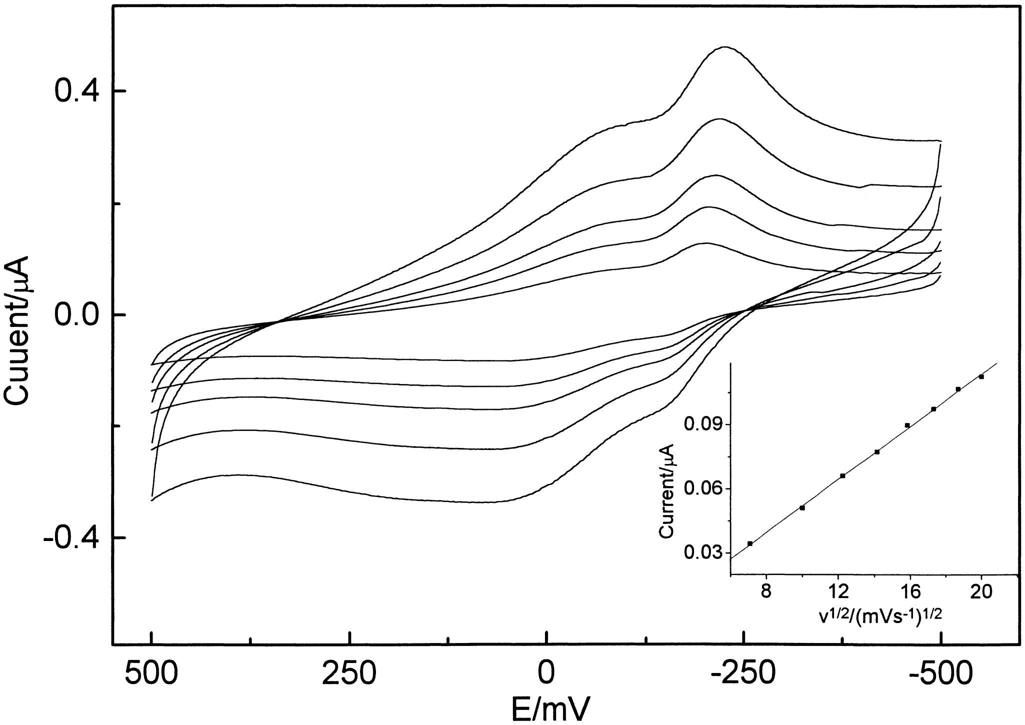

Fig. 2. Cyclic voltammograms of the HRP/poly(thionine) modified electrode in pH 7.0 PB at 50, 100, 150, 250 and 400 mV s^{-1}. Inset: plot of cathodic peak current vs. $v^{1/2}$.

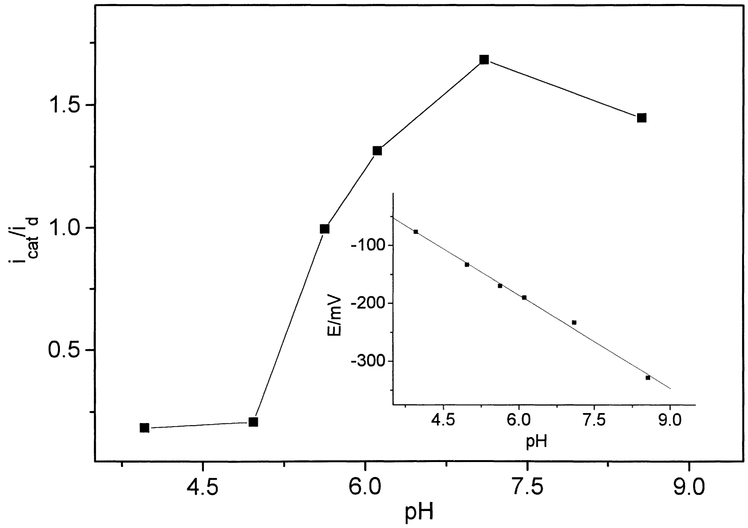

Fig. 3. Effect of pH on the amperometric response (i_{cat}/i_d) at the HRP/poly(thionine) modified electrode in PB with various pHs in the presence of 0.19 mM H_2O_2. Inset: plot of cathodic peak potential vs. pH.

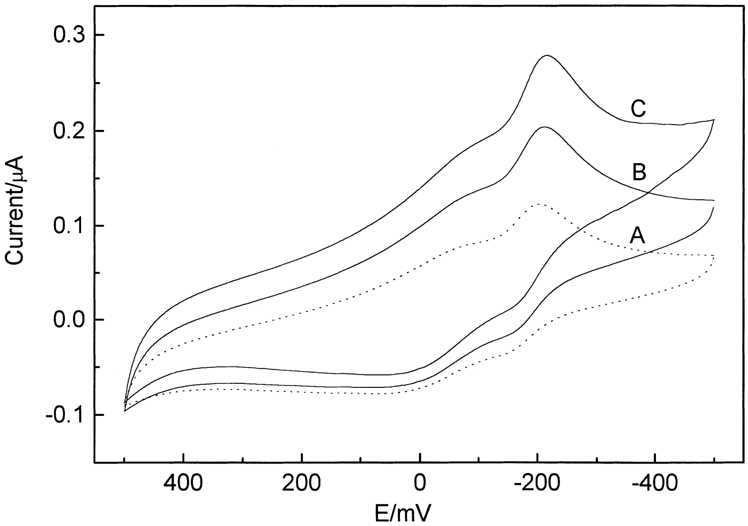

Fig. 4. Cyclic voltammograms of the HRP/poly(thionine) modified electrode in pH 7.0 PB at 50 mV s^{-1} in the absence of H_2O_2 (A) and in the presence of 0.16 mM H_2O_2 (B) and 1.4 mM H_2O_2 (C).

$$\mathrm{poly(thionine)(Ox)} + 2e^- + 2H^+ \rightarrow \mathrm{poly(thionine)(Red)}$$

The cathodic current increased with increasing scan rate. However, when the scan rate was greater than 80 mV s^{-1}, the oxidation peak current rose again, which resulted from the direct oxidation of the remaining reduced form of poly(thionine) at the electrode surface. Thus, the oxidation rate of poly(thionine) by the oxidized HRP was also rather limited [32].

3.3. Determination of H_2O_2 and apparent Michaelis–Menten constant

The dependence of the steady-state current on the applied voltage is shown in Fig. 5. The steady-state current gradually increases with decreasing negatively applied voltage from −50 to −300 mV, indicating that the more negatively applied voltage facilitates the reduction of the oxidized state of poly(thionine). Thereby, the ability of mediating the electron transfer between compound I and II is increased. We have chosen a working potential of −100 mV for the amperometric determination of H_2O_2. A more positively applied voltage could avoid or decrease interference of oxygen and other electroactive substances in the solution.

The chronoamperomeric curves of the HRP/poly(thionine) modified electrode with successive additions of H_2O_2 are shown in Fig. 6. The excellent bioelectrocatalytic response of the HRP/poly(thionine) modified electrode to the reduction of H_2O_2 is shown in Fig. 6A(a), together with the poly(thionine) modified electrode without the entrapment of HRP (Fig. 6A(b)). These results indicated that the amperometric response of the HRP/poly(thionine) modified electrode resulted from only the catalytic activity of HRP entrapped into the polymer film. A considerably faster response was obtained, with a 95% of steady-state current being achieved in less than 5 s, for the H_2O_2 concentration at 13 mM. There was a linear relation between the steady-state current and H_2O_2 concentration in the range of 6.2 µM–9.4 mM with a correlation coefficient of 0.999 (n=17). The detection limit of 3 µM was estimated at a signal-to-noise ratio of 3. The sensor showed good reproducibility for the determination of H_2O_2 concentration in its linear range. The variation coefficients were 3.8% and 4.5% for over 10 successive assays at the H_2O_2 concentration of 93 µM and 2.8 mM, respectively. By a calibration curve method, the recoveries of six H_2O_2 samples with concentrations from 78 µM to 5.1 mM were listed in Table 1. The recovery of the H_2O_2 sensor was in the range of 89% to 97%.

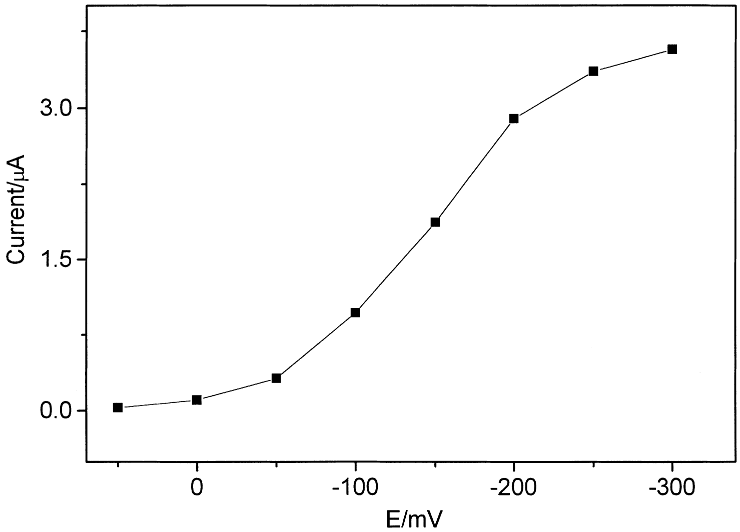

Fig. 5. Dependence of steady-state current on applied voltage at the HRP/poly(thionine) modified electrode in pH 7.0 PB in the presence of 39 µM H_2O_2.

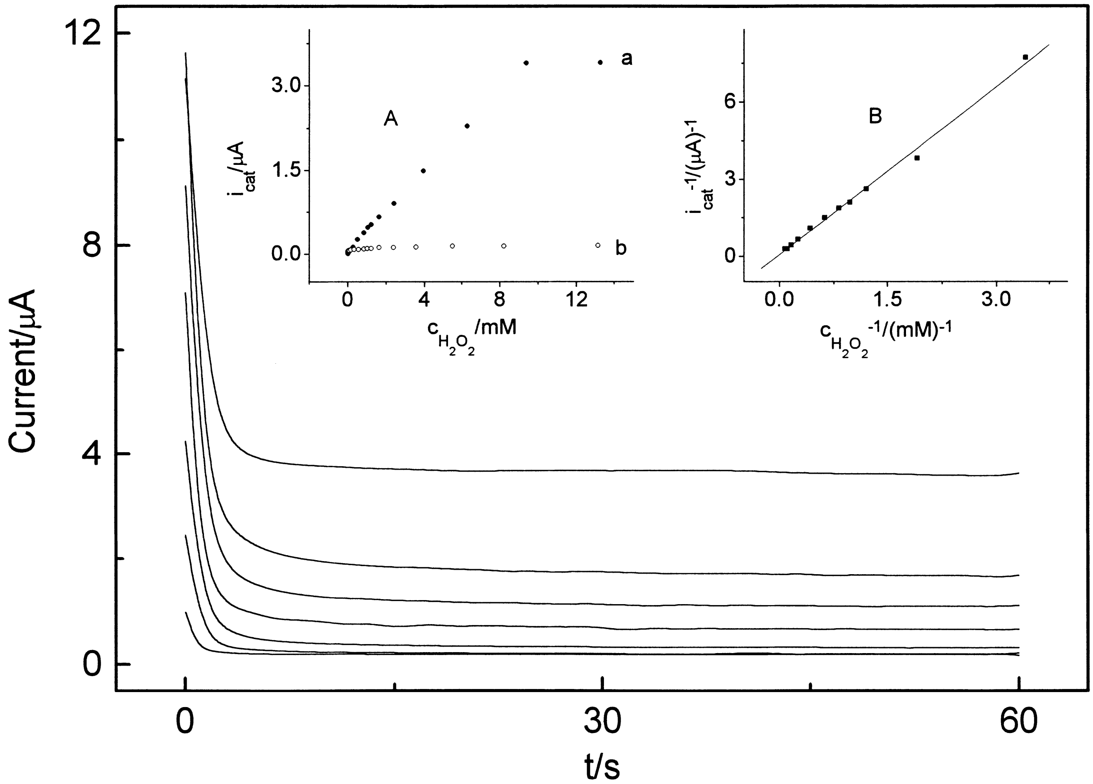

Fig. 6. Amperometric response of H_2O_2 sensor in pH 7.0 PB containing 0, 0.0062, 0.14, 0.84, 1.6, 2.4 and 6.3 mM H_2O_2 (from bottom to top) under steady-state condition. Inset: (A) plot of catalytic current vs. H_2O_2 concentration at the poly(thionine) modified electrode (a) with and (b) without the entrapment of HRP; (B) plot of i_{cat}^{-1} vs. reciprocal of H_2O_2 concentration.

Table 1
The recoveries of H_2O_2 determined at the HRP/poly(thionine) modified electrode

Added (mM)	0.078	0.23	0.47	1.2	2.8	5.1
Found[a] (mM)	0.076	0.21	0.42	1.1	2.5	4.7
Recovery (%)	97	91	89	92	89	92

[a] mean of three determinations.

When the concentration of H_2O_2 was higher than 9.4 mM, a plateau was observed, showing a characteristic of Michealis–Menten kinetic mechanism. The apparent Michealis–Menten constant (K_m^{app}) was determined by plotting the reciprocal of the steady-state current versus the reciprocal of H_2O_2 concentration [33] (Fig. 6(B)). The K_m^{app} value for the HRP/poly(thionine) modified electrode was found to be 28 mM. This value was higher than that obtained for the free enzyme ($K_m = 11$ mM), and markedly higher than that reported by Li and co-workers [29] for a sol–gel immobilized HRP biosensor, indicating that the affinity of the immobilized HRP to H_2O_2 was reduced presumably because of the presence of the poly(thionine) film.

3.4. Stability of the HRP/poly(thionine) electrode

The stability of the HRP/poly(thionine) electrode was studied in the linear range of H_2O_2. When the H_2O_2 sensor was stored in a pH 7.0 phosphate buffer for at least two weeks at 4°C, the sensor retained >85% of its initial sensitivity to H_2O_2. The deterioration of the sensor may be due to the deactivation of HRP. To maintain the bioactivity of HRP over a longer period of time, high concentrations of H_2O_2 (>10 mM) should avoided.

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