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A novel protocol for covalent immobilization of thionine on glassy carbon electrode and its application in hydrogen peroxide biosensor

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ABSTRACT

A novel protocol for effectively covalent immobilization of thionine (Th) was proposed, which was based on Schiff-base reaction between $-NH_2$ of Th and –COH which was in situ generated on glassy carbon electrode (GCE) via simple potentiostatic activation in diluted nitric acid. GCE pretreated by potentiostatic activation possessed CHO-riched surface and microporous structure with high distribution density of electron transfer sites, and thus it became a good candidate for effective immobilization of Th through imine bond with high stability. The application of the resulting Th modified electrode in hydrogen peroxide biosensor was also investigated and it exhibited rapid response to H_2O_2 within 3 s. The linear calibration ranged from 5.0×10^{-7} to 5.8×10^{-3} M with a detection limit of 1.0×10^{-7} M. The effective immobilization of Th on potentiostatically activated GCE surface has deep significance in mediator immobilization, on which further researches based are under way.

1. Introduction

Electron transfer between an electrode and the redox center of an enzyme is the basis for developing various enzyme-based biosensors (Ruan et al., 1998a). To achieve this goal, there are two typical methods. The commonly adopted strategy is to realize the direct electron transfer from the redox center of the enzyme to electrode with the assistance of nanoparticles (Wang et al., 2008), nanotubes (Yang et al., 2008), conductive polymers (Komathia et al., 2009), and ion liquids (Sun et al., 2007a), however, the sensitivity and detect limit are not ideal. Since direct electron transfer is relatively difficult to realize for most enzymes as their redox centers are located deep inside the insulated protein shells, mediators can establish an electrical communication between the redox centers and the electrode. Mediated electrochemical biosensor, the so-called second-generation biosensor, is always the focus people pay attention to. Furthermore, mediated enzyme-based biosensors display high sensitivity and low detect limit. The electron-shuttling mediators, hexacyanoferrates (Schubert et al., 1991), ferrocene and its derivatives (Hickman et al., 1991; Chidsey et al., 1990), tetrathiafulvalene (Wendzinski et al., 1997), quinine (Katz et al., 1994), o-phenylenediimine (Wang et al., 1991), meldola blue (Liu et al., 1996), methylene green (Lei and Deng, 1996), and thionine (Th) (Xu et al., 1998) have been extensively studied.

Th, one of the family of thiazine dyes, is particularly attractive among the mediators. As an electroactive and photoactive molecule, Th has achieved more attention in both electrochemical biosensors (Ou et al., 2007; Yang et al., 2004; Huang et al., 2005) and photochemical biosensors (Deng et al., 2008). In most Th-mediated electrochemical biosensors, Th was either used in substrate solution or immobilized on electrode surface. As the mediator presents in solution, the electrodes are unstable and irreproducible for continuous use (Xu et al., 1998). As a result, immobilization of Th on electrode surface has become a trend. As to the strategy for modifying Th on underlying substrate, there has been various methods, such as layer-by-layer, (Deng et al., 2008), electrostatic interaction (Shahrokhian and Zare-Mehrjardi, 2007), adsorption (Li et al., 2004), electropolymerization (Dempsey et al., 2004), sol–gel (Ding et al., 2004) and so on. It cannot be ignored that Th immobilized according to above protocols would suffer from loss as Th can easily diffuse from electrode surface to bulk solution. It is important to establish new ways to realize the effective immobilization of Th. Covalent linking can overcome this disadvantage. There are two typical ways for covalent immobilization of Th, including Schiff-base reaction with glutaraldehyde as the cross-linker (Ruan et al., 1998a,b; Xu et al., 1998; Zhang and Gorski, 2005; Chen et al., 2008a) and carbodiimide reaction (Shobha Jeykumari et al., 2007; Wu et al., 2007). Covalent attachment is preferable

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for its stability, however, glutaraldehyde may lead to low coupling efficiency and carbodiimide reaction often includes tedious procedures.

Usually, glassy carbon electrode (GCE) should be pretreated in order to obtain reproducible surface and improved electron transfer. The commonly used methods include mechanical polishing (Zhang and Coury, 1993), laser activation (Strein and Ewing, 1991), and electrochemical activation. It has been reported electrochemically pretreated GCE surface possessed microporous structure (Nagaoka and Yoshino, 1986) and oxygen-containing functionalities, including –COOH, –COH, $-C_6H_5OH$, –OH, etc. (Shi et al., 2007; Chen et al., 2009). The in situ generated –COH may has high activity towards $-NH_2$ to form imine bond and has the potential to be used in the immobilization of Th.

In this paper, Th was covalently immobilized on GCE surface which was pretreated by simple potentiostatic activation in diluted nitric acid (HNO_3). The $-NH_2$ of Th reacted with –COH in situ generated during the electrochemical activation process to form imine bond. Due to the microporous structure and in situ generated –COH of the electrode surface, the amount and stability of immobilized Th were highly strengthened. The electrochemical property of the as-prepared Th–COH/GCE was also demonstrated by its obvious electrochemical catalysis towards H_2O_2. The biosensor exhibited fast amperometric response to H_2O_2 with wide linear range, low detection limit, high sensitivity, good reproducibility, and long-term stability.

2. Experimental

2.1. Reagents

Thionine (Th) was purchased from Guo Yao Company (China). Phosphate buffer solutions (PBS) were prepared by mixing standard stock solutions of Na_2HPO_4 and NaH_2PO_4 and adjusted with HCl or NaOH to various pH values. All other chemicals were of analytical reagent grade and double distilled water (DDW) was used throughout the measurements.

2.2. Apparatus

Cyclic voltammetric (CV) and amperometric measurements were performed with a CHI 832B electrochemical analyzer (Shanghai CH Instrument Company, China). Electrochemical impedance spectroscopy (EIS) was performed on a CHI 660C electrochemical analyzer (Shanghai CH Instrument Company, China). A conventional three-electrode system was employed with GCE or modified GCE as the working electrode, Pt wire and Ag/AgCl (saturated KCl) as auxiliary and reference electrodes, respectively. All experiments were carried out under the protection of high-purity nitrogen.

2.3. Potentiostatic activation of GCE and immobilized Th on the pretreated GCE surface

Prior to modification, GCEs were polished with 1.0, 0.3, and 0.05 μm alumina slurry, respectively. Then, the electrodes were rinsed thoroughly with DDW between each polishing step and were cleaned by ultrasonication. Potentiostatic activation was carried out by anodization of the polished electrodes in 0.1 M HNO_3 at 1.8 V for 3 min, and then cathodization at −1.5 V for 1 min. For covalent immobilization of Th, the obtained electrode above was immersed in 2 mg/mL Th solution (0.1 M PBS pH 7.0) for 1 h, and then washed by ultrasonication for 10 min, followed by rinsing with DDW to remove non-specifically adsorbed Th. The as-prepared electrode was denoted as Th–COH/GCE and stored at 4 °C when not in use. For comparison, unpretreated GCE immersed in the same Th solution for the same time was also prepared.

3. Results and discussion

3.1. Immobilization of Th on potentiostatically activated GCE surface

Herein, a simple but effective protocol of covalent immobilization of Th was proposed, which was based on Schiff-base reaction between $-NH_2$ of Th and –COH in situ generated on GCE surface via a potentiostatic activation process in diluted HNO_3. Schematic representation of the fabrication process is given in Fig. 1. Potentiostatically activated GCE surface possessed microporous structure (Nagaoka and Yoshino, 1986), leading to an increased electrode area available for loading of Th. The in situ generated –COH reacted with Th to form imine bond, leading to stable attachment of Th on electrode surface. Thus the amount and stability of immobilized Th can be highly strengthened.

3.2. Characterization

CV was employed to probe the electrochemical characteristics of modified electrodes during stepwise modification. Fig. 2 shows CVs of different modified electrodes over the potential range from 0.2 to −0.6 V in 0.1 M PBS (pH 6.0) containing 0.1 M KCl at a scan rate 50 mV s^{-1}. Compared with bare GCE (Fig. 2 a), there was very small redox peaks of Th appearing at the unpretreated electrode immersion in Th solution for 1 h (Fig. 2b). The peaks disappeared in the subsequent scans, which can be attributed to physical adsorption towards Th. After the GCE was electrochemically activated, the resulting electrode had larger background current (Fig. 2c), which is probably due to the significant increment of electrode surface area as a result of the microporous structure formed during the pretreatment process. Obviously, a pair of well-defined redox peaks appeared at Th–COH/GCE (Fig. 2d), with the anodic (Epa) and cathodic potential (Epc) of −0.18 and −0.22 V, respectively. The peak-to-peak separation (ΔEp) is 40 mV, indicating a fast electron transfer process. This could be definitely attributed to the redox reaction of Th, which demonstrated the successful immobilization of Th on the surface of pretreated electrode.

EIS is a powerful tool for probing the interface features of the modified electrodes. Fig. 3 shows the typical Nyquist plots obtained on the bare GCE (a), the pretreated GCE (b), and Th–COH/GCE (c) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6^{3-}$ and 1 mM $Fe(CN)_6^{4-}$. For the bare GCE (Fig. 3a), the value of electron transfer resistance (R_{et}) was found to be 400 Ω. After electrochemical activation, the pretreated electrode exhibited an almost straight line (Fig. 3b), which was just the characteristic of the diffusion limiting step of the electrochemical process (Chen et al., 2008b), indicating that the resulting electrode represented high conductivity due to its microporous architecture. When Th was covalently immobilized onto the surface of pretreated GCE, as we expected, the value of R_{et} further decreased (Fig. 3c), due to the strong electrostatic adsorption between the negatively charged $Fe(CN)_6^{3-/4-}$ and the positively charged Th molecules.

The effect of scan rate on the response of immobilized Th at the Th–COH/GCE was also investigated (see supplementary material). It was obvious that CVs of Th–COH/GCE gave nearly symmetric anodic and cathodic peaks. With the increasing of scan rate, anodic peak potential and cathodic peak potential were shifted slightly toward the positive and the negative direction of potential, respectively. Both the anodic and the cathodic peak currents of the Th–COH/GCE were proportional to the scan rate between

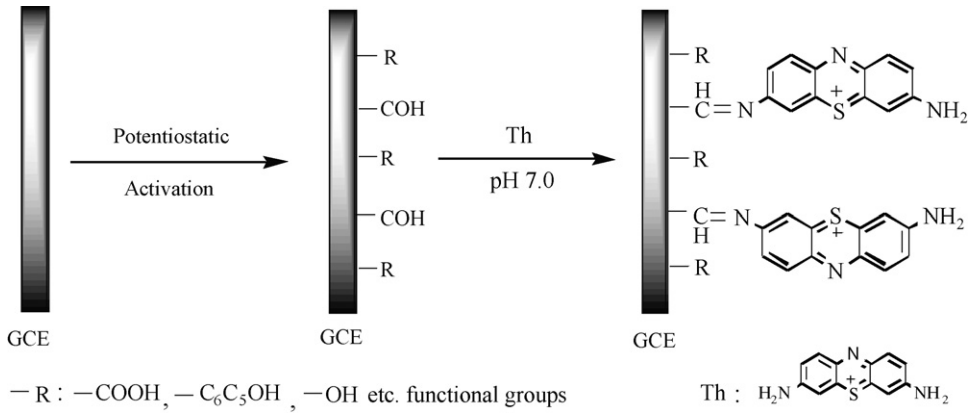


Fig. 1. Schematic representation for the immobilization of Th onto GCE.

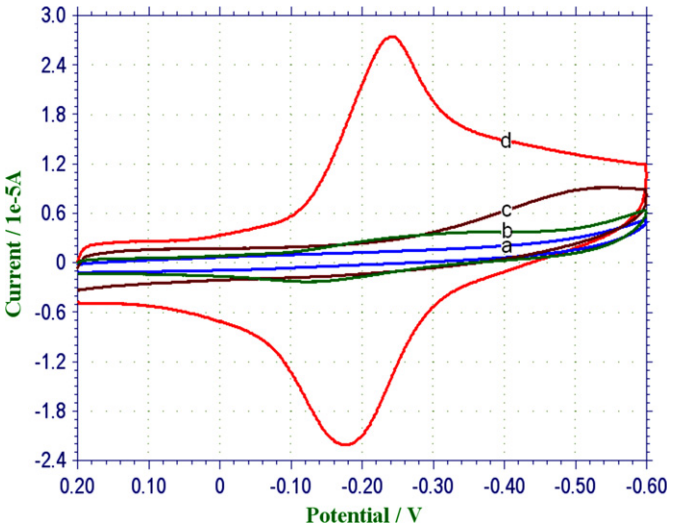


Fig. 2. CVs of (a) bare GCE, (b) the unpretreated GCE immersion in Th solution for 1 h, (c) the pretreated GCE, and (d) Th–COH/GCE in 0.1 M PBS (pH 6.0) at a scan rate of 50 mV s^{-1}.

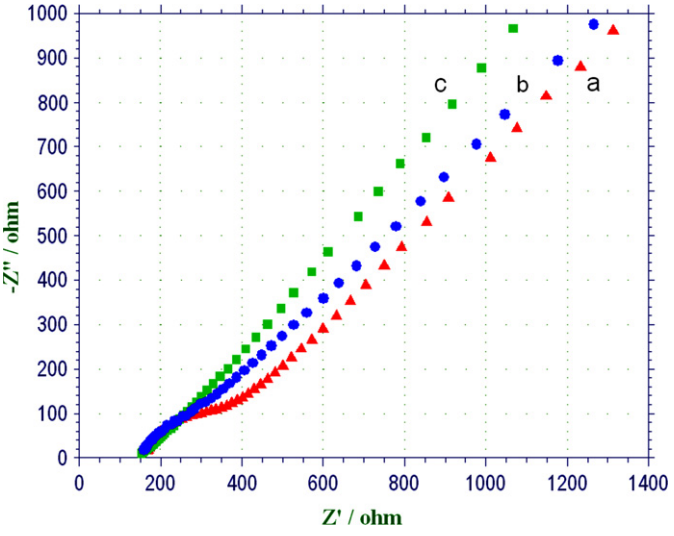


Fig. 3. EIS for (a) the bare GCE, (b) the pretreated GCE and (c) Th–COH/GCE in a solution of 0.1 M KCl containing 1.0 mM $Fe(CN)_6^{3-}$ and 1.0 mM $Fe(CN)_6^{4-}$.

30 and 400 mV s^{-1}, indicating a surface-controlled electrode process.

Fig. 4 depicts the electrocatalytic response of Th–COH/GCE in 0.1 M PBS (pH 6.0) without (a) and with (b) 2.0 mM H_2O_2 at a scan rate of 50 mV s^{-1}. Upon the addition of H_2O_2 to the substrate solution, the reduction peak current increased, while the oxidation current decreased (Fig. 4b), suggesting a typical electrocatalytic reduction process towards H_2O_2.

3.3. Influence of pH and applied potential on the developed biosensor responses

Th is a pH-dependent dye (Nicotra et al., 2008), so the electrochemical behavior of Th modification on GCE can be influenced by solution pH. CVs of the Th–COH/GCE in 0.1 M PBS from pH 3.0 to 8.0 at a scan rate of 50 mV s^{-1} are shown in Fig. 5. It was found that both Epc and Epa shifted to a negative direction with the increase of pH, but the ΔEp changed slightly, indicating a good reversibility of Th on the well-confined surface. The redox current increased from pH 3.0 and reached a maximum at pH 6.0. Then, the current response was found to be decreased from pH 7.0 to 8.0. E_0 which is estimated as the midpoint between Epa and Epc had a linear relationship with pH from 3.0 to 5.0 with a slope of −57.5 mV pH^{-1}, which was very close to the anticipated Nernstian value of 59 mV,

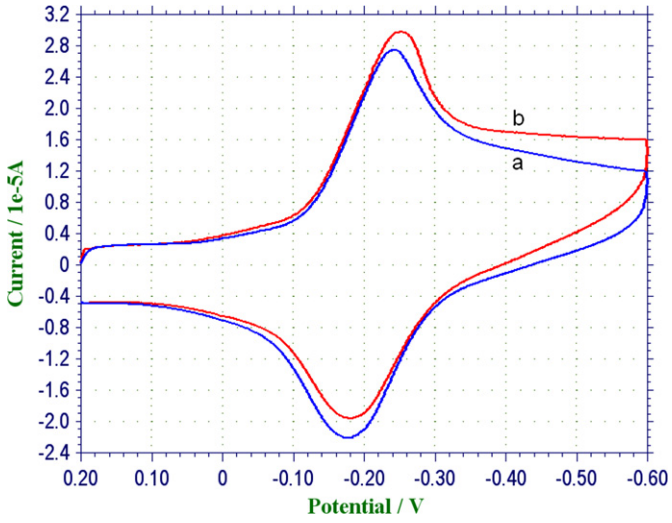


Fig. 4. CVs of the Th–COH/GCE (a) without H_2O_2 and (b) with 2.0 mM H_2O_2 in 0.1 M PBS (pH 6.0) at a scan rate of 50 mV s^{-1}.

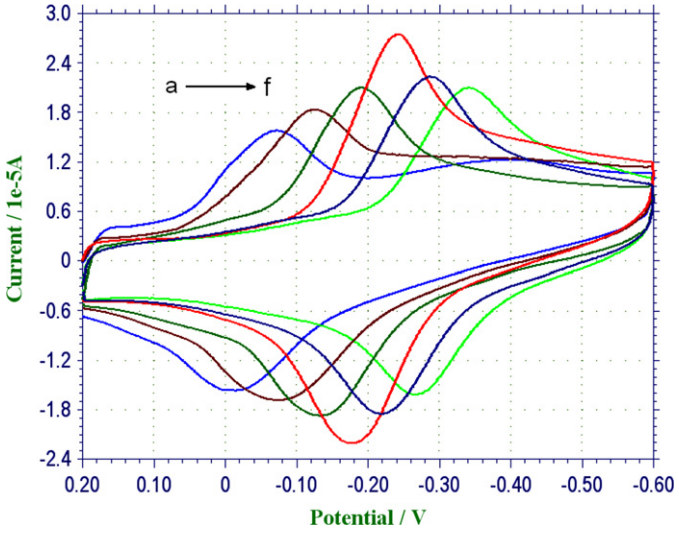


Fig. 5. CVs of the Th–COH/GCE in 0.1 M PBS containing 0.1 M KCl at pH (a) 3.0, (b) 4.0, (c) 5.0, (d) 6.0, (e) 7.0, (f) 8.0, and (g) 9.0 at a scan rate of 50 mV s^{-1}.

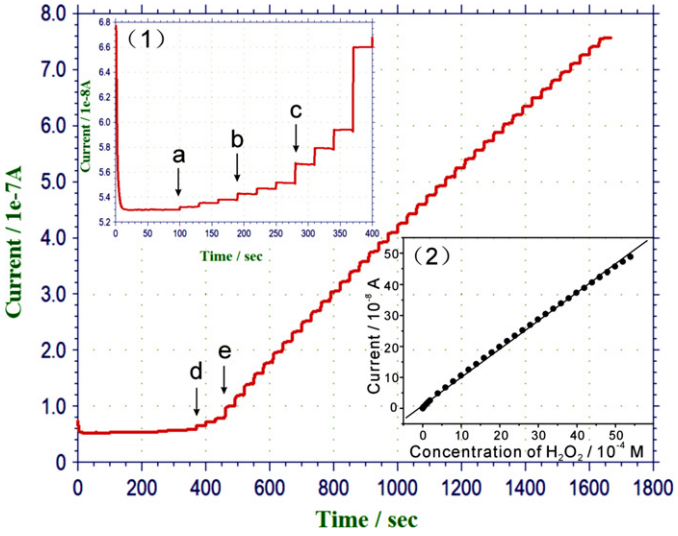
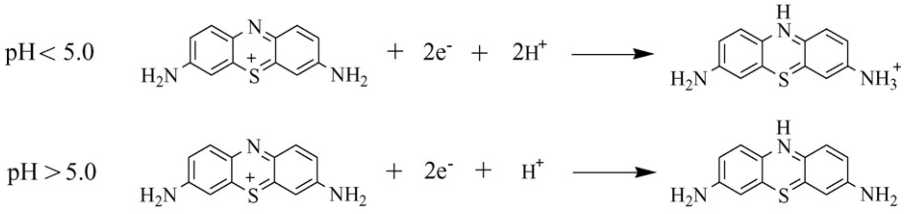


Fig. 6. Typical amperometric response of the Th–COH/GCE to successive addition of H_2O_2 (a) 0.5 μM, (b) 2.0 μM, (c) 10.0 μM, (d) 50 μM, (e) 200 μM into stirring 0.1 M PBS (pH 6.0) containing 0.1 M KCl. Inset showed the amperometric response to H_2O_2 in 400 s (1) and the linear part of the calibration curve between the current and the concentration of H_2O_2 (2).

indicating a reversible two protons coupled two electrons transfer during electrochemical reduction (Sun et al., 2007b). Besides, another straight line with a slope −46 mV pH^{-1} was obtained from pH 5.0 to 8.0, indicating a two electrons and one proton process (Clavilier et al., 1995). The electrochemical process of TH modified GCE can be expressed as follows:



Taking the response and the lifetime of the biosensor into consideration, we selected the suitable pH with the maximum response of the Th–COH/GCE at pH 6.0.

The effect of applied potential on the biosensor performance was also investigated from −0.40 to −0.15 V (see supplementary material). The current increased rapidly with the applied potential shifting from −0.40 to −0.25 V. At a potential more negative than −0.25 V, a significant decrease in the current was observed. The current approached a maximum value at −0.25 V, thus an operating potential of −0.25 V was chosen for further amperometric determination of H_2O_2.

3.4. Amperometric response of the developed biosensor

Amperometric response of the Th–COH/GCE to successive addition of H_2O_2 in 0.1 M PBS (pH 6.0) with an applied potential of −0.25 V is given in Fig. 6. Upon addition of an aliquot of H_2O_2 to the stirring solution, the reduction current increased steeply to reach a stable value. The modified electrode achieved 95% of the maximum steady-state current in less than 3 s (Fig. 6(1)). Such a rapid response could attribute to the fast diffusion of H_2O_2 to the porous surface of the pretreated GCE. Under the optimized experimental conditions, the proposed biosensor displayed increasing amperometric responses to H_2O_2 with very wide linear range from 5.0×10^{-7} to 5.8×10^{-3} M with a relative coefficient 0.9965 ($n = 40$, inset (2) in Fig. 6). The linear range of four orders of magnitude of the developed biosensor can be comparable to or even be wider than some enzyme-based biosensors (Xu et al., 2006; Zhao et al., 2008; Kafi et al., 2008; Wang et al., 2009). The detection limit was estimated to be 1.0×10^{-7} M defined from a signal-to-noise ratio of 3.

3.5. Reproducibility and stability

The repeatability and stability of the proposed biosensor were examined by amperometric measurements in the presence of 1.0 mM H_2O_2. The proposed biosensor showed an acceptable repeatability with a relative standard deviation (R.S.D.) of 2.6% for 11 successive assays. The fabrication reproducibility was investigated by preparing six biosensors independently using the same procedure and a R.S.D. of 3.1% was obtained. The long-term stability of the electrode was investigated over a 30-day period and the biosensor retained more than 98.4% of its initial response to the reduction of H_2O_2, which may be attributed to the covalent immobilization of Th on electrode surface, leading to avoidance of suffering from loss.

4. Conclusions

Electroactive Th molecule was successfully immobilized on potentiostatically activated GCE surface through imine bond. The resulting CHO-riched and microporous structure of pretreated electrode surface allows efficient electron transfer, wide linear range, low detection limit, high sensitivity, good reproducibility and long-term stability of the fabricated biosensor. The proposed protocol is simple, enzymeless, but effective. The constructed sensing plat-

form has potential in H_2O_2 determination and a series of biosensors fabrication with Th as the mediator.

Acknowledgements

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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.2010.03.027.

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