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Electrochemical sensing platform based on covalent immobilization of thionine onto gold electrode surface via diazotization-coupling reaction

Feng Li*, Yan Feng, Limin Yang, Shufeng Liu*

Shandong Provincial Key Laboratory of Biochemical Analysis, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao 266042, PR China

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ABSTRACT

A novel electrochemical sensing platform by modification of electroactive thionine (Th) onto gold electrode surface was constructed, which was realized by diazotization of 4-aminothiophenol (ATP) self-assembled monolayer, followed by coupling of Th with the diazonium group to form a covalent diazo bond. A pair of well-defined redox peaks of Th was observed in the cyclic voltammetric measurement. The resulting diazo-ATP monolayer displayed superior electrical conductivity, which contributed to the sensitive detection of hydrogen peroxide (H_2O_2). The immobilized Th also showed a remarkable stability, which may benefit from the π–π stacking force and the covalent diazo bond between diazo-ATP and Th molecules. Under the optimized experimental conditions, the current fabricated non-enzyme and reagentless sensor could show a rapid response to H_2O_2 within 3 s and a linear calibration plot ranged from 1.0×10^{-6} to 6.38×10^{-3} M with a detection limit of 6.7×10^{-7} M. The current fabrication strategy of electroactive interface is expected to be used as a versatile route for the immobilization of more electroactive molecules and offer more opportunities for the applications in electrochemical sensor, biosensor, electrocatalysis, etc.

1. Introduction

Since direct electron transfer is usually difficult to realize between redox enzymes and the electrode surface, the participation of mediators can easily establish the electrical communication between the redox center of enzymes and the electrode due to their excellent electron transfer ability. Mediated electrochemical sensors also have the advantages of higher sensitivity, lower detection limit, lower operational potential, and less interference from oxidizable species over unmediated sensors [1]. Thus, the well-confinement of electron mediators on the electrode surface is of mandatory importance for the development of mediator-based electrochemical sensors, especially for the achievement of electrochemical enzyme biosensors.

Various strategies have been designed to entrap or bind different electron mediators on the electrode surface. These include direct electrochemical polymerization [2,3], electrostatic interaction [4,5], adsorption [6], covalent binding [7,8], etc. Among the various mediators, thionine (Th) is particular attractive and has received much attention in both electrochemical [1,10,9] and photochemical sensors [11–14]. Th is a small planar molecule and contains one heterocyclic nitrogen atom and two amine groups symmetrically distributed on each side. Various strategies have been devised to confine Th onto the electrode surface for the achievement of electrochemical sensor fabrication. For example, Yuan and co-workers developed a novel amperometric immunosensor based on layer-by-layer assembly of gold nanoparticles and multi-walled carbon nanotubes-Th multilayer films on polyelectrolyte surface [11]. Niu group prepared nanotube/Th/Au composite film by electrostatic interaction, and then incorporated oppositely charged horseradish peroxidase (HRP) into the composite film to fabricate a H_2O_2 biosensor [1]. Recently, Shen and co-workers are devoted to exploiting the electropolymerization of Th for the application in biosensor fabrication [15,2]. Ding group employed a sol–gel method to trap Th in silica solid matrix and explored it for solid-state optical sensor applications [13].

In the above immobilization strategies, Th was co-immobilized with nanomaterials to improve the electrical conductivity of the system or with enzymes to enhance the electocatalysis capability. However, the participation of nanomaterials and/or enzymes makes the detection procedures become complicated, and the dissociation of Th from electrode surface to bulk solution cannot be neglected. Covalent binding is always preferable due to its intrinsic stability. It is common to see Th was immobilized on underlying substrates by using glutaraldehyde via Schiff-base reaction [16] or using EDC/NHS via carbodiimide reaction [17], however, the above two methods have the disadvantage of low coupling efficiency or complicated procedure. Therefore, the development of simple and

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* Corresponding authors. Tel.: +86 532 84023927; fax: +86 532 84023927.
E-mail addresses: lifeng@qust.edu.cn (F. Li), shufengliu77@yahoo.com (S. Liu).

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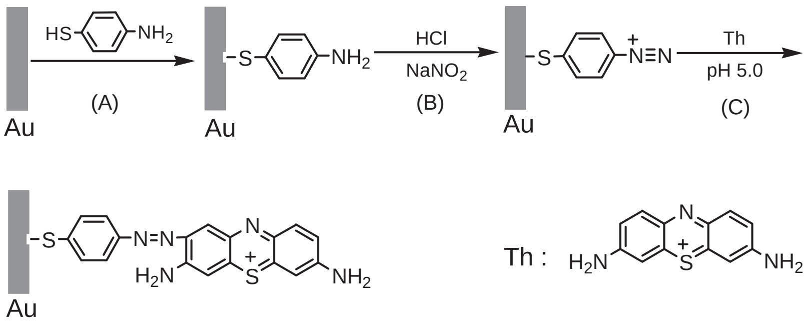

Fig. 1. Schematic representation of the fabrication of H_2O_2 sensor based on covalent immobilization of Th on self-assembled diazo-ATP modified Au electrode by diazotization-coupling reaction.

cost-effective covalent approaches for the stable immobilization of Th on the electrode interface is highly desirable for the applications in electrochemical sensor, biosensor, and electrocatalysis.

Diazonium salts have been received substantial attention in recent years due to its simple procedure but versatile surface derivatization [18–22]. It has been reported that diazonium salts can react with phenolic, imidazole, or amino groups to form covalent diazo bonds for the achievement of different types of surface derivatization [23]. The diazonium salts modified electrode has also been reported to possess high electrical conductibility [19,20]. Inspired by it, the immobilization of electron mediator via a simple diazotization-coupling reaction that could improve the electron transfer ability of electron mediator should be an effective approach toward the fabrication of many electrochemical sensors or biosensors. As a proof of notion, herein, Th was chosen as a model to be covalently immobilized on ATP modified Au electrode surface by a simple diazotization-coupling reaction. The as-prepared Th/diazo-ATP film was demonstrated to have good direct electrocatalysis toward H_2O_2 without the use of peroxidase. The sensor also exhibited fast amperometric response to H_2O_2 with high sensitivity, good reproducibility, and long-term stability.

2. Experimental

2.1. Chemicals and materials

4-Aminothiophenol (ATP) was obtained from Sigma–Aldrich. Th was purchased from Qingdao Guoyao Chemical Reagent Company (China). They can be used without further purification. 0.1 M Phosphate buffer solutions (PBS) were prepared by mixing standard stock solutions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4, and adjusted with HCl or NaOH to various pH values. All other chemicals were of analytical 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) measurement was performed on a CHI 660C electrochemical analyzer (Shanghai CH Instrument Company, China). A conventional three-electrode system was employed with Au electrode or modified Au electrode as the working electrode, Pt wire and Ag/AgCl (saturated with KCl) as the auxiliary and the reference electrode, respectively. Electrochemical experiments were carried out under the atmosphere of high-purity nitrogen.

2.3. Immobilization of Th onto self-assembled ATP monolayer modified electrode via diazotization-coupling reaction

Prior to modification, the working Au electrode was polished to a mirror-like surface with 1.0, 0.3, and 0.05 μm alumina slurry on microcloth pads, and sonicated in ethanol and water, respectively. Then, the Au electrode was dipped in piranha solution (H_2SO_4/H_2O_2, 7:3, v/v) and electrochemically treated by cycling the potential between −0.2 and +1.5 V in 0.5 M H_2SO_4 at a scan rate of 100 mV s^{-1} until a stable CV was obtained.

In present work, the self-assembly of ATP monolayer on a clean Au electrode and its further diazotization was conducted according to the reported procedure in the literature [20]. In brief, the clean Au electrode was firstly immersed in ethanol solution containing 0.5 mM ATP for 10 h to form ATP monolayer modified electrode. Then, the obtained electrode was washed thoroughly with ethanol and water and then dried. Afterwards, the diazonium derivative was achieved by transferring the ATP modified Au electrode into 0.1 M HCl solution at 2–4 °C, and 100 mg $NaNO_2$ was slowly added to give a total concentration of about 0.05 M. After 30 min incubation, the Au electrode was removed and immediately rinsed with icy water [22]. For covalent coupling of Th, the above obtained electrode was immersed in 2 mM Th (0.1 M PBS pH 5.0) solution at 2–4 °C for 30 min, and then rinsed with DDW to remove non-specifically adsorbed Th. The as-prepared electrode was denoted as Th/diazo-ATP/Au and stored at 4 °C when not in use.

3. Results and discussion

3.1. Immobilization of Th onto self-assembled ATP monolayer modified electrode via diazotization-coupling reaction

Schematic representation of the fabrication of H_2O_2 sensor is given in Fig. 1. As seen, three steps were involved in the covalent immobilization of Th. Firstly, the monolayer of ATP was formed on the electrode surface via the self-assembly technique [22]. Secondly, the amine group of self-assembled ATP monolayer on Au electrode was converted into diazo-derivation. Finally, the diazonium groups were reacted with Th to form covalent diazo bonds for the achievement of Th immobilization. Usually, the diazonium derivative is relatively poor in stability, but the formation of diazo bonds with Th could improve the stability remarkably, which is probably due to the π–π stacking force between diazo-ATP and Th molecules.

3.2. Electrochemical characterization of the fabricated H_2O_2 sensor

The electrochemical performance of the fabricated sensor during stepwise modification was evaluated by CV. Fig. 2 shows typical

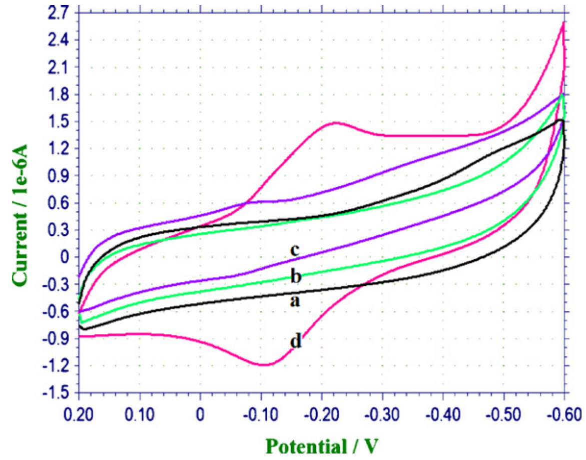

Fig. 2. CVs of bare Au electrode (a), ATP/Au (b), diazo-ATP/Au (c), and Th/diazo-ATP/Au (d) in 0.1 M PBS (pH 6.0) at a scan rate of 50 mV s^{-1}.

CVs obtained for bare Au electrode (a), ATP/Au (b), diazo-ATP/Au (c), and Th/diazo-ATP/Au (d) in 0.1 M PBS (pH 6.0) at a scan rate of 50 mV s^{-1}. No redox peaks were observed at the bare Au (Fig. 2a) and ATP/Au (Fig. 2b) electrodes over the working potential window from 0.2 to −0.6 V. After diazo-derivation of the self-assembled ATP monolayer, a pair of small peaks appeared at around −82 mV (Fig. 2c), which could be ascribed to the reduction and oxidation of diazonium group. When Th was covalently immobilized onto diazonium functionalized Au electrode, a pair of stable and well-defined redox peaks corresponding to the oxidation and reduction of Th was observed (Fig. 2d), with the anodic peak potential (Epa) and cathodic peak potential (Epc) positioned at −118 and −228 mV, respectively. It also strongly indicated that Th had been immobilized onto diazo-ATP film effectively by the current method.

EIS could provide further information on the impedance changes of the electrode surface during the modification process. Fig. 3 shows the Nyquist plots obtained on bare Au electrode (a), ATP/Au (b), diazo-ATP/Au (c), and Th/diazo-ATP/Au (d) in a solution of 0.1 M KCl containing 1 mM $Fe(CN)_6{}^{3-}$ and 1 mM $Fe(CN)_6{}^{4-}$. As seen, significant differences in the electron transfer resistance (*R*et) were observed upon the stepwise modification. For comparison, the *R*et value of ATP/Au (Fig. 3b) was found to be 610 Ω, which is much larger than that of bare Au electrode (Fig. 3a), implying that the self-assembled ATP film could obstruct the electron transfer from the electrochemical probe toward electrode surface. After diazo-derivatization, the plot of diazo-ATP/Au was almost a straight line (Fig. 3c), which was consistent with the result in our previous report [19,20], indicating that the diazo-ATP film had an excellent conductivity for easier electron transfer between the electrode surface and the redox probe. As is expected, the covalent couple of Th to diazo-ATP resulted in a slight decrease of the value of *R*et to 0.005 Ω (Fig. 3d), which further demonstrated that Th molecules had been successfully attached to the electrode surface. The decrease of *R*et valve may be ascribed to the strong electrostatic adsorption between the positively charged Th and negatively charged $Fe(CN)_6{}^{3-/4-}$ redox couple.

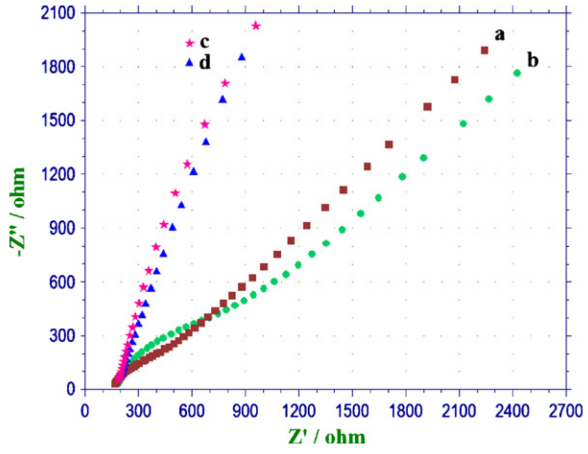

Fig. 3. EIS for bare Au electrode (a), ATP/Au (b), diazo-ATP/Au (c), and Th/diazo-ATP/Au (d) in a solution of 0.1 M KCl containing 1.0 mM $Fe(CN)_6{}^{3-}$ and 1.0 mM $Fe(CN)_6{}^{4-}$.

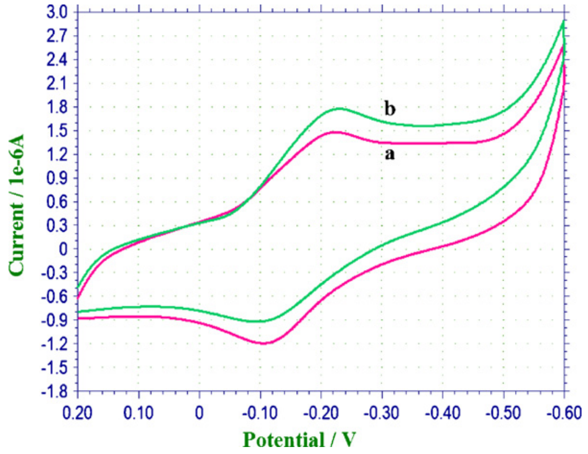

Fig. 4. CVs of Th/diazo-ATP/Au (a) without H_2O_2 and (b) with 1 mM H_2O_2 in 0.1 M PBS (pH 6.0) at a scan rate of 50 mV s^{-1}.

The effect of scan rate on the response of immobilized Th at the Th/diazo-ATP/Au was also investigated (see Supplementary material Fig. S1). With the increase of scan rate, Epa and Epc were shifted slightly toward the positive and the negative direction of potential, respectively. Anodic peak current and cathodic peak current were proportional to the scan rate between 30 and 400 mV s^{-1}, suggesting a surface-controlled electrode process for the immobilized Th.

It had been reported that proteins containing heme groups, such as HRP, hemoglobin, and myoglobin, could be used as a biocatalyst to catalyze the electrochemical reduction of H_2O_2 [24–26]. However, the major drawback of enzymatic biosensors is their lack of stability due to the intrinsic nature of enzymes. To address this problem, many attempts have been made to develop the non-enzyme electrochemical sensors. In current study, the application of the Th modified electrode as a sensor was further investigated for the determination of H_2O_2 by an electrocatalysis mechanism. Fig. 4 shows the CVs of the Th/diazo-ATP/Au (a) without H_2O_2 and (b) with 1 mM H_2O_2 in 0.1 M PBS (pH 6.0) at a scan rate of 50 mV s^{-1}. As shown in Fig. 4, with the addition of H_2O_2, the reduction peak current of Th increased while the oxidation peak current decreased (Fig. 4b), showing a typical electrocatalytic reaction behavior. The mechanism of H_2O_2 catalyzed by Th can be described as follows:

$$\text{thionine (red)} + H_2O_2 \xrightarrow{\text{pH 6.0}} \text{thionine (Ox)} + H_2O$$

$$\text{thionine (Ox)} + 2e^- + H^+ \xrightarrow{\text{pH 6.0}} \text{thionine (red)}$$

3.3. *Influence of pH and applied potential on the sensor responses*

The responses of the fabricated Th/diazo-ATP/Au toward pH changes from pH 3.0 to 8.0 in 0.1 M PBS were also investigated (see Supplementary material Fig. S2). Obviously, both the Epc and Epa shifted to a negative direction with the increase of pH, indicat-

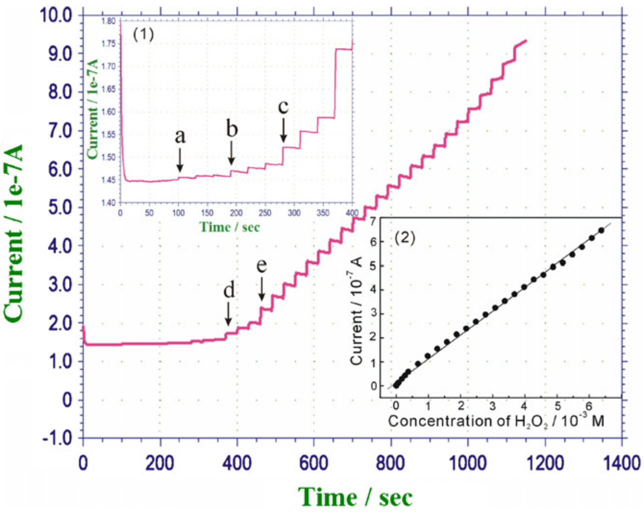

Fig. 5. Typical amperometric response of the Th/diazo-ATP/Au to successive addition of H_2O_2 (a) 1.0, (b) 4.0, (c) 20.0, (d) 100, (e) 300 μM into stirring 0.1 M PBS (pH 6.0). Inset (1) shows the amperometric response to H_2O_2 in 400 s and (2) displays the linear part of the calibration curve between the current and the concentration of H_2O_2.

ing both proton and electron transfer process. 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. It is attributed that appropriate amount of protonized Th assisted the coupling reaction between Th and diazonium salts for the optimized response [27]. Taking the response and the lifetime of the sensor into consideration, we selected pH 6.0 as a suitable solution pH value based on the maximum response of the Th/diazo-ATP/Au.

The applied potential is also a key parameter that could greatly influence the sensor performance. Amperometric responses of the proposed sensor toward H_2O_2 at different potentials were examined and the results revealed that applied potential produced a significant effect on the response of the sensor. With the increase of applied potential from −0.20 V, the current increased and reached a maximum value at −0.25 V. While the applied potential was over −0.40 V, the current response decreased slightly. 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 sensor*

Fig. 5 displays a typical amperometric response of the Th/diazo-ATP/Au electrode on successive addition H_2O_2 into stirring 0.1 M PBS (pH 6.0) at the applied potential of −0.25 V. Inset (1) shows the amplified amperometric response curve to H_2O_2 in 400 s and inset (2) displays calibration curve of the amperometric response of the sensor to the concentration of H_2O_2. The sensor exhibited a rapid response to H_2O_2 within 3 s and a linear calibration plot ranged from 1.0×10^{-6} to 6.38×10^{-3} M with a correlation coefficient of 0.9991, and a detection limit of 6.7×10^{-7} M. The linear range of the developed sensor was wider than some Th based sensor, such as $2.5 \times 10^{-6} \sim 2.8 \times 10^{-3}$ M of Th-NWs/GCE [28] and $1.37 \times 10^{-6} \sim 3.44 \times 10^{-4}$ M of TH/MWCNT/Nf/PIGE [8].

3.5. *Sensor repeatability and stability*

The repeatability and stability of the proposed sensor was examined by amperometric measurements in the presence of 1.0 mM H_2O_2. The sensor showed an acceptable repeatability with a relative standard deviation (RSD) of 2.6% for 11 successive assays. The fabrication reproducibility was investigated by preparing six sensors independently using the same procedure and a RSD of 2.9% was obtained. The stability of the fabricated sensor was also investigated by monitoring the response current in 0.1 M PBS (pH 6.0) containing 1.0 mM H_2O_2 after the sensor stored in a refrigerator for 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150 days. Seen from Fig. 6, the sensor retained more than 97.9% of its initial response to the reduction of H_2O_2 after a 150 days storage. It is probable that the formation of diazo bonds between Th and diazo groups functionalized surface led to effective immobilization and preserved Th suffering from loss.

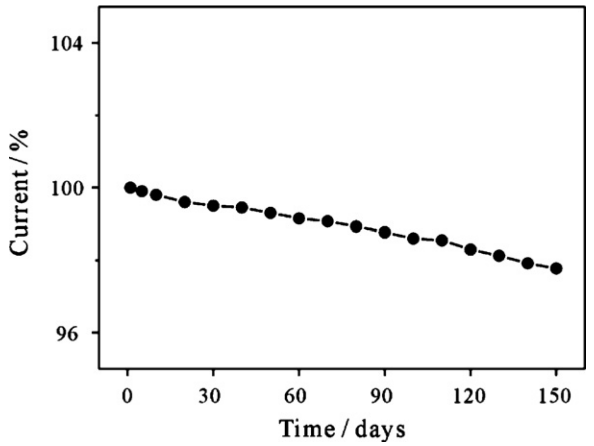

Fig. 6. Changes in current (%) of the sensor monitored in 0.1 M PBS (pH 6.0) containing 1 mM H_2O_2 during 150 days of usage The initial response was set as 100%.

4. Conclusions

In current study, a simple strategy was developed for the effective immobilization of Th on the electrode surface based on diazotization-coupling reaction mechanism. It could be easily extended for the fabrication of stable electroactive interface by other phenoxazine species. The resulting Th/diazo-ATP film exhibited excellent electrical conductivity and displayed rapid, stable and sensitive response toward the electrochemical reduction of H_2O_2. The current non-enzyme fabrication strategy also shows a considerable potential in the electrocatalytic analysis of more biorelevant substances.

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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.talanta.2010.09.007.

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