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Amperometric biosensor for hydrogen peroxide based on hemoglobin entrapped in titania sol–gel film

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Abstract

Hemoglobin (Hb) was entrapped in a titania sol–gel matrix and used as a mimetic peroxidase to construct a novel amperometric biosensor for hydrogen peroxide. The Hb entrapped titania sol–gel film was obtained with a vapor deposition method, which simplified the traditional sol–gel process for protein immobilization. The morphologies of both titania sol–gel and the Hb films were characterized using scanning electron microscopy (SEM) and proved to be chemically clean, porous, homogeneous. This matrix provided a biocompatible microenvironment for retaining the native structure and activity of the entrapped Hb and a very low mass transport barrier to the substrates. H_2O_2 could be reduced by the catalysis of the entrapped hemoglobin at −300 mV without any mediator. The reagentless H_2O_2 sensor exhibited a fast response (less than 5 s) and sensitivity as high as 1.29 mA mM^{-1} cm^{-2}. The linear range for H_2O_2 determination was from 5.0×10^{-7} to 5.4×10^{-5} M with a detection limit of 1.2×10^{-7} M. The apparent Michaelis–Menten constant of the encapsulated hemoglobin was calculated to be 0.18 ± 0.02 mM. The stability of the biosensor was also evaluated.

Keywords: Biosensors; Sol–gel; Hemoglobin; Hydrogen peroxide; Titania sol–gel

1. Introduction

The accurate determination of hydrogen peroxide is of great importance because it is an essential mediator in food, pharmaceutical, clinical, industrial and environmental analyses [1–3]. Many techniques such as titrimetry [4], spectrometry [5], chemiluminescence [6] and electrochemistry [7–9] have been employed for hydrogen peroxide analysis. The analytical methods for hydrogen peroxide based on the first three techniques are relatively time-consuming, subject to interferences and need expensive reagents. Electrochemical methods overcome these drawbacks. Many electrochemical methods based on the electrocatalysis of immobilized enzymes to H_2O_2 reduction have been developed. Horseradish peroxidase (HRP), in which the heme iron acts as the active center, is the most commonly used enzyme in the electrochemical detection of hydrogen peroxide [9–11]. However, the HRP is expensive and unstable in solution. Thus, the use of stable and cheap mimetic enzymes such as hemin [12], hematin [13] and the porphyrin complexes of Mn, Co, Fe and Mo [14–16] has become an attractive research area. They have been reported as peroxidase substitutes in the detection of H_2O_2. However, these simple natural or synthetic metalloporphyrins

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do not show satisfactory activity and selectivity because they lack the spatial structure of the natural enzyme for the special inclusion behavior between the enzyme and the substrate. Hemoglobin (Hb) is a natural macromolecular protein, containing four subunits of polypeptide and each polypeptide chain contains a heme group that serves as the active center, which is the same as the active center of HRP [17]. Thus, Hb can exhibit enzymatic activity towards hydrogen peroxide. Furthermore, its natural macromolecular structure results in a higher catalytic activity than hemin, β-CD-hemin and some metalloporphyrins [18,19]. The electrochemical behaviors of the immobilized Hb have been widely investigated [20,21]. It has been used as a mimetic enzyme of HRP for the construction of hydrogen peroxide biosensor [22,23].

The immobilization of enzyme or protein is an important step in the fabrication of a biosensor. In recent years, silica sol–gel material has emerged as one matrix well suited for the immobilization of enzyme [24,25], and the construction of hydrogen peroxide biosensor [26–28]. This kind of inorganic silica sol–gel material can be prepared under ambient conditions and exhibits tunable porosity, high thermal stability, chemical inertness and negligible swelling in both aqueous and non-aqueous solutions [29–31]. It is well biocompatible and can retain the catalytic activities of enzymes to a large extent [32–34]. However, silica sol–gel derived matrix is fragile and easy to shrink, crack and desquamate from the electrode surface [29,30]. Meanwhile, the silica sol–gel process is usually carried out in acidic condition, which is hostile to the activities of enzymes. New sol–gel materials are desired for biosensor construction. Recently, a titania sol–gel matrix has been reported to immobilize enzymes in conductive polymers for electrochemical biosensor constructions [35,36]. But these titania matrices involved in complex preparation processes. In order to overcome these drawbacks, our previous work developed a titania sol–gel material prepared with a new vapor deposition method in neutral medium at ambient temperature [37]. This method simplified the procedure of sol–gel synthesis, shortened the time needed in enzyme electrode preparation and also avoided the shortcomings caused by acidic catalyst and calcination step needed in traditional titania sol–gel process [38–40]. The new material has been used for the entrapment of HRP [37].

In this paper, we use the vapor deposition method to prepare a new Hb entrapped titania sol–gel film and to develop a mediator-free amperometric biosensor. This biosensor exhibits a fast response to hydrogen peroxide and possesses high sensitivity, good reproducibility and long-term stability.

2. Experimental

2.1. Reagents

Hb (bovine serum) was purchased from Sino-American Biotechnology Company (Shanghai, China) and used as received. Titanium isopropoxide [Ti(*i*-PrO)$_4$] was obtained from Aldrich. A stock solution of H_2O_2 (0.10 M) was prepared by diluting 5.5 ml of 30% (v/v) hydrogen peroxide to 500 ml with water. The solution was standardized by titration with potassium permanganate. Testing standard solutions were prepared daily by appropriate dilution of the stock solution. All of the other chemicals were of analytical grade and were used without further purification. All solutions were made up with twice-distilled water.

2.2. Preparation of Hb entrapped titania sol–gel film

Glassy carbon electrodes (diameter of 4 mm) were polished before each experiment with 1.0, 0.3 and 0.05 μm α-alumina powder (Beuhler), respectively, rinsed thoroughly with doubly distilled water between each polishing step, and then sonicated in 1:1 nitric acid, acetone and doubly distilled water successively. After these pretreatments the electrode was allowed to dry at room temperature.

For fabrication of a Hb entrapped titania sol–gel film (titania/Hb), 2.5 mg ml^{-1} Hb solution was first prepared with pH 7.4 phosphate buffer solution. Ten microliters Hb solution was dropped onto the surface of a pretreated glassy carbon electrode. The electrode was then suspended vertically above titanium isopropoxide in a sealed flask kept at a constant temperature of 25 °C for 6 h. This resulted in absorption of saturate titanium isopropoxide vapor at 25 °C by the Hb solution

and slow formation of a titania sol–gel membrane through hydrolysis of titanium isopropoxide on the electrode surface, while the Hb was entrapped in the membrane.

2.3. Apparatus

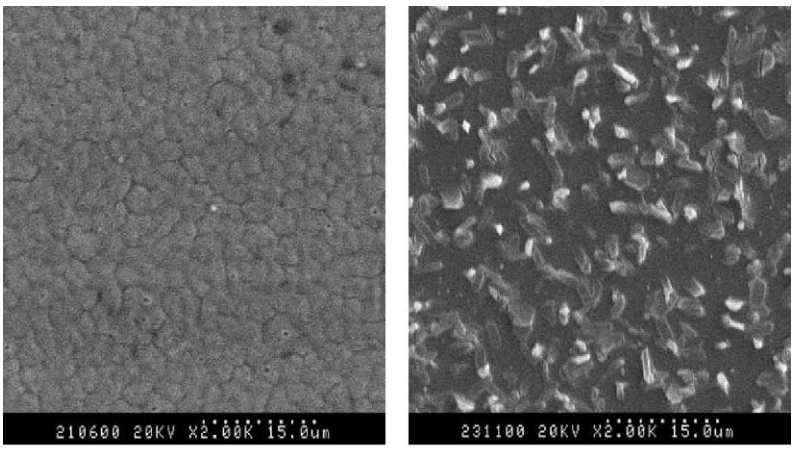
Electrochemical measurements were performed with a conventional three-electrode system using the titania/Hb modified electrode as working electrode, a platinum wire as auxiliary electrode, and a saturated calomel electrode (SCE) as reference against which all potentials were measured. The electrodes were connected to a BAS-100B electrochemical analyzer (Bioanalytical System, USA). Cyclic voltammetric measurements were done in a thermostated and unstirred electrochemical cell at $25 \pm 0.2\,^{\circ}C$. Amperometric experiments were carried out in a stirred cell at $25 \pm 0.2\,^{\circ}C$ by applying a potential of -300 mV to the working electrode and successively adding aliquots of hydrogen peroxide standard solution to the cell. Current–time data were recorded after a steady-state current achieved. All solutions were deoxygenated by highly pure nitrogen before electrochemical experiments.

Scanning electron micrographs of titania sol–gel and titania/Hb membranes were obtained with a Hitachi X-650 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) at an acceleration voltage of 20 kV.

3. Results and discussion

3.1. Morphologies of titania sol–gel and titania/Hb films

The response of the titania/Hb modified electrode is related to its physical morphology. Thus, the surface morphology of the titania sol–gel matrix is an important factor affecting its performance. Fig. 1 shows the morphologies of titania sol–gel and titania/Hb films characterized with scanning electron microscopy (SEM). The SEM of titania sol–gel membrane displays a chemically clean three-dimensional uniform porous structure (Fig. 1a). This uniform and open structure provides a significant increase of effective electrode surface for Hb loading and a good preparation reproducibility of the titania/Hb modified electrode. When Hb is entrapped in the titania sol–gel matrix, the uniform open structure is retained and bright particles of Hb are observed (Fig. 1b). The aggregates of the trapped Hb molecules are distributed regularly and show an island-like structure. This structure facilitates substrate access to the Hb and results in a good amperometric enzyme electrode response.



(a) (b)

Fig. 1. Scanning electron micrographs of the glassy carbon electrodes coated with (a) titania sol–gel film and (b) Hb entrapped titania sol–gel film.

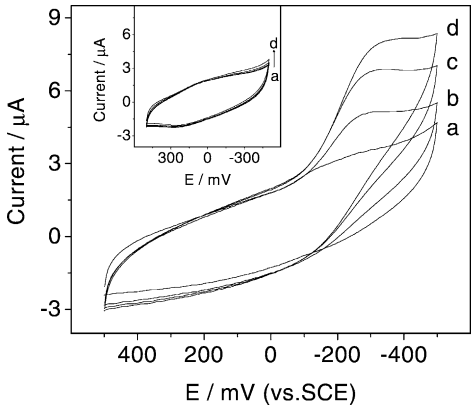


Fig. 2. Cyclic voltammograms of the titania/Hb and titania sol–gel modified electrodes (inset) in 0.2 M pH 4.5 acetate buffer without (a), and with 3.0×10^{-5} M (b), 5.0×10^{-5} M (c) and 1.2×10^{-4} M (d) H_2O_2 at 100 mV s^{-1}.

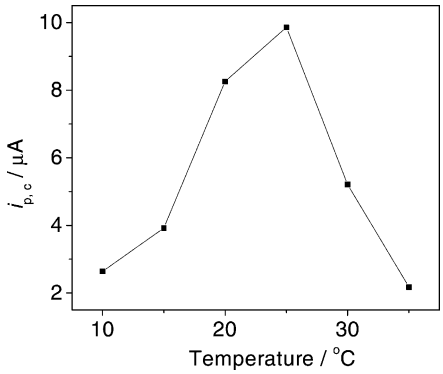


Fig. 3. Effect of temperature for sol–gel film preparation on cathodic peak current of the titania/Hb modified electrode in 0.2 M pH 4.5 acetate buffer containing 1.5×10^{-4} M H_2O_2 at 100 mV s^{-1}.

3.2. Cyclic voltammetric behavior of titania/Hb modified electrode

The cyclic voltammogram of the titania/Hb modified electrode in 0.2 M pH 4.5 acetate buffer solution shows no detectable signal (Fig. 2a). Upon addition of H_2O_2 to the buffer solution, the cyclic voltammogram gives a reduction current with the shape of catalytic wave and a maximum value occurring at the potential of −300 mV. The reduction current increases proportionally to the H_2O_2 concentration (Fig. 2b–d). While the cyclic voltammogram of the titania sol–gel film without presence of Hb does not display any detectable reduction current of H_2O_2 with the same concentration as those at the titania/Hb modified electrode (inset in Fig. 2). Thus, the increasing reduction current at the titania/Hb modified electrode results from the electrocatalytic reduction of H_2O_2. Theses phenomena illustrate that the entrapped Hb has a catalytic function similar to peroxidase, which is the native property of Hb. This catalytic property results from its native structure. That is to say, the entrapping process of Hb in titania sol–gel matrix does not damage its native structure. In other words, titania sol–gel matrix provided a biocompatible microenvironment for retaining the native structure and catalytic activity of hemoglobin. In the scan rate range of 10–300 mV s^{-1}, the electrocatalytic reduction current of H_2O_2 is proportional to the square root of scan rate, thus the electrocatalytic rate is rather fast and the electrode process is controlled by the diffusion of H_2O_2 from solution to electrode surface.

3.3. Optimization of titania/Hb modified electrode preparation

Titanium isopropoxide is much more reactive to water than tetraethyl orthosilicate. In case of touching with water, the precipitate of titanium dioxide forms immediately. So vapor deposition method is engaged to make the hydrolysis process slow down to form sol–gel but not titanium dioxide powder. The performance of the Hb electrode mainly results from two aspects: preparation temperature and the amount of Hb dropped on the electrode surface.

Temperature value directly dominates the vapor pressure of titanium isopropoxide, which controls the hydrolysis rate. Fig. 3 shows the effect of the electrode preparation temperature on the reduction peak current ($i_{p,c}$). The Hb electrode gets a best performance at the preparation temperature of 25 °C. Too high temperature results in the formation of titanium powder on the electrode surface due to a high vapor pressure and a rapid hydrolysis rate of titanium isopropoxide, which leads to the formation of titanium powder. Thus, Hb is not effectively entrapped. Low temperature results in a very slow gas deposition rate, which cannot catch up with the rate of water volatilization. This brings on a poor yield of the hydrolysis product of titania sol–gel. At the temperature of 25 °C, hydrolysis rate

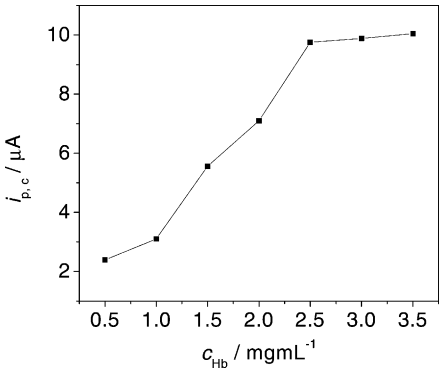


Fig. 4. Effect of Hb concentration for sol–gel film preparation on cathodic peak current of the titania/Hb modified electrode in 0.2 M pH 4.5 acetate buffer containing 1.5×10^{-4} M H_2O_2 at 100 mV s^{-1}.

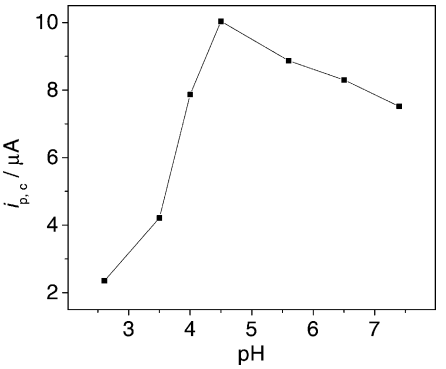


Fig. 5. Dependence of cathodic peak current of the sensor in 1.5×10^{-4} M H_2O_2 on solution pH at 100 mV s^{-1}.

matches with water volatilization rate and results in a largest loading of Hb. On the other hand, the amount of Hb is an important parameter in the preparation of titania/Hb modified electrode. Fig. 4 shows the effect of Hb amount on the $i_{p,c}$ of the titania/Hb modified electrode. The cathodic peak current increases with increasing concentration of Hb solution dropped onto the electrode surface. The curve gets a plateau at Hb concentration of 2.5 mg ml^{-1}. This indicates the Hb loading capacity of titania sol–gel thin film has been saturated.

3.4. Optimization of the experimental parameters

pH value is one of the parameters which affect the response of the titania/Hb modified electrode to H_2O_2. Fig. 5 illustrates the effect of pH value on the reduction peak current of 1.5×10^{-4} M H_2O_2 at the titania/Hb modified electrode. When pH $\geq$4.5, the modified electrode has a good response that reaches the maximum at pH 4.5. At the pH values less than 3.5, the reduction peak current decreases steeply. This results from the influence of pH on protein denaturation due to the dissociation of the heme–globin complex between 4.3 and 2.5 [41]. So we select pH 4.5 acetate buffer as the supporting electrolyte for the determination of H_2O_2.

The dependence of the sensor on applied potential for amperometric determination of H_2O_2 is shown in Fig. 6. The reduction of H_2O_2 is already observed at around −100 mV, and the steady-state current increases rapidly as the applied potential moves negatively from −100 to −300 mV. The current approaches a plateau at −300 mV, which is selected as the working potential.

3.5. Amperometric response of the biosensor

Fig. 7 illustrates a typical current–time plot for the sensor on successive step additions of H_2O_2 concentration at −300 mV. When an aliquot of H_2O_2 is added into the buffer solution, the reduction current rises steeply to reach a stable value. The titania/Hb modified electrode achieves 96% of steady-state current in less than 5 s. The response rate without any mediator corresponds to the result obtained by entrapping peroxidase in copolymer grafting silica sol–gel membrane with the mediator of methylene blue [42], and

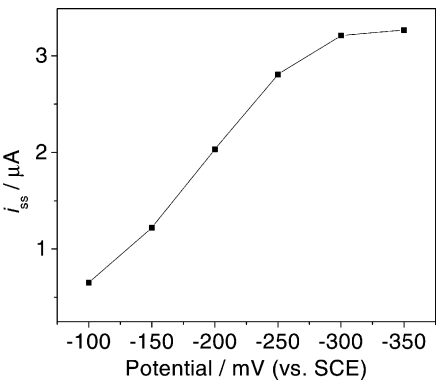


Fig. 6. Influence of applied potential on amperometric response of the sensor to 1.0×10^{-5} M H_2O_2 in 0.2 M pH 4.5 acetate buffer.

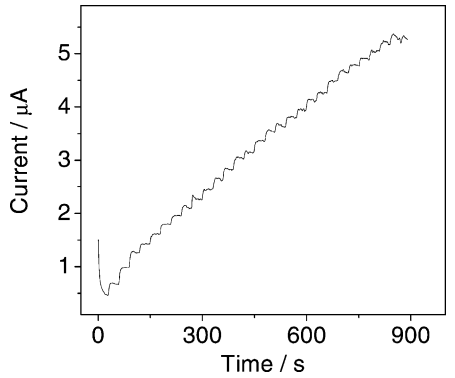


Fig. 7. Typical current–time response curve of the sensor upon successive additions of 2.0×10^{-6} M H_2O_2 in 0.2 M pH 4.5 acetate buffer at −300 mV.

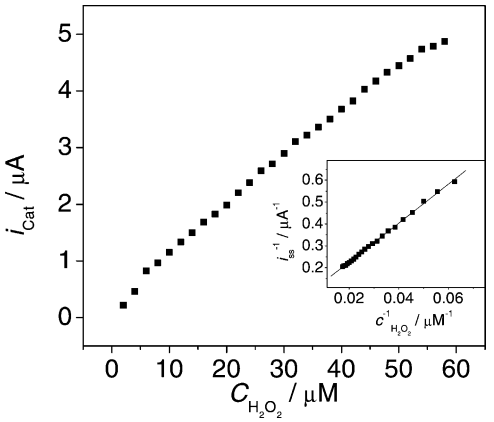


Fig. 8. Calibration curve of the titania/Hb electrode for H_2O_2 determination in 0.2 M pH 4.5 acetate buffer at −300 mV. Inset: the Lineweaver–Burk plot.

is faster than that of around 15 s reported in the pure silica sol–gel matrix with the mediator of ferrocene [43] and Os-polymer [44]. The faster response rate comes from the different structures between titania gel membrane and pure silica sol–gel matrix, which are produced from the different synthesis routes and conditions. Hb entrapped in the titania sol–gel network has enough spatial freedom in its orientation, which makes it much easier for the electroactive center of Hb to unfold. Thus, it is possible to facilitate the electron transfer. Such a short response time further proves that the vapor deposition derived titania sol–gel material is a promising material for the biosensor fabrication.

Fig. 8 displays the calibration curve of the titania/Hb modified electrode under the optimal experimental conditions. The linear range spans the concentration of H_2O_2 from 5.0×10^{-7} to 5.4×10^{-5} M with a correlation coefficient of 0.997 ($n = 27$). It has a sensitivity of 1.29 mA mM^{-1} cm^{-2}, which is more than 7.5 times that of soybean peroxidase entrapped in silica sol–gel film with methylene blue as a mediator [42], and 5 times that of horseradish peroxidase in silica sol–gel carbon paste matrix with hexacyanoferrate(II) as a mediator [7]. This illustrates titania sol–gel matrix is more suitable for protein loading and Hb can be used as an effective mimetic peroxidase for the catalytic reduction of H_2O_2. The H_2O_2 sensor gives a detection limit of 1.2×10^{-7} M at a signal-to-noise ratio of 3. At high H_2O_2 concentrations a platform response is observed, showing a characteristic of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_M^{app}), a reflection of the enzymatic affinity, is calculated to be 0.18 ± 0.02 mM according to the Lineweaver–Burk equation [45] (inset in Fig. 8). The K_M^{app} value is 12 times smaller than that of 2.1 mM for peroxidase in silica sol–gel with methylene as mediator [46], indicative of a high affinity of the entrapped Hb to H_2O_2.

The reproducibility of the sensor was examined at a H_2O_2 concentration of 1.0×10^{-4} M. The mean steady-state current is 4.2 μA with a relative standard deviation of 3.1% for nine determinations. The fabrication reproducibility of six sensors, independently constructed based on the same bare electrode, shows an acceptable reproducibility with a relative standard deviation of 2.5% for the steady-state current obtained at the H_2O_2 concentration of 1.0×10^{-4} M.

3.6. Stability of the titania/Hb modified electrode

When the titania/Hb modified electrode was stored in the refrigerator at 4 °C, it retained 89% of initial current response after 80 days. If its current response was detected once per 10 days, after the intermitted use over the 80-day period it retained 78% of its initial current response. These results demonstrate titania sol–gel film is very efficient for retaining the activity of Hb and its spatial orientation. Good long-term stability is attributed to two elements. One of them is that vapor deposition sol–gel method provides a mild immobilization process. This process does not involve

any additive that results in chemical modification and fouling of the Hb molecules, so Hb can maintain its biological activity to a large extent. The other is attributed to the large quantities of hydroxyl groups in the sol–gel film, which can form strong hydrogen bonds with Hb and sol–gel cages for enzyme loading. The cages in the film have rigidity and protective nature [32], which are beneficial for preventing the enzyme from leaking out of the titania sol–gel thin film.

4. Conclusions

This work develops a novel biosensor for H_2O_2 by entrapping Hb in titania sol–gel matrix with a vapor deposition method. The suggested sol–gel method provides a mild immobilization process for Hb and a biocompatible microenvironment around the Hb, thus can retain its biological activity as a mimetic enzyme. The porous structure of the titania sol–gel matrix results in a low mass transport barrier to the H_2O_2, a fast response rate and a good loading and high catalytic activity of the Hb. This film is very efficient for preventing leakage of the Hb out of the film, which results in a long-term stability and good reproducibility of the sensor.

Acknowledgements

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