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A third-generation hydrogen peroxide biosensor fabricated with hemoglobin and Triton X-100

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Abstract

A new film of Triton X-100 to incorporate hemoglobin for biosensor fabrication is presented in this paper. In this film, hemoglobin displays a pair of redox peaks in pH 6.0 NaAc–HAc buffer solution with a formal potential of −0.257 V (versus SCE) and shows a thin-layer behavior. Without electron mediator or promoter, the modified electrode has an electrocatalytic activity to the reduction of hydrogen peroxide (H_2O_2), which shows a linear dependence on the H_2O_2 concentration ranging from 1.0×10^{-6} to 1.0×10^{-4} mol/L. The detection limit of 3.0×10^{-7} mol/L is estimated when the signal-to-noise ratio is 3. The K_M^{app} value of hemoglobin in Triton X-100 film has been determined to be 4.27 mmol/L. Ascorbate, uric acid, dopamine, catechol, cystine and epinephrine will not interfere with the sensitive determination of H_2O_2.
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1. Introduction

As an important step in fabrication of biosensor, the immobilization of enzyme or protein has been an active research topic in amperometric biosensor design [1–7]. Whereas, since the prosthetic group of many proteins is buried deeply in the protein shell and the electron transfer (ET) rate is determined by the ET distance, electron transfer rate between protein and electrode surface is usually low [8,9]. To break through this obstacle, "electrochemical" mediators were introduced, both natural enzyme substrates or products (first-generation biosensors, mostly oxygen), and artificial redox mediators (second-generation biosensors, mostly dye molecules, conducted polymers, etc.). However, most electrochemical mediators lack selectivity and make the reaction system complicated. Enzymes are famous for their selectivity and high affinity with substrates. Therefore, many efforts have been taken to obtain the direct electron transfer of proteins, which is the basis of the third-generation biosensors [10]. And, supramolecule self-assembling has been found to be a feasible method in facilitating electron exchange at electrode surface. According, some third-generation biosensors have been fabricated.

Some non-ionic surfactant, such as Triton X-100, is usually chosen to preserve the functional state of proteins and to accelerate electron transfer in the supramolecular complex [11,12]. Since the formation of intrachain hydrophobic aggregation can prevent electron transferring/exchanging from protein to electrode, associating protein with Triton X-100 may lead to the destruction of intrachain hydrophobic aggregation and replace it with mixed alkyl/surfactant one. So using non-conducting polymers with permselective property to entrap protein is an effectively alternative method for constructing interference-free biosensors [13–18].

The hydrogen peroxide (H_2O_2) determination plays an important role in many areas, including food, pharmaceutics, industry, clinical laboratory and the environment. However, conventional methods for the H_2O_2 determination are not satisfied in many facets, such as sensitivity, reliability, and operational simplicity. Based on electron transfer between protein and electrode, the third-generation sensor

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is promising in fabricating sensitive and linearly responding devices. And many oxidases are frequently used in third-generation sensors for the determination of H_2O_2 [19–23].

Hemoglobin (MW ~ 67,000) (Hb), which contains four electroactive iron hemes, is a physiologically oxygen transport protein. Although Hb does not function physiologically as an electron carrier, it is an ideal molecule to be used as a model for the study of electron transfer reactions of heme proteins. The protein has certain intrinsic peroxidase, but readily preserves its structure than the oxidases [24]. It is easily obtained, and is cheaper for commercial purposes. So, Hb has been received more and more interests for sensors preparations. In this report, Hb is incorporated with Triton X-100 at PG electrode surface. The electrochemical characteristics of the modified electrode with respect to the effect of pH and the operational and storage stabilities of the biosensor have been studied. These results indicate that Hb, incorporated in Triton X-100, can efficiently exchange electron with electrode. What is more, since Hb has certain intrinsic peroxidase activity due to its close similarity with peroxidases, it might be possible to employ Hb to catalyze the reduction of H_2O_2. So, based on the direct electrochemistry of Hb, a third-generation biosensor for H_2O_2 can be constructed.

2. Experimental

Bovine hemoglobin and Triton X-100 were obtained from Sigma and used as received. Other chemicals were all of analytical grade. All solutions were prepared by double distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of >16 MΩ cm^{-1} and stored in the refrigerator at the temperature of 4 °C.

Electrochemical experiments were carried out with a PAR 283 Potentiostat/Galvanostat (EG&G, NJ, USA) and a three-electrode system. A one-compartment glass cell with a modified pyrolytic graphite (PG) working electrode, a saturated calomel reference electrode (SCE) and a platinum wire auxiliary electrode were used for the measurements, with a working volume of 5 mL. All the following potentials reported in this work were versus SCE.

The PG electrode was prepared by putting a PG rod into a glass tube with fixing it by epoxy resin. Electrical contact was made by adhering a copper wire to the rod with the help of Wood alloy.

The PG electrode was firstly polished on rough and fine sand papers. Then its surface was polished to mirror smoothness with an alumina (particle size of about 0.05 μm)/water slurry on silk. Eventually, the electrode was thoroughly washed by ultrasonicating in both double distilled water and ethanol for about 5 min.

A mixture of 10 μL 0.1 mmol/L Hb and 10 μL 1% Triton X-100 was spread on the PG electrode surface. The film was then dried overnight at room temperature. The modified electrode was thoroughly rinsed with nanopure water and was ready for use. When they were not in use, the modified electrodes were stored in a 0.1 mol/L NaAc–HAc buffer solution with pH 6.0 at 4 °C.

The test buffer solution was firstly bubbled thoroughly with high purity nitrogen for at least 5 min. Then a stream of nitrogen was blown gently across the surface of the solution in order to maintain the solution anaerobic throughout the experiment. Cyclic voltammetry (CV) was carried out in the range from 0.2 to −0.8 V. All experiments were carried out at room temperature of 25 °C.

3. Results and discussion

The cyclic voltammogram obtained at a PG electrode for an Hb solution (0.1 mmol/L) was similar to that for the buffer solution alone. Essentially, no peak of interest was observed. This confirmed that the heterogeneous electron transfer rate between Hb and the electrode was very slow. However, after the protein was entrapped in Triton X-100 film and was then modified onto the PG electrode surface, electron transfer of Hb and PG electrode was achieved. Fig. 1d displays the cyclic voltammograms obtained at an Hb/Triton X-100 film modified electrode in a 0.1 mol/L NaAc–HAc buffer solution with pH 6.0. A pair of well-defined peaks was observed in the CV cycles with the apparent formal potential of −0.257 V (versus SCE). No corresponding peak was observable at Triton X-100 film (free of Hb) or Hb modified PG electrode in the same potential range. This result clearly demonstrates that Triton X-100 film formed on the electrode surface provides desirable environments for the electron transfer process between Hb and the PG electrode, which makes the direct electrochemistry of Hb possible. A likely explanation for this feasibility is that Triton X-100 conducts the proper orientation of Hb on the electrode surface, and then it facilitates electron transfer between Hb and the electrode. The plot of peak current

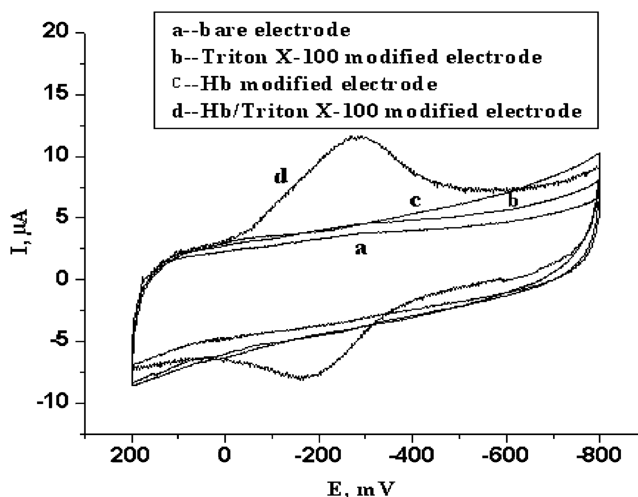

Fig. 1. Cyclic voltammograms obtained at (a) bare PG electrode; (b) Triton X-100 modified electrode; (c) Hb modified electrode; (d) Hb/Triton X-100 modified electrode, in a pH 6.0 NaAc–HAc buffer solution. Scan rate: 100 mV/s.

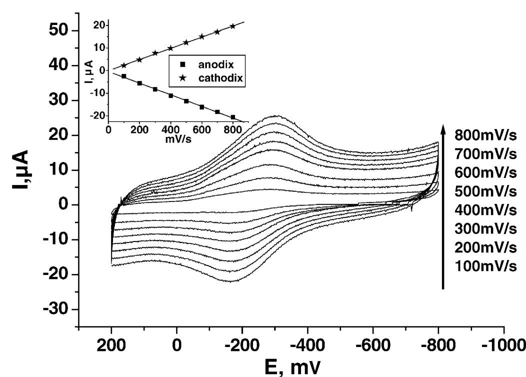

Fig. 2. Series cyclic voltammograms obtained at an Hb/Triton X-100 modified electrode in a pH 6.0 NaAc–HAc buffer solution. Scan rate: from 100 to 800 mV/s.

(anodic peak at −0.281 V and cathodic peak at −0.233 V) versus scan rate is linear up to 800 mV/s (Fig. 2), indicating a thin-layer electrochemical behavior [25]. With the pH increase in the solution from 3.0 to 11.0, good voltammetric responses were always obtained (Fig. 3). Considering the effect of pH and the stability of Hb, we selected pH 6.0 as an optimum condition in the following experiments. Further experiments demonstrated that the modified electrode was very stable. The peak current decreased only approximately 5% after a 2-week storage.

When an aliquot of H_2O_2 was added into the buffer solution (0.1 mol/L NaAc–HAc buffer solution with pH 6.0), the current of cathodic peak increased gradually (Fig. 4). Since no electrochemical signal corresponding to H_2O_2 was observed in the potential range of interest at a bare PG or a Triton X-100 alone-modified electrode, the increase of the cathodic peak was obviously related to the electrocatalytic reduction of H_2O_2 in the presence of Hb modified at the electrode. Meanwhile, the diagram of the height of the cathodic peak versus the concentration of H_2O_2 in Fig. 4 inset shows a typical linear curve.

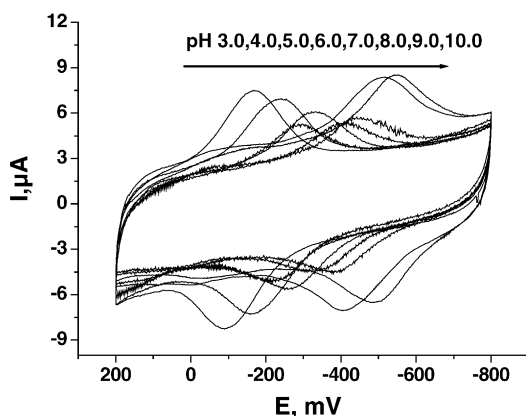

Fig. 3. Series cyclic voltammograms obtained at an Hb/Triton X-100 modified electrode in different pH NaAc–HAc buffer solutions.

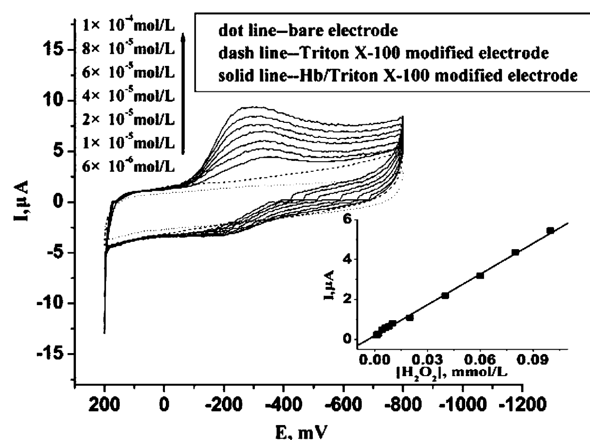

Fig. 4. Cyclic voltammograms obtained at an Hb/Triton X-100 modified electrode, a Triton X-100 modified electrode and a bare electrode in a pH 6.0 NaAc–HAc buffer solution containing different concentrations of H_2O_2. Scan rate: 100 mV/s. The inset is the linear range response of this biosensor.

Further studies revealed that the catalytic peak currents were proportional to the concentration of H_2O_2 in the range from1.0 × 10^{-6} to 1.0 × 10^{-4} mol/L. The linear regression equation was $y = 1.12 + 38.59x$, $r = 0.999$. The detection limit is estimated to be 3.0 × 10^{-7} mol/L when the signal-to-noise ratio is 3. As an attempt to estimate the enzyme activity of Hb in this system, we calculated the Michaelis–Menten constant according to the electrochemical version of the Lineweaver–Burk equation: $1/I_{ss} = 1/I_{max} + K_M^{app}/I_{max}C$, where I_{ss} is the steady-state current after the addition of a substrate, C the bulk concentration of the substrate, I_{max} the maximum current measured under the saturated substrate condition and K_M^{app} is the apparent Michaelis–Menten constant. Consequently, K_M^{app} was obtained as 4.27 mmol/L by an analysis of the slope and intercept for the double reciprocal plot of the steady-state current versus the H_2O_2 concentration. So, Hb showed a high peroxidase activity and a good sensitivity in this film. Certainly, it should be noted that the real peroxidase catalysis obeys a complicated ping–pong mechanism, instead of the Michaelis–Menten model. This value is thus a simple estimation and only useful when compared with similar electrochemical sensing system. The sensitivity and detection limit reported here are similar to some recent reports involving Hb with direct electron transfer. [26,27] The direct electrical communication between the heme site of Hb in the film and the electrode might contribute to this high electrocatalytic efficiency.

The stability of this H_2O_2 biosensor has been examined as well. After being exposed to air in the refrigerator for 7 days, signals decreased by less than 5% and after 30 days by less than 10% (data from five similarly prepared modified electrodes).

The selectivity of this H_2O_2 biosensor was evaluated by H_2O_2 determinations in the presence of some potentially co-existing compounds of H_2O_2 in biological systems. These include ascorbate, uric acid, dopamine, catechol, cystine and

Table 1
Interference from other substances for H_2O_2 determination[a]

Substance	Concentration ($\times 10^{-5}$ mol/L)	Selectivity ratio (%)
No interference	–	100
Ascorbate	1	102
Dopamine	1	100
Catechol	1	97
Uric acid	1	99
Cystine	1	100
Epinephrine	1	103

[a] Measurements in a 1.0×10^{-5} mol/L H_2O_2 solution with pH 6.0.

epinephrine. Table 1 shows that the detection of H_2O_2 is not influenced by these potential interference compounds at concentrations 1–2 orders higher than expected in biological systems. We propose that Hb in the Triton X-100 film can exhibit a fine peroxidase character and a high catalytic activity to H_2O_2, which makes this Hb-based H_2O_2 biosensor to have a good selectivity.

4. Conclusion

Hb can achieve electron exchange with the electrode through its incorporation with a Triton X-100 film. Furthermore, this modified electrode linearly responds to H_2O_2 without electron mediator or promoter. Accordingly, a third-generation H_2O_2 biosensor has been prepared. It has advantages of simple construction, good sensitivity and stability, and shows potential utility in the trace H_2O_2 determination.

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