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RESEARCH ARTICLE

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Electrochemical behavior of hydrogen peroxide sensor based on new methylene blue as mediator

Abstract A novel amperometric hydrogen peroxide sensor was proposed by co-immobilizing new methylene blue (NMB) and Horseradish peroxidase (HRP) on glassy carbon electrode through covalent binding. The electrochemical behavior of the sensor was studied extensively in 0.1 mol/L phosphate buffering solution (pH = 7.0). The experiments showed NMB could effectively transfer electrons between hydrogen peroxide and glassy carbon electrode. The electron transfer coefficient and apparent reaction rate constant were determined to be 0.861 and 1.27 s^{-1}. The kinetic characteristics and responses of sensor on H_2O_2 were investigated. The Michaelis constant is 8.27 mol/L and the linear dependence of current on H_2O_2 is in the range of 2.5−100 μmol/L. At the same time, the effects of solution pH, buffer capacity, and temperature on the sensor were examined.

Keywords new methylene blue, biosensor, covalently bonding, hydrogen peroxide

1 Introduction

The determination of hydrogen peroxide and its practical application is an important study, especially in some fields such as environmental monitoring, biomedical engineering, clinical examination, and food. Many methods have been adopted for the detection of hydrogen peroxide, and using conventional electrode has been a feasible way to carry out the direct electrochemical detection of H_2O_2. However, it induced many disturbing substances for its high overpotential [1]. The direct detection, by catalyzing reduction of H_2O_2, could be taken through immobilizing peroxidases on the electrode. However, its application is limited for its low sensitivity [2]. Recently, many electron mediators have been employed to enhance the electron transfer between the active center of the enzyme and the electrode surface. Many researches on this have focused on the choice of mediator and the immobilization of both the enzyme and the mediator. The mediators include ferrocene and its derivatives [3,4], polymers [5] and redox dye [6]. And covalent binding [7,8], adsorption [9], ion exchange method [10] and self-assembled membrane [11] are the conventional immobilization methods.

New methylene blue is a substance with a conjugated complanate structure that possesses excellent electrochemical activity and is a good electron transfer mediator. In this paper, a novel amperometric hydrogen peroxide sensor was prepared by co-immobilizing new methylene blue (NMB) and Horseradish peroxidase (HRP) on a glassy carbon electrode through covalent binding, and the kinetic characteristics have been researched. The experimental tests indicate that the sensor provides some advantages including simple operation, quick response, high sensitivity, good stability and reproducibility.

2 Experimental

2.1 Instruments and reagents

M273 potentiostat/galvanostat (EG&G PARC, USA) and constant temperature instrument (Shanghai Laboratory Instrument General Works) were employed. The three-electrodes system included glassy carbon electrode (GCE) as working electrode, saturated calomel electrode (SCE, all the potential in this paper versus SCE) as reference and platinum wire as auxiliary electrode. Phosphate buffer solution (PBS) (KH_2PO_4-Na_2HPO_4), glutaraldehyde (Beijing Chemical Reagents Company), new methylene blue (Aldrich Company), horseradish peroxidase (Beijing Dingguo Biotech. Co. Ltd), and H_2O_2 (30%, Beijing Beihua Fine Chemical Sco., LTD.) were purchased. The water used in the experiment was double distilled.

2.2 Pretreatment of GCE

The GCE (3-mm diameter) was polished to a mirror finish using 0.05 μm alumina slurry followed by thoroughly rinsing

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with double distilled water. After ultrasonicating successively in 7 mol/L NaOH + 30% H_2O_2, acetone and double distilled water for 10 min, the electrode was pretreated electrochemically by applying a potential of + 1.75 V in 0.1 mol/L pH 5.0 PBS for 300 s and scanned between + 0.3 and + 1.3 V until a steady-state current-voltage curve was observed [10]. Then a blue film was formed on the surface.

2.3 Preparation of NMB/HRP/GCE modified electrode

The pretreated electrode was then soaked in 5% glutaraldehyde for 12 h to form a glutaraldehyde-modified GCE. After the modified GCE was thoroughly rinsed with double distilled water to remove physically adsorbed glutaraldehyde, it was immersed in a mixture of 30 μL of 5 mmol/L HRP solution and 30 μL of 0.2 mmol/L NMB solution for 5 h. Then, a NMB/HRP-modified hydrogen peroxide sensor was prepared, and the sensor was rinsed thoroughly with double distilled water and kept on the surface (in the top part) of (0.1 M, pH = 7.0) PBS at 4°C prior to the electrochemical experiments. The procedure is shown in Fig. 1.

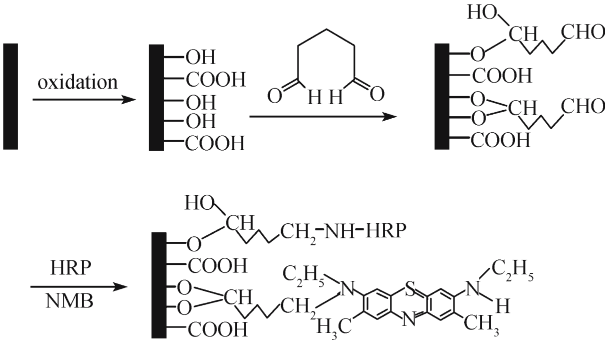

Fig. 1 Procedure for the preparation of the biosensor oxidation pretreatment

3 Results and discussion

3.1 Electrochemical behavior of NMB/HRP/GCE-modified electrode

Figure 2 shows the cyclic voltammograms of different modified electrodes in 0.1 M (pH = 7.0) PBS at scan rate, v, of 50 mV/s. No peaks were observed with bare and pretreated GCEs during –0.1–0.6 V (curves a and b). However, the background current of the pretreated GCE rose for the formation of —OH and —COOH groups on the GCE surface [12]. The glutaraldehyde-modified electrode had no peaks in the same PBS (Fig. 2(c)), which indicated that the glutaraldehyde reacted with those —OH on the surface [13] and prevented the electron transfer. After NMB and HRP immobilized on the glutaraldehyde-modified electrode, the modified electrode gave a reversible redox peaks at −0.300 and −0.326 V (Fig. 2(d)), and the dispersion of the anodic and cathodic peak potentials ΔEp = 26 mV, which belongs to the two-electron reaction behavior of NMB in solution (the anodic and cathodic peak potentials of NMB are −0.217 V and −0.246 V and ΔEp = 29 mV in solution, so the potentials shifted to the negative potentials). The results implied that NMB was bound with the glutaraldehyde on the surface of the electrode.

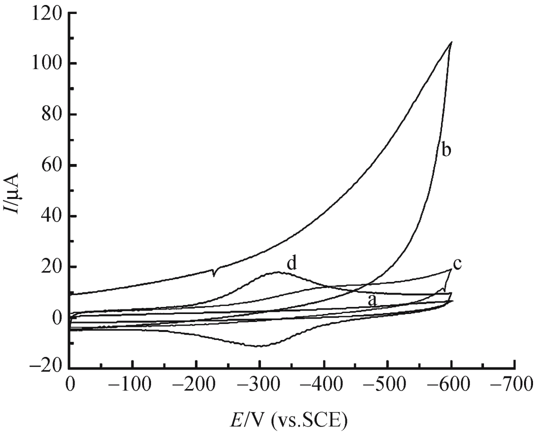

Fig. 2 Cyclic voltammograms of (a) unpretreated GCE; (b) electrochemical pretreated GCE; (c) glutaraldehyde modified GCE; (d) NMB/HRP/GCE sensor in 0.1 mol/L PBS (pH = 7.0) at 50 mV/s

Figure 3 shows the cyclic voltammograms of the sensor in 0.1 M PBS (pH = 7.0) at different scan rates. At low scan rate (10−120 mV/s), the ratio of the peak current was about one ($i_{pa}/i_{pc} \approx 1$) and the peak potential values did not change with the different scan rates, indicating better reversibility. In addition, a plot of cathodic peak current versus the scan rate was linear (Fig. 3 inset) and the correlation coefficient (R) was 0.999, which indicated that the reaction was a surface-controlled electrode process. When the potential scan rate was higher than 300 mV/s, ΔEp increased dramatically and was not reversible. When $\Delta E_p > 100$ mV, it was proportional to log*v* (Fig. 4) and in accordance with the Laviron equation [14].

$$\text{Log Ks} = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log(RT/nFv) - \alpha(1-\alpha)nF\Delta E_p/2.3\,RT$$

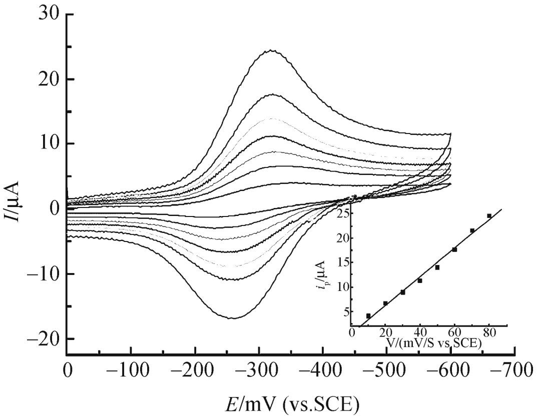

Fig. 3 Cyclic voltammograms of sensor in 0.1 mol·L^{-1} PBS (pH = 7.0) at different scan rates (from inner curve to outer one: 10, 20, 30, 40, 50, 60, 70, 80 mV/s) inset: plot of peak current versus scan rate

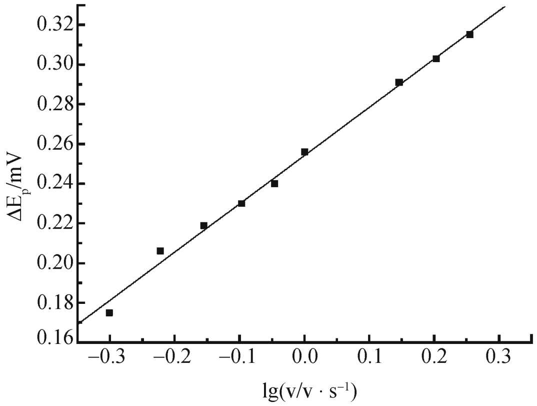

Fig. 4 Dependence of ΔEp on scan rate

where α is electron transfer coefficient, K_s is the apparent electron-transfer rate constant, n is the number of electrons involved in an electrode. Figure 4 shows that in the equation $\Delta E_p = 0.254\,2 + 0.243\,3\log vV$ ($R = 0.997\,4$), the apparent electron-transfer rate constant of 1.27 s^{-1} and the electron transfer coefficient (α) of 0.861 were obtained. The charge $Q = 74.56$ C from the area of the peak of cyclic voltammograms was obtained. Then the surface coverage (Γ) of NMB was calculated to be 5.46×10^{-9} mol/cm^2, which was in accordance with the equation: $\Gamma = Q/nFA$.

3.2 Response mechanism and the detection of dynamics parameter of the NMB/HRP/GCE to H_2O_2

3.2.1 Response mechanism of the sensor to H_2O_2

Horseradish peroxidase can catalyze the reduction of H_2O_2. However, because the interaction between the enzymatic active center and the electrode surface is slow, a mediator to enhance the transfer of electrons is needed [15]. NMB can increase the transfer of electron due to its conjugated complanate structure so that it can catalyze the reduction of H_2O_2. Figure 5 shows the cyclic voltammetric behavior of the sensor in 0.1 M PBS (pH = 7.0). Upon the addition of H_2O_2 to the solution, the reduction peak current increased and the oxidation peak current of the sensor decreased. However, no changes were found at the same solution when the HRP was not immobilized on the surface of the electrode (Fig. 5, inset). This indicated that the HRP on the electrode surface could exhibit excellent catalytic activity in H_2O_2. The mechanism for the whole electrode process could be expressed in the following equation

$$\mathrm{HRP} + \mathrm{H_2O_2} \longrightarrow \text{Compound I} + \mathrm{H_2O}$$

$$\text{Compound I} + \mathrm{NMB(Red)} \longrightarrow \text{Compound II} + \mathrm{NMB(Ox)}^*$$

$$\mathrm{NMB(Ox)}^* + \text{Compound II} + 2\mathrm{H}^+ \longrightarrow \mathrm{HRP} + \mathrm{NMB(Ox)} + \mathrm{H_2O}$$

$$\mathrm{NMB(Ox)} + 2e^- + 2\mathrm{H}^+ \longrightarrow \mathrm{NMB}$$

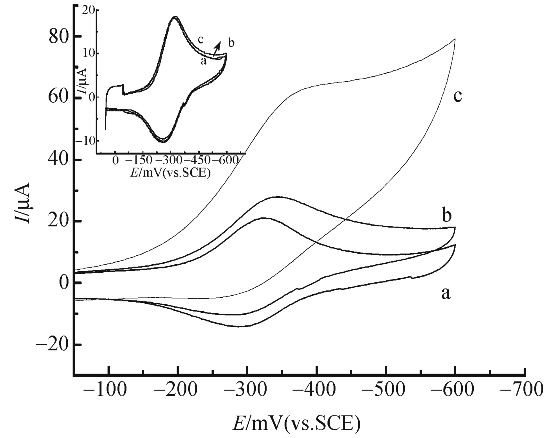

Fig. 5 Cyclic voltammograms responses of sensor in (a) PBS (pH = 7.0); (b) (a) + 0.15 mmol/L H_2O_2; (c) (a) + 2 mmol/L H_2O_2; Inset: Cyclic voltammograms responses of NMB/ GCE in (a) PBS (pH = 7.0); (b) (a) + 0.15 mmol/L H_2O_2; (c) (a) + 2 mmol/L H_2O_2 (scan rate: 50 mV/s)

3.2.2 Response of the sensor to H_2O_2

Figure 6 shows that the reduction peak current increased with the increases in the concentration of H_2O_2. It is the typical curve of enzymatic catalytic dynamics which is the first order of reaction at the lower concentration of the substrate while it exhibited the second order of reaction at the higher concentration. When the concentration of H_2O_2 was less than 100 μmol/L, the response current exhibited excellent linearity with the concentration of the substrate. The equation of linearity is: $I_p = 0.048\,79c + 22.51$ ($R = 0.996\,3$) and the linear range was 2.5−100 μmol/L. The lower detection limit was 2.07 μmol/L from the ten times detection value of the blank solution. And the response time is less than 20 s.

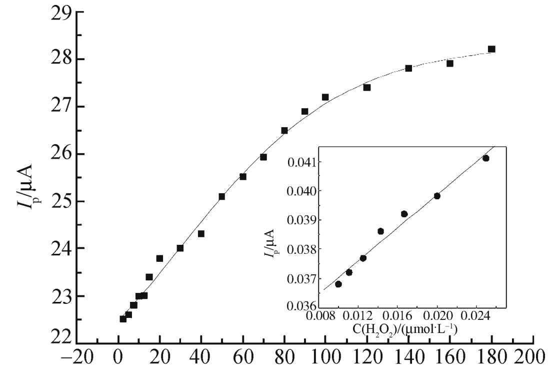

Fig. 6 The relationship between the peak current and the H_2O_2 concentration (0.1 mol/L PBS with pH = 7.0, at 20°C. Inset shows the determination of the apparent Michaelis-Menten constant: K_m)

3.2.3 The dynamics parameter of the sensor

The sensor response to H_2O_2 exhibited the characteristics of the enzymatic catalytic wave, and the apparent

Michaelis-Menten constant K_m indicated the affinity between enzyme and substrate. According to the Michaelis-Menten equation

$$1/i_{ss} = (K_m/i_{max}) \times (1/C) + 1/i_{max}$$

where i_{ss} is the steady-state catalytic current, i_{max} represents the maximum current, C is referred to the H_2O_2 concentration, we could obtain the Lineweave-Burk figure when the $1/i_{ss}$ as y-axis and 1/C as x-axis (Fig. 6, inset). The linear equation is $1/i_{ss} = 0.034\,2 + 0.283\ (1/C)$, and then $i_{max} = 29.24$ μA, $K_m = 8.27$ μmol/L.

3.3 Factors of effects on the sensor

3.3.1 Effect of pH

The pH dependence of the sensor in PBS containing 2 mmol/L H_2O_2 is illustrated in Fig. 7. The experimental results showed that the current response was higher in the range of pH 7.0−7.5. When pH > 7.5, the current response became smaller. This was attributed to the higher activity of HRP in neutral or alkalescent solution. Furthermore, the dispersion of the anodic and cathodic peak potentials increased with increasing pH, which agrees with the fact that the redox reaction of NMB follows a mechanism of two-electrons and two-protons.

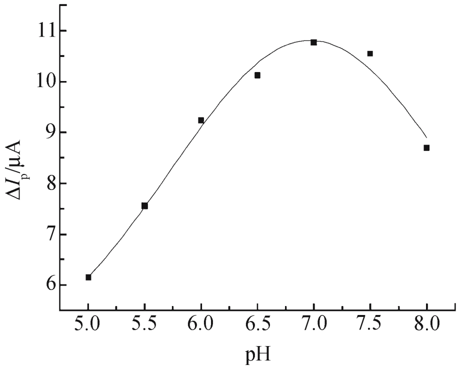

Fig. 7 Effect of pH on peak current in 2 mmol/L H_2O_2 and 0.1 mol/L PBS at 100 mV/s

The experiment in PBS at various concentrations showed that the reversibility was best in 0.1 mol/L PBS. Therefore, we selected 0.1 mol/L PBS (pH = 7.0) as the working solution in order to optimize the response of the sensor to H_2O_2.

3.3.2 Effect of temperature

The effect of temperature on the sensor had been examined between 15 and 50°C. The current response on H_2O_2 and sensitivity increased with the increasing temperature in the same solution. However, further increase in the temperature led to a decrease in the response of the current because of the partial denaturation of the enzyme. For the purposes of convenience we chose the temperature 25°C as the detection temperature.

3.4 Reproducibility and stability of the sensor

The reproducibility of the current response of the sensor was examined at H_2O_2 concentration of 2 mmol/L, and the relative standard deviation was 3.86% ($n = 10$). It indicated that the sensor possessed good reproducibility. After those experiments, the sensor was kept on the surface (in the top part) of PBS at 4°C in order to keep the activity of HRP. We used the sensor to detect H_2O_2 for two times every day, and the results showed that the activity only decreased about 1.04% after six days. This indicated that the sensor has good stability and it can be used repeatedly.

4 Conclusions

The H_2O_2 sensor based on new methylene blue as mediator possesses a variety of characteristics including high sensitivity, good stability and multiple usage. The method can overcome the disadvantage of modified film, which is basically its instability. We hope that the method described here can be applicable in the preparation of a glucose biosensor or a cholesterin biosensor. Moreover, may it pave the way for the study of immunoassay through immobilized labeled antibody.

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