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Nickelocene-mediating Sensor for Hydrogen Peroxide Based on Bioelectrocatalytic Reduction of Hydrogen Peroxide

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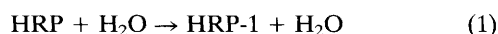
An amperometric H_2O_2 sensor using nickelocene as the electron transfer agent between immobilized horseradish peroxide and a glassy carbon electrode was fabricated. The sensor was highly sensitive to H_2O_2 with a detection limit of 5.0×10^{-7} mol l^{-1} H_2O_2 and a response time of less than 20 s. The effect of applied potential and temperature on the Michaelis–Menten constant was calculated and the influence of various experimental parameters such as pH, temperature and applied potential were explored for optimum analytical performance.

Horseradish peroxidase (HRP) has been used in the catalytic reduction of hydrogen peroxide to amplify the amperometric response of the nickelocene-mediating sensor for hydrogen peroxide. HRP reacts vigorously with H_2O_2, up to 600 units per milligram of protein solid[1] and each molecule can efficiently convert about 25 000 H_2O_2 molecules to H_2O per minute. The structure and function of HRP have previously been reported.[2,3] The reaction between hydrogen peroxide and peroxidase involves HRP-1 (containing Fe^{IV} and a porphyrin-radical cation) and HRP-2 (containing only Fe^{IV}) in the following sequential reaction:[4–6]



$$\text{HRP} + H_2O \rightarrow \text{HRP-1} + H_2O \quad (1)$$

$$\text{HRP-1} + \text{AH} \rightarrow \text{HRP-2} + \text{A} \quad (2)$$

$$\text{HRP-2} + \text{AH} \rightarrow \text{HRP} + \cdot\text{A} \quad (3)$$

where AH refers to the hydrogen donor and ·A the free radical formed during the reaction. The radicals are bound to the enzyme throughout the enzymatic reaction, and dimerize before they are reduced at a certain potential. Although HRP enzyme electrodes have been reported to undergo direct electron transfer reaction,[7–10] they exhibit a sensitivity much lower than that of peroxidase electrodes with electron transfer *via* soluble or immobilized mediators[11] which include (2-aminoethyl)ferrocene,[12] and $[Ru(NH_3)_5py]^{2+}$,[12] *o*-phenylenediamine,[13] ferrocene,[14] tetrathiafulvalene,[15] and organic dyes[16,17] such as Meldola Blue, phenazine methosulfate, New Methylene Blue N (3,7-diethylamino-2,8-dimethylthiaphenazine), Methylene Violet, Toluidine Blue and Methylene Blue. In this paper, cyclic voltammetry and constant applied potential measurements are, for the first time, employed to demonstrate the feasibility of electron transfer *via* nickelocene between immobilized peroxidase and a glassy carbon electrode. The response of the sensor to H_2O under N_2 saturation shows an apparent Michaelis–Menten constant, K_m^{app}, of 1.49 and 1.74 mmol l^{-1} at applied potentials of 270 and 300 mV, respectively, and reaches 95% steady-state current within 20 s.

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Experimental

Reagents

Peroxidase (POD) from horseradish (EC 1.11.1.7, type VI) was obtained from Sigma (St. Louis, MA, USA) and nickelocene was purchased from Aldrich (Milwaukee, WI, USA). Hydrogen peroxide (30% m/v solution) was purchased from Shanghai Chemical Reagent (Shanghai, P.R. China). The concentration of more dilute peroxide solutions prepared from this stock solution was determined by titration with cerium(IV) to a ferroin end-point.[18] Silk fibroin solution was prepared according to the literature.[17] All other chemicals were analytical-reagent grade. All solutions were prepared with doubly distilled water.

Apparatus

All experiments were performed with a three-electrode system comprising a H_2O_2 sensor as working electrode, a saturated calomel reference electrode and a platinum wire auxiliary electrode. The electrodes were connected to an FDH 3204 and FDH 3206 cyclic voltammetry apparatus (Scientific Equipment, Fundan University, Shangai, P.R. China) and the signal was recorded on a type 3086 *x-y* recorder (Yokogawa Electronic Works, Tokyo, Japan) for separate cyclic voltammetric and amperometric measurements. All experiments were carried out in a thermostated, stirred electrochemical cell containing 5 ml of 0.1 mol l^{-1} phosphate buffer (pH 7.0) at 25.0 ± 0.5 °C. All experimental solutions were thoroughly dexoygenated by bubbling nitrogen through the solution for at least 10 min. In the constant potential experiments, successive additions of stock H_2O_2 solution in the buffer were made and the current–time data were recorded after a constant residual current had been established. Changes in the measured reduction current were recorded as a function of time, following the addition of H_2O_2.

Fabrication of H_2O_2 sensor

Glassy carbon eletrodes were polished to a mirror-like finish with 0.3, 0.1 and 0.05 μm Al_2O_3 paste, rinsed thoroughly with de-ionized water between each polishing step, sonicated in nitric acid–acetone (1 + 1) and doubly distilled water consecutively, and dried in air before use. Nickelocene was coated on the electrode by pipetting 8 μl of 0.1 mol l^{-1} nickelocene–acetone solution onto the electrode surface and letting it dry in air. Peroxidase (10 mg) in 0.5 ml of regenerated

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silk fibroin was completely mixed. Aliquots (25 μl) of the mixture were deposited on the electrode and allowed to dry in ambient conditions for 20 h. A 5 μl aliquot of 75% ethanol was syringed onto the sensor and allowed to air dry for about 5 min.

Results and Discussion

Cyclic Voltammetry

Cyclic voltammograms of the sensor in 0.1 mol l^{-1} phosphate buffer (pH 7.0) without H_2O_2 indicate that the enzyme contributes no response and only nickelocene at the electrode gives quasi-reversible voltammograms as the cathodic and anodic peak separation (ΔE_p) of the cyclic voltammogram is 80 mV and the peak current is proportional to the square root of the scan rate.

Electrocatalytic Reduction of H_2O_2 at the Sensor

There is no electrocatalytic reduction current at the nickelocene-modified electrode when H_2O_2 is added to the phosphate buffer. Fig. 1 shows characteristic cyclic voltammetric results for the H_2O_2 sensor. Without H_2O_2 only the typical oxidation and reduction peak for nickelocene at the electrode in solution is observed [Fig. 1(*a*)]. The addition of H_2O_2 to the cell led to an enhanced cathodic current. Comparison of the voltammograms with and without the presence of H_2O_2 illustrates that nickelocene can enhance electron communication between immobilized peroxidase and glassy carbon electrode. The mechanism of the sensor is summarized in Fig. 2.

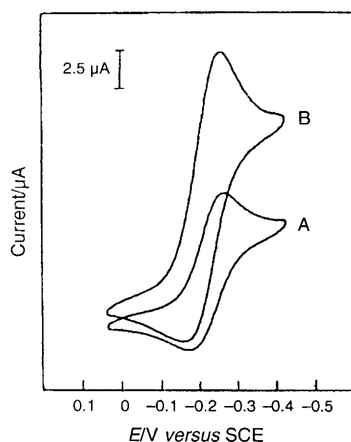

Fig. 1 Cyclic voltammograms of the sensor at a scan rate of 60 mV s^{-1} in 0.1 mol l^{-1} phosphate buffer, pH 7.0, A, without H_2O_2, and B, with 0.5 mmol l^{-1} H_2O_2

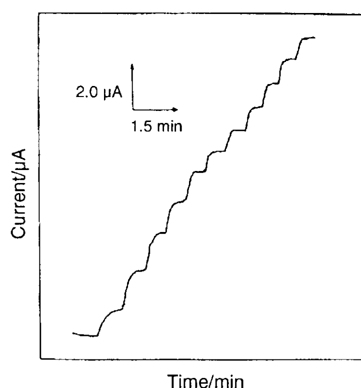

Fig. 2 Typical response of the H_2O_2 sensor to the successive increase of 0.1 mmol l^{-1} H_2O_2 in solution at an applied potential of −0.3 V

The POD reduces hydrogen peroxide penetrating the membrane to water, $H_2O_2 + POD_{red} \rightarrow H_2O + POD_{ox}$ then oxidized peroxidase converts nickelocene (Nc) to nickelocene ion (Nc^+), $POD_{ox} + 2Nc \rightarrow POD_{red} + 2Nc^+$. Finally Nc^+ is reduced at the sensor, bringing about a cathodic current $2Nc^+ + 2e \rightarrow 2Nc$.

Steady-state Amperometric Response of the Sensor to H_2O_2

Fig. 3 displays a typical trace of the steady-state current–time response of the senior at 0.30 V with successive injections of H_2O_2. The trace clearly demonstrates the fast response and high sensitivity of the sensor to H_2O_2 and the time required to reach 95% of a maximum response. The response time is fast and calculated to be within 20 s. The linear response is observed up to 0.7 mmol l^{-1} of H_2O_2. An extremely low detection limit of 5.0×10^{-7} mol l^{-1} of H_2O_2 can be estimated at a signal-to-noise ratio of 3. The apparent Michaelis–Menten constant $K_M{}^{app}$ can be obtained from a Michaelis–Menten analysis of the H_2O_2 calibration plot. The data fitted the electochemical Lineweaver–Burk form of the Michaelis–Menten equation.[19]

$$\frac{1}{i_s} = \frac{1}{i_{max}} + \frac{1}{c} \times \frac{K_M^{app}}{i_{max}}$$

where i_s is the ready-state current, i_{max} refers to the maximum current measured under saturating substrate conditions, c represents H_2O_2 concentration and K_M^{app} is the apparent Michaelis–Menten constant of the system as a whole (and not that of an intrinsic property of peroxidase). The Michaelis–Menten constant is dependent on the working potential and its values at −0.27, −0.30 V are 1.49 and 1.74 mmol l^{-1}, respectively.

Effect of pH and Temperature on the H_2O_2 Sensor

The pH dependence of the sensor was investigated between pH 4.0 and 8.0. The result shows that the sensor displayed an optimum response between pH 6.0 and 7.5.

The effect of temperature on the sensor was examined between 15–55 °C. The current response increased with temperature, reaching a maximum value at 45 °C. Further increase in temperature resulted in a decrease of the response current because of the partial denaturing of the enzyme. The dependence of Michaelis–Menten constant to temperature is summarized in Table 1.

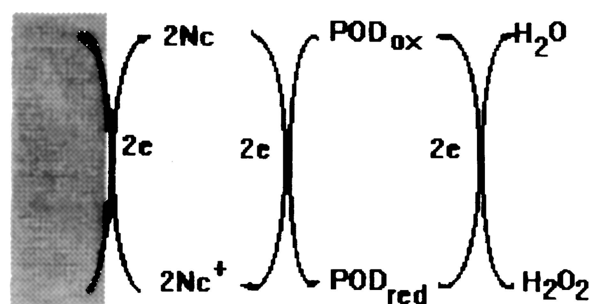

Table 1 Effect of temperature on Michaelis–Menten constant at −0.3 V

Temperature/°C	20	30	40	50
Michaelis–Menten constant/mmol l^{-1}	1.56	1.79	1.92	1.87

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