An amperometric Meldola Blue-mediated sensor high sensitive to hydrogen peroxide based on immobilization of horseradish peroxidase in a composite membrane of regenerated silk fibroin and poly(vinyl alcohol)

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H. Y. Liu, X. Zhang, J. Wei, X. Wu, D. Qi, Y. Liu, M. Dai, T. Yu, and J. Q. Deng, "An Amperometric Meldola Blue-mediated Sensor: High Sensitive to Hydrogen Peroxide Based on Immobilization of Horseradish Peroxidase in a Composite Membrane of Regenerated Silk Fibroin and Poly (vinyl alcohol)" *Analytica Chimica Acta*, 1996, 329, 97-103.

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Made available courtesy of Elsevier: https://doi.org/10.1016/0003-2670(96)00130-4

Abstract:

A new composite membrane of poly(vinyl alcohol) (PVA) and regenerated silk fibroin (RSF) was successfully employed to immobilize horseradish peroxidase (HRP) and infrared (IR) was used to get insight in the structure of the composite membrane. An amperometric HRP-based sensor highly sensitive to hydrogen peroxide was fabricated, which was based on Meldola Blue as a mediator to facilitate efficient electron transfer between immobilized HRP and a glassy carbon electrode. Performance and characteristics of the sensor were evaluated with respect to response time, detection limit, selectivity, operating and storage stability, and dependence on temperature, pH, applied potential and mediator concentration. The sensor displayed high sensitivity to hydrogen peroxide with low detection of limit of 0.1 μM.

Keywords: Sensors | Poly(vinyl alcohol) | Regenerated silk fibroin | Meldola Blue | Horseradish peroxidase | Hydrogen peroxide

Article:

1. Introduction

The importance of bioelectrocatalytic reduction of hydrogen peroxide formed by oxidases via horseradish peroxidase (HRP) has inspired studies of the performance of HRP on graphite, metal and polymermodified electrode surfaces. HRP (EC 1.11.1.7, H_2O_2 oxidoreductase) has been utilized in the catalytic reduction of hydrogen peroxide to amplify the amperometric response of the sensor because of its extremely high activity for H_2O_2, i.e. up to 600 units per milligram protein solid, of which one molecule efficiently converts about 25 000 H_2O_2 molecules to H_2O per minute [l]. Electron transfer between the electrode and the active site of HRP can be achieved by two pathways: (1) direct electron transfer and (2) electron transfer via soluble or immobilized mediators. In the absence of any mediator, HRP has been reported to undergo direct electron reaction with carbon black [2], spectrographic graphite [3], nonplatinized activated carbon electrodes [4] and pyrolytic graphite [5]. Entrapment of horseradish peroxidase in carbon paste [6] or in polypyrrole [5] has been investigated. Generally, direct electron transfer between common electrode materials and immobilized HRP is a slow process and electron transfer via a mediator is more effective in the bioelectrocatalytic reduction of peroxides at HRP based sensors with detection limit as low as 10^{-8}~10^{-7} M. Many mediators have been used to enhance electron transfer between immobilized HRP and the electrode. They are ferrocene and its derivatives [7,8] hexacyanoferrate (II) [9], quinone [10], $[Ru(NH_3)_5py]^{2+}$ [11], *o*-phenylenediamine [12], tetrathiafulvalene [13], oxmium bipyridine conjugated to poly(vinylpyridine) polymer [14], nickelocene [15] and tetracyanoquinodimethane salts [16,17]. In this paper, an amperometric Meldola Blue-mediated sensor highly sensitive to hydrogen peroxide is fabricated, based on the immobilization of HRP in a novel composite of poly(vinyl alcohol) (PVA) and regenerated silk fibroin (RSF). Infrared (IR) is employed to elucidate the structure of the composite membrane. Cyclic voltammetry and amperometric measurement were utilized for the first time to demonstrate the feasibility of electron transfer between immobilized HRP and the electrode in bioelectrocatalytic reduction of hydrogen peroxide via Meldola Blue. High sensitivity of the sensor to hydrogen peroxide results from high efficiency of bioelectrocatalytic reduction of hydrogen peroxide via Meldola Blue. Dependence of the Michaelis-Menten constant on the applied potential and the mediator concentration is investigated and its results are presented.

2. Experimental

2.1 Materials

Peroxidase from HRP (EC 1.11.1.7, type VI) was obtained from Sigma. Meldola Blue was purchased from Aldrich. Hydrogen peroxide (30%, w/v solution) and PVA (MW=75000-79000) were purchased from Shanghai Chemical Reagent Company. The concentration of these dilute peroxide solutions prepared from this material was determined by titration with cerium(IV) to a ferroin endpoint [18]. The RSF solution was prepared [19]. All other chemicals employed were of analytical grade and used without further purification. Doubly distilled water was used to prepare the solutions.

Membranes were cast by using the RSF solution or the solution with the given weight of silk fibroin, PVA and HRP on glass plates at room temperature in air.

2.2 Apparatus

All experiments were performed with a three electrode configuration consisting of an H_2O_2 sensor as a working electrode, a saturated calomel reference electrode and a platinum wire auxiliary electrode. The electrodes were connected to FDH 3204 and FDH 3206 cyclic voltammetry apparatus (Scientific Equipment of Fudan University, China) and the signal was recorded on a type 3086 x-y recorder (Tokyo, Japan) for cyclic voltammetric and amperometric measurements, separately. All experiments were carried out in a thermostated, stirred electrochemical cell containing 5 ml of 0.1 M phosphate buffer (pH 6.5) at 20.0±0.5°C. All experimental solutions were thoroughly deoxygenated by bubbling nitrogen through the solution for 6 min to reduce the background current. In the constant potential experi ments, 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 function of time, following the addition of H_2O_2. The sensor response was measured as the differences between total and residual current.

IR spectra of the dried membranes were recorded by the transmission method on a MAGNA-IR 550 (Nicolet) spectrometer at room temperature.

2.3 Construction of hydrogen peroxide sensor

Glassy carbon electrodes (3.5 mm in diameter) were polished with 0.3, 0.1 and 0.05 μM Al_2O_3, rinsed thoroughly in de-ionized water between each polishing step, successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and dried in air before use. 20 mg peroxidase was completely dissolved in 0.6 ml composite solution with 1:5 ratio of regenerated silk fibroin to PVA. Aliquots (35 μl) of the mixture were spread on a glassy carbon electrode and allowed to dry under ambient conditions for 20 h. Then 5 μl of 75% ethanol solution was pipetted onto the surface of the sensor and let dry. The sensor was kept dry in air at 4°C in a refrigerator between the measurements.

2.4 Calculation of Michaelis-Menten constant

The apparent Michaelis-Menten constant K_M^{app} can be determined from the electrochemical Eadie-Hofstee form of the Michaelis-Menten equation [20]

$$j_{ss} = j_{max} - K_M^{app}(j_{ss}/C),$$

where j_{ss} is the steady-state catalytic current, j_{max} represents the maximum current measured under saturated substrate conditions, C is referred to the H_2O_2 concentration and K_M^{app} stands for the apparent Michaelis-Menten constant of the system as a whole, not that of an intrinsic property of peroxidase.

3. Results and discussion

3.1 IR Spectra of the samples

Before ethanol treatment, RSF possesses a silk structure I with absorption bands at 1653 (amide I), 1543 (amide II) and 1243 cm^{-1} (amide III). PVA displays absorption bands at 1446 and 1332 cm- 1 (O-H bending), 1143 and 1094cm- 1 (C-O stretching). RSF in a composite membrane with 5:1 ratio of PVA to RSF maintains its silk I structure, displaying absorption bands at 1651 (amide I), 1539 (amide II), and 1238 cm^{-1} (amide III). HRP has absorption II) and 1236 cm^{-1} (amide III). The composite membrane with 5:1 ratio of PVA to RSF shows absorption bands at 1626 (amide I), 1528 (amide II) and 1234 cm^{-1} (amide III), a characteristic of silk structure II of RSF. The blend membrane of RSF and HRP exhibit absorption bands at 1627 (amide I), 1531 (amide II) and 1235 cm^{-1} (amide III), suggesting that ethanol treatment results in structure transition of RSF from silk structure I to silk structure II. Ethanol treatment does not affect the structures of PVA and HRP because their absorption bands are the same as those before ethanol treatment. The composite membrane of RSF, PVA and HRP displays absorption bands at 1651, 1538, 1443, 1333, 1237, 1142 and 1093 cm^{-1} indicating that ethanol treatment makes RSF in composite membrane change its structure from silk structure I to silk structure II, and that there are intermolecular interactions among them. Immobilization of HRP in the composite membrane is accompanied by a structural transition.

3.2 Cyclic voltammetry

Fig. 1 shows dependence of cyclic voltammograms on scan rate. In the absence of hydrogen peroxide, Meldola Blue in 0.1 M phosphate buffer exhibits diffusion-controlled reversible oxidation behavior for a two-electron couple since both the cathodic and the anodic peaks of the cyclic voltammogram given by Meldola blue were proportional to the square root of the potential scan rate ranging from 20 to 160 mV/s and the peak-to-peak separation (ΔE_p) of the cyclic voltammogram is 30 mV. Meldola Blue displays a formal potential (E^0) of -110 mV versus SCE at pH 7.0.

3.3 Hydrogen peroxide biosensing

No electrocatalytic reduction of hydrogen peroxide is observed at a glassy carbon electrode in 0.1 M phosphate buffer after hydrogen peroxide is added to the solution. The cyclic voltammograms in Fig. 2 demonstrate the bioelectrocatalytic reduction of hydrogen peroxide at the sensor via Meldola Blue. Compared with a cyclic voltammogram obtained in the absence of hydrogen peroxide (curve a in Fig. 2), it can be seen that there is a great increase in the cathodic peak current and a decrease in the anodic peak current (curve b in Fig. 2). The mechanisms of HRP-catalyzed reactions have been widely investigated. HRP is a glycoprotein with a molecular weight of approximately 44 000 [21] containing ferroprotoporphyrin as the strongly bound cofactor. HRP has been employed to catalytic reduction of hydrogen peroxide and certain organic peroxides to amplify the amperometric response of the sensor. In a first two electron step, hydrogen peroxide involves the oxidation of the ferriheme prosthetic group of HRP(Fe^{3+}), giving an unstable intermediate with the

oxyferryl iron (Fe^{4+}) and a porphrin π cation radical, compound I,

$$HRP(Fe^{3+}) + H_2O_2 \rightarrow \text{compound I} + H_2O.$$

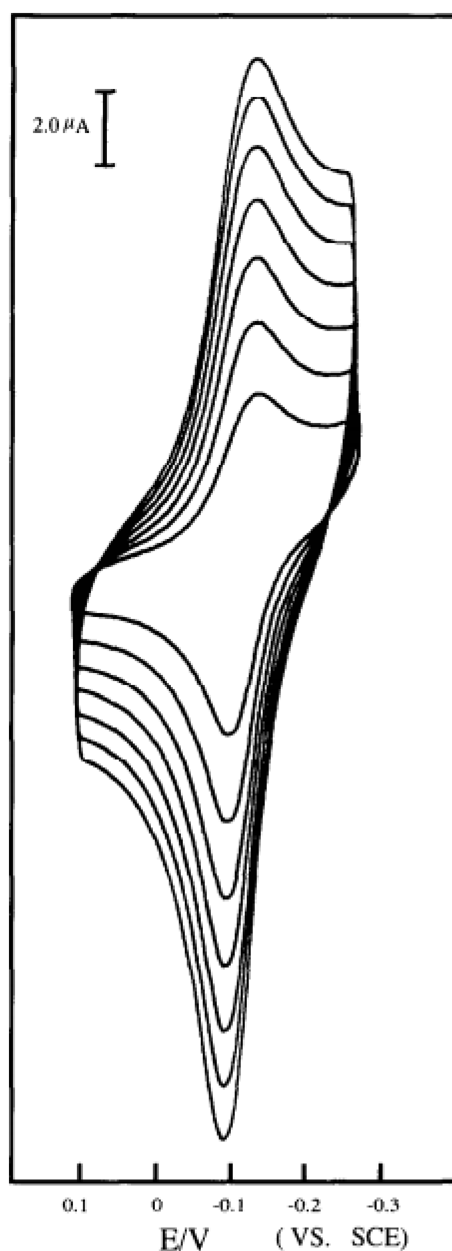

Fig. 1. Cyclic voltammograms of the H_2O_2 sensor at various scan rates of 25, 45, 65, 85, 105, 125, 145 mV/s (from inner curve to outer one) in 0.1 M phosphate buffer containing 0.5 mM Meldola Blue.

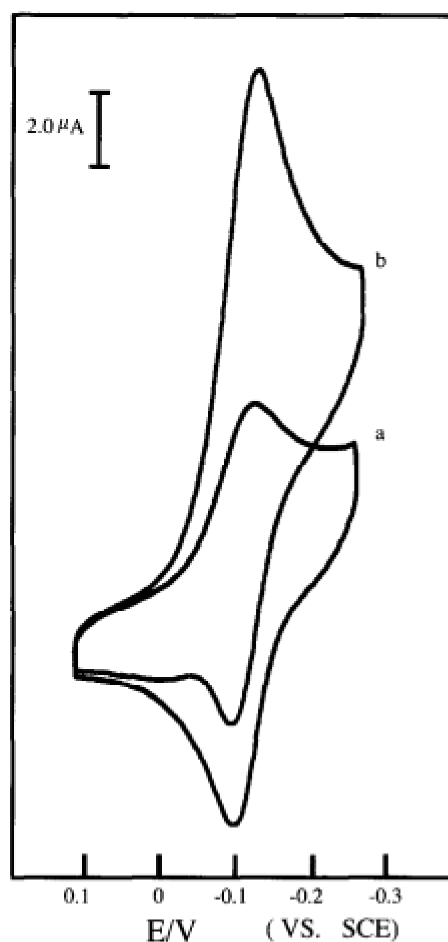

Fig. 2. Cyclic voltammograms of the H_2O_2 sensor at a scan rate of 35 mV/s in 0.1 M phosphate buffer (pH 7.0) containing 0.5 mM Meldola Blue in the absence of H_2O_2 (a) and the presence of 0.3 mM H_2O_2 (b).

The reduction of compound I to HRP can be achieved through two successive one-electron steps [22,23] via an electron transfer mediator or by direct electron transfer from the electrode to the heme site of the HRP in intimate contact to the conducting surface without a mediator.

$$\text{compound I} + Med_{red}(\text{or } e^-) \rightarrow \text{compound II} + Med_{ox},$$

$$\text{compound II} + Med_{red}(\text{or } e^-) \rightarrow HRP(Fe^{3+}) + Med_{ox}.$$

The oxidized mediator can be reduced at the electrode, bringing about a reduction current. In general, direct electron transfer between common electrode materials (such as platinum or graphite electrodes) and HRP is a slow process and electron transfer via a mediator is more efficient in the bioelectrocatalytic reduction of hydrogen peroxide at a HRP-based sensor.

Fig. 3 depicts a typical trace of the steady-state current-time response of the sensor. A well-defined and fast amperometric response is observed at -0.15V with successive injections of H_2O_2 and the time required to reach 95% of maximum response is less than 25s. Fig. 4 shows the calibration plot of the sensor response. A detection limit of 1.0×10^{-7} M can be estimated at a signal-to-noise ratio of 3 (a detection limit as low as 10^{-8} M has been reported [24,25]). Michealis-Menten constants of the sensor at applied potential of 150, 200 and 250 mV are 1.45, 1.94, 2.4 mM in 0.1 M phosphate buffer containing 0.5 mM Meldola Blue. The enhanced sensitivity and linear range of sensor with decreasing applied potential can be due to increasing driving force for the fast reduction of compound I and II.

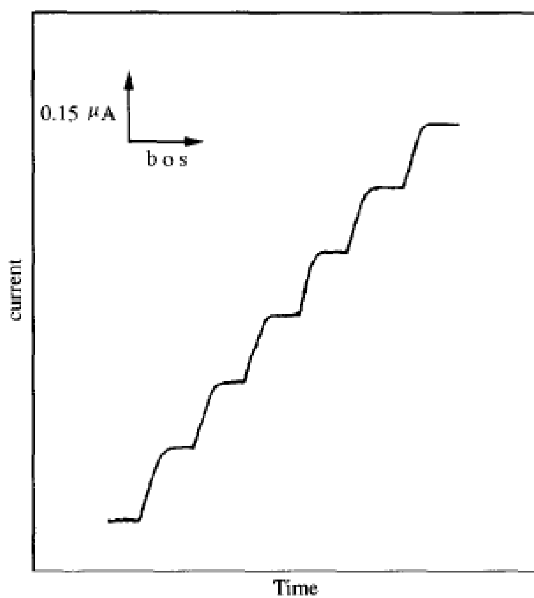

Fig. 3. Dynamic response of the H_2O_2 sensor to successive additions of H_2O_2 in 0.1 mM steps in a solution containing 0.5 mM Meldola Blue at the applied potential of −150 mV.

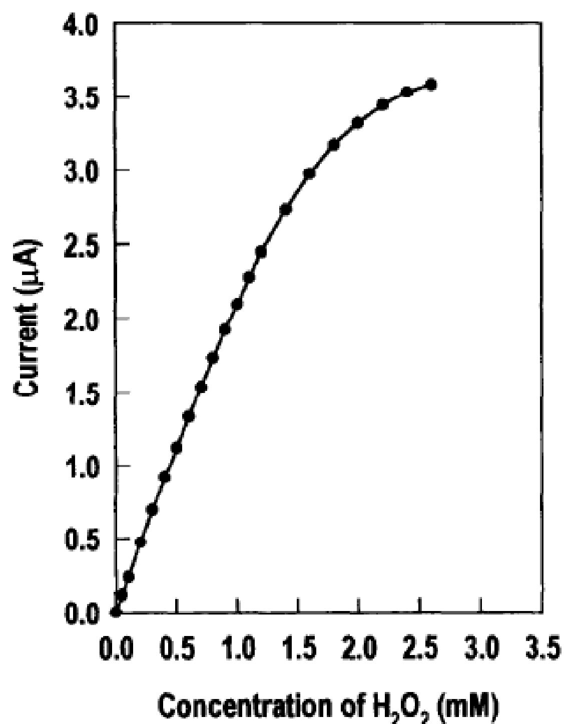

Fig. 4. Calibration plot for the H_2O_2 sensor, Steady-state current was measured in 0.1 M phosphate buffer (pH 6.5) containing 0.5 mM Meldola Blue at 20°C.

3.4 Effect Meldola Blue concentration on Michaelis-Menten constant

Dependence of the Michaelis-Menten constant on the Meldola Blue concentration is given in Table 1. Michaelis-Menten constant increases with an increase of the mediator concentration from 0.5 to 2.5 mM, showing the enhanced mediating ability with mediator concentration.

Table 1
Effect of the concentration of Meldola Blue (MB) on Michaelis–Menten constant at applied potential of −150 mV

Concentration of MB (mM)	0.5	1.0	1.5	2.0	2.5
K_M^{app} (mM)	1.45	2.12	2.62	3.15	3.38

3.5 Effect on pH temperature on the sensor

The pH affects not only the electrochemical behavior of Meldola Blue but also the activity of HRP, resulting in the dependence of the sensitivity and Michaelis-Menten constant of the sensor on pH. A formal potential (E^0) of Meldola Blue shifts negatively as pH increases. The sensor displays an optimum sensitivity of response at pH 6.5. The effect of pH on the Michaelis-Menten constant is summarized in Table 2.

Table 2
Effect of pH on Michaelis–Menten constant at −0.15 V in 0.1 M phsophate buffer containing 0.5 mM Meldola Blue

pH	5.5	6.0	6.5	7.0	7.5	8.0
Michaelis–Menten constant (mM)	1.24	1.38	1.45	1.32	1.20	1.09

Table 3
Effect of temperature on Michaelis–Menten constant at −0.15 V in 0.1 M phsophate buffer containing 0.5 mM Meldola Blue

Temperature (°C)	15	20	30	35	40	45
Michaelis–Menten constant (mM)	1.18	1.45	1.76	2.02	2.32	2.25

The effect of temperature on the sensor has been investigated between 15°-55°C. The sensitivity of the sensor to hydrogen peroxide increases with temperature and reaches a maximum value at 40°C which is characterized by the complete disappearance of oxidation peak in the cyclic voltammogram (compared with the result at 20°C shown in Fig. 2). The increased sensitivity of the sensor with temperature may be due to the enhanced speed of electron transfer between immobilized HRP and the electrode via Meldola Blue. At 35°C the sensor loses 9.5% of its activity in 5 h for 40 successive assays of 0.1 mM H_2O_2. Further increase of temperature results in a decrease of the response current because of loss of enzyme. In addition, temperature higher than 45°C leads to partial dissolution of PVA in the composite membrane. The dependence of Michaelis-Menten constant of temperature is summarized in Table 3.

3.6 Study of interferences

13 substances were used to evaluate the selectivity of the sensor. The current obtained for each possible interferants at given concentrations in the presence of 0.5 mM hydrogen peroxide was used as an indicator for the sensor selectivity in comparison with the hydrogen peroxide reading alone. L-Tyrosine (0.2 mM), galactose (5.0mM), L-leucine (0.2 n&I), L-cysine (0.2 mM), L-tryptophan (0.2 n&I), L-lactate (0.5 mM), glucose (5.0 n&l), uric acid (0.2 mM), Lcysteine (0.2 mM), L-aspartic acid (0.2mM), L-histidine (0.2mM) and L-glutamic acid (0.2mM) do not cause any observable interference to the determination of H_2O_2. However, addition of ascorbic acid (0.5 mM) causes a decrease of bioelectrocatalytic reduction currents (about 13.8%), which may result from the consumption of hydrogen peroxide reduced by ascorbic acid or from the competition of ascorbic acid with Meldola Blue for the reduction of compound I and II.

3.7 The stability of the sensor

The operational stability has been studied by recording over 20 successive assays of 0.5 and 0.08 mM H_2O_2, the relative standard deviations are 2.2% and 2.5%, respectively. The storage stability of the sensor stored at 4°C has been investigated by checking its relative activity every 3 days. The sensor maintained 95.6% of its activity during a month and 89.4% during two months' storage.

4. Conclusion

Entrapment of HRP in novel composite membrane of RSF and PVA offers a useful immobilization of the enzyme. An amperometric Meldola Blue-mediated sensor provides favorable analytical features for hydrogen peroxide biosensing including high stability, sensitivity and low detection limit. This results from the high efficiency of bioelectrocatalytic reduction of hydrogen peroxide via Meldola Blue. These features ensure the possibility of detecting hydrogen peroxide formed by a variety of oxidases combined with the H_2O_2 sensor. In addition, we have for the first time found that a series of compounds such as Methylene Blue, Phenazine Methosulphate, Cresyl Fast Biolet, Methylene Green, Catechol Violet, Brilliant Cresyl Blue, Toluidine Blue, 3-pnaphthoyl-Nile Blue A and New Methylene Blue N can also enhance electron transfer between immobilized HRP and a glassy carbon electrode.

Acknowledgements

This work is supported by the National Key Topics of China, the Natural Science Foundation of Shanghai for Young Scientists, the National Natural Science Foundation of China and the Open Electroanalytical Chemistry Laboratory of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences.

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