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ORIGINAL PAPER

A Prussian blue-based amperometric sensor for the determination of hydrogen peroxide residues in milk

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Abstract A highly selective, sensitive, fast, and stable amperometric sensor for the determination of hydrogen peroxide residues in aseptic milk is presented. To fabricate this amperometric sensor, a thin film of Prussian blue was first electrodeposited on a glassy carbon electrode and then a Nafion polymer layer was formed on the top. It was found that Nafion film greatly improves the anti-interference ability and the stability of the Prussian blue-modified electrode. Factors that influence the overall analytical performance of the sensor, such as the concentration of Nafion drop and pH value of the electrolyte, were examined. Results show that the prepared sensor possesses efficient electrocatalytic activity towards hydrogen peroxide with the detection limit as low as 0.2 μM and linear range from 0.8 μM to 0.12 mM. The developed sensor was applied to the determination of hydrogen peroxide in milk with satisfactory results.

Keywords Hydrogen peroxide · Prussian blue · Nafion · Amperometric sensor · Milk

Introduction

Hydrogen peroxide (H_2O_2) is one of the products of reactions catalyzed by several oxidase in many biological and environmental processes [1, 2]. Meanwhile, because of its inherent sporicidal and bactericidal properties [3, 4], H_2O_2 is also used for the cleaning of instruments and equipment including mixing, transporting, bottling, and packing in food industry [5]. If the subsequent washing and drying process is incomplete, the foodstuff could be contaminated with H_2O_2 residues, which would be irritative to the skin and affect human health. Thus, the determination of H_2O_2 is of great importance in food industry [6–8].

There are many analytical methods that can be used for the determination of H_2O_2, but most of them show limitations such as low sensitivity, less anti-interference ability, and a lack of stability. The use of the electrochemical method could overcome these drawbacks because of its simplicity, fast response, and high sensitivity [9, 10]. It is well known that H_2O_2 is an electroactive species, which can be easily oxidized or reduced. By modifying an electrode surface with Prussian blue (PB), it is in fact possible to easily detect H_2O_2 at an applied potential around 0 V versus Ag/AgCl, making it avoid or reduce the possible electrochemical interferents [11–13]. Recently, papers have appeared on constructing oxidase enzyme-based biosensors for the determination of choline [14], cholesterol [15], glutamate [16], lactate [17], glucose [18], etc.

In this paper, a PB-based amperometric sensor is present for the determination of H_2O_2 in commercially aseptic milk. In the manufacturing process of milk, H_2O_2 is commonly used as a stabilizer [19–21], in well-defined concentrations, in some European and American countries, and particularly in certain preparations of hard cheeses as an alternative to pasteurization. In general, sufficient sterilization is obtained at 0.1% H_2O_2. Nevertheless, the H_2O_2 residues in milk would affect its quality and consumption. So far, only few papers have been reported for the determination of H_2O_2 residues in milk [22–24]. All these measurements are based on the catalysis decomposition role of enzymes including

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catalase and horseradish peroxidase. However, enzyme-based biosensors are expensive, easily trapped by other substances, and inherently unstable. Here, a non-enzyme sensor based on PB mediator-modified electrode was employed to detect H_2O_2. In some cases, especially in food quality control, it is necessary to detect low levels of analytes in the presence of 50–500 higher concentration of interferents. Also, in milk, there are many grain suspended substances, ions, and macromolecular compounds that can adsorb to the electrode surface. Therefore, a Nafion film was required to protect the PB film. Nafion is a commercially available perfluorinated sulfonate ionomer widely used in biosensor working as a protective coating material against electrode surface fouling and as a support for enzyme immobilization [25, 26]. At the same time, the Nafion film can exclude macromolecular and anionic species by charge and/or size from reaching the electrode surface [27, 28]. In this way, no sample pretreatment is required. The developed sensor shows high sensitivity, selectivity, and long-time stability. To our knowledge, there is no paper on such PB-based sensor for the determination of H_2O_2 residues in milk up to now.

Experimental

Chemicals

All reagents were prepared with chemicals of analytical grade. A solution of Nafion (5% *w*/*v*, in 90% light alcohol), catalase (EC 1.11.1.6, 4,000 U mg^{-1}), and H_2O_2 (30% *w*/*w*) was purchased from Sigma and used as received. Millipore-Q (18.2 MΩ cm^{-1}) water was used for all experiments. The Nafion solution was diluted with ethanol (100%) to 0.25%, 0.50%, 0.75%, and 1.0%. H_2O_2 solution was prepared using double-distilled water and stored in a refrigerator (4 °C), and the concentration of dilute hydrogen peroxide was determined by titration with potassium permanganate. Aseptically packaged milk was obtained from a local market in Hangzhou, China.

Preparation of sensor

Before surface modification, glassy carbon electrode (GCE, 3 mm diameter) was polished with successively finer grade aqueous alumina slurries (grain size 5–0.5 μm) on a polishing cloth. The composite films were prepared in two stages. The first modification step was the deposition of PB film accomplished by applying a working potential of 0.4 V for 40 s in a solution containing 3.0 mM $FeCl_3$, 3.0 mM $K_3Fe(CN)_6$, 0.1 M KCl, and 0.1 M HCl. After washing with water, the electrode was dried at 100 °C for 1 h. Then the modified electrode was immersed in a phosphate buffer solution (PBS, pH=5.8, 0.05 M K_2HPO_4/KH_2PO_4 and 0.1 M KCl) for an electrochemical activation by keeping it at −0.05 V versus Ag/AgCl for 600 s. Nafion membrane was prepared by syringing an appropriate amount (usually 3.0 μl) and allowing the solvent to evaporate at ambient temperature. Thereafter, the film-modified electrode was placed in PBS for 10 min to achieve equilibrium of the layers.

Apparatus and procedures of measurement

All the electrochemical measurements were carried out on CHI 440 electrochemical workstation (CH Instruments USA). A conventional three-electrode system, consisting of a GCE coated with PB/Nafion film as a working electrode, a saturated Ag/AgCl electrode as a reference electrode, and a platinum wire as an auxiliary electrode, respectively, was used. The amperometric measurements of H_2O_2 response were performed at 0 V versus Ag/AgCl, and the solution was stirred gently. The milk samples were prepared by mixing packaged milk and PBS (pH5.8) with a volume ratio of 1:1. Before the test, catalase was employed to confirm whether the milk samples contain H_2O_2. For the determination of H_2O_2 in milk samples, a series of standard concentration of H_2O_2 was injected into the working solution when a steady state of the current response was obtained. The difference between the baseline and the steady-state current was used to calculate the current responses relative to concentrations of H_2O_2. All the measurements were carried out at room temperature.

Results and discussion

Electrochemistry of the PB/Nafion film-modified GCE

Figure 1 shows typical cyclic voltammograms of PB/Nafion film-modified GCE recorded in the absence and presence of 4.0 mM H_2O_2 in unstirred PBS. In the absence of H_2O_2, a well-defined reversible electrochemical behavior of PB was observed (Fig. 1a). After the addition of H_2O_2, a clear increase was found for the reduction peak, whereas the oxidation peak decreased (Fig. 1b). Moreover, the redox peaks moved towards more negative potential. Comparing with these two curves, it was found that the PB/Nafion composite film possesses high electrocatalytic activity towards H_2O_2, which provides an effective way of using it as a sensor to detect H_2O_2.

Determination of H_2O_2

Optimization of experimental variables

To improve the performance of the sensor, various factors influencing the response of the sensor were investigated,

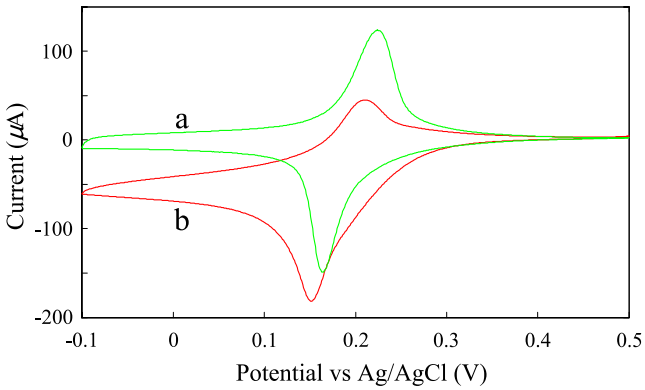

Fig. 1 Cyclic voltammograms of the PB/Nafion film-modified GCE in 0.05 M PBS (pH 5.8) in **a** the absence of H_2O_2 and in **b** the presence of 4.0 mM. Scan rate: 0.05 V/s

including the concentration of Nafion drop and pH of the solution. The effect of the concentration of Nafion drop on the response of the sensor is illustrated in Table 1. The optimization of the concentration of Nafion drop was studied based on the amperometric responses of H_2O_2 as well as the response time and the ability of the anti-interferents. The current response towards 10.0 μM H_2O_2 decreases obviously with the increasing concentration of the Nafion drop. Also, the response became slower for the thicker Nafion film probably due to the diffusion problem. However, it was found that the ability of anti-interferents decreases remarkably when the PB film was coated with low concentration or none of the Nafion membrane. Such a decrease in the ability of anti-interferents is attributed to the fact that the lesser amount of Nafion molecules on the surface of PB film has not enough negative charge to electrostatically exclude the interferents such as ascorbic acid and uric acid. Therefore, the level of 0.50% for the Nafion drop was chosen in further experiments.

Another study was conducted to examine the influence of the pH of the solution on the sensitivity and stability of the sensor. It is well known that PB film is stable in acidic and neutral solution while unstable in alkaline solution. Results showed that the sensor has the maximum response toward H_2O_2 in the pH range 4.8–6.8.

Table 1 The influence of the concentration of Nafion droplets on the response and detection limit of the sensors

Nafion concentration[a]	Current (μA)[c]	Detection limit (M)
PB[b]	2.01	1×10^{-7}
0.25%	1.85	1×10^{-7}
0.50%	1.72	2×10^{-7}
0.75%	1.52	4×10^{-7}
1.00%	1.34	5×10^{-7}
1.25%	1.11	6×10^{-7}

Applied potential=0 V

[a] The volume of every drop was 3.0 μl

[b] GCE/PB without modified Nafion film

[c] H_2O_2 concentration was 10.0 μM, n=6

Amperometric response of H_2O_2

Figure 2 displays a typical current–time plot of the PB/Nafion film-modified GCE on successive addition of 3.0 μM H_2O_2 into stirring PBS at the applied potential of 0 V. It was found that the current increases sharply with the addition of H_2O_2 and reaches maximum steady-state current in 3–5 s. The fast response is attributed to the fact that H_2O_2 can penetrate the Nafion film smoothly and be reduced under the electrocatalysis of PB. A plot of current versus H_2O_2 concentration is given in Fig. 2 (inset) and the correlation coefficient is 0.9992. For the prepared sensor, the linear range is from 0.8 μM to 0.12 mM with a detection limit of 0.2 μM based on a signal-to-noise ratio of 3. This detection limit is low enough to satisfy the requirement for the determination of H_2O_2 residues in food.

The reproducibility of single electrode was investigated by 30 injections of 10.0 μM H_2O_2 in PBS. The relative standard deviation (RSD) was 2.8%. Moreover, the electrode-to-electrode reproducibility was also checked using the amperometric response of the electrodes to 10.0 μM H_2O_2. Ten GCEs were used to test and the RSD is 4.3%. Thus, the reproducibility of the prepared sensor is acceptable.

Here, we also investigated the possible interferents for the determination of H_2O_2. The evaluation of selectivity is based on calculating the ratio of current response toward 5.0 mM interferents to current response toward 0.01 mM H_2O_2. The amperometric measurements were performed at 0 V versus Ag/AgCl (Supporting Information). As showed in Table 2, the sensor was highly selective for H_2O_2, and

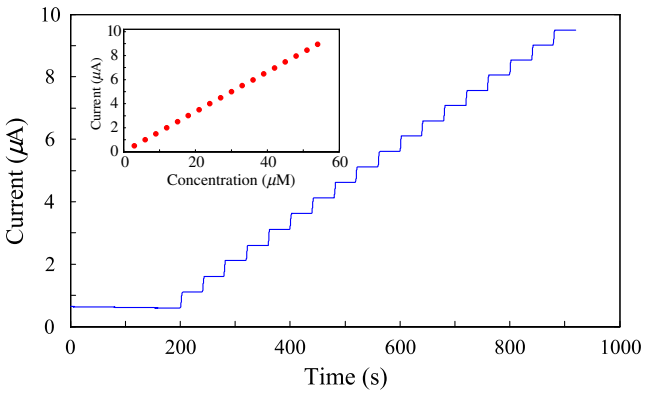

Fig. 2 Amperometric response of the PB/Nafion film-modified GCE on successive injection of 3.0 μM H_2O_2 into stirring PBS. Applied potential=0 V. Concentration of Nafion drop=0.5%. *Inset* linear plots of i versus H_2O_2 concentration

Table 2 The ratio of the response towards 5.0 mM interferents to the response towards 0.01 mM H_2O_2 on the PB/Nafion film-modified GCE

Interferent	Response ratio (%)
Ascorbic acid	<1.33
Glucose	<0.02
Sucrose	<0.03
L-Glutamic acid	<0.10
Uric acid	<1.20
D-Fructose	<0.06
Citric acid	<0.21

Applied potential=0 V; concentration of Nafion drop=0.5%

various interferents only produced minor interference (<1.5%).

The storage stability was examined by comparing the changes of the current response of 10.0 μM H_2O_2 (Supporting Information). The sensors were stored in a dry state in a refrigerator (4 °C) when not in use. No obvious decrease in the response to H_2O_2 was observed after 30 days of storage. The sensor retains 90% of the initial current response after a 40-day storage period. These results imply that the prepared sensor possesses good operational stability and long-time stability, which would be very suitable for the sensing application.

These good performances of the investigated sensor can be ascribed to the novel combination of PB and Nafion film. PB molecule has high spin divalent iron ion, which possesses catalytic activity for the reduction of H_2O_2 [29]. Thus, the modification of PB onto GCE can greatly enhance the sensitivity for the H_2O_2 detection. The negatively charged Nafion film only allows the cations and neutral molecules to pass through. In this way, it can slow down the dissolution of PB from electrode surface into solution, which prolongs the stability of PB film. Furthermore, some macromolecules such as protein are barred from absorbing to the electrode surface due to the size exclusion of Nafion film.

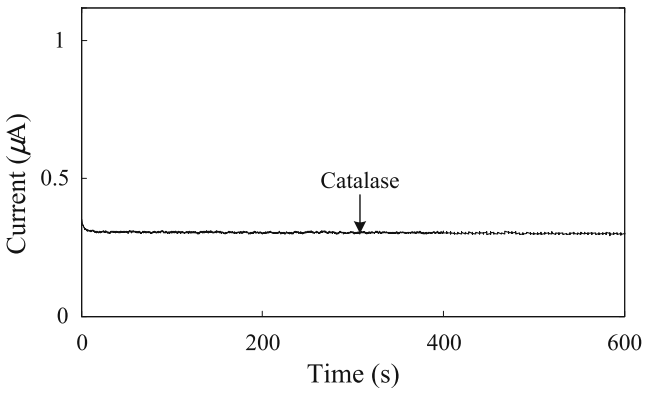

Fig. 3 Amperometric response of the PB/Nafion film-modified GCE on the milk sample with no addition of H_2O_2. Catalase: 40.0 U/l. Applied potential=0 V. Concentration of Nafion drop=0.5%

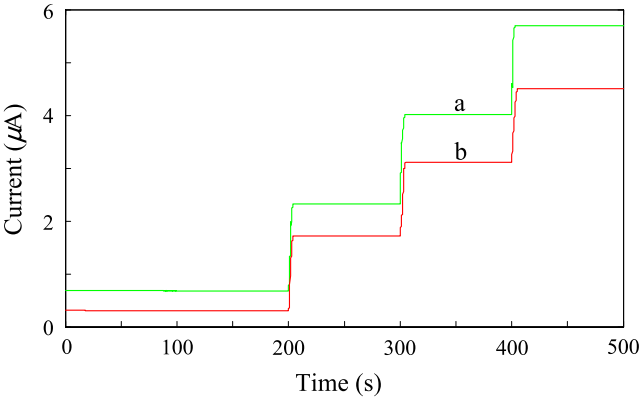

Fig. 4 Amperometric responses of the PB/Nafion film-modified GCE towards 10.0 μM H_2O_2 in **a** PBS pH 5.8 and **b** milk sample. Applied potential=0 V. Concentration of Nafion drop=0.5%

Real sample analysis

The feasibility of the proposed amperometric sensor for use in measuring H_2O_2 in milk sample was investigated. It should be noted that the only sample treatment required in all cases is the appropriate sample dilution with the PBS solution. Prior to the test, catalase was employed to confirm whether the milk samples contain H_2O_2. As shown in Fig. 3, no changes in current response of the milk sample were observed on the injection of catalase (40.0 U/l), which indicates that there are no H_2O_2 residues in the samples. After verifying the absence of endogenous H_2O_2 in the aseptic milk samples, the performance of the sensor for the analysis of H_2O_2 in these samples was carried out by standard addition method. Figure 4 shows the typical amperometric response on the determination of H_2O_2 in milk sample using the PB/Nafion-modified GCE. The baseline of the current response in milk sample (Fig. 4b) is lower than the one in PBS (Fig. 4a), suggesting the

Table 3 The recovery of H_2O_2 in milk sample

H_2O_2 concentration (μM)		Recovery (%)
Added	Found[a]	
0.75	0.74	98.7
1.50	1.49	99.3
2.50	2.58	103.2
7.50	7.53	100.4
10.0	9.98	99.8
15.0	15.2	101.3

Applied potential=0 V; concentration of Nafion drop=0.5%

[a] The average value of the six measurements

fouling of electrode surface in the milk. On the injection of H_2O_2, the current responds quickly and reaches the maximum current in 4–6 s. The current value in milk sample is a bit smaller than the one in PBS, which can also be attributed to the fouling of electrode surface in the milk samples. In the optimized conditions, the prepared sensor exhibited a linear range from 0.75 to 100.0 μM H_2O_2 with a detection limit of 0.55 μM in milk sample. The feasibility of the developed sensor for the determination of H_2O_2 in milk sample was further examined by the recovery tests. The results (see in Table 3) indicated that the sensor possesses reasonable selectivity and produces satisfactory recovery results with an average recovery of 100.45% and the RSD was less than 4.7%. These findings demonstrate that the sensor prepared in this paper could be used for the measurement of H_2O_2 in aseptic milk samples.

Conclusions

This paper demonstrates the feasibility of PB/Nafion film-modified GCE for the detection of H_2O_2 in aseptic milk. The Nafion film could greatly improve the selectivity and stability of the PB-modified GCE. No pretreatment was performed in the milk sample. The sensor developed in this paper shows efficient electrocatalytic ability to the reduction of H_2O_2. In comparison with conventional enzyme-based H_2O_2 sensors, PB-based sensors have some advantages such as long-time stability, low cost, and simplicity. In real sample analysis, PB/Nafion film could eliminate the effect of interferents and decrease the fouling of electrode surface from the sample electrolyte. This is the first attempt to apply PB-based sensor to trace analysis of H_2O_2 residues in aseptic milk.

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