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ORIGINAL PAPER

A mediator-free amperometric hydrogen peroxide biosensor based on HRP immobilized on a nano-Au/poly 2,6-pyridinediamine-coated electrode

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Abstract A mediator-free amperometric hydrogen peroxide biosensor was prepared by immobilizing horseradish peroxidase (HRP) enzyme on colloidal Au modified platinum (Pt) wire electrode, which was modified by poly 2,6-pyridinediamine (pPA). The modified process was characterized by electrochemical impedance spectroscopy (EIS), and the electrochemical characteristics of the biosensor were studied by cyclic voltammetry, linear sweep voltammetry and chrono-amperometry. The biosensor displayed an excellent electrocatalytical response to reduction of H_2O_2 without the aid of an electron mediator, the linear range was 4.2×10^{-7}–1.5×10^{-3} mol/L ($r = 0.9977$), with a detection limit of 1.4×10^{-7} mol/L. Moreover, the performance and factors influencing the resulted biosensor were studied in detail. The studied biosensor exhibited permselectivity, good stability and good fabrication reproducibility.

Keywords 2,6-Pyridinediamine · Colloidal Au · Horseradish peroxidase · Biosensor · Electropolymerization

Introduction

The determination of H_2O_2 is becoming of practical importance in chemical, biological, pharmaceutical, clinical, environment analyses and in many other fields. Various methods, such as titrimetry, spectrometry, chemiluminescene and electrochemistry [1–9], have been employed for the determination of hydrogen peroxide. The first three techniques have obvious drawbacks because they are time-consuming and expensive. The electrochemical technique coupled with the intrinsic selectivity and sensitivity of enzymatic reactions is promising for the fabrication of simple and low-cost enzyme sensors [10].

Recently, conducting polymers have attracted much interest in the development of biosensors. They are used to enhance speed, permselectivity, sensitivity and versatility of biosensors in diagnostics to measure vital analytes. As a suitable matrix of proteins, incorporating enzymes or protein molecules into conducting polymeric films permit the localization of biologically active molecules on electrodes of any size or geometry and is particularly appropriate for the fabrication of multianalyte micro-amperometric biosensors [11–15]. Moreover, conducting polymers have the ability to efficiently transfer electric charge produced by the biochemical reaction to electronic circuit [16]. The electrically conducting polymers are known to possess such numerous features, which allow them to act as excellent materials for immobilization of biomolecules and rapid electron transfer for the fabrication of efficient biosensors [17, 18].

Colloid Au (nano-Au), because of their large surface area, low electron transfer impedance, good biocompatibility and suitability for many surface immobilization mechanism, have been effectively used for the immobilization of various biomolecules such as enzymes, proteins, DNA and so on [19–21] and have

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proven to be excellent substrates in many fields ranging from biosensors to interfacial interaction studies.

Until now, horseradish peroxidase (HRP) has been the commonly used enzyme for the construction of a H_2O_2 biosensor with [22] or without [23–25] mediators. The enzyme contains heme as a prosthetic group, which is also the protein active site with the resting state of the heme-iron: Fe(III), and it can catalyze the H_2O_2 dependent one-electron oxidation of a great variety substrates [26]. Among these applications, the construction of the third-generation biosensor caused much attention, as the direct electron transfer (DET) between an enzyme and an electrode surface will permit the sensitive and convenient electrochemical measurement of the enzyme substrate without the addition of a mediator.

In this study, a mediator-free amperometric hydrogen peroxide biosensor has been prepared. First, The poly 2,6-pyridinediamine (pPA) film was formed on platinum (Pt) electrode by utilizing electropolymerization, which yield an interface with amine groups for the assembly of colloidal Au. Then, HRP was chemiadsorbed onto the surface of nano-Au/pPA-coated electrode. Compared with HRP immobilized on gold nanoparticles using cysteine self-assembled monolayer on gold electrodes, the conducting polymer has the ability to fast immobilize HRP, enhance direct, reversible electron transfer between HRP and electrode without the aid of an electron mediator. Thus, the biosensor is stabler than the electrode modified with self-assembled layers and an electron mediator. On the other hand, the conducting polymer has permselectivity. The biosensor displayed an excellent electrocatalytical response to reduction of H_2O_2, the linear range was 4.2×10^{-7} –1.5×10^{-3} mol/L ($r = 0.9977$), with a detection limit of 1.4×10^{-7} mol/L. Comparing the result with some literatures, which have reported that the linear range were 5.0×10^{-7}–1.6×10^{-3} mol/L and so on [3, 4, 8, 10, 27, 28]. The biosensor showed broader linear range, lower detection limit. Moreover, the studied biosensor exhibited strong permselectivity, good stability and good fabrication reproducibility.

Materials and methods

Materials

Horseradish peroxidase (HRP, EC 1.11.1.7, type VI) was obtained from Sigma. 2,6-Pyridinediamine (PA) was purchased from Bailingwei Chemical Regant Co, China. H_2O_2 (30%, m/v solution) was purchased from Shanghai Chemical Regant Co, China, and fresh solutions of H_2O_2 were prepared daily. All other chemicals were of analytical grade and were used as received. Doubly distilled water was used throughout this study. Spherical colloidal gold nanoparticles were prepared by citrate reduction of $HAuCl_4$ in aqueous solution [29]. The average nanoparticle diameter is 16 nm as measured by TEM (not shown here).

Apparatus

Amperometric measurement and cyclic voltammetric experiment were performed on an electrochemical workstation CHI 660A (Shanghai Chenhua Instrument Co, China). All electrochemical measurements were performed in the three-electrode system. An HRP/nano-Au/pPA-modified Pt electrode was a work electrode. A Pt wire and a saturated calomel electrode (SCE) were used as a counter and reference electrode, respectively. The AC impedance of the electrode membrane was measured with a Model IM6e (ZAHNER Elektrick, Germany).

Electrode modification

Platinum wires with a diameter of 1.0 mm were polished carefully with 0.6 μm diamond powder, rinsed thoroughly two times with water, then boiled in nitric acid (1:1) for 10 min, ultrasonicated in acetone and washed in water two times, respectively.

The pPA film was formed on cleaned Pt electrode by utilizing electropolymerized voltage between –0.8 and +1.6 V versus SCE in the solution of 1×10^{-2} mol/L 2,6-pyridinediamine + 0.1 mol/L $NaNO_3$ (pH 6.0). Then the electrode was washed by water and dried in air. After that, the electrode was immersed in the colloid Au solution for 6 h at 4 °C. On this way, the electrode was assembled with sufficient Au particles for the enzyme immobilization. Finally, the electrode was immersed in the HRP (3 mg/mL, pH 6.5 Phosphate) at 4 °C overnight. Thus, the pPA membrane/nano-Au monolayer/HRP layer was formed on the Pt electrode surface. Figure 1 showed the illustration of multiplayer structure. All resulting electrodes were washed with water and stored at 4 °C when not in use.

Experimental measurements

Electrochemical experiments were performed in a conventional electrochemical cell containing a three electrode arrangement, and the potential swept from –0.4 to 0.7 V (vs. SCE) with a sweeping rate of 100 mV/s in 5 mL Britton–Robinson (B.R) buffer solution (pH 6.5) at room temperature. The electrochemical

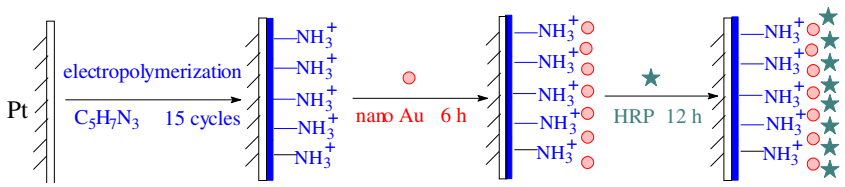


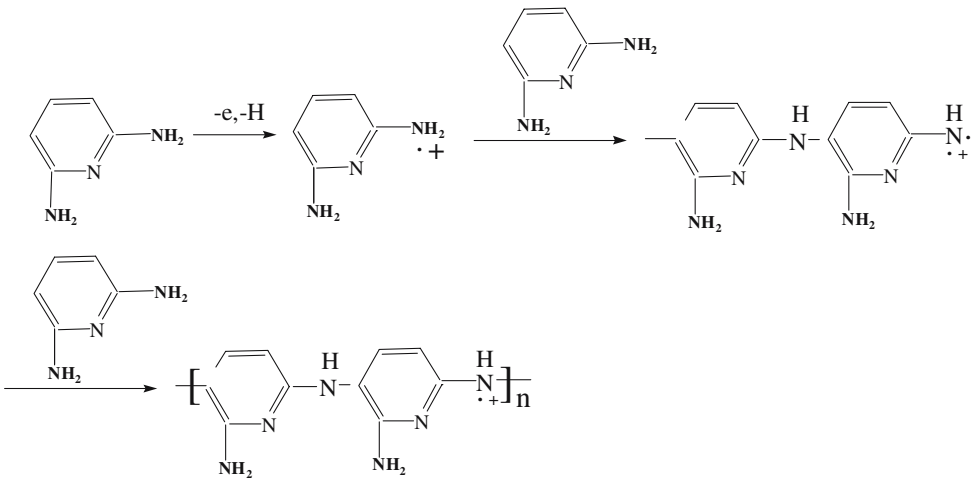
Fig. 1 Peparation process of the HRP biosensor on the Pt electrode

characteristics of the modified electrode were characterized by using impedance measurements (electrochemical impedance spectroscopy, EIS). It was performed in the presence of a 1.0×10^{-2} mol/L $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as a redox probe in 1.0×10^{-2} mol/L phosphate buffer (containing 0.1 mol/L KCl, pH 5.5). Impedance measurements were performed at the frequency range from 10^{-2} to 10^6 Hz at the formal potential of 220 mV, using alternating voltage of 10 mV.

Results and discussion

Polymerization of 2,6-pyridinediamine

The Pt electrode was modified with a permselective conducting polymer by electropolymerization of 2,6-pyridinediamine to reject interferences. According to the heterocyclic compound's orientation rule and oxidation law [30, 31], we assume that the electropolymerization of 2,6-pyridinediamine is as follows:



The pPA film has abundant amine groups which can absorb colloidal Au to immobilized HRP. Moreover, the conducting polymers have the ability to efficiently transfer electric charge produced by the biochemical reaction to electronic circuit. The electropolymerization process was performed by cyclic voltammetry from –0.8 to +1.6 V versus SCE on bare Pt electrode from 1×10^{-2} mol/L 2,6-pyridinediamine in 0.1 mol/L $NaNO_3$ (pH 6.0) as shown in Fig. 2. Generally, the thickness of the electropolymerized film influences the current response of the electrode. As a thicker film would reject the interferences, the sensitivity to analyte would also be lowered and response time increased, while too thin a film, the permselective behavior would not be satisfactory [32]. The polymerized film formed by electropolymerization 2,6-pyridinediamine in the solution of 1×10^{-2} mol/L 2,6-pyridinediamine for 15 cycles can bring a relatively stable current response for the biosensor, which is applied in all following experiments.

Cyclic voltametry

The electrocatalytic behavior of the biosensor towards the electrochemical reduction of H_2O_2 was studied using cyclic voltammetry. Figure 3 shows the bioelectrocatalytic behavior of the biosensor in 5 mL B.R (pH 6.5). In the blank Britton–Robinson buffer, no obvious current was found (Fig. 3a), but an obvious catalytic current was caused in the presence of 8.0×10^{-5} mol/L H_2O_2 (Fig. 3b). It demonstrated that the biosensor had a good response to H_2O_2. To verify whether the current was due to nonenzymatic reduction of hydrogen peroxide, a control experiment in the

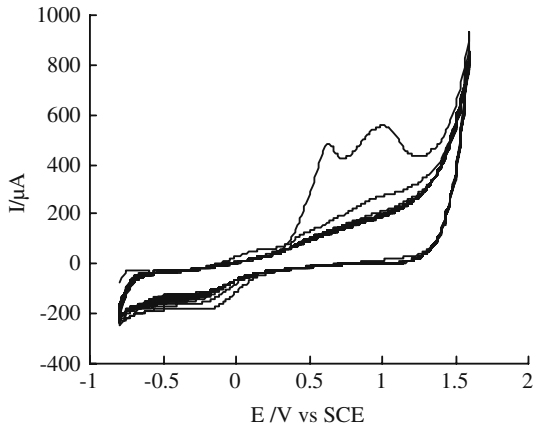


Fig. 2 Electropolymerization of PA at Pt electrode from 1×10^{-2} mol/L 2,6-pyridinediamine in 0.1 mol/L $NaNO_3$ (pH 6.0) with scans from –0.8 to +1.6 V at a scan rate of 100 mV/s

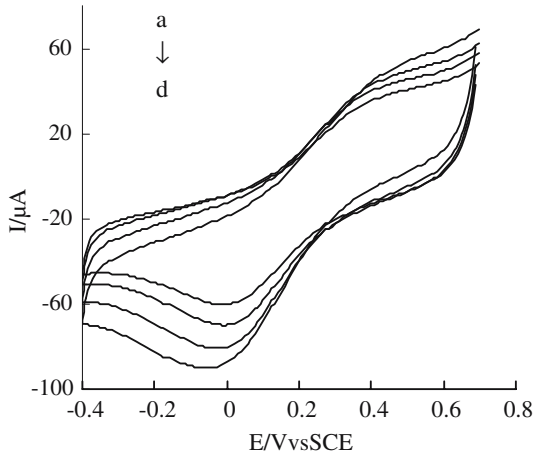


Fig. 3 Cyclic voltammograms of the HRP biosensor in 5 mL B.R (pH 6.5) without (**a**) and with 6.1×10^{-7} mol/L H_2O_2 (**b**); 8.9×10^{-6} mol/L H_2O_2 (**c**); 7.4×10^{-5} mol/L H_2O_2 (**d**), respectively. Scan rate 100 mV/s

absence of HRP was performed. At the colloidal Au/pPA/Pt electrode, the catalytic current response to H_2O_2 decreased significantly, and the reduction current starts at a more negative potential (Fig. 4b). The advantage of using the colloidal Au could also be seen from the comparison in Fig. 4c, when colloidal Au is not assembled on the electrode, at the HRP/pPA/Pt electrode, the electrocatalytic current of H_2O_2 also decreases markedly. From these results, we confirmed that the catalytic current was mainly due to the direct electron transfer between the HRP molecules and the electrode, that is colloidal Au could act as a bridge to link HRP molecules and pPA/Pt electrode. The presence of colloidal Au allowed efficient electron tunneling and assisted the electron transfer between the redox protein and the bulk electrode surface [10].

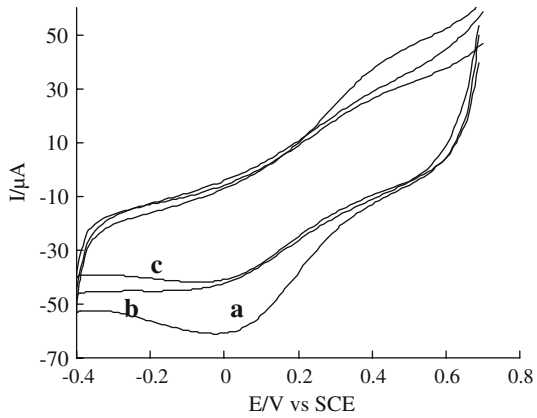


Fig. 4 Cyclic voltammograms of different electrodes in 5 mL B.R (pH 6.5) and 4.0×10^{-5} mol/L H_2O_2 (**a**) HRP/colloidal Au/pPA/Pt electrode; (**b**) colloidal Au/pPA/Pt electrode; (**c**) HRP/pPA/Pt electrode. Scan rate, 100 mV/s

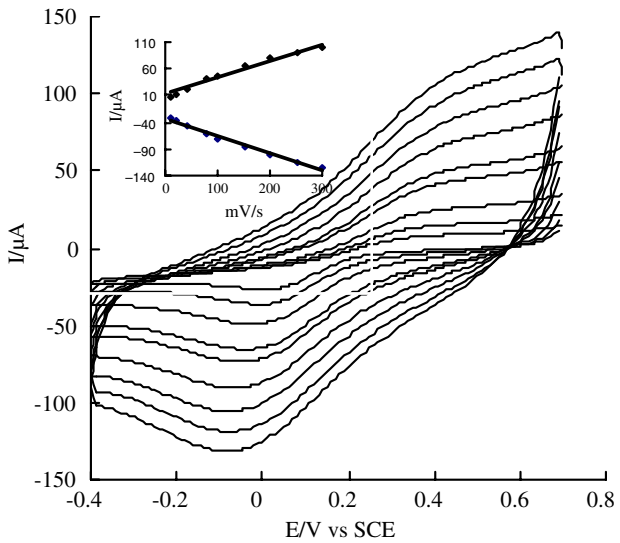


Fig. 5 Cyclic voltammograms of the modified electrode at different scan rates (from inner to outer): 10, 20, 40, 80, 100, 150, 200, 250 and 300 mV/s in 5 ml B.R. buffer solution (pH 6.5) under room temperature. All potentials are given versus SCE. The *inset* shows the dependence of redox peak currents on the potential sweep rates

The CVs of the resulting biosensor in B.R buffer solution (pH 6.5) at different scan rates were shown in Fig. 5. The finding that the biosensor represents reversible surface waves in cyclic voltammetry, indicates that the HRP, which entrapped in colloidal Au/pPA membrane, is efficiently connected on Pt electrode for facile charge transfer. Furthermore, both the anodic and cathodic peak currents were directly proportional to the potential scan rates in the range of 10–300 mV/s, as shown in the inset of Fig. 5, suggesting a surface confined redox process [33].

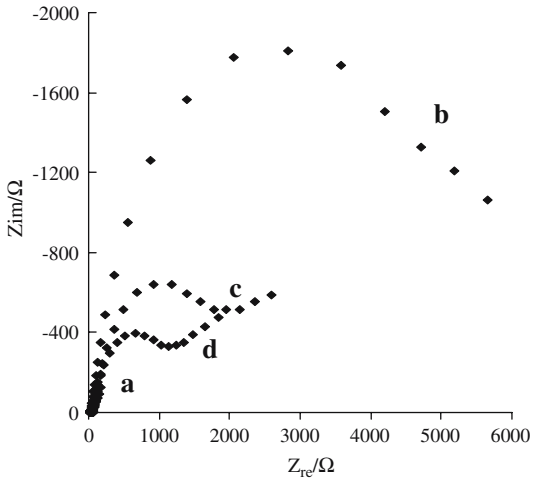


Fig. 6 EIS of the different electrodes: **a** a bare Pt electrode; **b** pPA-modified Pt electrode; **c** nano-Au/ pPA-modified Pt; **d** HRP/ nano-Au/pPA-modified Pt. Supporting electrolyte, 1.0×10^{-2} mol/L PBS + 0.1 mol/L KCl + 1.0×10^{-2} mol/L $Fe(CN)_6^{4-/3-}$ solution (pH 6.5). The frequency range is at 1×10^{-2}–1×10^{6} Hz at 25 °C (Z′ vs. Z″ at 220 mV vs. SCE)

Electrochemical impedance characterization of HRP/nano-Au/pPA modified Pt electrode

The EIS is an effective method to probe the interface properties of surface-modified electrodes [34–36]. Thus, the stepwise assembly of the biosensor was characterized by EIS, and the results are presented in Fig. 6. The complex impedance can be presented as the sum of the real, Z_{re} (Ω), and imaginary, Z_{im} (Ω), components originating mainly from the resistance and capacitance of the cell, respectively. The semicircle portion, observed in the EIS, corresponds to the electron-transfer-limited process. The semicircle diameter in the impedance spectrum equals to the electron-transfer resistance, R_{et}.

As shown in Fig. 6, curve a is almost a straight line which implied the characteristic of a diffuse limiting step of the electrochemical process on a bare Pt electrode in 1.0×10^{-2} mol/L ferricyanide solution. After the bare Pt electrode was coated with pPA, the EIS of the resulting film shows a interfacial R_{et} (Fig. 6, curve b), indicating that the pPA membrane has some impedance in the ferricyanide solution, although the pPA is conducting polymer. Then adsorption of colloid Au, the R_{et} decreased (Fig. 6, curve c). The reason may be that the nanometer-sized gold colloids immobilized on the platinum electrode play an important role similarly to a conducting wire or electronconduction tunnel, which makes it easier for the electron transfer to take place [37–39]. After absorption of the HRP to the electrode surface, a further decrease of the R_{et} is observed in Fig. 6d. This decrease is attributed to the direct electron transfer between the HRP molecules and the electrode [10]. From the results, we confirmed that the HRP was successfully immobilized on the electrode.

Optimization of experimental variables

Effect of pH on the response of the biosensor

The pH of catalytic medium shows a strong effect on the activity of the HRP. The influence of pH was studied between 4.5 and 8.5 using a constant concentration of H_2O_2 at 25 °C. The biosensor response increases with increasing pH from 5.5 to 6.5 and then decreases as the pH increases further. It is well known that at relatively high pH, the activity of the enzyme is inhibited [40]. The experimental results show that the maximum response occurs at pH 6.5. Therefore, a B.R (pH 6.5) was used as the medium for enzyme reaction.

Effect of temperature on the response of the biosensor

The effect of temperature on the function of the enzyme has been reported to be very important. An increase of temperature had a favorable effect on the enzyme. The effect of temperature on the HRP was examined at the range from 10 to 50 °C. It was found that the current response increased with the increasing temperature up to 35 °C. However, temperature more than 40 °C caused irreversible behavior (denaturation

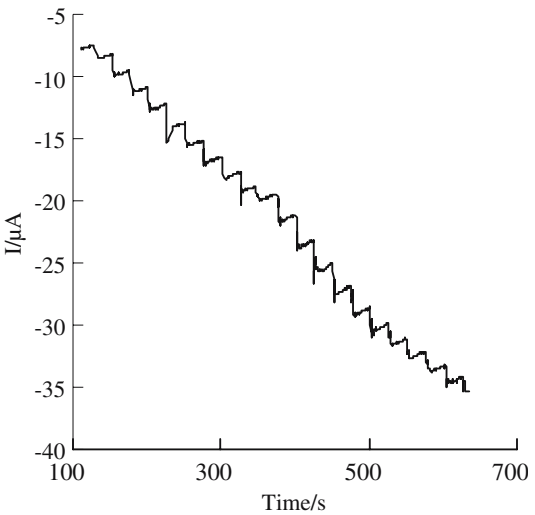


Fig. 7 Typical steady-state response of the biosensor on successive injection of 1.0×10^{-2} mol/L H_2O_2 into 5 ml of stirring B.R. Applied potential 0 mV; supporting electrolyte, pH 6.5 B.R

of proteins) involved in the process [41]. As is well known, an optimal temperature of enzyme would be 37 °C, which supports this observation at this temperature; however, a long incubation time might decrease the activity of enzyme, leading to the deterioration of response signals. Thus, the temperature of 25 °C (room temperature) was selected as a compromise [40].

Electrode response characteristics

An increasing amperometric response on the HRP/nano-Au/pPA-modified Pt electrode can easily be observed in the presence of hydrogen peroxide compared with the background current without hydrogen peroxide. The reduction current began to rise at a potential of 0 mV. These results indicate that HRP catalyzed the reduction of hydrogen peroxide at this potential. On the basis of the results in Fig. 3, a potential of 0 mV was selected as the working potential for this enzyme electrode. Figure 7 displays the typical current–time response of the biosensor on successive step changes of H_2O_2 concentration under the optimized experimental conditions. When an aliquot of H_2O_2 was added into the buffer solution, the reduction current rose steeply to reach a stable value. The biosensor could achieve 95% of the steady state current within 5 s. As mentioned previously, such a fast response can be attributed to the following facts: H_2O_2 can diffuse to the enzyme freely for the HRP molecules are exposed to the surface of colloidal Au and colloidal Au are favorable to the orientation of the HRP molecule on the electrode in the process of bioelectrocatalysis, so they can transfer electrons more conveniently [10]. Figure 7 also indicated that the immobilized HRP could well catalyze the reduction of H_2O_2, which was due to the fact that the nano-Au could effectively retain the bioactivity of the HRP. The response of the biosensor is linear in the range between 4.2×10^{-7} and 1.5×10^{-3} mol/L with a correlation coefficient of 0.9977 (Fig. 8). The detection limit of the sensor was found to be 1.7×10^{-7} mol/L, based on a signal-to-noise ratio of 3.

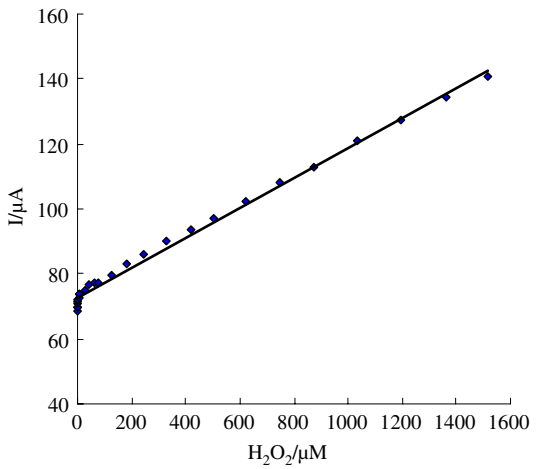


Fig. 8 Calibration plot for the biosensor in 5 mL B.R (pH 6.5) containing different H_2O_2 concentration

Table 1 Possible interference tested with the HRP biosensor

Potential interfering substance	Current ratio (%)
Glucose	0.98
Ethanol	0.99
Acetic acid	1.00
Oxalic acid	1.02
Citric acid	1.00
Ascorbic acid	1.03

Selectivity against interferences

Selectivity is an important factor in the performance of an inhibition-based enzyme inhibition sensor. Six kinds of possible interfering substances were used to evaluate the selectivity of the electrode. The current ratios are calculated from the current reading of the biosensor in the assay solution containing 4.0×10^{-5} mol/L H_2O_2 and 8.0×10^{-5} mol/L interfering substance compared with the current reading of the biosensor in the same assay solution containing 4.0×10^{-5} mol/L H_2O_2. The results of the interference study are listed in Table 1. It shows that they do not cause interference under the experimental conditions. This is largely associated with the low working potential of 0 mV as no electrochemical reactions occur at this potential and the conducting polymer which has permselectivity itself.

Reproducibility and stability of biosensor

The reproducibility of the current response of the biosensor was examined at an H_2O_2 concentration of

Table 2 Application of the biosensor for determining H_2O_2 in plant samples

Samples[a]	Found[b] 10^{-5}mol/L (H_2O_2)	Add	Found[b]	Recovery (%)
Sample 1	7.08 ± 0.15	5.00	11.48 ± 0.23	95
		25.00	30.16 ± 0.24	94
Sample 2	6.20 ± 0.13	5.00	10.75 ± 0.12	96
		25.00	30.58 ± 0.56	98
Sample 3	5.86 ± 0.11	5.00	10.43 ± 0.18	96
		25.00	29.93 ± 0.45	97

[a] Samples were the extraction solution of plant leaves

[b] Mean ± SD of three measurements

8.0×10^{-5} mol/L and the relative standard deviation was 2.2% ($n = 6$). The fabrication reproducibility of six electrodes, independently constructed based on the same bare electrode, showed an acceptable reproducibility with a relative standard deviation of 4.3% for the steady-state current obtained at the H_2O_2 concentration of 8.0×10^{-5} mol/L.

The steady state current of the biosensor will gradually decrease after storage and usage for some time; this is unavoidable because of the slow deactivation and loss of the enzyme on the electrode [42]. The stability of the biosensor was studied by amperometric measurements in the presence of 4.0×10^{-5} mol/L H_2O_2. The electrode was tested every other day for 1 month. When not in use, the enzyme electrode was stored in 0.1 mol/L PB (pH 7.0) at 4 °C in a refrigerator. As a result, the sensitivity of the electrode response maintained about 84.2 % of the original value after 1 month testing. This could be due to the good biocompatibility of nano-Au and strongly electrostatic interaction between nano-Au (the surface of Au nano particle was negatively charged) and HRP (at pH 7.0, the HRP molecule was positively charged); thus the lifetime of the HRP biosensor could be prolonged [43].

Application

The applicability of this enzyme electrode was assessed by the determination of the H_2O_2 in the extraction solution of the plant leaves, with satisfactory recoveries as listed in Table 2. The extraction solution of the plant leaves was prepared as follows: the fresh plant leaves were first clear, then the clear leaves were triturated, centrifuged. The solution was diluted by B.R buffer solution (pH 6.5). The recovery was in the range 94–98%, which demonstrate the feasibility of using the proposed biosensor for the testing of H_2O_2 assay in plant samples.

Conclusions

A mediator-free amperometric hydrogen peroxide biosensor was developed with the first time using pPA as a permselective polymer and the colloidal Au nanoparticles were successfully constructed as the immobilization architecture for the massive entrapment of HRP. Such immobilized HRP electrode exhibited direct electrochemical behavior toward the reduction of hydrogen peroxide and had its own advantages. The resulted biosensor exhibited fast amperometric response, low detection limit and broad linear range to H_2O_2. Moreover, the studied biosensor exhibited strong permselectivity, high sensitivity, good reproducibility, and long-term stability.

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