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Hydrogen peroxide biosensor based on the immobilization of horseradish peroxidase onto a poly(aniline-*co*-*N*-methylthionine) film

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ABSTRACT

In this present work, we developed a novel hydrogen peroxide (H_2O_2) biosensor fabricated using electrochemical doping to immobilize hydrogen peroxidase (HRP) in a new conducting polymer, poly (aniline-*co*-*N*-methylthionine) (PAN-PNMThH). Amperometric detection of H_2O_2 was evaluated by holding the PAN-PNMThH HRP electrode at −0.25 V (versus saturated calomel electrode (SCE)). PAN-PNMThH showed excellent redox activity and high porosity and acted as an electron transfer mediator. The biosensor had a wide linear response range from 5.0 μM to 60.0 mM H_2O_2 with a sensitivity of 35 mA M^{-1} cm^{-2}, a detection limit of 3.2 μM (signal-to-noise ratio of 3) and an apparent Michaelis constant (K_M) of 2.79 mM. The biosensor possessed good analytical performance and storage stability.

1. Introduction

Nowadays, some polymers are replacing traditional materials in many applications due to their low density, low cost, and specific properties [1–3]. Over the last few decades, conducting polymers, such as polyaniline and polypyrrole, have become of increasing interest for their novel optical, electrical, and electrochemical properties and promising applications in secondary batteries, supercapacitors, electrochromic devices, anticorrosive coatings, electrochemical sensors, and biosensors [4–6]. Advantages of using the conducting polymers in biosensors are acceleration of charge transfer, impressive signal amplification, and elimination of electrode fouling [7]. A conducting polymer film can be directly electrodeposited on a solid electrode surface. The film adheres strongly to the electrode surface, and its thickness and characteristics can be easily controlled from the charge consumed during the electropolymerization [8]. Moreover, the film offers an appropriate environment for immobilization of enzymes [9]. Thus, these polymers have been widely employed as enzyme immobilization materials in biosensors.

Three methods for the enzyme immobilization with a conducting polymer are commonly used [10], namely, electrochemical entrapment, covalent bonding, and electrochemical doping. An important advantage of the electrochemical doping in a biosensor fabrication is that the enzyme immobilization can be performed under a mild condition, which has no influence on the enzyme nature [11].

Fast and easy determination of hydrogen peroxide (H_2O_2) is of practical importance in clinical, pharmaceutical, biochemical, environmental and food analysis [12]. H_2O_2 has been determined by titrimetry, volumetry, colorimetry, chemiluminescence, and pectrophotometry. However, these methods generally suffer from various interferences and are complicated and time-consuming [8,13]. The sensitive determination of H_2O_2 can be achieved with the use of peroxidase enzyme-modified electrodes, since the enzymes possess excellent selectivity and high sensitivity [14]. Currently, numerous efforts have been made to develop the enzyme-based biosensors for the detection of H_2O_2, which is the basis of the measurement of many small biological molecules like glucose [15,16], choline [10,17], and cholesterol [18,19].

It is known that the optimum pH for most enzymes usually ranges from 4.0 to 10.0 [20]. However, this pH range is not suitable for polyaniline because it almost loses its electroactivity in solutions of pH greater than 4.0 [21–23]. Therefore, it is necessary to improve the electroactivity of polyaniline at high pH values through structural modifications. An effective approach to its structural modifications is the copolymerization of aniline with a suitable monomer.

Methylene blue and other phenothiazine dyes have been commonly used as redox mediators in sensors and biosensors [24,25]. However, they can easily diffuse away from the electrode surface into the solution bulk during the continuous measurement, which would result in great signal loss and significantly affect the performance of the biosensor. The electropolymerized polymeric

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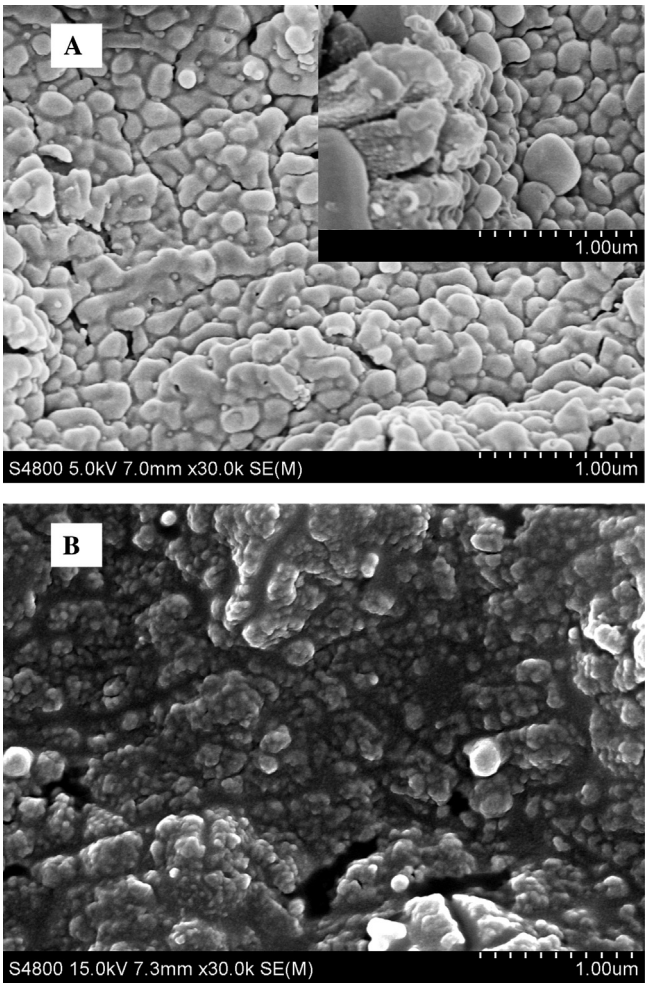

Fig. 1. SEM images of PAN-PNMThH (A) and the copolymer HRP electrode polyaniline (B).

dye film is able to efficiently overcome these problems [26]. Furthermore, such a polymer film with a three-dimensional distribution of redox mediators is preferable to monolayer of the dye monomer because of the much larger catalytic response of the polymer due to the volume effect [27]. In recent years, considerable attention has been focused on tailoring the structure, morphology and properties of these polymers to achieve new improved biosensors based on polyphenothiazine dyes [25].

Recently, three-dimensional flower-like microparticles of a new conducting polymer, poly(aniline-*co*-*N*-methylthionine) (PAN-PNMThH), were electrosynthsized in our laboratory [28]. The copolymer, which contains phenothiazine units, exhibits a high electroactivity in acidic, neutral and basic solutions, which is favorable to enhancing the redox activity and charge transfer ability of the polymer at high pH. Taking into account that polythionine and other polyphenothiazine dyes can serve as redox mediators for the electrochemical sensors and biosensors [26–30], the use of the copolymer as a mediator would be of special interest for biosensing application.

In this study, we used PAN-PNMThH to immobilize horseradish peroxidase (HRP) and studied effects of some experimental variables such as applied potential, pH, and temperature on the bioelectrochemical response of the hydrogen peroxide biosensor.

2. Experimental

2.1. Reagents and solutions

N-Methylthionine and HRP (EC 1.11.1.7, type VI, 250–300 units mg^{-1}) were bought from Sigma–Aldrich (Shanghai, China). H_2O_2 (30%, v/v) was purchased from Beijing Beihua Fine Chemicals (Beijing, China). Ascorbic acid, uric acid, ethanol, sodium chloride, hydrochloric acid, sodium hydroxide, sodium dihydrogen phosphate, and disodium phosphate were obtained from Sinopharm Chemical Reagent Corporation (Shanghai, China) and were of analytical grade. Aniline was distilled before use. All the solutions were prepared using deionized water. The phosphate buffer solution (PBS) was prepared using 0.20 M sodium dihydrogen phosphate, 0.20 M disodium phosphate and 0.20 M sodium chloride and adjusting the pH values with hydrochloric acid or sodium hydroxide, and fresh solutions of hydrogen peroxide were prepared daily.

2.2. Apparatus

All electrochemical experiments were carried out using a CHI 660C electrochemical workstation (Chenhua, Shanghai, China). Cyclic voltammetry and chronoamperometry measurements were performed in a conventional three-electrode cell with a platinum foil and a saturated calomel electrode (SCE) as counter and reference electrodes, respectively. Another platinum foil was used as a working electrode. The area of each platinum foil was 4 mm × 4 mm. All potentials were quoted relative to the SCE. The pH values of solutions were determined using a PHS-3C pH meter (Rex, Shanghai, China).

2.3. Electrosynthesis of PAN-PNMThH

PAN-PNMThH was synthesized using cyclic voltammetry in a potential range from −0.20 to 1.15 V at a scan rate of 60 mV s^{-1} for 100 cycles in a 0.20 M pH 2.0 PBS containing 0.10 M aniline and 2.5 mM *N*-methylthionine [28]. After electrodeposition, the PAN-PNMThH film was thoroughly rinsed with deionized water to remove unreacted monomer.

2.4. Fabrication of the PAN-PNMThH HRP electrode

Enzyme immobilization on the surface of a conducting polymer modified electrode was performed using the electrochemical doping as described elsewhere [31–33]. The PAN-PNMThH modified electrode was first immersed in a 0.20 M pH 6.8 PBS and reduced at a potential of −0.60 V for 30 min until a steady state was achieved. Then, the reduced copolymer modified electrode was immediately moved into a solution of HRP at a potential of 0.60 V for 30 min. The enzyme solution is a 0.20 M pH 6.8 PBS containing 0.1 mg ml^{-1} HRP. Since the isoelectric point of HRP is 5.5 [32], the HRP carries a negative charge in the PBS. Under the electrostatic interaction, the negatively charged HRP was doped into the positively charged copolymer film to fabricate a conducting polymer enzyme electrode. The copolymer HRP electrode was rinsed thoroughly with de-ionized water to remove any enzyme not tightly bound to the copolymer, and used immediately or kept in 0.20 M phosphate buffer solution (pH 7.0) at 4 °C if not in use.

2.5. UV–vis spectra

The UV–vis spectra of HRP solutions were recorded on a U-3010 UV–vis spectrometer (Hitachi, Japan). The amount of HRP incorporated within the copolymer was determined by comparing