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An amperometric hydrogen peroxide biosensor based on the immobilization of HRP on multi-walled carbon nanotubes/electro-copolymerized nano-Pt-poly(neutral red) composite membrane

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ABSTRACT

This work described a convenient and effective strategy to construct a highly sensitive amperometric biosensor for the detection of hydrogen peroxide (H_2O_2). This novel biosensor relied on electro-copolymerization of Pt nanoparticles (nano-Pt) and neutral red (NR) on a multi-walled carbon nanotubes (MWCNTs) modified glass carbon electrode (GCE), followed by immobilization of horseradish peroxidase (HRP) on the surface. The introduction of MWCNTs and copolymerized membrane not only enhanced the surface area of the modified electrode for enzyme immobilization but also facilitated the electron transfer rate, resulting in a high sensitivity of the biosensor. Several technologies such as cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS) and scanning electron microscopy (SEM) have been used to characterize the fabrication process of the sensing surface. The apparent surface electron transfer rate constant (k_s) was 1.83 s^{-1} and the surface coverage (Γ) of HRP was 4.9×10^{-9} mol cm^{-2}. This novel biosensor showed an excellent electrocatalytic activity for H_2O_2, and the response was proportional to H_2O_2 concentration in the range of 3.6×10^{-6}–4.3×10^{-3} mol/L with a detection limit of 1.1×10^{-6} mol/L. Moreover, the proposed biosensor possessed a good stability and reproducibility. The factors affecting the performance of the resulted biosensor were also studied.

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1. Introduction

The rapid and accurate determination of hydrogen peroxide (H_2O_2) is of great importance in pharmaceutical, clinical, industrial, and environmental analyses. Many techniques have been employed for the determination of H_2O_2, such as titrimetry, photometry, chemiluminescence, high performance liquid chromatography and electrochemistry [1]. Among these techniques, amperometric enzyme-based biosensors have received considerable attention due to its convenience, high sensitivity and selectivity [2]. In order to prepare excellent biosensors, many materials were employed to improve the microenvironment around proteins, provide suitable orientation, and accelerate the electron transfer between protein and the electrode surface [3]. Generally, the electrodes were modified with biomolecules films [4], conducting polymers [5], redox dye [6] and nanoparticles [7]. Conducting polymers and nanomaterials [8–10] have attracted great research interest in biosensor due to their versatility of the physical and chemical properties.

Carbon nanotubes (CNTs) have been widely used in the preparation of biosensors [11–13], owing to their high surface area, high electrical conductivity and good chemical stability. Previous studies have demonstrated that CNTs had fast electron-transfer kinetics [14] and could catalyze the electrochemical reaction of NADH [15,16], glucose [17–19], neurotransmitters [20], and dopamine [21,22]. It was also reported that functional CNTs with dyes or nano-materials could lead to the formation of specific composites and enrich the application of CNTs [23–25]. In our laboratory, different kinds of biosensors based on functional CNTs-modified electrodes, which possessed a high electrocatalytic effect and a fast electron transfer rate have been prepared in recent years [2,12]. Meanwhile, there has been a growing interest in studying the application of metal nanoparticles in constructing a biosensor. These nanoparticles include nano-Pt [26], nano-Cu [27], nano-Au [24] and nano-Ag [28]. Pt nanoparticles have been widely studied, due to their stability in electrochemical reactions and its prominent catalytic activities to H_2O_2.

Mediators have an extensive application in biosensor fabrication, because of their unique electronic structure, which is considered to be responsible for their electrical conductivity, low ionization potentials and high electron affinity. The conventional methods used to immobilize the mediators on the electrode sur-

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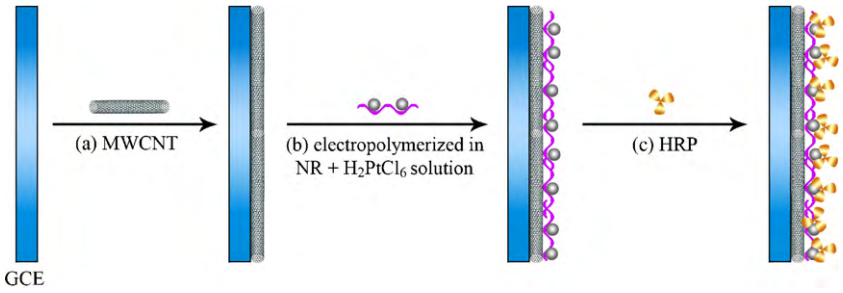

Scheme 1. The schematic illustration of stepwise fabrication process of the biosensor: HRP/nano-Pt-PNR/MWCNTs/GCE.

face involved in the entrapment [29], absorption [17], covalent-link [30], electropolymerization [31] or self-assembly [32] of mediators. Among these approaches, electropolymerization is one of the most convenient and advantageous methods. During electropolymerization, the monomers are oxidized to form radical cations, followed by coupling reactions to form oligomers. This eventually leads to deposition of the polymers on the electrode surface to produce a phenazine-like chain structure, which can be controlled by electrode design and improved stability of the resulting enzyme biosensor. The representative examples of this type of conducting polymers are tyrosine [33], neutral red [34], thiophene [35] and pyrrole [36]. The electropolymerization of neutral red (NR) from aqueous solution and its catalytic activity towards biomolecules, different inorganic and organic compounds have been reported in recent years [37–39]. Neutral red exhibited a fast reversible electrochemical response at negative potentials, which made it a convenient redox mediator for electrochemical investigation of biological systems. Many biosensors have been developed and applied by NR, such as MWCNTs/NR [18,19], Pt/PNR [39]. However, to our knowledge, a new electro-copolymerization film of nano-Pt and NR on MWCNTs is not examined as an enzyme immobilization matrix to date.

In this work, a highly sensitive amperometric biosensor for the detection of H_2O_2 based on the electro-copolymerization of nano-Pt and NR on MWCNTs matrix is reported. Our aims were to improve the stability and bioactivity of enzyme biosensors and combine the advantageous features of nano-Pt-PNR film and MWCNTs to develop a simple, stable, economical, and sensitive H_2O_2 biosensor using HRP as a model enzyme. First, MWCNTs were successfully immobilized on the surface of glassy carbon electrodes. Then nano-Pt-PNR film was constructed onto the resultant electrode surface by electro-copolymerization. Later, HRP was immobilized onto the electro-copolymerization film. Such fabrication scheme not only simplified the procedure to prepare biosensor but also enhanced stability and reproducibility of the biosensor. This novel nano-Pt-PNR/MWCNTs composite membrane with a good conductivity, a high electrochemical signal and biocompatibility can provide a remarkable synergistic augmentation to facilitate electron transfer between the active redox center of the enzyme and the electrode surface. In the fabrication process, nano-Pt can cause nanoparticle size distribution by electro-copolymerization with NR on GCE surface modified with MWCNTs. Moreover, the combination of nano-Pt and MWCNTs can improve the sensitivity of the biosensor because of their good electrocatalytic activity to H_2O_2.

2. Experimental

2.1. Materials

Horseradish peroxidase (HRP) (250 U/mg and isoelectric point of pH 7.2) and neutral red were obtained from Shanghai Chemical Company, China. Hydrogen hexachloroplatinate (IV) hydrate (H_2PtCl_6), was purchased from Sigma Chemical Co. (St. Louis, MO, USA). They were used directly without further purification. Hydrogen peroxide (30%, w/v solution) was bought from Chemical Reagent Company, Chongqing, China. The concentration of the more diluted hydrogen peroxide solutions prepared from 30% hydrogen peroxide was determined by titration with potassium permanganate. The multi-walled carbon nanotubes (MWCNTs) were obtained from Chengdu Organic Chemicals Co. Ltd. Prior to use, MWCNTs were refluxed in concentrated nitric acid for about 7 h, then filtered and washed with double-distilled water until the filtrate became neutral, and finally dried under vacuum. Phosphate buffer solutions (PBS) with various pH were prepared with 0.1 M KH_2PO_4 and 0.1 M Na_2HPO_4. The supporting electrolyte was 0.1 M KCl. All chemicals and solvents used were of analytical grade and were used as received. Double distilled water was used throughout this study.

2.2. Preparation of MWCNTs suspension

Two mg MWCNTs in 1.0 mL of *N,N*-dimethylformamide (DMF) was sonicated for 3 h to obtain a black suspension, subsequently 1 mL of ethanol was added to the suspension and the resulting suspension was sonicated for 1 h to give a homogeneous black suspension. Then MWCNTs suspension of 1.0 mg/mL was obtained, which was sonicated for 5 min immediately before preparing the films.

2.3. Preparation of hydrogen peroxide biosensor

A glassy carbon electrode (GCE) (4 mm in diameter) was firstly polished to a mirror-like surface repeatedly with 0.3 and 0.05 μm alumina slurry, followed by successive sonication in ethanol and double-distilled water for 5 min, respectively and dried in air. Subsequently, 10 μL of this black suspension was cast on a cleaned GCE surface. After that, the electrode was dried in air, formed the MWCNTs-modified GCE, denoted as MWCNTs/GCE. Then the MWCNTs/GCE was immersed in 0.5 M H_2SO_4 solution containing 5.0 mM neutral red and 0.1% (v/v) H_2PtCl_6 for electrochemical copolymerization by CV between −0.2 and 1.4 V for 25 cycles at a scan rate of 50 mV/s, forming the nano-Pt-PNR/MWCNTs modified electrode (Fig. 2a and b). In addition, PNR/MWCNTs-modified electrode was prepared similarly during the electrochemical copolymerization process in the absence of H_2PtCl_6 (Fig. 2c and d).

The obtained electrode was thoroughly washed with double-distilled water to remove any physisorbed and unreacted materials from the electrode surface. At last the resulting electrode was immersed in 0.5 mL HRP solution (1 mg/mL, 0.1 mol/L pH 6.0 PBS) at 4 °C for 8 h to produce a HRP/nano-Pt-PNR/MWCNTs/GCE. The preparation process of the modified electrode is shown in Scheme 1. For comparison, a HRP/nano-Pt-PNR/GCE without MWCNTs and a HRP/PNR/MWCNTs/GCE without nano-Pt were prepared through

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