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A novel hydrogen peroxide biosensor based on horseradish peroxidase immobilized on gold nanoparticles–silk fibroin modified glassy carbon electrode and direct electrochemistry of horseradish peroxidase

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ABSTRACT

The preparation of horseradish peroxidase (HRP)–gold nanoparticles (AuNPs)–silk fibroin (SF) modified glassy carbon electrode (GCE) by one step procedure was reported for the first time. The direct electrochemistry of HRP at the modified electrode was investigated. The enzyme electrode showed a quasi-reversible electrochemical redox behavior with a formal potential of −210 mV (vs. SCE) in 0.1 M phosphate buffer solution at pH 7.1. The response of the biosensor showed a surface-controlled electrochemical process with one electron transfer accompanying with one proton. The cathodic transfer coefficient was 0.42, the electron transfer rate constant was 1.84 s^{-1} and the surface coverage of HRP was 1.8×10^{-9} mol cm^{-2}. The experimental results indicated that AuNPs–SF composite matrix could not only steadily immobilize HRP, but also efficiently retain its bioactivity. The biosensor displayed an excellent and quick electrocatalytic response to the reduction of H_2O_2.

1. Introduction

The heterogeneous direct electron transfer (DET) reactions between redox enzyme and electrode surface has attracted considerable interest [1,2]. Understanding of these reactions fundamentally has important significance in providing insight into the theoretical research of electron exchange among enzymes in biological systems. Moreover, it lays technical foundation for fabricating biofuel cell, biochip and the third generation biosensor based on the direct electrochemistry of redox enzymes [3]. HRP is an important heme-containing enzyme, which has been studied for more than one century [4]. Although HRP has been intensively studied with electrochemical methods, its direct electrochemistry is relatively difficult to be investigated at a bare electrode with two reasons [5,6]. Firstly, HRP molecules would be adsorbed on electrode surface, resulting in denaturation and loss of electroactivity and bioactivity. Another reason is that the active sites of HRP are deeply buried in a thick protein shell, the long distance between the active sites and electrode surface would slow the electron transfer. Therefore, a promoter is required to realize the direct electron transfer between HRP protein and electrode. It is necessary to find new electrode modified materials and suitable methods of immobilization of HRP enzyme onto electrode surface to obtain the direct electrochemistry. An ideal promoter used for the direct electron transfer of protein should provide a suitable interface between protein and electrode surface to partly or completely eliminate the denaturation of enzyme molecule on electrode surface [5,7]. Up to now, a variety of materials have been employed to modify electrode as a bridge of electron transfer between the redox center of HRP and electrode surface, such as AuNPs [1,2,8–15], carbon nanotube [16], dye [17], conducting polymer [18], lipid [19], biopolymer chitosan [20], and so on. Among them, AuNPs have attracted enormous interest for that they can strongly adsorb some proteins and effectively retain their biological activity [8–11]. Moreover, AuNPs can provide an environment similar to that of redox proteins in a native system and allow protein molecules more freedom in orientation, thus reducing the insulating property of protein shell and facilitating the electron transfer through the conducting tunnel of AuNPs.

SF is a natural protein with molecular weight at about 350 kDa, which can be extracted from silkworm cocoon. Due to the unique properties of SF with good thermal stability, avirulence, hygroscopicity, microbial resistance and biocompatibility, it has been widely used as a substrate for enzyme immobilization [21]. The tyrosine residue component in SF, having strong electron donating property, can reduce some metal ions to form nano-metal particles [22]. So AuNPs could be *in situ* produced by the reduction of SF solution at room temperature, in which SF acts as both reducing agent and

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protector [23]. It is well known that amine, hydroxyl, carbonyl, aromatic, or sulphydryl in protein can easily bind with metal surface and form the bioconjugate [22]. Thus it can be concluded that AuNPs could react with SF to form a bioconjugate. This kind of AuNPs–SF colloid should possess a novel core–shell structure with a stable and highly dispersed nature [22,23].

To our knowledge, the direct electron transfer of HRP at HRP–AuNPs–SF/GCE has not yet been reported. In this paper, we report a novel methodology for construction of HRP-based H_2O_2 biosensor by one step procedure, which combine the merits of SF and AuNPs to immobilize HRP. The direct electron transfer of immobilized HRP and its electrocatalytic response to the reduction of H_2O_2 were studied by cyclic voltammetry and amperometry. The performance and factor influencing the fabricated biosensor were studied in detail. The experimental results indicated that this H_2O_2 biosensor can be directly used for determination of H_2O_2 concentration in practical samples with an electrochemical method without the aid of an electron mediator. The biosensor exhibited high sensitivity, good repeatability and long-term stability.

2. Experimental

2.1. Reagents and apparatus

HRP (EC 1.11.1.7, 250 U mg^{-1} and isoelectric point of pH 7.2) was purchased from sigma (USA) and used as received. $HAuCl_4$ was obtained from the National Pharmaceutical Group Chemical Reagents Company (China). Silkworm cocoon was obtained from a local market. Phosphate buffer solution (PBS) was prepared by mixing stock solution of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 and adjusting the pH with 0.1 M H_3PO_4 or 0.1 M NaOH. All of the chemicals were of analytical reagent grade and all the solutions were prepared with redistilled deionized water from quartz.

Electrochemical experiments were performed with CHI832A electrochemical workstation (Shanghai Chenhua Co., China) with a conventional three-electrode cell. A HRP–AuNPs–SF modified glassy carbon electrode was used as working electrode. A saturated calomel electrode (SCE) and a platinum wire were used as reference electrode and auxiliary electrode, respectively. UV–vis absorbance spectroscopy was performed using a UV-1601 spectrophotometer (Shimadzu Co., Japan). The pH measurements were carried out on a PHS-3C exact digital pH meter (Shanghai KangYi Co. Ltd., China), which was calibrated with standard pH buffer solutions. All the measurements were carried out at room temperature. All the experimental solutions were deaerated by bubbling nitrogen for 20 min.

2.2. Preparation of AuNPs–SF colloid

AuNPs–SF colloid was prepared as the method described elsewhere [22,23] with minor modification. silkworm cocoon was degummed three times to remove silk sericin with 0.05% Na_2CO_3 solution at 98–100 °C for 30 min. The obtained SF was washed thoroughly with distilled water and dried at room temperature. After that, the dried SF was dissolved in the mixture solution of $CaCl_2/CH_3CH_2OH/H_2O$ (1:2:8 mol ratio) at 98 °C for 1 h and then filtrated under reduced pressure. The obtained SF solution was dialyzed against distilled water for 3 days to remove $CaCl_2$ using a cellulose semipermeable membrane. Then the purified SF solution was obtained with the concentration of 3% (mass%). In order to prepare AuNPs and SF composite matrix, SF aqueous solution with the concentration of 3% was added into 1 mM $HAuCl_4$ aqueous solution by dropping. The pH of the mixed solution was adjusted to 9–10 with 0.1 M NaOH solution to prevent aggregation of SF. Finally, the purple AuNPs–SF colloid was obtained after the mixed solution was placed for 24 h without light.

2.3. Preparation of HRP–AuNPs–SF modified glassy carbon electrode

A glassy carbon electrode (GCE, 3 mm in diameter) was polished to a mirror-like with metallography abrasive paper (No. 5), followed by 0.3 and 0.05 μm alumina slurry on micro-cloth pads, rinsed thoroughly with redistilled deionized water between each polishing step, then washed successively with redistilled deionized water, acetone, 1 M NaOH, 1:1 HNO_3 (v/v) and redistilled deionized water in an ultrasonic bath and dried in air before use.

The immobilization of HRP in the AuNPs–SF composite matrix was accomplished by addition of 0.4 mg HRP into 80 μL AuNPs–SF mixed solution. With a microinjector, aliquots (4 μL) of such a mixed solution were deposited on a GCE surface and allowed to dry for 1 day in refrigerator. The enzyme electrode was then immersed into 80% methanol (v/v) for 30 s, dried under ambient condition and rinsed with redistilled deionized water to remove the unimmobilized mixture. The obtained electrode was denoted as HRP–AuNPs–SF/GCE. For comparing with HRP–AuNPs–SF/GCE, SF/GCE, AuNPs–SF/GCE, HRP/GCE and HRP–SF/GCE were fabricated with the similar procedures. Both the modified electrodes were stored at 4 °C in a refrigerator before use.

3. Results and discussion

3.1. UV–vis spectroscopy

UV–vis spectra can reflect the characteristic structure of proteins. The position of Soret absorption band of heme may provide information about the denaturation of heme proteins [24]. Fig. 1 shows UV–vis spectra of AuNPs–SF (a), SF (b), HRP (c) and HRP–AuNPs–SF (d). The AuNPs–SF solution showed a distinct surface plasmon absorption band of AuNPs at 524 nm and a weak absorption peak at 276 nm (Fig. 1a). The absorption peak of AuNPs at 524 nm is in agreement with that obtained by Na–citrate reduction method [25]. The SF solution showed an absorption peak at 276 nm (Fig. 1b). Comparing with the AuNPs–SF, this absorption peak was more stronger. These results demonstrated that AuNPs was successfully synthesized. The UV–vis spectrum of native HRP gave a typical heme band at 403 nm (Fig. 1c), while AuNPs–SF has no absorption at this band. For the mixture of HRP–AuNPs–SF, the

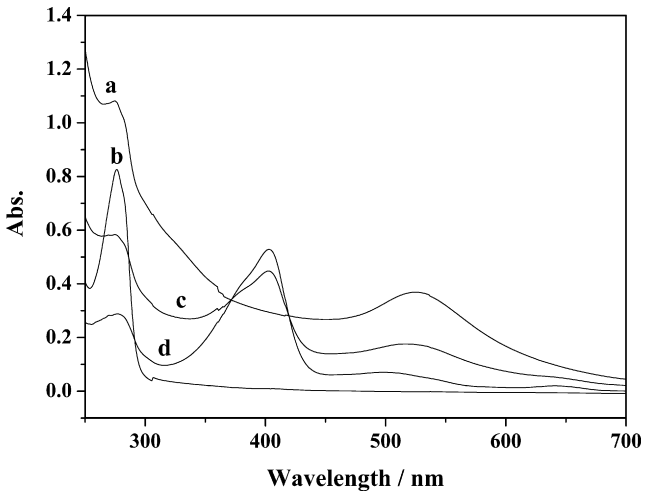

Fig. 1. UV–vis absorption spectra of AuNPs–SF (a), SF (b), HRP (c), HRP–AuNPs–SF (d).