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Study on the bioelectrochemistry of a horseradish peroxidase-gold nanoclusters bionanocomposite

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ABSTRACT

A horseradish peroxidase (HRP)-Au nanoclusters (AuNCs) bionanocomposite was prepared simply by mixing HRP and $NaAuCl_4$ in an alkaline aqueous solution (pH 12) at 37 °C for 12 h under vigorous stirring. Cast-coating the HRP-AuNCs bionanocomposite and then Nafion on a glassy carbon electrode (GCE) yielded a Nafion/HRP-AuNCs/GCE. This electrode exhibited a pair of well-defined cyclic voltammetric peaks of the immobilized HRP with a formal potential of −0.389 V vs SCE and an electron transfer rate constant of 3.19 s^{-1} in pH 7.0 phosphate buffer. The HRP also exhibits a high bioactivity. This electrode showed high electrocatalytic activity for reduction of both O_2 and H_2O_2, as a result of the synergistic effects of HRP and nearby AuNCs. This electrode gave a linear amperometric response to H_2O_2 from 0.01 to 3.27 mmol L^{-1} with a sensitivity of 0.86 μA mM^{-1} cm^{-2} and a limit of detection (LOD, S/N = 3) of 0.99 μM.

1. Introduction

Simultaneous realization of high electroactivity and high bioactivity of a redox enzyme (or a redox protein) is an interesting and important topic in bioelectrochemistry, especially for the development of mediator-free third generation amperometric biosensors [1–4]. However, a redox enzyme can be easily adsorbed onto a solid electrode surface, and the adsorption can lead to protein-configuration change and thus partial or full loss of its natural bioactivity. In addition, the active center of a redox enzyme is often buried deeply inside its large three-dimensional structure and thus the electron transfer of the enzyme to the electrode is blocked [5–8]. To date, many efforts have been made for such bioelectrochemical studies. To our knowledge, five kinds of protocols have been reported for studying the direct electrochemistry of a redox enzyme (or protein). (1) Selecting an appropriate promoter to connect the electrode and the enzyme molecule mainly by electrostatic interaction and/or hydrogen bond [9–10]. (2) Modifying the enzyme with an electron mediator or a metal nanomaterial to facilitate the electron transfer between enzyme and electrode [11–12]. (3) Modifying the electrode surface to adjust the absorbed state of the enzyme [6,13]. (4) Entrapping an enzyme into a conducting polymer to realize its direct electron transfer [14–15]. (5) Reconstituting an apo-protein on a cofactor-Au nanoparticle modified electrode [16–18]. Despite of so many efforts in the field, the simultaneous realization of bioactivity and electroactivity of a redox enzyme is still a challenging task to date. For example, we have quantitatively discovered that most of glucose oxidase (GOx) molecules adsorbed on carbon nanomaterials become electroactive but the electroactive GOx molecules become almost bioinactive [3], and such findings have been supported by a series of more recent studies [4,8,19–20]. Therefore, more fundamental data are needed for in-depth understanding of the topic on simultaneous realization of high electroactivity and high bioactivity of a redox enzyme, including studies on many redox enzymes other than GOx, such as horseradish peroxidase (HRP) [7,14,21–23].

Noble metal nanoclusters (NMNCs) are a kind of (sub)nanomaterials consisting of several to tens of noble metal atoms, which show interesting electrical, optical, catalytic and chemical properties [24–27]. Noble metal nanoclusters exhibit promising application potential for fuel cells [28–29] and chemo-/biosensors [24,30–32]. Due to the tiny size and good electron-conductivity, NMNCs can be plugged into an enzyme (or protein) molecule to act as a relay unit for the promotion of electron transfer [33]. The AuNCs are one of the most important NMNCs, which have been widely studied for catalysis and analytical applications [34]. The synthesis of AuNCs usually involves the reduction of a gold salt (e.g. $NaAuCl_4$) by a reducing agent (e.g. $NaBH_4$) in the presence of a stabilizing ligand (e.g. small thiol molecules) [35]. Recently, some proteins are used as both reducing agent and stabilizing ligand to synthesize high-quality bifunctional protein-AuNCs [27,36].

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Herein, we report on the preparation of a HRP-AuNCs bionanocomposite, simply by mixing HRP and $NaAuCl_4$ in an alkaline aqueous solution (pH 12) under vigorous stirring at 37 °C overnight. A Nafion/HRP-AuNCs/glassy carbon electrode (GCE) is prepared by the cast-coating method. The direct electrochemistry and high bioactivity of HRP are well achieved. This electrode shows high catalytic activity for electroreduction of both O_2 and H_2O_2, which is also used as a biosensor for H_2O_2 assay.

2. Experimental

2.1. Apparatus and chemicals

All electrochemical experiments were performed on a CHI660A electrochemical workstation (CH Instrument Co.). A conventional three-electrode electrolytic cell was used. A disk GCE (3 mm diameter) or its modified electrodes served as the working electrode. A KCl-saturated calomel electrode (SCE) and a graphite rod were used as the reference electrode and the counter electrode, respectively. Ultraviolet-visible (UV–vis) spectra were recorded on a UV-2450 spectrophotometer (Shimadzu Co., Japan). A Hitachi F-4500 fluorescence spectrophotometer was used for fluorescent measurements. Scanning electron microscopy (SEM) images were collected on a JEM-6700F field emission scanning electron microscope. Transmission electron microscopy (TEM) images were collected on a Hitachi 800 transmission electron microscope.

HRP (EC 1.11.1.7, 250 U mg^{-1}, isoelectric point at pH 7.2) and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma (USA). Chloroauric acid ($HAuCl_4$) was obtained from Shanghai Chemicals Station (Shanghai, China). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Nafion (5 wt%, in a mixture of water and lower aliphatic alcohols) was purchased from Aldrich (USA). A 30 wt% H_2O_2 solution was purchased from Shanghai Taopu Chemical Factory, and a fresh solution of H_2O_2 was prepared daily. 0.1 M phosphate buffer was prepared by mixing 0.04 M NaH_2PO_4 and 0.06 M Na_2HPO_4 and adjusting to the specified pH with 0.1 M H_3PO_4 or 0.1 M NaOH. All chemicals were of analytical grade or better quality and used as received. Milli-Q ultrapure water (Millipore, ≥18 MΩ cm) was used throughout. All experiments were performed at room temperature around 25 °C.

2.2. Synthesis of HRP-AuNCs bionanocomposite

The glassware for the experiments was cleaned with aqua regia (highly toxic and corrosive, treat with great care). The preparation of HRP-AuNCs was performed as reported earlier, with a slight modification [37]. In a typical experiment, aqueous $NaAuCl_4$ (0.5 mL, 10 mM, 37 °C) was added to a HRP solution (0.5 mL, 10 mg mL^{-1}, 37 °C) under vigorous stirring. After 10 min, NaOH solution (0.12 mL, 1 M) was dropwise introduced to adjust the solution pH to ca. 12, and the reaction was allowed to proceed under vigorous stirring at 37 °C for 12 h. The color of the solution turned blackish green slowly, indicating the formation of Au nanoclusters. Then, the obtained HRP-AuNCs solution was adjusted to neutral pH with H_2SO_4 and kept in the freezer and stored at 4 °C for further use. The HRP-AuNCs solution was stable at least for one year. As a control, the BSA-AuNCs solution was similarly obtained by the same method. Furthermore, a HRP-$NaAuCl_4$ mixture was prepared by similarly mixing HRP and $NaAuCl_4$ but without adding NaOH to adjust the pH, by which the AuNCs cannot be generated, and the mixture was used for comparative examination of the direct electrochemistry of HRP.

2.3. Electrode modifications

Prior to modification, the GCE was abraded with fine SiC paper and polished with 0.3 and 0.05 μm alumina slurry, respectively, then sonicated sequentially in ultrapure water, ethanol, and ultrapure water for 5 min each to remove residual alumina powder. The treated electrode was dried under a stream of nitrogen. The cleaned GCE was subject to continuous potential cycling (from −1 to 1 V, 100 mV s^{-1}) in 0.50 M aqueous H_2SO_4, until reproducible cyclic voltammetry (CV) curves were obtained. The Nafion/HRP-AuNCs/GCE was prepared as follows. 5.0 μL HRP-AuNCs solution (final concentrations: 4 mg mL^{-1} HRP and 4 mmol L^{-1} Au) was cast on the GCE surface and air-dried, and then 2.0 μL Nafion (1 wt%) solution was cast and air-dried. Finally, the electrode was rinsed with ultrapure water three times to remove loosely attached enzyme. For comparison, Nafion/GCE, Nafion/HRP/GCE, Nafion/HRP-$NaAuCl_4$/GCE, Nafion/BSA-AuNCs/GCE, and Nafion/HRP/Au_{plate}/GCE were prepared following the same procedures as above. Au_{plate}/GCE was obtained by electroplating Au on the cleaned GCE by multi potential step electrolysis from 1.1 to 0 V, with a pulse width of 0.25 s and 300 steps in 0.50 M aqueous H_2SO_4 containing 1.0 mM $HAuCl_4$. When not in use, the prepared enzyme electrodes were stored in phosphate buffer at 4 °C (refrigerator).

2.4. Measurements

CV measurement of the direct electrochemistry of HRP was performed in the electrolyte solution bubbled with nitrogen gas for 30 min, and a nitrogen atmosphere was maintained over the electrochemical cell to protect the solution from oxygen during the experiment. The electrocatalytic reduction of oxygen was characterized by CV from −0.8 to 0.2 V in 0.1 M phosphate buffer saturated with air. In the amperometric biosensing experiment of H_2O_2, the enzyme electrodes were tested by potentiostating them at −0.4 V in 0.1 M phosphate buffer saturated with nitrogen under solution-stirred conditions. The response current was recorded as a variation between the steady state current after substrate addition and the initial background current without substrate.

Fluorescent emission spectra were recorded using the fluorescence spectrophotometer operating at the excitation wavelength of 365 nm, with both excitation and emission slit widths of 10 nm. The enzymatic specific activity (ESA) of HRP and HRP-AuNCs were measured on the UV–vis spectrophotometer as follows. Into a stirred colorimetric system of 3 mL 0.1 M citrate-phosphate buffer (pH 4.0) containing 0.1 mM TMB and 1 mM H_2O_2, 1 μL native HRP (1 mg mL^{-1}) or HRP-AuNCs dispersion (the same HRP amount as the native HRP) was added to trigger the colorimetric reaction. After reaction for 1 min, 3 mL 0.5 M H_2SO_4 was immediately added to terminate the enzyme reaction. Finally, absorbance at 450 nm was recorded [38].

3. Results and discussion

3.1. Synthesis and characterization of HRP-AuNCs

The HRP-AuNCs bionanocomposite is prepared simply by mixing HRP and $NaAuCl_4$ in an alkaline aqueous solution (pH 12) at 37 °C for 12 h under vigorous stirring. According to the previous reports [36–37], $AuCl_4^-$ is entrapped into the interior of HRP molecule to undergo progressive reduction to form AuNCs in situ by the tyrosine residual of HRP above pH 12. The AuNCs are stabilized by Au—S bonding with the cysteine residual of HRP, and the AuNCs are thus most likely to be implanted in the interior of HRP molecule and stand close to the active center of HRP. The HRP-AuNCs bionanocomposites are characterized by TEM, as shown in Fig. 1A. The TEM image clearly shows the presence of AuNCs with diameter ca. 1–2 nm. The result implies that AuNCs are successfully synthesized, which are mostly likely located into the interior of the enzyme molecules [37]. The HRP-AuNCs bionanocomposites modified electrodes are also characterized by SEM, as shown in Fig. 1B–D. The SEM images show that the HRP-AuNCs modified electrode is rougher than the bare electrode (Fig. 1C vs Fig. 1B), and a rough surface with