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Enhancing sensitivity of hemoglobin-based electrochemical biosensor by using protein conformational intermediate

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ABSTRACT

Redox proteins-based electrochemical biosensors possess high selectivity and biological compatibility but suffer from the low voltammetric response because of the deep bury of heme groups in proteins. Herein, conformational intermediate based on accurately modulating three-dimensional structure of hemoglobin (Hb) was proposed to construct an electrochemical biosensor for improving the response sensitivity. Hb was entrapped in dimethyldidodecylammonium bromide (DDAB) film to modify glassy carbon electrode (DDAB/Hb/GCE). The unfolding processes of Hb were then monitored by UV–vis spectroscopy, fluorescence, circular dichroism, and electrochemistry to obtain the appropriate conditions for maintaining intermediate. Under the optimized conditions, the sensor exhibited accelerated direct electron transfer and high sensitivity for sensing hydrogen peroxide (H_2O_2) and nitrite ($NaNO_2$). The sensitivities for detecting H_2O_2 and $NaNO_2$ were improved as 2.3 and 4.4 times higher than those on the native DDAB/Hb/GCE. The electrocatalytic reduction of nitrite showed a wider linear range from 0 to 225.0 μM with a high sensitivity of 0.4731 μA μM^{-1} and a low limit of detection of 0.069 μM. The unfolded Hb-based sensor possesses the advantages of excellent stability, good reproducibility, and satisfactory recovery. The results provide a new strategy to utilize the unique properties of proteins intermediate in the development of electrochemical biosensors.

1. Introduction

Electrochemical biosensors based on redox proteins or enzymes have been extensively studied because of their facile fabrication, high sensitivity, and biological compatibility. The direct electrochemistry between redox proteins and conventional electrodes undoubtedly is mainly concerned due to the deep burying of electroactive groups in hydrophobic cavity of proteins, which greatly hinders electron transport, decreases sensitivity, and limits the performance of the sensors [1,2]. Some modified methods such as adsorption, various polymer coatings, carbon surface reactions, and different immobilization materials including hydrogel polysaccharide, amphiphilic polymer, mesopore-structured organic peroxide, lipids, and various nanoparticles have been reported to construct a more ideally modified layers to provide suitable microenvironment for native proteins on the electrode surface [3–5]. These sensors based on natural proteins on the surface electrodes obviously realized good direct electrochemistry and obtained good sensitivities. However, their amperometric responses were not fully improved to the maximum due to the greater distances and unfavorable orientation between the deep buried electroactive groups and electrode surfaces. Therefore, it remains a great challenge to obtain more high improved sensitivity.

Heme proteins such as hemoglobin (Hb), myoglobin (Mb), and cytochrome *c* (cyt *c*) have long been used as the paradigms for understanding the structure–function relationships of proteins, studying the direct electron transfer (DET), and constructing the electrochemical biosensors in the past several decades [6–8]. Researchers mainly focused on keeping their natural conformation and biological activity on the surface of modified electrodes. Therefore, a surfactant of dimethyldidodecylammonium bromide (DDAB) film was mostly used to maintain native state of heme proteins and facilitate their DET to electrode [2,9,10]. However, these native proteins with deep buried heme groups still show low voltammetric response in DDAB film [9]. It is known that the biological activities of proteins relate with their three-dimensional structure closely. Structural alterations of proteins, namely the unfolding or denaturation, frequently result in the loss of the activities and a wide range of diseases [11]. But, even so, proper unfolding of protein to a most exposure intermediate state without losing heme groups can greatly promote its biological activities or electrocatalytic ability [12], therefore extremely enhance the sensing sensitivity.

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Hb is a typical heme protein that stores and transports molecular oxygen in mammalian blood with a molecular weight of 67,000. Hb comprises four polypeptide subunits and each subunit has a heme group within molecularly hydrophobic cavity [13,14]. Each heme group consists of an iron atom (Fe^{2+} or Fe^{3+}) attached to a planar organic structure belonging to porphyrin compounds. The heme serves as the active site to which oxygen may be bound and also offers Hb the ability to reduce different targets such as H_2O_2 and nitrite [1]. Heme proteins entrapped in DDAB film on the surface of electrode can retain its native conformation and its electrochemical response in such a film can be greatly enhanced, which is ascribed to the formation of uniform and ordered structure as Hb incorporated in DDAB film. More importantly, the rapid diffusion of Hb in DDAB layer and the inhibition of macromolecular adsorptions enhance the direct electron transfer between Hb and electrode [2,9,15,16]. However, when Hb was unfolded to be intermediate, it could exhibit high electrocatalytic activity and fast response time [12]. This motivates us to investigate and utilize the electrochemical properties of protein intermediate.

Herein, Hb was chosen as a model heme protein to investigate the relationship between its electrocatalytic properties and confrontational alterations. UV–vis spectroscopy, fluorescence, circular dichroism (CD), and electrochemistry were used to monitor the conformational changes of Hb induced by urea or acid in solution, which was investigated to obtain the optimized conditions for maintaining intermediate. After being unfolded to a suitable intermediate, Hb on the DDAB/Hb/GCE exhibited extremely high sensitivity and low limit of detections for nitrite and H_2O_2. Therefore, the improved electrocatalytic activity based on Hb intermediate conformation has potential application in the construction of biosensor.

2. Experimental

2.1. Chemicals and reagents

Hemoglobin (Hb) and dimethyldidodecylammonium bromide (DDAB) were obtained from Sigma and used without further purification. Hydrogen dioxide (H_2O_2, 30%) was purchased from Beijing Chemical Reagent Factory (China). Nitrite ($NaNO_2$), urea, and other chemicals were of analytical grade and used as received. Phosphate buffered saline (0.1 M PBS containing 0.1 M KCl) with different pH were prepared from the stock solutions of 0.1 M KH_2PO_4 and Na_2HPO_4 and adjusted by NaOH and HCl solutions. Urea solutions were prepared by using 0.1 M PBS. Doubly distilled water was used to prepare all the aqueous solutions at ambient temperature.

2.2. Preparation of DDAB/Hb/GCE

Before surface modification, the glassy carbon electrode (GCE, 3 mm in diameter) was polished with 0.05 μm Al_2O_3 powders, and then was sequentially sonicated in 8 mol L^{-1} nitric acid solution, absolute ethanol, and doubly distilled water for 30 s, respectively. The cleaned GCE was dried by high purity nitrogen. 5.0 mg mL^{-1} Hb stock solution was prepared by dissolving 5.0 mg Hb in 1.0 mL 0.1 M pH 7.0 PBS. 10 mM DDAB suspension was sonicated for 30 min to produce homogeneous vesicle dispersion, and it was then mixed with 5 mg mL^{-1} Hb solution at a ratio of 1:1 (V:V). After that, 10 μL of the mixture solution was spread on the well-cleaned GCE and dried at 4 °C, The modified electrode is taken as DDAB/Hb/GCE.

2.3. Apparatus and measurements

UV–vis absorption spectra were recorded on a UV-3150 spectrophotometer (Shimadzu, Japan) using 1.0 cm quartz cells. Fluorescence spectra were obtained on a RF-5301PC spectrophotometer (Shimadzu, Japan) with a 1.0 cm quartz cell. The excitation wavelength was selected at 280 nm and the emission spectra were recorded between 290 and 450 nm with the slit width of 5/5 nm. Circular dichroism (CD) measurements were carried out with a J-810 spectrometer (Tokyo, Japan). The CD spectra of 1.0 μM Hb were recorded over a wavelength range of 200–250 nm with a scan speed of 50 nm min^{-1} and a band width of 1.0 nm. Each CD spectrum was the average of three scans.

Electrochemical experiments were performed by using a CHI660C electrochemical workstation (Chenghua, Shanghai, China). A conventional three-electrode system, including a platinum wire counter electrode, an Ag/AgCl reference electrode (3 M KCl), and a working modified electrode (DDAB/Hb/GCE), were employed. Cyclic voltammetry was carried out in the corresponding potential ranges at the scan rate of 100 mV s^{-1}. Prior to the electrochemical experiments, solutions were purged with high purity nitrogen for 15 min to remove oxygen and a nitrogen environment was kept over the measurements. $NaNO_2$ and H_2O_2 solutions were added into a sealed electrochemical cell using micro-injector and stirred carefully before detection. The real sample analysis was performed by amperometric current–time technique at the applied potential of −1.12 V. The collected river water samples were filtrated using a 0.45 μM membrane filter and then injected in 0.1 M PBS and 6.0 M urea mixed solution by micro-injector. The concentrations of nitrite in the water samples were analyzed by standard addition method using the proposed sensor.

All experiments were carried out at room temperature.

3. Results and discussions

3.1. Conformational change of Hb

Different conformational transitions of Hb molecules such as aggregation, disassembling of subunits, or exposure/burial of amino acid residues and active centers can occur under different unfolding conditions by typical denaturants such as urea, acid, alkali, salts, and guanidine hydrochloride (GdnCl). GdnCl and acid has strong subunit- and heme-dissociating ability and they can induce the dissociation of the heme groups from the hydrophobic cavity of Hb [6,17]. Urea has chaotropic and mild effects on the Hb conformation [18], which was thus used in this research to optimize the conditions for regulating Hb conformation to a most unfolded state. Fig. 1A shows the UV–vis spectra of 2.0 μM Hb in 0.1 M pH 7.0 PBS with different concentrations of urea from 0 to 8.0 M. The sharp Söret absorption at 405 nm is ascribed to the heme monomer coordinating to His-F8 of native Hb in neutral PBS (pH 7.0) [6]. As shown in Fig. 1B, with increasing the concentration of urea from 1.0 to 8.0 M, three different degrees of decreases in the absorbance occurred. When the concentration of urea increased from 0 to 2.0 M, the UV–vis absorbance at 405 nm decreased dramatically due to the partial loss of the native conformation of Hb. When the concentration of urea was in the range of 2.0–6.0 M, the absorbance decreased slowly because of the structural rearrangement of Hb during unfolding processes. After the concentration of urea was higher than 6.0 M, obvious absorbance change was observed again. The results indicate that the capability of secondary bonds (i.e., hydrogen bond, van der Waals forces, electrovalent bond) for maintaining three-dimensional conformation decreased dramatically at high concentration of denaturants. Moreover, the maximal absorption wavelength remained unchanged even as the urea concentration up to 8.0 M, suggesting that urea can induce the unfolding of Hb conformation but cannot cause the dissociation of heme groups [19].