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Sensitivity enhancement of electrochemical biosensor via cobalt nanoflowers on graphene and protein conformational intermediate

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ABSTRACT

The nanocomposite of cobalt nanoflowers on graphene (Co-GE) was synthesized by a facile, one-pot ultrasonic electrochemical method. The scanning electron microscopy measurements displayed that the synthesized Co-GE exhibited a related hierarchical structure of a flake GE homogeneous distribution as a matrix for the growth of Co nanoflowers. The chemical composition was confirmed by energy dispersive X-ray spectrograms and X-ray diffraction analysis. The electrochemical biosensors based on redox proteins or enzymes possess high selectivity and biological compatibility but suffer from the low voltammetric response due to the deep burial of electroactive center in proteins. Herein, two typical denaturants, urea and acid, were synergistically utilized to maintain hemoglobin (Hb) in its most unfolded state, while simultaneously maintaining the presence of the heme groups. A novel hydrogen peroxide (H_2O_2) biosensor was structured using nanocomposite and protein conformational intermediate. The unfolded Hb/Co-GE/GCE exhibited accelerated direct electron transfer for sensing H_2O_2 under optimal conditions. The sensitivity for detecting H_2O_2 improved as much as 8.3 times higher than those for the native Hb/Co-GE/GCE. The electrocatalytic reduction of H_2O_2 showed a wide linear range from 0.25 to 190.0 μM with a high sensitivity of 116.3 μA mM^{-1} and a low limit of detection of 0.08 μM (S/N = 3). The unfolded Hb-based biosensor possesses the advantages of excellent stability, good reproducibility, and satisfactory recovery. The current research provides a novel strategy to utilize the unique properties of protein conformational intermediates in the development of electrochemical biosensors.

1. Introduction

The advancement in nanotechnology has led to nanoparticles (NPs) playing a crucial role in improving sensor performance owing to their large specific surface areas and biocompatibilities [1–3]. A significant amount of attention has been paid to nanocarbon materials for supporting nanosized metallic particles, which are widely used in heterogeneous catalysis, electrocatalysis and the enhancement of catalyst dispersion and stability [4,5]. Their widespread applications are due to their extraordinary electronic conductivity and availability of accessible surface area for the nanosized catalyst ability to induce enhanced catalytic activity [6–8]. In recent years, graphene (GE), a monolayer of graphite, has elicited considerable attention for its superior chemical stability, large surface-to-volume ratio, tunable band gap, and great electronic, mechanical, and thermal stabilities [9,10]. Owing to the strong planar stacking of sheets from π–π* interaction, GE prefers to self-assemble and forms two-dimensional (2D) agglomerates [11]. Furthermore, convenient and low-cost fabrication procedure for large scale production of GE makes it a perfect alternative electrode material to carbon nanotubes.

The enzyme sensors have been extensively studied because of their facile fabrication, high sensitivity, and biocompatibility. However, deep burial of electroactive groups in hydrophobic cavity of proteins greatly hinders the direct electrochemistry between redox proteins and conventional electrodes, in process, decreasing sensitivities of the sensors [12,13]. A few 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 NPs have been reported to construct relatively ideal modified layers to provide suitable microenvironment for native proteins on the electrode surface [14–16]. These sensors based on natural proteins on the surfaces of electrodes established better direct electrochemistry, in turn, demonstrating decent sensitivities. However, their amperometric responses were not completely enhanced to the maximum due to a large distance between the deeply buried electroactive groups and electrode surfaces. The previous researches have shown that the unfolding proteins can obtain higher sensitivities [17,18].

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In the past several decades, heme proteins have been used as the paradigms for understanding the relationships between structure and function, studying direct electron transfer (DET), and constructing electrochemical biosensors [19–21]. The researchers have primarily kept protein natural conformation and biological activity on the surface of modified electrodes. However, these native proteins with deep buried heme groups still show low voltammetric response [22]. It is widely known that the biological activities of proteins closely relate with their three-dimensional structure. The structural alterations of proteins, namely the unfolding or denaturation, frequently results in the loss of activities and a wide range of diseases [23]. Nevertheless, appropriate unfolding of proteins to intermediate state with most exposure without losing heme groups can greatly boost their biological activities or electrocatalytic ability [24], in process enhancing the sensing sensitivity.

Hemoglobin (Hb), a physiological carrier of oxygen in red blood cells, comprises of two pairs of α- and β-subunits. Also, each subunit of Hb contains a heme group. Additionally, 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 can bind and also offers Hb the ability to reduce different targets such as H_2O_2 and nitrite [25]. However, when Hb is unfolded to be intermediate, it can exhibit higher electrocatalytic activity and faster response time due to the greater exposure of electroactive center [26,18]. These results motivated us to investigate and utilize the electrochemical properties of protein intermediate.

In the current work, Co-GE with related hierarchical structures on a glassy carbon electrode (GCE) was successfully synthesized via one-pot ultrasonic electrochemical method. In addition, Hb was chosen as a model heme protein to investigate the relationship between its electrocatalytic properties and conformational alterations. The UV–vis spectroscopy, fluorescence, and electrochemistry were used to monitor the conformational changes of Hb induced by urea or acid in solution. The monitoring of conformational changes was required to obtain the optimized conditions for maintaining an intermediate. After being unfolded to a suitable intermediate, Hb on Co-GE/GCE exhibited extremely high sensitivity and low limit of detection for H_2O_2. Therefore, the excellent electrocatalytic activity based on Hb intermediate conformation has potential application in the construction of biosensor.

2. Experimental

2.1. Chemicals and reagents

Bovine Hb (M_w = 64,500) was purchased from Sigma and used without further purification. Urea was obtained from Fluka. Cobaltous acetate ($Co(Ac)_2{\cdot}4H_2O$) was purchased from Xi'an Chemical Reagent Plant (Xi'an, China). Graphene oxide (GO) was purchased from Pioneer Nanotechnology Co. (Nanjing, China). Other reagents were of analytical reagent grade.

Phosphate buffer saline (0.1 M, pH 7.0, containing 0.1 M KCl) was prepared with the stock solutions of Na_2HPO_4 and NaH_2PO_4, and adjusted to the pH value.

2.2. Apparatus and measurements

A DL-180 ultrasonic cleaning machine (35 kHz, Zhejiang Haitian Electron Instrument Factory, China) was used to dissolve and form homogeneous solutions. A GCE with 3 mm diameter, before use, was first polished to a mirror-like state with 1.0, 0.3 and 0.05 μm Al_2O_3 slurries on a polishing cloth, then ultrasonic treatment in ethanol and double-distilled water for 5 min, respectively. All electrochemical experiments were carried out on a CHI660D electrochemical workstation (Shanghai CH Instrument Co. Ltd., China) using a three-electrode system with a GCE as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum electrode as the counter electrode.

Indium oxide glass (ITO) has many advantages [27], such as good conductive ability, high optical transmittance, a robust nature, the ability for easy patterning, and an excellent adhesion property to substrates, which make it an ideal matrix for convenient sample preparation. Herein, ITO (Hebei Lingxian Gaoke Co. Ltd.) was used as the substrate for the investigation of the morphology and ingredients. Before use, it was cleaned by sonication treatment sequentially for 20 min in acetone, 10% KOH in ethanol and double-distilled water. Scanning electron microscopic (SEM) measurements were carried out on a scanning electron microscope (JEOL, JSM-6700F) at 15 kV. The chemical composition of the samples was investigated using an energy dispersive X-ray spectroscope (EDS) attached to a JSM-6390A SEM. All electrochemical experiments were conducted at room temperature (25 ± 2 °C).

The X-ray photoelectron (XPS) spectra were performed on a VG ESCALAB 220-IXL spectrometer using an Al K_α X-ray source (1486.6 eV). The energy scale was internally calibrated by referencing to the binding energy (Eb) of the C1s peak of a carbon contaminant at 284.6 eV. X-ray diffraction (XRD) patterns of the samples were obtained using a D/MAX-3C (Rigaku Japan).

UV–vis absorption spectra were recorded on a Specord 50 spectrometer (Jena, Germany). Fluorescence measurements were conducted on an F-2500 fluorescence spectrometer with xenon lamps (Hitachi Ltd., Japan).

For all spectral measurements, Hb was incubated in urea (1.0–8.0 M) for 24 h at 4 °C to reach equilibrium. In order to obtain better spectra, all Hb solutions were diluted before measurements, and the time between the measurements and dilution was controlled within seconds. The concentrations of Hb for UV–vis and fluorescence measurements were 80 and 300 mg L^{-1}, respectively. All spectral measurements were carried out at room temperature (25 ± 2 °C).

2.3. Preparation of uHb/Co-GE/GCE

GO was dispersed in 1/15 M, pH 9.18 phosphate buffer saline with ultrasonication for several minutes to form a homogeneous solution containing 10 mM of $Co(Ac)_2{\cdot}4H_2O$ and 1 mg mL^{-1} of GO. The electrochemical deposition of Co-GE on the bare GCE was performed from − 1.0 to 1.0 V for 20 scans at the scan rate of 10 mV s^{-1} under successive irradiation of ultrasonic waves. For comparison purpose, GE/GCE and CoNPs/GCE were prepared using the same procedure without 10 mM of $Co(Ac)_2{\cdot}4H_2O$ or 1 mg mL^{-1} of GO. The Hb conformational intermediate (noted as uHb), incubated in acidic urea, was cast onto the surface of Co-GE/GCE and the biosensor was noted as uHb/Co-GE/GCE. The fabrication processes of the biosensor were shown in scheme 1. The temperature of the solution was controlled at T = 25 ± 0.1 °C using a water bath. Prior to experiments, the solution was deoxygenated with high purity nitrogen gas.

3. Results and discussion

3.1. Preparation and characterization of Co-GE/GCE

The cyclic voltammograms (CVs) of the electrodeposition of Co, GE and Co–GE on the GCEs were shown in Fig. 1. It could be observed in Fig. 1A that Co^{2+} reduction occurred at − 0.962 V (vs. SCE) in the first cycle, and thereafter at − 0.950 V in the second cycle. The extraordinary potential positive shift was attributed to the overpotential of Co electrodeposition onto the GCE substrate in the initial phase of CoNPs formation. Along with the potential positive shift, the reduction current decreased step by step and tended to be constant from the fifth scan. A similar phenomenon has been described in the literature [28]. The oxidation peak of $Co(H_2O)_6{}^{2+}/Co^0$ couple occurred at − 0.312 V (vs. SCE) [29]. The above results also indicated that Co was formed on GCE surface. As shown in Fig. 1B, the CVs of GE electrodeposition

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