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Direct electrochemistry and electrocatalysis of cytochrome *c* immobilized on gold nanoparticles–chitosan–carbon nanotubes-modified electrode

Cuili Xiang [a,b], Yongjin Zou [a,b], Li-Xian Sun [a,*], Fen Xu [a,*]

[a] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China*
[b] *Graduate School of the Chinese Academy of Sciences, Beijing 100049, China*

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Abstract

A robust and effective nanohybrid film based on gold nanoparticles (GNPs)/chitosan (Chit)/multi-walled carbon nanotubes (MWNTs) was prepared by a layer-by-layer self-assembly technique. Cytochrome *c* (Cyt *c*) was successfully immobilized on the nanohybrid film modified glassy carbon (GC) electrode by cyclic voltammetry. The direct electron transfer between Cyt *c* and the modified electrode was investigated in detail. Cyt *c* shows a couple of quasi-reversible and well-defined cyclic voltammetry peaks with a formal potential ($E^{0'}$) of −0.16 V (versus Ag/AgCl) in pH 7.0 phosphate buffer solution (PBS). The Cyt *c*/GNPs/Chit/MWNTs modified GC electrode gives an improved electrocatalytic activity towards the reduction of hydrogen peroxide (H_2O_2). The sensitivity is 92.21 μA mM^{-1} cm^{-2} and the calculated apparent Michaelis–Menten constant (K_m^{app}) is 0.791 mM, indicating a high-catalytic activity of Cyt *c*. The catalysis currents increase linearly to the H_2O_2 concentration in a wide range of 1.5×10^{-6} to 5.1×10^{-4} M with a correlation coefficient 0.999. The detection limit is 9.0×10^{-7} M (at the ratio of signal to noise, S/N = 3). Moreover, the modified electrode displays rapid response (5 s) to H_2O_2, and possesses good stability and reproducibility.

Keywords: Cytochrome *c*; Gold nanoparticles; Multi-walled carbon nanotubes; Chitosan; Nanohybrid; Direct electron transfer

1. Introduction

During the past few years, the direct electron transfer (DET) reaction between redox proteins and electrode surface has been extensively studied [1–4]. It can be applied to the study of physiological electron transfer processes and enzyme-catalyzed reactions in biological systems [5–7]. Among the various redox proteins, Cytochrome *c* (Cyt *c*) is an important heme-containing metallorotein, which exists in the cytosol between the inner and outer membranes of mitochondria. It plays an important role in the biological respiratory chain, whose function is to receive electrons from Cyt *c* reductase and deliver them to Cyt oxidase [8]. In recent decades, a substantial amount of research work has been carried out on the direct electrochemistry and electrocatalysis of Cyt *c* irreversibly immobilized on inert substrates [9,10], However, the voltammetric response of Cyt *c* is quite poor at the conventional electrodes, most likely due to protein denaturation at the metal electrode surface leading to extremely slow electron transfer kinetics or in the light of its three dimensional structure which hinders interaction with the electrode [11]. Recently, nanohybrid composites were used to investigate the direct electrochemical property between the Cyt *c* and the electrode [12,13]. The materials of these modified electrodes were found to promote the direct electron transfer of Cyt *c* at electrode surfaces [14].

Carbon nanotubes (CNTs), which can be divided into multi-walled carbon nanotubes (MWNTs) and single-walled carbon nanotubes (SWNTs) [15], are of great interests for the fabrication of new classes of advanced materials. The unique electronic properties show that CNTs have the ability to promote electron transfer reactions when used as a modifier on electrode in chemical reactions [16–18]. These properties make them extremely attractive for fabricating sensors and biosensors [19–21]. However, it is difficult to perform the adhering of protein on CNTs owing to CNTs hydrophobic properties [5]. Though direct electrochemistry of Cyt *c* on MWNTs modified electrodes has been

* Corresponding authors at: Materials & Thermochemistry Laboratory, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China. Fax: +86 411 84379213.
E-mail addresses: lxsun@dicp.ac.cn (L.-X. Sun), fenxu@dicp.ac.cn (F. Xu).

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reported, the electrochemical response of the electrode is very weak [8].

Just recently, the research interest has extended to modify CNTs with nanomaterials to prepare nanohybrid composite materials so as to optimize the use of them in studying the DET reaction of proteins [22–25]. The nanohybrid film can lead to new composite materials possessing the properties of each component with a synergistic effect that would be useful in particular applications. As good biocompatible materials, gold nanoparticles (GNPs) provide a mild microenvironment similar to that of redox proteins in native systems and give the protein molecules more freedom in orientation [26]. They also can develop conducting channels between enzyme and electrode surface [27,28], thus improving the electron transfer rate between the enzymes and electrode.

Chitosan (Chit) is a naturally occurred biopolymer product found in the exoskeleton of crustaceans. It is an attractive biocompatible, biodegradable, nontoxic natural and high mechanical strength biopolymer that exhibits excellent film-forming ability [29]. Because of its desirable properties, chitosan has been widely used as an immobilization matrix for biosensors and biocatalysis. Considering its relatively poor conductivity, chitosan was usually combined with carbon nanotubes, redox mediators and metal nanoparticles for electrochemical biosensing platforms [30].

Though MWNTs is a suitable matrix for immobilizing GNPs, it is difficult to absorb the GNPs on the MWNTs owing to the poor film-forming of GNPs. On the other hand, a layer-by-layer (LBL) self-assembly technique for fabricating multilayer film has attracted much attention because of its simplicity in procedure and wide choice of materials. Especially, the advantage of the LBL method lies on the adsorption process is carried out in aqueous solutions under mild conditions, which minimized the possibility of protein denaturing. In the present paper, a new method for fabricating GNPs/Chit/MWNTs nanohybrid film was developed by a LBL self-assembly technique. Cyt *c* was successfully immobilized on the resultant electrode by cyclic voltammetry. The resulted electrodes shows improved direct electrochemical behaviors of Cyt *c* and displays excellent electrocatalytical responses to the reduction of H_2O_2. The catalysis currents increase linearly to the H_2O_2 concentration in a wide range of 1.5×10^{-6} to 5.1×10^{-4} M with a correlation coefficient 0.999. The detection limit is 9.0×10^{-7} M (at the ratio of signal to noise, S/N = 3). Moreover, the modified electrode displays rapid response (5 s) to H_2O_2, and possesses good stability and reproducibility.

2. Experimental

2.1. Chemicals and reagents

Horse heart cytochrome *c* (M_W 12,384) was purchased from Sigma and used without further purification. Chitosan (M_W $(1.9–3.1) \times 10^5$; 92.5% deacetylation) was purchased from Nantong Shuanglin (China). MWNTs (95%, 20–60 nm) purchased from Shenzhen Nanotech. Port. Co. Ltd. (Shenzhen, China). The MWNTs were treated with nitric acid during purification process and then filtered, rinsed with double-distilled water and dried. The MWNTs charged negatively after treatment [31]. A fresh H_2O_2 aqueous solution was prepared prior to use. $AuCl_3 \cdot 3H_2O$ and other chemicals were of analytical grade and used as received. The 0.1 M pH 7.0 phosphate buffer solution (PBS), which was made from Na_2HPO_4 and NaH_2PO_4, was always employed as a supporting electrolyte. All the solutions were prepared with deionized water and were deaerated with high purity nitrogen before experiments.

2.2. Preparation of the nanohybrid film modified GC electrode

One milligram of purified MWNTs was dispersed in 5 ml dimethylformamide (DMF) with the aid of ultrasonic bath to give a 0.2 mg ml^{-1} black suspension. A 1 wt.% Chit solution was prepared by dissolving Chit in 2 wt.% acetic acid solution with magnetic stirring for about 2 h, then the pH of the solution was adjusted to pH 5.0 with a concentrated NaOH solution. Chit is soluble in acidic aqueous solutions in which it behaves as a cationic polyelectrolyte. GNPs were prepared according to the literature [32].

The GC (3 mm in diameter) electrode was polished carefully with 1.0, 0.3 and 0.05 μm alumina slurry, utrasonicated successively in 1:1 nitric acid, acetone and deionized water and then allowed to dry at room temperature. Under the optimized condition, the cleaned GC electrode was treated by dropping a suspension (10 μl) of the MWNTs in DMF and then dried under an infrared lamp. The negatively charged MWNTs modified GC electrode was immersed in a positively charged Chit solution for 1 h at 4 °C. After thoroughly rinsed with water, the obtained electrode was immersed in a negatively charged GNPs solution for 10 h at 4 °C and then rinsed with water to remove weakly adhered GNPs on the chitosan surface. The above electrode was immersed in 0.02 M deoxygenated L-cysteine (Cys) aqueous solution for 2 h at 4 °C. Again, the modified electrode was rinsed fully with water. It has been showed that L-cysteine plays a promoter role for the electron transfer reaction of Cyt *c*. It can provide negatively charged sited and interact with the hydrophilic surface of Cyt *c*. Furthermore, the carboxyl and ammo of L-cysteine interact with the lysine residues surrounding the heme edge of Cyt *c* [33]. In the present paper, we assembled the L-cysteine on the GNPs. Finally, the Cys/GNPs/Chit/MWNTs-modified electrode was placed in a Cyt *c* solution (1 mg/ml, pH 7.0 PBS) and a consecutive cyclic potential scans was performed in the potential range from −0.7 to 0.3 V with a scan rate 50 mV/s up to obtain a stable CV curve. Then the electrode was removed from the solution, washed with deionized water and stored in pH 7.0 PBS at about 4 °C. The fabrication of the Cyt *c*/Cys/GNPs/Chits/MWNTs/GC electrode is sketched in Scheme 1.

2.3. Apparatus and measurements

Scanning electron microscopy (SEM) images were obtained by using JSM-6360LV SEM (JEOL, Japan). Cyclic voltammetric (CV), electrochemical impedance spectroscopy (EIS)