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Direct electron transfer of cytochrome *c* at mono-dispersed and negatively charged perylene–graphene matrix

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ABSTRACT

Mono-dispersed 3,4,9,10-perylene tetracarboxylic acid (PTCA) functionalized graphene sheets (PTCA-graphene) were fabricated by a chemical route and dispersed well in aqueous solution. PTCA-graphene with plenty of –COOH groups as electrostatic absorbing sites were beneficial to the loading of Cytochrome c (Cyt *c*). Cyt *c*, which was tightly immobilized on the PTCA-graphene modified glassy carbon electrode, maintained its natural conformation. Direct electron transfer of Cyt *c* and the electrocatalytic activity towards the reduction of H_2O_2 were also achieved. It has been substantiated that PTCA-graphene is a preferable biocompatible matrix for Cyt *c*.

1. Introduction

Cytochrome *c* (Cyt *c*), whose function is to receive electrons from Cyt *c* reductase and deliver them to Cyt *c* oxidase, plays an important role in the respiration chain. Just like other heme-containing proteins, such as myoglobin and horseradish peroxidase, Cyt *c* has the ability to electro-catalyze the reduction of hydrogen peroxide (H_2O_2) [1]. It has been previously reported to detect some other small molecules including nitrite salt [2], nitric oxide [3], superoxide radical anion [4] etc. Furthermore, Cyt *c* can be a powerful tool to make sensitive electrochemical biosensor devices [5]. To realize this application, electrochemical research on the electron transfer of Cyt *c* at electrode/solution interfaces is indispensable. However, Proteins are huge molecules and the electro-active centers are usually embedded deeply into the large three dimensional structures of enzyme molecules [6]. It is difficult for electrons to transfer from the redox center of protein to the surface of the electrode. To efficiently achieve the direct electron transfer (DET) of Cyt *c* and realize its potential applications for electrochemical biosensors, different kinds of nanomaterials have been employed as media or matrix, such as gold nanoparticles (GNPs) [7,8], cobalt oxide nanoparticles [9], silicon nanotubes [10], single-walled carbon nanotubes (SWNT) [11,12], and multi-walled carbon nanotubes (MWNT) [13], etc. In fact, it has been investigated and concluded that these nanomaterials acted as "tiny conducting wires" leading between the protein center and the electrode.

Meanwhile, how to immobilize Cyt *c* to the electrode is another key challenge to its application for electrochemical biosensors. The importance of binding between protein and electrode as a prerequisite for electron transfer has been previously discussed [14]. In order to conquer the unfavorable orientation of Cyt *c* at the surface of the electrode and maintain its natural activity, researchers have tried to find several effective approaches to immobilize Cyt *c* onto the surface of the electrode [15–18]. The electrostatic interaction between Cyt *c* and electrode surface plays a key role during the redox reaction of Cyt *c* [19–22]. Cyt *c* with a high PI (>10) is positively charged in neutral conditions. Chen and coworkers mixed negatively charged DNA with carbon nanotubes to perform a preferable matrix which could strongly adsorb Cyt *c* [21]. Armstrong found that the electron transfer rate of Cyt *c* on the edge which has higher surface O/C ratio was 2-folds higher than that on the basal plane of pyrolytic graphite [19]. Kasmi and coworkers have investigated the changes of the electron transfer rates when $-COO^-$ terminated self-assembled monolayer surface mixed with hydroxyl groups [16]. According to these reports, introducing negatively charged –COOH groups to the substrate might be an effective way to promote the loading of Cyt *c* and facilitate the redox reaction of Cyt *c*.

Among different kinds of nanomaterials, graphene is an ideal candidate to achieve the functions as "conducting wire" and serve as good substrate simultaneously. Graphene has the unique structure of single layer of carbon atoms in a closely packed

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honeycomb two-dimensional lattice. This structure helps graphene expose itself completely to the analyte and provide more active sites essential for the redox reaction. According to the literature, graphene has a higher surface area which can afford a higher loading capacity for enzyme comparing with carbon nanotubes [23]. Meanwhile, its excellent conductivity can facilitate the electrons transfer from the biomolecules [24]. And its biocompatibility was demonstrated by Chen and coworkers based on the cell culture experiments [25]. These exceptional properties of graphene provide it potential applicability in the applications in electrochemical biosensors. Till now, researchers have developed the graphene-based glucose biosensor [26,27], H_2O_2 biosensor [28,29], NADH biosensor [30], Cholesterol biosensor [31] etc. However, the advantages of graphene are only associated with individual sheets. Due to the high cohesive van der Waals energy and strong π–π stacking, graphene sheets will agglomerate irreversibly without any protections. Therefore, in order to obtain graphene materials with good dispersibilities as well as excellent properties, covalent modifications [32,33] or noncovalent functionalizations [24,34,35] of graphene have been performed.

In this work, –COOH groups have been introduced to graphene sheets by functionalization with 3,4,9,10-perylene tetracarboxylic acid (PTCA) which contains a π-conjugated perylene ring and four carboxyl groups. The composite has plenty of electrostatic interaction sites for the loading of Cyt *c*. Meanwhile it has the superb dispersibility which can provide high conductivity and high surface area. During the electrochemical investigations, a pair of well-defined and symmetric redox peaks corresponding to the oxidation and reduction of the iron in the heme group of Cyt *c* was obtained at the PTCA-graphene modified glassy carbon electrode (GCE). Cyt *c* on the modified electrode showed its native properties. It electrochemically catalyzed the reduction of H_2O_2. PTCA-graphene was proved to be a preferable substrate and "conducting wire" simultaneously. It played an important role in the DET between active center of Cyt *c* and electrode surface. The monodispersibility and negative charge make this material a suitable candidate for the substrate of other biosensors.

2. Materials and methods

2.1. Chemicals

Horse heart Cyt *c* (96%, Mw 12,384) and 3,4,9,10-perylenetetracarboxylic dianhydride (PTCDA,97%) were obtained from Sigma. Graphite powders (spectral requirement) were purchased from Shanghai chemicals, China. Hydrazine solution (50 wt%) was purchased from Beijing Yili Chemicals, China. Ammonia solution (28 wt%) and hydrogen peroxide solution (30 wt%) were obtained from Beijing Chemicals, and a fresh solution of H_2O_2 was prepared daily. Phosphate buffer solutions (PBS, 20 mM) with various pH values were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4. Acetic acid-sodium acetate buffer solution was prepared with stock standard solutions of HAc and NaAc. Unless otherwise stated, reagents were of analytical grade and used as received. All aqueous solutions were prepared with doubly distilled water from a Millipore system (>18 MΩ/cm).

2.2. Apparatus

UV–vis absorption spectra were recorded using a Cary 500 UV/vis/near-IR spectrometer. Fourier transform infrared spectroscopy (FTIR) was performed on a Bruker Vertex 70 spectrometer. Scanning electron microscopy (SEM) pictures were imaged by a field emission scanning electron microscopy (FE-SEM, XL30ESEM-FEG). AFM images were obtained with a Digital Instruments nanoscope IIIa (Multimode, Veeco), operating in tapping mode.

Cyclic voltammetry (CV) measurements were performed with CHI 660A electrochemical workstation (CHI, USA). Electrochemical impedance spectroscopy (EIS) measurements were carried out with Solartron 1255B Frequency Response Analyzer (Solartron Inc., UK). All the electrochemical experiments were performed using a conventional three electrode cell with bare or modified GCE ($d=3$ mm) as the working electrode, a Pt coil as the counter electrode and Ag/AgCl (3 M KCl) as the reference electrode. Before use, the GCE was mechanically polished with alumina slurries (1, 0.3, 0.05 μm) to a mirror successfully, and sonicated in ultrapure water for several times.

2.3. Electrochemical measurements

Unless otherwise stated, the electrolyte solution used for all electrochemical experiments was a 0.02 M, pH 7.4 PBS. EIS measurements were performed in the presence of 5 mM $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ (1:1) and 0.1 M KCl by applying an alternating current (AC) voltage with 5 mV amplitude in a frequency range from 0.1 Hz to 10 kHz under open circuit potential conditions. Prior to electrochemical experiments, all the solutions were deaerated with high purity nitrogen and maintained under nitrogen atmosphere during measurements. All the electrochemical experiments were carried out at room temperature.

2.4. PTCA-graphene fabrication

Functionalization of graphene with PTCA was conducted according to our previous work [36,37]. First, graphene oxides (GO) were synthesized from natural graphite powder by a modified Hummers method which was originally presented by Kovtyukhova and colleagues [38]. The PTCA solution was made by hydrolyzing PTCDA in a minimal volume of 1.0 M sodium hydroxide [39]. Red depositions appeared in the yellow–green solution and were collected by centrifugation and dried under vacuum at room temperature. 21.2 mg graphene oxide and 5.2 mg PTCA were dissolved in 20.0 mL water by ultrasonication, and then stirred at 40 °C overnight. Subsequently, 26.8 μL hydrazine solution and 0.30 mL ammonia solution were added to the above solution and the resulting mixture was held at 95 °C for 30 min under vigorous agitation. The product was subsequently filtered by a Nylon membrane with *ca.* 0.22 μm pores and thoroughly washed with ultrapure water and dried under vacuum at room temperature. As controlled experiment, pristine graphene was obtained by the similar procedure without addition of PTCA to the blank sample.

2.5. Preparation of modified electrode

Cyt *c* solution (10 mg/mL in PBS, 20 mM, pH 7.4) and PTCA-graphene suspension (0.4 mg/mL) were mixed by the volume ratio of 2:3 at the room temperature with constant stirring for 5 min. Then 5 μL of the mixed solution was drop-cast on the surface of the electrodes which were allowed to be dried at room temperature.

3. Result and discussion

3.1. Characterizations of PTCA functionalized graphene

The preparation of PTCA-graphene is illustrated in Scheme 1. The PTCA molecules carried –COOH groups and interacted with the basal plane of graphene sheets by π–π stacking and hydrophobic