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Direct electrochemistry and electrocatalysis of hemoglobin protein entrapped in graphene and chitosan composite film

Huifeng Xu, Hong Dai, Guonan Chen*

Ministry of Education Key Laboratory of Analysis and Detection for Food Safety, Fujian Province Key Laboratory of Analysis and Detection for Food Safety, and Department of Chemistry, Fuzhou University, Fuzhou, Fujian 350002, China

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ABSTRACT

A novel, biocompatible sensing strategy based on graphene and chitosan composite film for immobilizing the hemoglobin protein was firstly adopted. The direct electron transfer and bioelectrocatalytic activity of hemoglobin after incorporation into the composite film were investigated. A pair of reversible redox waves of hemoglobin was appeared, and hemoglobin could exhibit its bioelectrocatalytic activity toward H_2O_2 in a long term. Such results indicated that graphene and chitosan composite could be a friendly biocompatible interface for immobilizing biomolecules and keeping their native structure. Furthermore, the appearance of graphene in the composite film could facilitate the electron transfer between matrix and the electroactive center of hemoglobin. Hence, this graphene and chitosan based protocol would be a promising platform for protein immobilization and biosensor preparation.

1. Introduction

The understanding of electron transfer mechanism of biomolecules in vivo is still an important event. So the investigation about the direct electron transfer of proteins has attracted considerable attention in life science and analytical chemistry since 1970s [1]. However, the direct electron transfer to metal centers of proteins is generally difficult to occur due to the embedment of the electroactive centers within the protein structure or the denaturation of proteins adsorbed on solid electrode surfaces. Hence various modified electrodes were developed for such purpose and the immobilization of proteins was widely studied [2–7]. Especially, when the nanometer size material is employed in fabricating various biosensing interfaces, the nanometer size hybrids based biosensors then become promising platform for the direct electron transfer investigation. Due to the unique properties of nanometer size material, the nanostructures can offer the electron transfer interface to load high quantitive biomolecules and exhibit the properties of inherent catalysis, penetrability and resistance to biodegradation [8–10].

Owing to the numerous unexpected properties of graphene, such as quantum Hall effect [11], the two-dimensional structure of graphene has become a hot research topic in both the experimental and theoretical scientific communities [12,13]. Recently, various graphene based sensors have been developed so as to explore its inherent properties. Wang et al. adopted the graphene-modified electrode to selectively detect dopamine in a large excess of ascorbic acid [14]. Li et al. fabricated a graphene and Nafion based electrochemical platform for ultrasensitive detecting of cadmium [15]. Niu and co-workers constructed a novel polyvinylpyrrolidone-protected graphene/polyethyleneimine-functionalized ionic liquid/glucose oxidase electrochemical biosensor [16]. Based on the exfoliated graphite nanoplatelets, Worden and co-workers [17,18] successfully developed a high performance glucose biosensor. These results indicate that graphene displays a tremendous potential for fabricating various biosensors, it may be an inexpensive alternative to carbon nanotube so as to design a new generation of bioelectronic devices with high sensitivity and selectivity. While, there is still no much effort has been made to investigate the direct electron transfer of protein in the graphene based biosensor, and the application of graphene in electrochemistry research is still limited. Later, Lin and co-workers immobilized glucose oxidase to the hybrid nanocomposite of graphene–chitosan and succeeded in realizing DET [19]. This work substantiated that this graphene–chitosan nanocomposite film can provide a favorable microenvironment for biomolecule and effectively keep their activities.

In the present communication, the direct electron transfer of hemoglobin (Hb) was found in the graphene–chitosan based biosensor and the results also showed that the proposed modi-

* Corresponding author. Tel.: +86 591 87893315; fax: +86 591 83713866.
E-mail address: gnchen@fzu.edu.cn (G. Chen).

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fied film could retain the bioactivity of Hb, indicating that this modified film could provide a favorable microenvironment for immobilization of Hb. Especially, this strategy for immobilizing the proteins based on graphene–chitosan composite film was quite simple and stable. Therefore, graphene–chitosan composite film would be a sort of biomaterial being suitable for protein immobilization and preparation of the new generation biosensors.

2. Experimental

2.1. Reagents and apparatus

Hemoglobin (bovine blood) was purchased from Sigma Chemical Co. (USA) and used as received. Chitosan of low molecular weight from the shrimp shell with a degree of deacetylation of 83.3% was obtained from Sigma–Aldrich. All other chemicals were of analytical grade. All the solutions were prepared with doubly distilled water. A series of 0.1 mol/L PBS was used as supporting electrolyte. Graphene was synthesized according to previous reports [20,21].

Cyclic voltammetric experiments and electrochemical impedance spectroscopy were both performed on a CHI 760C electrochemical analyzer (Shanghai CH Instrumentation, China). The three-electrode system was composed of an Hb modified glassy carbon electrode as working electrode, a KCl-saturated silver–silver chloride (Ag/AgCl) as reference electrode and a platinum wire as auxiliary electrode. All the test solutions were thoroughly deoxygenated with pure nitrogen for about 15 min prior to experiments. UV–visible (UV–vis) absorption spectrum was carried out by using a Cary 550 spectrophotometer (Varian, USA). Transmission electron microscope (TEM) and XPS measurements were conducted using Tecnai G2 F20 S-TWIN microscope (FEI Company, The Netherlands, 200 KV) and VG ESCALAB 250(Thermo Nicole) respectively.

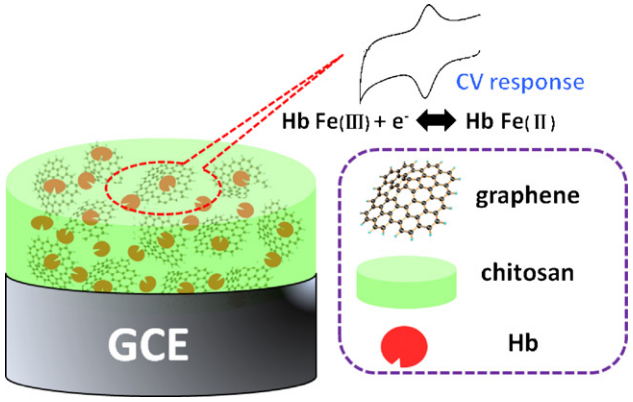

Fig. 1. Schematic of the construction of Hb-graphene–chitosan/GCE.

2.2. Electrode modification procedure

The schematic principle for fabrication of this biosensor is shown in Fig. 1. The glassy carbon electrodes (GCE, 4 mm in diameter) were polished to a mirror-like with 1.0, 0.3, and 0.05 μm alumina slurry. The electrodes were successively sonicated in 1:1 nitric acid, acetone and doubly distilled water, and then dried in nitrogen.

A 0.5 wt.% Chi solution was prepared according to the reported method [22]. 5 mg Chi was dissolved in 1 mL of 1% acetic acid and pH of solution was adjusted to 5.0 with concentrated NaOH. The graphene–chitosan solution was prepared by dispersing 15 mL graphene solution with 10 mL of 0.5 wt.% chitosan solution. Then dissolve 10 mL Hb PBS (3 mg/mL, pH 5.5) into proposed graphene–chitosan solution to form the modified solution. The modified film was achieved by dropping 5 μL suspensions onto the surface of GCE and the solvent was evaporated in the air for 4 h. In order to form a uniform Hb-CS-graphene composite film at the

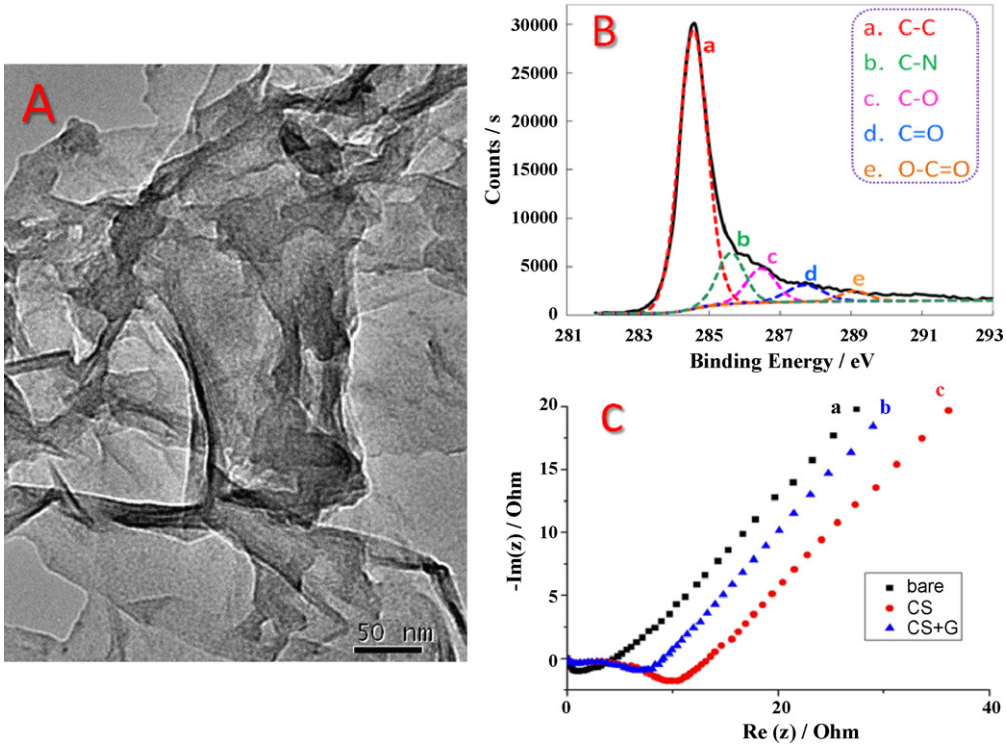

Fig. 2. Characterization of graphene: (A) TEM image of graphene; (B) C 1s XPS spectra of graphene; (C) electrochemical impedance spectroscopy for bare GCE (a), chitosan/GCE (b) and graphene–chitosan/GCE in a solution of 5.0 mM $K_4Fe(CN)_6/K_3Fe(CN)_6$ + 0.1 M KCl (c).

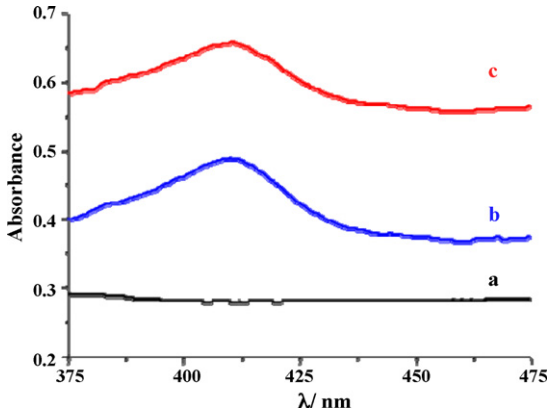

Fig. 3. UV–vis spectra of the graphene–chitosan (a), Hb (b) and Hb-graphene–chitosan films (c) on indium tin oxide sides.

GCE surface, a beaker was covered over the electrode so that water can evaporate slowly. The dried Hb-CS-graphene/GCE was stored at 4 °C in a refrigerator when not in use. For comparison, Hb-CS/GCE, CS-graphene/GCE, and Hb-graphene/GCE were prepared with the same procedures as described above.

3. Results and discussions

3.1. Characterization of graphene

The state of the dispersed graphenes was observed using transmission electron microscopy (TEM). As shown in Fig. 2A, large flakes of graphene were dispersed. There were some monolayer graphene with slightly scrolled edges. Some graphene flakes folded together.

The graphene was also characterized by XPS in Fig. 2B. XPS analysis can provide additional information about the oxygen-containing surface groups. The dominant peak structure for C 1s core level of graphene at a binding energy of 284.5 eV was quite similar to the carbon shells of CNTs [23]. A raised bump around 289.1 eV, similar to that for oxidized carbon nanotubes (CNTs), resulted from the COO functional group [23]. And the rest of the absorbance peaks of graphene at 286.5 and 287.7 eV could be owned to C–O and C=O respectively.

The interface properties of the modified electrodes have been characterized by electrochemical impedance spectroscopy (EIS), and the diameter of the semicircle corresponds to the interfacial electron transfer resistance (Ret) [24]. The bare glassy carbon electrode gave an almost straight line (Fig. 2Ca), which demonstrates the characteristic of a diffusion-controlling step of the electrochemical process. The immobilization of chitosan on the GCE would further hinder the access of the redox probe to the electrode, due to the resistance of the chitosan membrane, causing a further increase in Ret. While, when graphene was doped into the chitosan membrane, an obvious decrease in Ret was observed which was related to the unique properties of graphene. The two-dimensional structure of graphene may act the role of accelerating the electron transfer in the sensing interface.

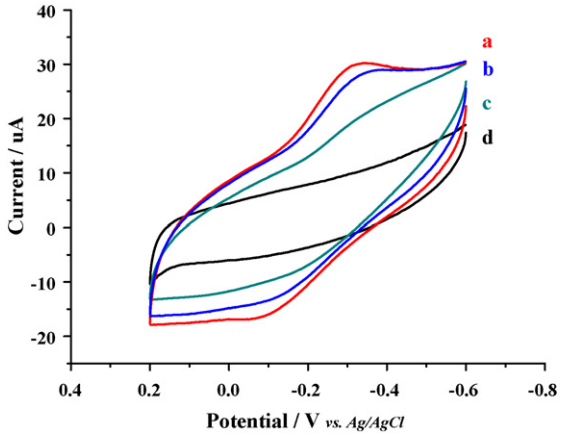

Fig. 4. Cyclic voltammograms of Hb-graphene–chitosan/GCE (a), Hb-graphene/GCE (b), Hb-chitosan/GCE (c) and graphene–chitosan/GCE (d) in PBS (pH 7.0); scan rate: 100 mV s^{-1}.

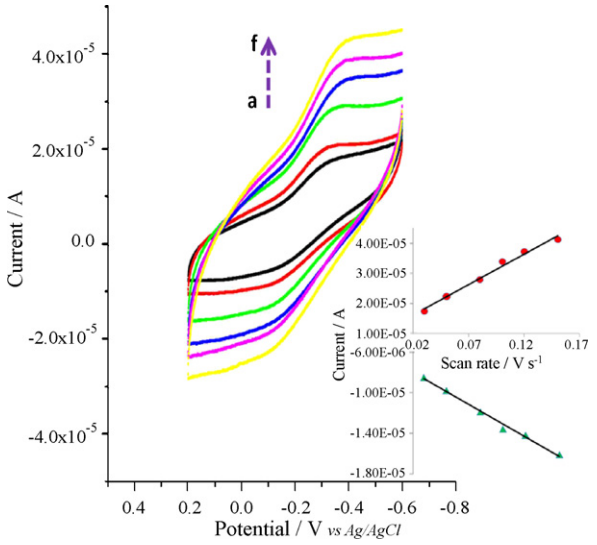

Fig. 5. Cyclic voltammograms of graphene–chitosan/GCE in PBS (pH 7.0) under different scan rates: (a) 30 mV s^{-1}, (b) 50 mV s^{-1}, (c) 80 mV s^{-1}, (d) 100 mV s^{-1}, (e) 120 mV s^{-1}, and (f) 150 mV s^{-1}; inset: plots for corresponding anodic and cathodic peak current against scan rate.

3.2. Characterization of graphene–chitosan and Hb-graphene–chitosan composite film

The position of Soret absorption band of heme may provide information about possible denaturation of proteins [25]. To investigate the activity of Hb immobilized on the graphene–chitosan modified film, Hb absorbed onto the surface of this composite film was then performed by spectroscopic analysis. As shown in Fig. 3, the Soret band of the Hb entrapped in graphene–chitosan films was observed at 411 nm, shifting only 1 nm toward the red with the Soret band of 410 nm for native Hb film. Thus it can be concluded that the Hb entrapped in the graphene–chitosan composite films could retain its native structure because the graphene–chitosan film might have good biocompatibility.

3.3. Direct electrochemistry of Hb on the graphene–chitosan/GCE

The cyclic voltammogram of Hb on the graphene–chitosan/GCE displayed a couple of stable redox peaks, while no obvious electrochemical response was observed at graphene–chitosan/GCE (Fig. 3). Thus these peaks were attributed to the redox reaction of the electroactive center of Hb. In comparison, we found that slightly unconspicuous redox peaks appeared on graphene/GCE (Fig. 3b) and no remarkable redox peaks at Hb-chitosan/GCE (Fig. 3c) were obtained, suggesting that it was difficult to realize the direct electron transfer of the Hb at the pristine chitosan modified electrode. It indicated that large surface-to-volume ratio and high conductivity of graphene cooperates with good biocompatibility of chitosan that leads to enhancing the enzyme absorption and promotes direct electron transfer between redox enzymes and the surface of

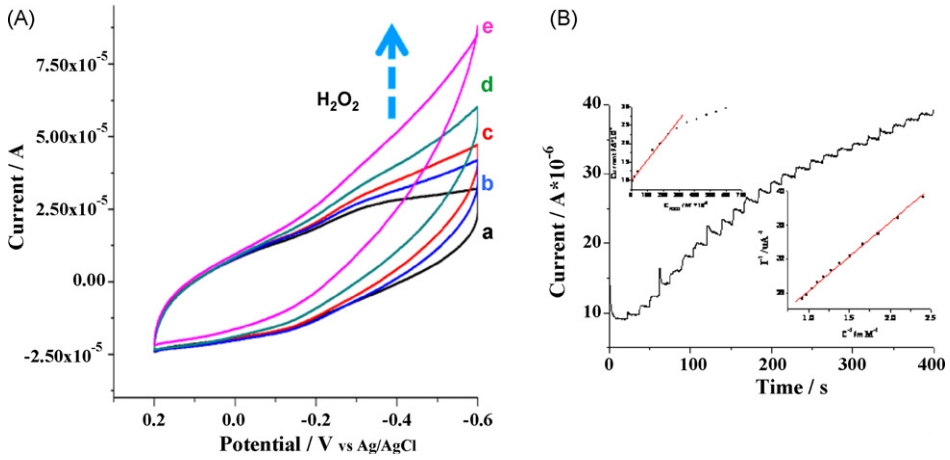

Fig. 6. Cyclic voltammograms of Hb-graphene–chitosan/GCE in 0.1 M PBS (pH 7.0) containing 0 M H_2O_2 (a), 2.0×10^{-5} M H_2O_2 (b), 6.0×10^{-5} M H_2O_2 (c), 1.6×10^{-4} M H_2O_2 (d), and 3.6×10^{-4} M H_2O_2 (e) at 100 mV s^{-1} (A) and amperometric response of the biosensor in 0.1 M PBS (pH 7.0) at −400 mV upon successive additions of H_2O_2 (B); insets are the calibration curve (top left) and corresponding Lineweaver–Burk plot (right side).

electrodes. Graphene in the sensing interface may act as enhancing agent for effective acceleration of electron transfer between matrix and Hb, leading to more rapid current response for Hb [26]. Moreover, at Hb-graphene–chitosan/GCE, both i_{pc} and i_{pa} increased linearly with scan rates from 30 to 150 mV s^{-1} (Fig. 4), indicating a surface-controlled electrochemical process. By integrating the CV oxidation peaks of the biosensor, the surface concentration of electroactive Hb on the modified electrode was estimated to be 3.1×10^{-10} mol cm^{-2}, which was much greater than the theoretical monolayer coverage of Hb [27]. It might be mainly ascribed to the following reasons: firstly, the chitosan-dispersed graphene film electrode might possess a three-dimensional architecture; and secondly Hb was absorbed on the graphene nanoplatelets, leading to a better loading of the Hb in the hybrid nanocomposite matrix. Additionally, the chitosan and graphene hybrid film could prevent the leakage of Hb during the electrochemical measurement. Simultaneously, chitosan allows the Hb maintain its suitable conformation and activity. Hence, the chitosan and graphene composite film not only acts as the role of electronic signal transduction, but also provides a shelter for the biomolecules to retain their bioactivity.

3.4. Biocatalytic activity of immobilized Hb

The bioelectrocatalytic reduction behavior towards H_2O_2 was further explored by CV and amperometric techniques. Upon addition of H_2O_2 to 0.1 M PBS (pH 7.0), the cyclic voltammogram of the Hb-graphene–chitosan/GCE for the direct electron transfer of Hb changed dramatically with an increase of reduction peak current and a decrease of oxidation peak current (Fig. 5a). So the electrocatalytic response could be used as an efficient biosensor for detection of H_2O_2.

At an applied potential of −400 mV, Fig. 4B shows the amperometric response of the Hb-graphene–chitosan/GCE to successive addition of H_2O_2 in 0.1 M PBS (pH 7.0). It can be known from the inset of Fig. 5B that the linear range of this biosensor to H_2O_2 concentration was between 6.5 and 230 μM which can be described by a linear regression equation of I (μA) = 10.07 + 0.056C (μM) (R = 0.993). From the slope, a limit of detection for H_2O_2 at a signal-to-noise ratio of 3 was estimated to be 5.1×10^{-7} M. Furthermore, the experiments showed that the electrocatalytic response was very fast; the biosensor achieved 95% of the maximum steady-state response to H_2O_2 in less than 5 s, which demonstrated that the chitosan and graphene composite film might provide a well geometric structure for investigating the fast electrode process kinetics. When the concentration of H_2O_2 was higher than 230 μM, a response plateau was observed, representing the feature of the Michaelis–Menten kinetic mechanism. According to the Lineweaver–Burk form of the Michaelis–Menten equation [28]: $1/I_{ss} = (1/I_{max}) + (K_m^{app}/I_{max}C)$. Here I_{ss} was the steady-state current after the addition of a substrate and obtained from amperometric experiments, I_{max} was the maximum current under saturated substrate conditions, C was the concentration of the substrate, and K_m^{app} was the apparent Michaelis–Menten constant and was independent of the enzyme concentration. A value of 0.344 mM for K_m^{app} was obtained by the analysis of slope and intercept of the reciprocals of the steady-state current versus H_2O_2 concentration. The value was smaller than that of biosensors based on some carbon nanotubes composite film [29,30]. The low value of K_m^{app} indicates that Hb immobilized in CS-graphene/GCE film retains its bioactivity and has a high biological affinity to H_2O_2 (Fig. 6).

When the resultant sensor was not in use, it was stored at room temperature. Electrode stability was tested daily over a 14-day period. It could maintain 80.1% of its initial electrochemical response after 2 weeks. By using the same process for preparation of three proposed biosensors, the response of these biosensors almost has no difference.

4. Conclusions

In summary, the strategy utilizing chitosan and graphene composite film for encapsulation of Hb could provide a favorable microenvironment around the protein to retain the native structure and bioactivity of immobilized protein. Benefited from the two-dimensional structure and the high quality of the sp^2 conjugated bond in the carbon lattice of graphene, the proposed biosensor exhibited some excellent analytical performance, such as wider linear range, low detection limit, good stability etc. This methodology described here can be applicable to develop other graphene based biosensors. Moreover, the chitosan and graphene composite film would be useful for investigation of the electron transfer properties of proteins.

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