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Electrochemical behaviour of haemoglobin on glassy carbon nanotubes – a review

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ABSTRACT

In this study, we have demonstrated that haemoglobin can be coupled to acid-treated multiwall carbon nanotubes in the presence of 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) and assembled as haemoglobin–carbon nanotube (Hb–CNT) composites. Our observations of the electrochemical studies demonstrate that the electrochemical response of Hb–CNT assembled in the presence of EDC is much higher than that in the absence of EDC. It is evident that the direct electron transfer of haemoglobin could be effectively accelerated in the Hb–CNT assembly by using EDC on a glassy carbon electrode (GCE), and the relative electron transfer rate constant *K*s is found to be 1.02 ± 0.05 s−1. The results of our studies illustrate that the assembly of haemoglobin–multiwall carbon nanotubes using EDC could provide a novel strategy to effectively facilitate the direct electrochemistry of heme-containing proteins, which could be further utilized as a promising biosensor for some specific biological substrate and related biological process.

Introduction

Electron transfer in biological system is one of the leading areas in biochemical and biophysical research fields [1–3].The redox mechanism of proteins and the respective metabolic process of enzymes could be revealed on the basis of the direct electron transfer of proteins or enzymes, which could play a fundamental role in the life sciences and biomedical engineering [4–7]. Since haemoglobin is a physiological oxygen transport protein and acts as an important protein in life, it could be utilized as an ideal model protein to explore the relative biological process and redox behaviour of heme proteins or enzymes. However, studies on the structure–function relationship of haemoglobin have intrigued researchers over the past years [8,9]. It is well known that the redox centers are deeply immersed in haemoglobin, so the direct electron transfer between haemoglobin and the electrode surface is rather difficult, which could cause the low rate of electron transfer between haemoglobin and the solid electrode. Recently, some methods have been explored for facilitating the relative electron transfer of haemoglobin through combining with the mediators, promoters, and some special electrode materials [10,11]. Polymers such as poly(diallyldimethylammonium) (PDDA) and clay modified electrodes have been reported to be utilized for achieving the direct electrochemistry of heme proteins such as haemoglobin and cytochrome *c* [12–14]. Besides, metal and oxide nanoparticles have been introduced in this area and a glassy carbon electrode modified with haemoglobin-immobilized gold nanoparticles has been adopted to serve as a sensitive biosensor [15,16]. Moreover, it is noted that three-dimensional, ordered, macro porous gold film electrode could significantly enhance the amount of adsorbed haemoglobin at the monolayer and provide a good microenvironment for retaining the biological activity of haemoglobin [17,18].

Carbon nanotubes have become the subject of intensive investigations since their discovery, due to their unique mechanical and electrical properties. The large length-to-diameter aspect ratios of carbon nanotubes could provide for high surface- to-volume ratios. Recently, carbon nanotubes have been widely utilized in biosensing and bioelectroanalysis [19–21]. Some reports have illustrated that carbon nanotubes could promote the electron transfer of some proteins and enhance the relative electrochemical reactivity through physical adsorption onto their surfaces [22–24]. The direct electrical connection to heme proteins attached to the ends of carbon nanotube array electrodes has been reported in the literature [25]. The oppositely charged haemoglobin and single-wall nanotubes could be successfully assembled into layer-by-layer films on some electrodes by electrostatic interaction, and the built multilayer films show a direct and reversible cyclic voltammetric response for hemoglobin [26]. On the basis of these observations, in this study we have explored a new strategy for coupling haemoglobin to acid treated carbon nanotubes as a haemoglobin–carbon nanotube (Hb–CNT) assembly using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Our results illustrate that the direct electrontransfer of hemoglobin could be effectively facilitated in the Hb–CNT assembly on glassy carbon electrodes (GCE).

Materials and methods

Reagents

Haemoglobin and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide),Multiwall carbon nanotubes and suspended in ultrapure water. All other reagents were analytical grade. HEPES buffer solution (0.1 M, pH 7.1) was prepared in ultrapure water.

Purification and preparation of the carbon nanotubes

The collected multiwall carbon nanotubes (diameter 30 nm) were purified in a mixture of concentrated sulfuric acid and nitric acids (3:1 HNO3/H2SO4) by sonication for 24 h. Afterwards, the resultant nanotubes were thoroughly washed

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using doubly distilled water and then dispersed in ultrapure water by sonication for 1 h.

Preparation of hemoglobin–carbon nanotubes (Hb–CNT) composites

Haemoglobin (2 mg) was coupled to multiwall carbon nanotubes (2 mg) using EDC (5 mM) solution in 1 ml 0.1 M HEPES buffer (pH 7.1) at room temperature for 3 h. Afterwards, the solution was treated by sonication for 5 min and then centrifuged for 5 min at 12,000 rpm. This sonication and then centrifuging procedure was repeated three times to remove the physically adsorbed haemoglobin. At last the deposition of the centrifugal Hb–CNT composites was washed and suspended in 1 ml ultrapure water. In control experiments, haemoglobin (2 mg) was coupled to a multiwall carbon nanotubes (2 mg) solution in 1 ml 0.1 M HEPES buffer (pH 7.1) at room temperature for 3 h in the absence of EDC. The following procedure was the same as that above.

Electrochemical study

The suspension of Hb–CNT composites (10 μl) was dropped onto a glassy carbon electrode (area 0.07 cm2) and was dried naturally in air. All electrochemical measurements were carried out in HAc–NaAc buffer solution (0.1 M, pH 5.0) on a CHI 660B electrochemical workstation at room temperature (22 ± 2 ◦C). The electrochemical station is set under nitrogen steam and the solutions were purged with nitrogen prior to use. A three-electrode system was used in the relative electrochemical study, including cyclic voltammetry and square wave voltammetry [27], which contains a glassy carbon electrode (GCE) as the working electrode, a Pt wire as the counter electrode, and a saturated calomel electrode as the reference electrode.

UV–vis absorption spectroscopy and TEM characterization

UV–vis absorption spectroscopy was performed on a Hitachi U-4100. All the relative measurements were carried out at ambient temperature (22 ± 2 ◦C) under experimental conditions identical to those of the electrochemical studies. TEM images were obtained using a JEM2000EX (JEOL).

Results and Discussion

Characterization of the Hb–CNT assembly using TEM and UV–vis absorption spectroscopy

Since the multiwall carbon nanotubes were purified by acid treatment [28,29], the respective carbon nanotubes were first carboxyl functionalized by sonication in 3:1 HNO3/H2SO4. The carboxyl group at the carbon nanotubes could readily interact with the *N*-terminal valine residues of α- and β-globin chains of haemoglobin [18]. Our TEM images indicate that the apparent aggregation could be observed when using EDC to couple haemoglobin with carbon nanotubes, while it is not obvious in the absence of EDC. As shown in Fig. 1, it is noted that much larger aggregation of the Hb–CNT composites appears when they are prepared in the presence of EDC than in the absence of EDC (TEM of pure carbon nanotubes is shown in Fig. S1 of the supplementary material), suggesting that the presence of EDC could efficiently facilitate the coupling of hemoglobin with carbon nanotubes. In order to reveal the relative binding affinity of haemoglobin coupled to the multiwall carbon nanotubes, UV–vis absorption spectroscopy was utilized to explore the respective absorption changes of the supernatant solution of hemoglobin after incubated with carbon nanotubes in the presence and absence of EDC. As shown in Fig. 2, it appears that the peak absorption of the supernatant solution of hemoglobin after incubation with carbon nanotubes in the absence of EDC is relatively weaker than that of pure hemoglobin solution, indicating that some hemoglobin has already been partly adsorbed on the carbon nanotubes. Moreover, it is observed that the peak absorption of the supernatant solution of hemoglobin after incubation with carbon nanotubes in the presence of EDC is much weaker than that of the supernatant solution of hemoglobin after incubation with carbon nanotubes in the absence of EDC, suggesting that much more hemoglobin has been coupled to the carbon nanotubes by using EDC. Based on these observations, a calibrated plot of the absorption of hemoglobin at different concentrations of hemoglobin has been performed to estimate the amount of hemoglobin coupled to the carbon nanotubes. Our results illustrate that when hemoglobin is coupled to the carbon nanotubes in the presence of EDC, the relative adsorption amount is 0.17 μg hemoglobin per mg carbon nanotubes, while it is 0.04 μg hemoglobin per mg carbon nanotubes after incubation in the absence of EDC. These observations indicate that when incubated in the presence of EDC, hemoglobin could be readily coupled to the side wall of the acid-treated carbon nanotubes through the interaction between the carboxyl groups of the nanotubes and the *N*-terminal valine residues in globin chains of hemoglobin.

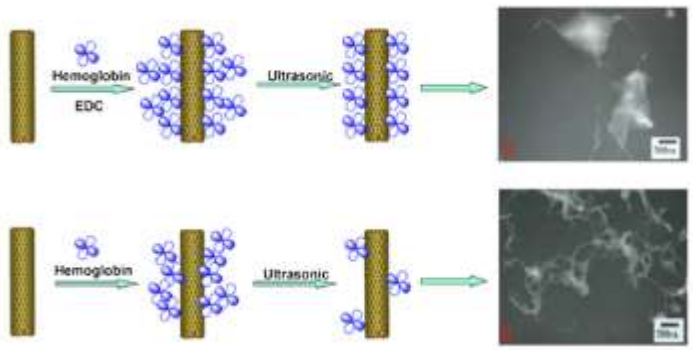

Fig. 1. Schematic draw of the preparation of Hb–CNT and typical TEM images of Hb–CNT assembled in the presence (A) and in the absence (B) of EDC.

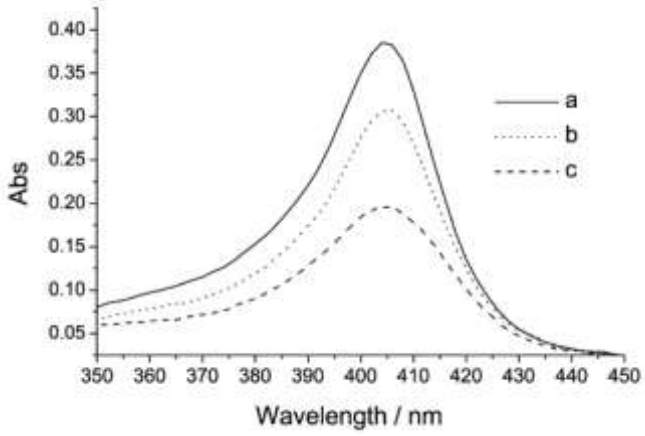

Fig. 2. UV–vis absorption spectra of (a) pure hemoglobin solution (2 mg/ml),(b) the supernatant solution of hemoglobin (2 mg/ml) after incubated incubation with carbon nanotubes in the absence of EDC, and (c) the supernatant solution of hemoglobin (2 mg/ml) after incubation with carbon nanotubes in the presence of EDC.

Electrochemical behavior of Hb–CNT assembly on glassy carbon electrode

Some recent report indicates that carbon nanotubes have promotion effects on the direct electron transfer of haemoglobin [22].Thus, in this study, we have explored the possibility of coupling haemoglobin to acid-treated carbon nanotubes as the

haemoglobin–carbon nanotube (Hb–CNT) assembly using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Our results of cyclic voltammetry illustrate that the direct electron transfer of haemoglobin could be effectively accelerated in this Hb–CNT assembly on a glassy carbon electrode (GCE), which shows a direct and reversible cyclic voltammetric response for haemoglobin. As shown in Fig. 3, it is noted that a pair of well defined redox peaks could be detected at −0.300 and −0.236 V, and the peak current ratio at these two potentials is about 1, indicating that haemoglobin has reversible redox activity at this Hb–CNT assembly film on GCE. Also, our studies of square wave voltammetry also demonstrate that the electrochemical response of haemoglobin in Hb–CNT composites assembled in the presence of EDC is considerably higher than that after incubation in the absence of EDC (see Fig. 4), which is consistent with our cyclic voltammetric studies. The rationale behind this may be attributed to the relatively high electrocatalytic activity of carbon nanotubes coupled with haemoglobin using ED, which could readily facilitate the direct electron transfer of haemoglobin and lead to much a stronger electrochemical response for haemoglobin in Hb–CNT assemblies. To further reveal the nature of the direct electron transfer of haemoglobin in Hb–CNT assembly on GCE, we have explored the relative kinetic mechanism of haemoglobin in this assembly film. Fig. 5a shows a cyclic voltammetry study of haemoglobin in the Hb–CNT modified glassy carbon electrode. The calibration plot of peak current vs scan rate is illustrated in Fig. 5b, which indicates a good linear relationship between the peakcurrent and scan rate from 10 to 500 mV/s (r = 0.9989). According to the Laviron equation [30,31], the peak current (Ip) and the surface coverage (Γ) could be expressed as the equation

$$I_p = \frac{n^2F^2vA\Gamma}{4RT} = \frac{nFQv}{4RT}, \quad (1)$$

where n represents the number of electrons transferred, Γ (mol/cm2) is the amount of haemoglobin on the electrode, A is the surface area of the electrode, Q is the quantity of charge, and v is the scan rate. From the plot of peak current vs scan rate, the number of electrons transferred for the direct electron transfer reaction of haemoglobin was calculated to be n = 0.99, suggesting that a single electron-transfer reaction occurred at the Hb–CNT modified glassy carbon electrode. Besides, the average surface coverage of haemoglobin at the modified electrode was calculated to be 4.7×10−9 mol/cm2.The formula to calculate the direct electron transfer rate constant Ks when the _Ep is lower than 200 mV [32] is

$$\log K_s = \alpha \log(1-\alpha) + (1-\alpha)\log\alpha - \log\frac{RT}{nFv} - \alpha(1-\alpha)\log\frac{nF\Delta E_p}{2.3RT}. \quad (2)$$

Taking the charge transfer coefficient α of 0.5 [4] and a scan rate of 200 mV/s (in this study, the _Ep at the scan rate of 200 mV/s is 82 mV), the direct electron transfer rate constant Ks of haemoglobin in the Hb–CNT assembly on GCE is 1.02 ± 0.05 s−1, which is larger than that reported in the literature [9].

pH influence

The electrochemical response of the Hb–CNT assembly on GCE shows a strong dependence on the pH values of the buffer solutions. It is observed that the peak potential of haemoglobin in the Hb–CNT assembly on GCE shifted negatively with the increase of pH values. Fig. 6 illustrates a cyclic voltammetric study of the Hb–CNT assembly at different pH values. There is a linear relationship between the potentials and the pH values, which is from 5.0 to 7.6 with a slope of −54 mV/pH. This slope value is reasonably consistent with the theoretical value of −57.6 mV/pH at 18 ◦C for a reversible one-electron transfer coupled with a single proton [33], indicating that the respective electrode reaction of haemoglobin in this Hb–CNT assembly is a single-proton electrode reaction process.

Catalytic reactivity

It was reported that proteins containing heme groups are capable of reducing hydrogen peroxide (H2O2) [17]. In this work, the catalytic effect of haemoglobin in the Hb–CNT assembly was also observed for hydrogen peroxide (H2O2) at the Hb–CNT film on glassy carbon electrodes. The voltammogram changed upon addition of hydrogen peroxide, with an increase in the reduction current and a decrease in the oxidation current (as shown in Fig. S2 of the supplementary material), indicating a catalytic reduction reaction.

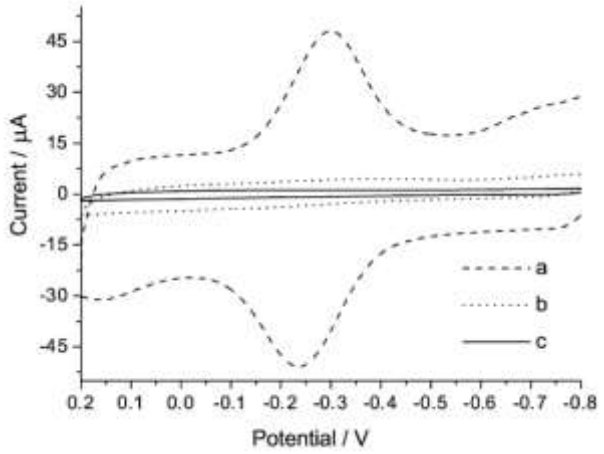

Fig. 3. Cyclic voltammetry study of Hb–CNT modified glassy carbon electrode in HAc–NaAc buffer solution (0.1 M, pH 5.0): (a) Hb–CNT assembled in the presence of EDC, (b) Hb–CNT assembled in the absence of EDC, and (c) bare GCE electrode. Scan rate 100 mV/s.

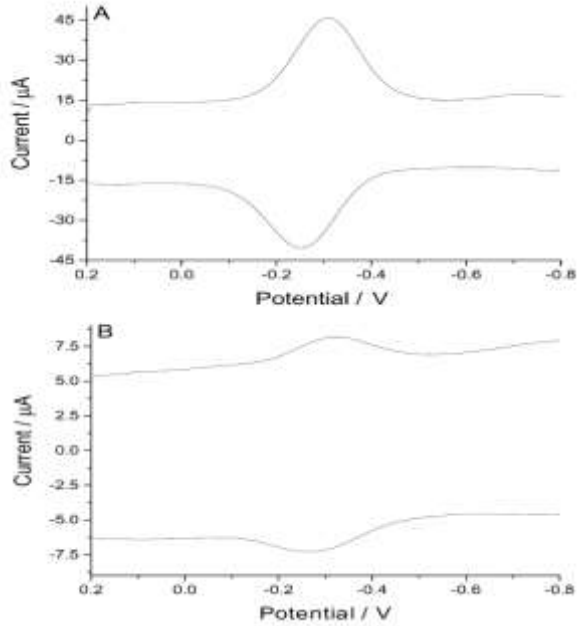

Fig. 4. Square wave voltammetry study of Hb–CNT modified glassy carbon electrode in HAc–NaAc buffer (0.1 M, pH 5.0): (A) Hb–CNT assembled in the presence of EDC, and (B) Hb–CNT assembled in the absence of EDC. Incr.4 mV, amplitude 25 mV, frequency 15 Hz.

Fig. 7 illustrates the relationship between the Hb peak currents at the Hb–CNT modified glassy carbon electrode and the H2O2 concentration. Our observations indicate that the respective peak current changes linearly with H2O2 concentration ranging from 2.5 × 10−7 to 1.4 × 10−4 M at the Hb–CNT modified GCE electrode, where the linear regression equation is *I* (μA) = 0.188[H2O2] (μM)+33.74 (μA), with a correlation coefficient of 0.9994. The relative detection limit of H2O2 is 1.8×10−7 M.

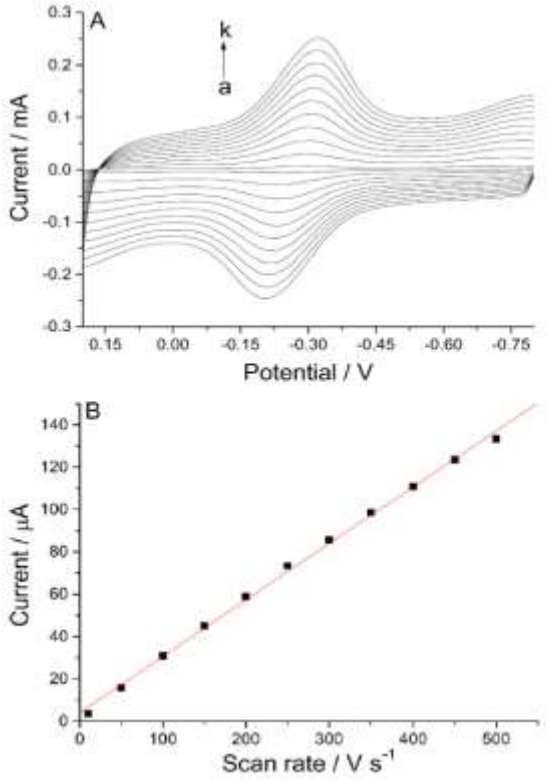

Fig. 5. (A) Cyclic voltammetry study of Hb–CNT modified glassy carbon electrode in HAc–NaAc buffer solution (0.1 M, pH 5.0) at scan rates of (a) 10, (b) 50, (c) 100, (d) 150, (e) 200, (f) 250, (g) 300, (h) 350, (i) 400, (j) 450, and (k) 500 mV/s. (B) Relationship between the scan rate and cathodic peak current for Hb–CNT modified glassy carbon electrode in HAc–NaAc buffer solution (0.1 M, pH 5.0)

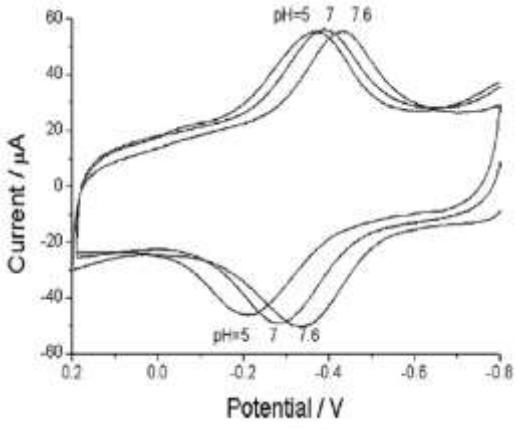

Fig. 6. Cyclic voltammetry study of Hb–CNT modified glassy carbon electrode at different pH values

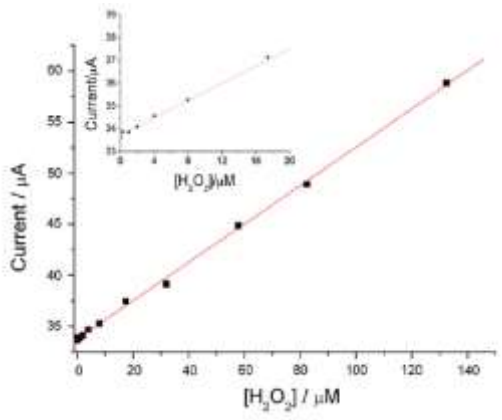

Fig. 7. Plot of the cathodic peak currents at Hb–CNT modified glassy carbon electrode in HAc–NaAc buffer solution versus H2O2 concentration. The insert shows the plot at low H2O2 concentration

Summary

We have demonstrated in this work that haemoglobin can be readily coupled to acid-treated multiwall carbon nanotubes using EDC and assembled as Hb–CNT composites. The direct electron transfer of haemoglobin was effectively accelerated in the relevant Hb–CNT assembly. Specifically, the heterogeneous electron transfer rate constant *K*s of haemoglobin in the Hb–CNT modified glassy carbon electrode is 1.02 ± 0.05 s−1.Our Observations of the cyclic voltammetry and square wave voltammetry demonstrate that the electrochemical response of Hb–CNT assembled in the presence of EDC is much higher than that in the absence of EDC, suggesting relatively faster electron transfer kinetics in the Hb–CNT assembly using EDC.Moreover, it was observed that the Hb–CNT modified electrode exhibits a peroxidase activity for the reduction of H2O2.The relative detection limit of the H2O2 is 1.8 × 10−7 M.Consequently, our observations indicate that the assembling of hemoglobin–multiwall carbon nanotubes using EDC could provide a novel strategy to effectively facilitate the direct electrochemistry of heme containing proteins, which could be further utilized as a promising biosensor for some specific biological process.

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