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Electrochemical Behavior of Cytochrome c on Exfoliated Graphite Electrode Modified with DNA

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Abstract:
DNA was immobilized on exfoliated graphite electrode to fabricate DNA-modified electrode. The direct electron transfer of horse heart cytochrome c on DNA-modified exfoliated graphite electrode was achieved. A pair of well defined redox peaks of cytochrome c appeared at E_{pc} = –0.018 V and E_{pa} = 0.09 V (vs. SCE) in 0.1 M phosphate buffer solution of pH 7.0 at a scan rate of 50 mV/s. The DNA- modified exfoliated graphite electrode could be applied to detect cytochrome c by means of cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The peak current increased with increasing amounts of DNA on the surface of an electrode, and then the magnitude of the peak current trended to be unvarying. It is suggested that the electrochemistry at the EG electrode is essentially governed by favourable electrostatic interactions.

Key words: *Exfoliated graphite, Cytochrome c, direct electrochemistry, DNA interaction, cyclic voltammetry*

1. Introduction

Electron transfer (ET) among biomolecules particularly through proteins plays a prominent role in both photosynthesis, that traps light energy, and metabolism, that oxidizes fuel. This efficient long-range ET has mostly been attributed to the well-controlled natural protein-protein interface [1]. Researchers particularly interested in DNA-mediated protein ET, which should not only focus on the interfacial protein ET but enhances our knowledge of protein-DNA interactions. Cyt *c* plays an important role in the biological respiratory chain, with a molecular weight of ca. 12,300 g/mol and approximately spherical shape with 34 Å in diameter, whose function is to receive electrons from cyt. c reductase and deliver them to cyt *c* oxidase, that leads to the electrochemical study of cyt. *c* is very important. This enzyme responsible for the final step in respiration: the four-electron reduction of O_2 to H_2O. The redox-active part of cyt. *c* is heme *c*, which is covalently linked to the protein via thioether bonds to cysteine residues.

In the protein, the central iron ion is also ligated by one histidine nitrogen, and one methionine sulphur. Cytochrome *c* is brightly colored because the heme absorbs strongly in the visible range of the electromagnetic spectrum. Few other studies suggested that direct electrochemistry of cyt. *c* was favourable at ds-DNA-modified gold electrodes [2], the exact ET mechanism remains ambiguous. Among the electrode materials investigated so far, chemically modified gold has received extensive attention since it is well-known that the surface modification using promoters results in the reversible, direct electrochemistry of cyt *c* [3,4].

Cytochrome *c* (Cyt c) is one of the well-studied redox proteins. The single iron atom in the Cyt *c* molecule alternates between the Fe (II) and Fe (III) oxidation states as it functions in the transport of electrons. Eddowes and Hill discovered well defined and reversible electrochemical behaviour of cytochrome *c* at a gold electrode in the presence of 4,4'-bipyridyl. Due to the significance in understanding the mechanism of biological electron transfer and the potential applications in the area of biosensors, the electrochemistry of redox proteins at a range of electrodes has been widely investigated [5-7]. Since that work, many studies on the electrochemistry of cytochrome c at modified gold and other electrodes have been reported [8,9], and many promoters, such as some small organic compounds [10], small peptides [11], amino acids together with some derived molecules [12,13] and conductive polymers [14] etc., have been found for promoting the direct electrochemistry of cytochrome c at the electrode surfaces.

DNA has offered a powerful tool in recognizing and monitoring many important compounds. DNA-modified electrode could be applied to explore a selective molecular interaction with DNA. It provides a route for rapid screening of compounds. The weak bonding between positively charged lysine residue of cytochrome *c* and the N atoms of the adsorbed species is likely for the interaction. DNA has negative charged phosphate, so it is possible to use a DNA-modified electrode to detect the cytochrome *c* in solution [15].

Carbon based electrodes have the advantage of being chemically very inert and electrochemically quite active. The functional groups on a carbon surface can be tuned to any particular application. Exfoliated graphite (EG) is a form of expanded graphite that has low density and high temperature resistance. It is a very good adsorption substrate owing to the near perfect crystallographic face and has better homogeneity of the surface than any other form of graphite. One of the main advantages of EG is that it can be compacted without the use of any binder. The resulting pellets are hard and possess chemical properties similar to that of natural graphite [16].

In this work, DNA was immobilized on exfoliated graphite electrode to fabricate DNA-modified electrode. The electrochemical behavior of cytochrome *c* on DNA-modified electrode was explored by cyclic voltammetry and differential pulse voltammetry. Each DNA modified electrode was characterized by using AC impedance spectra. The cytochrome *c* showed a pair of well-defined redox peaks on DNA-modified electrodes in PBS (pH 7.0). The related electrochemical parameters were obtained. The experimental results indicated that the electron transfer capability of cytochrome *c* on DNA modified electrodes was improved and that the redox reversibility of Fe(III)/Fe(II) couple as an electro-activated center embedded in cytochrome *c* was enhanced. DNA modified glassy carbon electrodes could be applied to determine the concentration of cytochrome *c*.

2. Experimental

2.1. Apparatus and Chemicals

Electrochemical measurements were performed on a CH Instruments (USA). The standard cell consisted of three electrodes assembly that include the working electrode, exfoliated graphite electrode, the reference electrode was a saturated calomel electrode (SCE) and platinum electrode was used as the auxiliary electrode.

The natural graphite particles were obtained from Stratmin Graphite Company (NJ, USA). The as-received material was sieved to result in particles of size 300 mm. Horse heart cytochrome c of AR grade (Type VI) obtained from Sigma, USA was used without any further purification. Phosphate buffer solutions (PBS) were prepared by 0.1 M Na_2HPO_4–NaH_2PO_4 (pH 7.0), and the pH was adjusted with dil. HCl and dil. NaOH. All the chemicals and reagents used were of AR grade. All the solutions including the supporting electrolyte solutions were prepared with doubly distilled water. The electrolyte solutions were deoxygenated with pure nitrogen gas for at least 20 minutes before each experiment. Stock solution of calf thymus-DNA (CT-DNA) was prepared by sonication at 0-5 ^{0}C. Concentration of DNA was determined by UV absorbance at 260 nm (ε_{260} = 6.6 x 10^3 $M^{-1}cm^{-1}$).

2.2. Electrode Modification

The EG electrode was polished using a piece of 1500 diamond paper, followed by 0.2 μm alumina, and was washed with absolute alcohol and doubly distilled water in an ultrasonic bath. Following that, 10 μl DNA solutions (0.1 mg/ml) was dropped onto the surface of the clean exfoliated graphite electrode and solvent was evaporated at 4 °C for 24 h [17]. By this means, a layer of DNA film was bound to the surface of each exfoliated graphite electrode. Then the DNA-modified electrode washed with doubly distilled water and stored in phosphate buffer solution of pH 7.

2.3. Preparation of Exfoliated Graphite Electrode

The EG particles were prepared according to a reported procedure [18]. The natural graphite flakes of particle size 300 mm, were intercalated with bisulphate anion for 24 hours using a mixture of concentrated H_2SO_4 and HNO_3 under ambient conditions. Acidified graphite washed thoroughly with plenty of water till it becomes neutral. The intercalated material was washed well and then subjected to thermal shock at 800 °C for about a minute. The intercalated material underwent a sudden transition to a vapour phase thereby leaving the graphite lattice by rupturing the layers.

The EG particles could be restacked to form pellets without any binder as reported [19,20]. The pellets were formed by compressing approximately 150 mg of EG under a pressure of 6 tons/cm^2 for 4 - 5 hours. Electrodes were then fabricated from these pellets using silver epoxy and copper wire. The surface of the working electrode was polished with SiC emery sheets of different grades (1500 grit followed by 4/0 and 5/0) to obtain a smooth surface. This polished EG surface served as working electrode in a three-electrode assembly.

3. Results and Discussion

3.1. Physicochemical Characterization of EG

The SEM pictures of exfoliated graphite particles show the expanded layers of graphite when compared to the natural graphite flakes (not shown). The restacked, binder less EG pellets are very strong and can be handled without any mechanical disturbance. The SEM pictures of polished and sonicated EG pellets are shown in Figure 1. The surface of the unpolished EG reveals preferential orientation of the basal plane though there are many line-shaped defects observed. The SEM micrographs of the DNA modified EG pellet surface shows the modified DNA covered the EG surface. SEMs of DNA film show very different patterned structure. The DNA film is

quite uniform and distributed thoroughly on EG surface. It suggests that, which may responsible for catalytic behavior of EG and the DNA film is stable for longer time.

4. Direct Electrochemistry of Cytochrome *c* at Exfoliated Electrode

Cytochrome *c* is an electron transfer protein responsible for shuttling electrons from cytochrome *c* reductase to cytochrome *c* oxidase. The Fe (III)/Fe (II) in the cytochrome *c* as the electrochemical redox center is embedded in a rigid 34 Å diameter shell with nine positive charges, and in neutral condition, the system has a considerable dipole moment. It is difficult for cytochrome *c* to exhibit well directed electrochemical activeness on an electrode because Fe (III)/Fe (II) redox center of cytochrome *c* could not come into contact with any surface of electrode. When the bare EG electrode was used in the PBS (pH 7.0) solution containing 0.2 mM cytochrome *c*, shows no obvious electrochemical response of cytochrome *c*. The electrochemical activeness of cytochrome *c* could not be exhibited considerably on a bare EG electrode.

The cyclic voltammograms of cyt *c* on polished EG electrode in 0.1 M phosphate buffer are shown in Figure 2. At bare EG electrode (fig.3a), cytochrome *c* has no obvious electrochemical response. However, a pair of redox waves for 0.2 mM cytochrome *c* is observed at 0.069 and – 0.042 V on EG electrode in 0.1 M PBS of pH 7.0 at a scan rate (*v*) of 50 mV/s. Whereas the cyclic voltammogram of Cyt *c* on EG modified with DNA (fig.3b) in 0.1 M phosphate buffer of pH 7, shows a pair of redox waves for 0.2 mM cytochrome *c* is observed at 0.081 and – 0.035 V at a scan rate of 50 mV/s. The reversibility of cytochrome *c* is greatly improved in comparison with the bare EG electrode, suggesting that the reversibility of cytochrome *c* is significantly improved. It is possible that carrying negative charge PO_4^{3-} on DNA could interact with the hydrophilic surface of Cyt. *c*, which carrys the positive charge Fe(III)/Fe(II) on cytochrome *c*. This phenomenon indicated that DNA plays a key role in promoting the redox behavior of cytochrome *c* on electrodes.

5. Optimization of analysis conditions

The experimental conditions affect the electrochemical behavior of cytochrome *c* on the DNA-modified electrode. The effects of the solution pH, electrolyte concentration and the amount of DNA on the surface of EG were explored. The peak currents of cytochrome *c* depend on the pH values of the buffer (PBS) (fig. not shown). The redox process was examined from pH 4.0 to 9.0. The peak potential did not shift much in these pH ranges. But the magnitude of peak current changed clearly under various pH conditions. The maximum peak current was obtained at pH 7.0, which is the same as the pH of biological media. When pH < 7.0, the electrostatic interaction between phosphate groups of DNA and cytochrome *c* decreases due to the protonation of phosphate. At pH > 7.0, the electrostatic interaction between phosphate groups of DNA and cytochrome *c* decreases due to the deprotonation of the lysine groups of cytochrome *c* [21]. The influence of the buffer solution concentration on redox peak current of cytochrome *c* was also examined. It was found that the redox peak current decreased with increasing the concentration of PBS. Hence, the 0.1 M PBS of pH 7.0 was selected in the experiments. It is possible that negative charge PO_4^{3-} of DNA could bind with positive charge of cytochrome *c* with DNA that was modified on EG electrode.

Different amounts of DNA were chosen to optimize the modified electrode. Fig. 3 explains the cyclic voltammograms of cyt *c* with different concentrations of DNA (2, 4, 6, 8. 10 µl) in 0.1 M PBS of pH 7.0 with the scan rate 50 mV/s. The peak current increased with increasing amounts of DNA on the surface of EG electrode is as shown in fig. 4, and then the magnitude of the peak current trended to be unvarying. While the amounts of DNA were further increased, the signal of voltammetry became gradually weak. The dense DNA film on an electrode blocked the electron transfer between electrode and cytochrome *c*. Thus, 10 µl DNA solutions (0.10 mg/ml) were chosen as optimum amounts of DNA in the detecting experiment [21].

6. Effect of Scan Rate

The redox peak currents vary linearly with the scan rate showing that cyt *c* is bound to the surface of the electrode. CVs of redox behavior of 0.2 mM cyt *c* with different scan rates (fig. not shown), an increasing the scan rate, peak potential does not shift but peak currents of both anodic and as well as cathodic increased linearly.

7. Differential Pulse Voltammetry (DPV)

Differential pulse voltammetry is used to make electrochemical measurements. It can be considered as a derivative of linear sweep voltammetry or staircase voltammetry, with a series of regular voltage pulses superimposed on the potential linear sweep or stair steps. The current is measured immediately before each potential change, and the current difference is plotted as a function of potential. By sampling the current just before the potential is changed, the effect of the charging current can be decreased. These measurements can be used to study the redox properties of extremely small amounts of chemicals because of the following two features: (1) In these measurements, the effect of the charging current can be minimized, so high sensitivity is achieved. (2) Faradaic current is extracted, so electrode reactions can be analyzed more precisely. The $E°$ obtained from DPV for cyt *c* is essentially the same as that found by CV, however that obtained by DPV for cyt *c* was significantly more negative. To explain this behavior we have to consider the different characteristic times *(T)* of CV and DPV experiments. The time scale for a CV experiment (at v= 100 mV/s) is $T = RT/nF$ = 257 ms [F is the faraday constant (96,485 C/eq) and *R* is the molar gas constant (8.314 J/mol^{-1} K^{-1})] while for a DPV experiment under the conditions here T = 17 ms. Therefore, for a system with heterogeneous electron transfer kinetic limitations as is the case for cyt *c*, more reversible behavior might be found with CV than with DPV [23].

The contrast experiment was performed with bare exfoliated graphite electrode under the same experimental conditions as explained above. It is difficult for Cyt. *c* to exhibit direct electrochemical activeness on bare electrode, because Fe(III)/Fe(II) redox center of Cyt. *c* could not contact with surface of electrode. However, DNA modified on surface of exfoliated graphite electrode can play a promoter role for the electron transfer reaction of Cyt. *c* on electrode. DNA could provide negatively charged sites and interact with the hydrophilic surface of Cyt. *c*, which has the positive charges. Therefore, Cyt. *c* could be adsorbed on the surface of the DNA modified exfoliated graphite electrode and possibly well arranged in proper orientation. Figure 4 (a & b) shows the comparison study of the electrochemical behavior of cyt *c* (0.2 mM cyt *c*) in PBS using DPV technique (anodic and cathodic direction) on bare EG and DNA modified exfoliated graphite electrode. Figure 5a shows that, for bare EG, Anodic peak potential observed at -0.025 V and the peak current is 4.69×10^{-5} A, cathodic peak potential observed at -0.002 V and peak current is -4.76×10^{-5} A for 0.2 mM cyt *c*, where as for DNA modified exfoliated graphite electrode, anodic peak potential observed at -0.034 V and the peak current is 5.22×10^{-5} A, cathodic peak potential observed at 0.002 V and peak current is -5.2×10^{-5} A.

8. Conclusion

A simple approach was used to fabricate DNA-modified exfoliated graphite electrode. Highly active surface for electron transfer reactions can be prepared from EG electrodes. The direct electron transfer of horse heart cytochrome *c* on DNA-modified electrode was achieved, without any intentional chemical modification. DNA plays a key role in promoting the redox of cytochrome *c* on DNA-modified electrodes. The DNA-modified exfoliated graphite electrode could be applied to detect cytochrome *c*.

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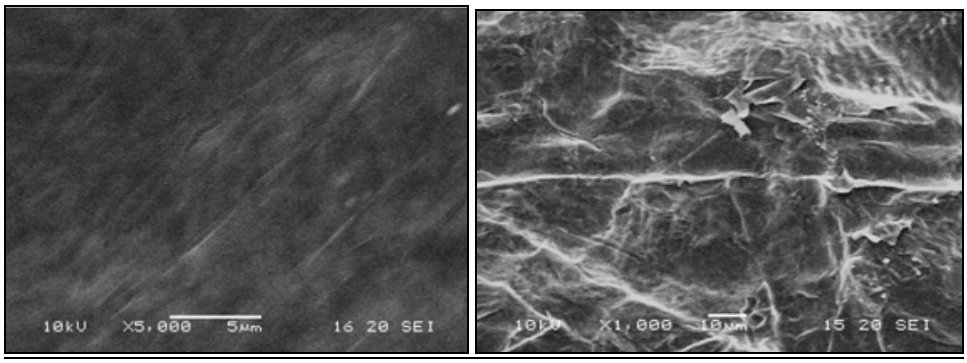

Figure 1: Scanning electron micrographs of (a) bare EG electrode, (b) DNA modified EG pellets

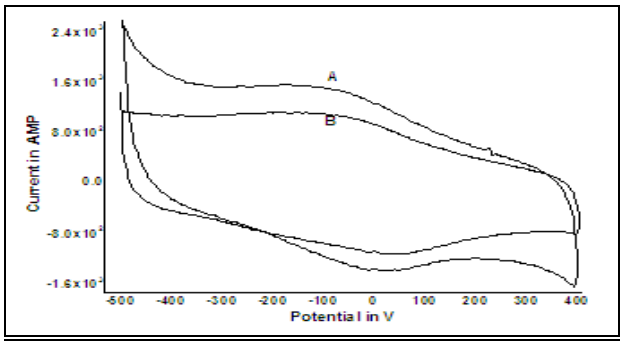

Figure 2: Cyclic voltammograms of cytochrome c in 0.1 M PBS of pH 7.0: (a) bare EG (b) DNA modified EG electrode

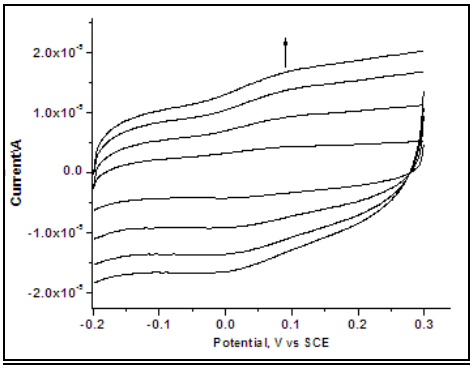

Figure 3: Cyclic voltammograms of 0.2 mM cyt c on DNA modified electrode with different concentrations of DNA (2, 4, 6, 8. 10 µl) in 0.1 M PBS, pH 7.0, and scan rate: 50 mV/s

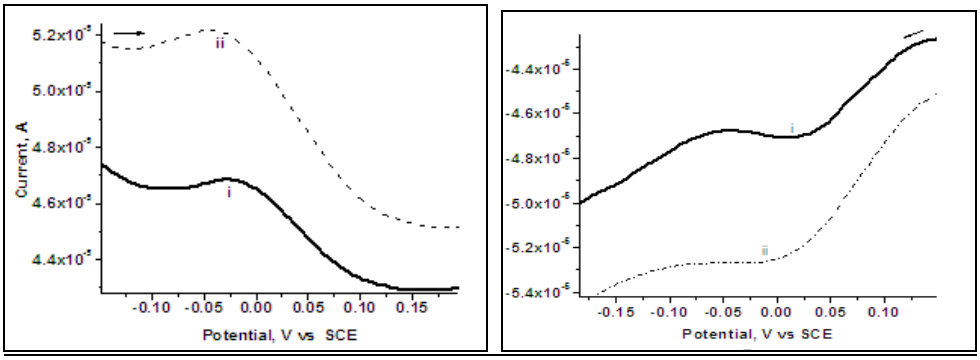

Figure 4: a) Anodic and b) Cathodic DPVs of 0.2 mM cyt c in 0.1M PBS of pH 7 On i) bare EG & ii) DNA modified exfoliated graphite electrode.

10. References

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