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Electrocatalytic studies of Cytochrome *c* functionalized single walled carbon nanotubes on self-assembled monolayer of 4-ATP on gold

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ABSTRACT

Direct electron transfer of redox proteins on electrode surfaces using surface immobilized protein films is gaining importance as it provides significant insight into the functioning of biological systems and in the development of biosensors. We have studied functionalized cytochrome *c* (cyt *c*) surfaces by covalently using glutaraldehyde as a cross linker and also non-covalently by a simple immersion process of physical adsorption. The third method described in this paper involves combining the affinity of the redox proteins for single walled carbon nanotube (SWNT) surface and its non-covalent binding to the 4-aminothiophenol (4-ATP) self-assembled monolayer on Au. The immobilized composite films facilitate the interfacial electron transfer and electrocatalytic activity of cyt *c*. The cyt *c* immobilized surfaces were characterized by scanning probe techniques, cyclic voltammetry, electrochemical impedance spectroscopy and chronoamperometry. The AFM images of the protein immobilized surface show dense distribution of SWNT- cyt *c* on the surface forming several clusters. A closer examination using STM shows dense distribution of proteins on 4-ATP surface. We have correlated the voltammetric results with the information obtained from AFM and STM images to explain the electrocatalytic activity of the protein layer on the electrode surface.

1. Introduction

A study of direct electron transfer between active sites of redox proteins and the electrode surfaces is of considerable research interest as it can provide some important information on the biological processes. Several groups have studied the electron transfer properties and electrocatalysis of DNA and proteins on single walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs) [1–18]. There are also several reports of the covalent functionalization of proteins on carbon nanotubes and study of their electron transfer properties [19–23]. The encapsulation and immobilization of phospholipids and proteins [24–27] in CNTs has been a subject of intense studies in recent times with particular emphasis on their ability to function as the intracellular transporters [25]. In addition, redox proteins are potentially useful for the development of amperometric biosensors and bio-electrocatalytic systems. Among them, the heme protein, cytochrome *c* (cyt *c*) is an ideal model protein, as it is electroactive with known three dimensional structure. Its electron transfer properties have been studied both from the solution [28] and after surface confinement on the self-assembled monolayer films formed on gold [29–33]. Immobilizing redox proteins without altering their conformations on electrode surfaces is a challenging task. The direct electrochemistry of cyt *c* adsorbed on the surface of the SWNT which is immobilized on the glassy carbon electrode was reported earlier [34,35]. However, there are no reports of electrochemical studies of surface immobilized cyt *c* functionalized SWNT on gold surface. To accomplish this, without denaturing protein, we have used a self-assembled monolayer of aromatic thiol molecules on the electrode surface as an electron transfer mediator. There is also another advantage of using aromatic thiols since they facilitate better electron transfer of the immobilized species due to their π-conjugation. We have, therefore, used 4-aminothiophenol (4-ATP), which forms a well-organized monolayer showing $\sqrt{3} \times \sqrt{3}\ R\ 30^\circ$ structure on gold for our study [36]. Recently we reported the behaviour of aminothiophenol modified SAM on gold under potential cycling and mixed SAM formation [37]. There are also some reports of 4-ATP self-assembled monolayer being used for immobilization of biomolecules [38,39]. The previous attempts to immobilize the protein on the self-assembled monolayer films used carboxyl, pyridine, cysteine and imidazole terminated alkanethiols [40–42].

We present in this paper, the results of the electrochemical study of immobilized cyt *c* on SAM of 4-ATP by covalent and non-covalent techniques. We also show that by combining the ability of the redox proteins affinity to SWNT surface and its non-covalent binding to the 4-ATP SAM on gold, it is possible to enhance the surface concentration significantly. We have prepared the cyt *c* and SWNT- cyt *c* modified electrodes by immobilization on 4-ATP SAM on gold in a phosphate buffer solution of pH 6.5.

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We have used cyclic voltammetry, electrochemical impedance spectroscopy to study cyt *c* electron transfer properties. We have also studied electrocatalytic properties of protein on the SAM of 4-ATP using chronoamperometry.

2. Experimental

2.1. Chemicals

We have used the following analytical grade reagents: cytochrome *c* (cyt *c*) from horse heart (Fluka) 4-aminothiophenol (Aldrich), sodium phosphate dibasic Na_2HPO_4 (Rankem), sodium dihydrogen Phosphate-1-hydrate (Merck), hydrogen peroxide (H_2O_2) solution 30% (Merck). All the solutions were prepared using millipore water of resistance 18 MΩ cm. cyt *c* solution was made by dissolving 2 mg of cyt *c* in 0.3 ml of phosphate buffer solution of pH 6.5.

2.2. Purification of single walled carbon nanotubes (SWNTs)

The single walled carbon nanotube obtained from Carbolex Co. USA has been purified according to the procedure described earlier [43]. In brief, the raw soot SWNT was subjected to air oxidation by heating the sample in air at 350 °C for 4 h to oxidize the non-tubular form of the carbon. The SWNTs were then refluxed with 6 N nitric acid for 30 min in order to dissolve the metal particles. After cooling, the sample was filtered using a cellulose nitrate filter paper with 0.2 μm pore size by applying vacuum suction. A clear green colored supernatant acidic solution was collected at the bottom of filtering unit. Successive washing with millipore water removes the substantial amount of trapped acid form the sediment. The SWNTs collected in the filter paper was dried in an air oven at 50 °C for 30 min.

2.3. Electrode pretreatment

All electrochemical experiments were carried out using gold disc of geometric area 0.002 cm^2 as a working electrode. Immediately before use, the poly crystalline gold disc electrode was polished with emery paper of grade 800 and 1500, followed by polishing in aqueous slurries of 1 μm, 0.3 μm and finally with 0.05 μm alumina slurries, ultrasonicated in water to remove alumina particles for 1 min and then cleaned with dil. aquaregia (3:1:4 mixture of conc. HCl, conc. HNO_3 and water) for one minute before each experiment. Finally, it was rinsed in distilled water thoroughly, followed by rinsing in millipore water and ethanol before SAM formation. The true surface area of the disc electrode was measured by potential cycling in 0.1 M $HClO_4$. The surface concentrations and the electron transfer rate constants reported here are after correcting for the roughness factor.

2.4. Self-assembled monolayer formation

The self-assembled monolayer of 4-aminothiophenol (4-ATP) was formed by placing the cleaned gold disc electrode in 1 mM ethanolic solution of 4-ATP for 12 h. The electrode was taken out from the thiol solution, rinsed with ethanol and finally in millipore water.

2.5. Covalent immobilization of cyt c on SAM: (cyt c/GA/4-ATP/Au)

Cytochrome *c* was covalently attached to the surface by immersing 4-ATP modified Au electrode in a 30% glutaraldehyde for 24 h. The electrode was taken out from glutaraldehyde, rinsed with water and placed in cytochrome *c* solution at 4 °C of pH 6.5 for 24 h. This is then washed with phosphate buffer solution for several times, followed by millipore water to remove any loosely bound enzyme on the surface.

2.6. Non-covalent immobilization of cyt c on SAM: (cyt c/4-ATP/Au)

Cyt *c* was adsorbed at 4 °C onto the 4-ATP self-assembled monolayer modified gold surface by immersing in a 2 mg of cyt *c* in a 0.3 ml of 0.1 M sodium phosphate buffer solution of pH 6.5. The electrode was taken out from the cyt *c* solution, washed with phosphate buffer solution followed by rinsing in millipore water.

2.7. Preparation of SWNT functionalized cyt c electrodes: (cyt c/SWNT/4-ATP/Au)

Homogeneous and well-dispersed CNTs in aqueous and non-aqueous media formed with the aid of polymers and surfactants were reported earlier [44–48]. Here we have used analogous method to disperse the SWNTs in pH 6.5 phosphate buffer solution. About 2 mg of cyt *c* was dissolved in 0.3 ml of 0.1 M phosphate buffer solution of pH 6.5. Dispersion of CNTs in protein solution by sonication has been reported recently [49,50]. In the present work, we find that following similar procedure helps to disperse SWNTs in phosphate buffer solution. We have transferred purified SWNTs into the cyt *c* solution which is then followed by sonication. The 4-ATP modified gold disc electrode was then placed in the freshly prepared dispersion of SWNT-cyt *c* for 24 h at 4 °C. The electrode was washed with phosphate buffer solution followed by rinsing in millipore water. During the formation of the dispersion, the cyt *c* functionalizes the SWNTs which is similar to the observations of other workers [25].

2.8. Electrochemical instrumentation

Cyclic voltammetry and chronoamperometry studies were carried out using EG&G potentiostat (model 263A), which was interfaced using a GPIB card (National Instruments). For electrochemical impedance studies, the potentiostat was used along with an EG&G 5210 lock-in-amplifier controlled by power sine software.

The three-electrode cell had a platinum foil of large surface area as a counter electrode, a saturated calomel reference electrode, and the modified gold surface as a working electrode. All the electrochemical experiments were done under N_2 atmosphere.

2.9. Evaporated gold surface

Evaporated gold (∼100 nm thickness) on glass with chromium under layers (2–5 nM thickness) was used. The substrate was heated to 350 °C during gold evaporation under a vacuum pressure of 2×10^{-5} mbar, a process that normally yields predominantly Au(111) orientation.

Evaporated gold surface was cleaned in a piranha solution (a mixture of 30% H_2O_2 and concentrated H_2SO_4 in a 1:3 ratio) before use. (*Caution! Piranha solution is very corrosive.*)

2.10. AFM and STM studies

All AFM and STM studies were carried out using evaporated gold on glass specimen. The images were taken using Pico plus (Molecular Imaging, USA) instrument in ac mode (Tapping mode) at a frequency 175 KHz with a cantilever of n^+-silicon of type PPP-NCL-50 from nanosensors, USA. All the STM images were obtained at ambient temperature in air in constant current mode. An electrochemically etched tungsten tip was used as the probe. The images shown here were plane corrected using scanning probe