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Immobilization and electrochemical redox behavior of cytochrome *c* on fullerene film-modified electrodes

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Abstract

Two different fullerene film-modified electrodes were prepared and used for surface immobilization and electrochemical property investigation of horse heart cytochrome *c* (cyt *c*). Both a pristine fullerene film and fullerene-palladium (C_{60}-Pd) polymer film-modified platinum, glassy carbon and indium–tin–oxide (ITO) electrodes were used. The immobilized cyt *c* was characterized by piezoelectric microgravimetry at a quartz crystal microbalance (QCM), UV–visible absorption, and X-ray photoelectron spectroscopy (XPS), as well as cyclic voltammetry (CV) techniques. The UV–visible spectral studies revealed a small blue shift of both the Soret and Q band of the heme moiety of cyt *c*, immobilized on the C_{60}-Pd polymer film-modified ITO electrode, as compared to the bands of cyt *c* in solution suggesting that molecules of cyt *c* are densely packed onto the surface of the modified electrode. The CV studies revealed a quasi-reversible electrode behavior of the heme moiety indicating the occurrence of kinetically hindered electron transfer. A good agreement was found between the values of cyt *c* electrode surface coverage determined by piezoelectric microgravimetry and cyclic voltammetry. For piezoelectric microgravimetry, these values ranged from 0.5×10^{-10} to 2.5×10^{-10} mol cm^{-2}, depending upon the amount of cyt *c* present in solution and the time allowed for immobilization, which compared with a value of $3.6\pm0.4\times10^{-10}$ mol cm^{-2} determined by CV. The possible mechanisms of cyt *c* immobilization on the C_{60} film and C_{60}-Pd film-modified electrodes are also discussed.

Keywords: Fullerene-modified electrode; Cytochrome *c*; Surface immobilization; Chemically modified electrode; Quartz crystal microbalance; Piezoelectric microgravimetry

1. Introduction

Fullerene and carbon nanotube modified electrodes have recently been widely used for catalytic and sensor applications [1] as well as for photoelectrochemical investigations [2]. Fullerenes and their derivatives are promising candidates for these applications due to rich reductive electrochemistry and the resulting electron accumulating properties [3]. Several procedures for fullerene-based electrode modification have been developed, which include using a fullerene solution of a volatile solvent for drop coating, vapor deposition of fullerene films, electro-oxidative deposition from solutions of fullerene anions, electrophoretic deposition of nanoscopic fullerene clusters, electropolymerization where the resulting fullerene entities are connected either by polymer side chain, or via epoxide formation, or by transition metals binding, as well as by self-assembling monolayers using either thiol or silane derivatives. Applications of some of these electrodes featuring unique properties have already been described in literature while others are being explored [1,2].

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In the present study, we report another application of the fullerene film-modified electrodes, which consists of immobilizing a redox-active protein on the modified electrode surface. Such an immobilization is of current interest for modeling charge transfer processes of redox-active proteins since the physiological redox partners are replaced with an electrode surface for the electron transfer. Protein immobilization on the electrode surface is important for developing a broad range of bioanalytical devices. In this regard, different strategies have been reported for specific electrode functionalization [4–7]. The redox-active protein employed in the present study, cytochrome *c* (cyt *c*) from horse heart [8], is a low-molecular weight (MW=12,400 D) redox-active heme protein with a single polypeptide chain of 104 amino acid residues covalently attached to the heme moiety. The shape of the cyt *c* molecule is roughly spherical with diameter of ca. 3.4 nm, which implies that the maximum surface coverage, i.e., that reached at hexagonal monolayer packing, of adsorbed cyt *c* is $\Gamma_{max} \approx 1.7\times10^{-11}$ mol cm^{-2}. In cyt *c*, there are 19 positively charged lysine residues and two arginine nucleic base clustered on one side, while 12 aspartic or glutamic acid residues are clustered together on the opposite side with an isoelectric point around pH ≈ 10. Therefore, one side of cyt *c* bears positive charges while the opposite side bears negative charges. In the literature, several procedures have been described to immobilize cyt *c* onto the electrode surface [9–14].

In the present work, we have utilized two different types of the fullerene film-modified electrodes for surface immobilization of cyt *c*. One involves an electrochemically conditioned fullerene drop-coated film electrode [15–19] and the other an electropolymerized fullerene cross-linked with palladium acetate complex (C_{60}-Pd) film electrode [20–22]. While both types of surface-modified electrodes are electrochemically active, as discussed below, the results obtained for the latter were much better defined.

2. Experimental

2.1. Reagents

Fullerene, C_{60} (+99.95% purity), was procured from SES Research (Houston, TX, USA). Tetra-*n*-butylammonium hexafluorophosphate, (TBA)PF_6, palladium acetate trimer, acetonitrile (in a 'sure-seal' bottle), toluene, 1,2-dichlorobenzene, cyt *c* from horse heart, and all other reagents were from Sigma-Aldrich Chemicals (Milwaukee, WI, USA). Tetra-*n*-butylammonium perchlorate, (TBA)ClO_4, was from Fluka Chemicals (Buchs, Switzerland). All chemicals were used as received.

2.2. Apparatus

The UV–visible spectroscopy measurements were carried out with a Shimadzu model 1600 spectrophotometer.

The cyclic voltammetry (CV) experiments were performed on an EG&G Model 263A potentiostat/galvanostat using a typical three-electrode cell. An insulator-shrouded 1.2-mm diameter platinum disk or 3.0-mm diameter glassy carbon disk of Bioanalytical Systems (West Lafayette, IN, USA) or 9×30 mm indium–tin–oxide (ITO) glass slide of Delta Technologies (Stillwater, MN, USA) were used as working electrodes. A platinum wire and a 3 M NaCl Ag/AgCl electrode served as the counter and reference electrode, respectively. For deaeration, all the solutions were purged with nitrogen gas prior to electrochemical measurements. The CV experiments in aqueous solutions were performed using the 0.03 M Na_2HPO_4, 0.009 M KH_2PO_4 phosphate buffer acidified to pH=7.4 with a few drops of HCl.

The piezoelectric microgravimetry experiments were performed on an electrochemical quartz crystal microbalance, EQCM 5510, of the Institute of Physical Chemistry (Warsaw, Poland) [23,24] by using 5-MHz, 14-mm diameter quartz crystal resonators with 5-mm diameter gold-over-titanium film electrodes. The frequency changes were measured with 1-Hz resolution.

Elemental composition of the cyt *c* films was analyzed by the X-ray photoelectron spectroscopy (XPS) with an ESCALAB-210 spectrometer of VG Scientific (East Grinstead, UK) using Al K_α ($h\nu$=1486.6 eV) X-ray radiation.

3. Results and discussion

The fullerene [15–19] and fullerene-palladium polymer film [20–22] modified electrodes were prepared according to the respective literature procedures, as follows.

The C_{60} film-modified electrodes were prepared by consecutive evaporation of several 10-μL droplets of dichloromethane solution of C_{60}, repeatedly dispensed on a glassy carbon or platinum working electrode surface. Fast evaporation of dichloromethane, facilitated by heating with a hot air gun, resulted in formation of fine microcrystalline C_{60} films. Then, multiscan CV curves for these electrodes immersed in 0.1 M (TBA)PF_6 in acetonitrile were recorded in order to condition the fullerene films. In effect, two major chemically reversible but electrochemically irreversible peaks of reduction and oxidation with steady-state currents and potentials were observed (Fig. 1a) in the potential range 0 to −1.2 V in accord with literature observations [15–19]. A potential window of this electrode extends between 0.4 and −0.6 V without occurrence of any electrode reaction of the fullerene film (dashed curve in Fig. 1a), thus making it suitable for probing electrode reactions of different redox species immobilized on top of the fullerene film. The electrodes prepared in this way were used directly for immobilizing cyt *c*.

The C_{60}-Pd polymer film-modified electrodes were prepared by performing multiscan CV runs for a solution of 0.35 mM C_{60} and 1.34 mM Pd-acetate trimer in 0.1 M (TBA)ClO_4, in 1,2-dichlorobenzene (Fig. 1b). The currents

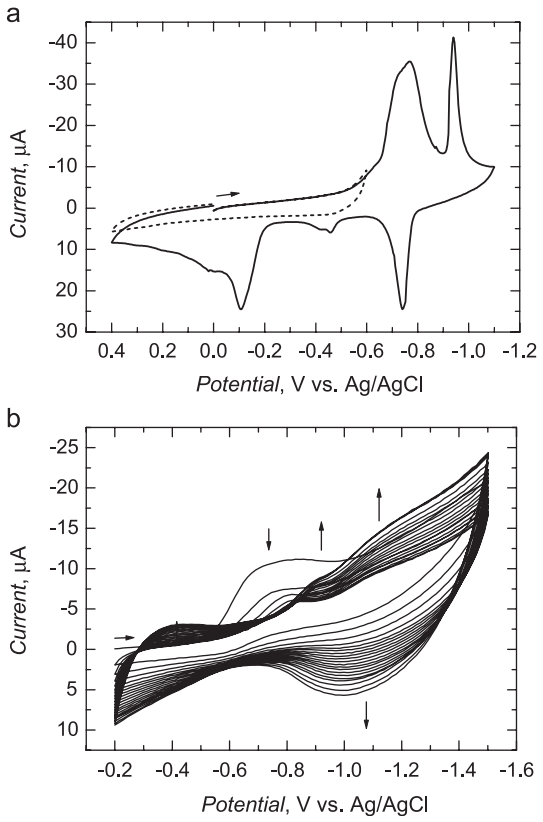


Fig. 1. (a) Cyclic voltammograms for the pristine C_{60} film-modified glassy carbon electrode in 0.1 M (TBA)PF_6, in acetonitrile; potential scan rate 100 mV/s. (b) Multiscan cyclic voltammograms for a 0.35 mM C_{60}, 1.34 mM Pd-acetate trimer, and 0.1 M (TBA)ClO_4, in 1,2-dichlorobenzene solution, for the initial 20 CV scans; potential scan rate, v=50 mV/s.

of the CV peaks corresponding to the solution C_{60} electroreduction decreased with the appearance of new CV peaks at more negative potentials corresponding to formation of the C_{60}-Pd polymer film on the electrode surface [20]. Thickness of this film was controlled with the number of CV scans performed. Virtually the same results were obtained for the inert metal (Pt disk or Au/Ti film-coated quartz) electrodes as well as for GCE and ITO electrodes. The modified electrodes were washed with the 1,2-dichlorobenzene electrochemical solvent in order to remove any unbound fullerene or palladium trimer and then immediately used for the cyt c immobilization studies.

The immobilization of cyt c on the fullerene film-modified electrodes was first examined by piezoelectric microgravimetry because this technique provides direct evidence of substance immobilization manifested by the resonator mass increase. Fig. 2 shows time dependence of the frequency change (Δf) at open circuit for the C_{60}-Pd polymer film-modified quartz crystal before and after addition of cyt c at various concentrations in the buffer solution, to the blank buffer solution while stirring with a magnetic bar. Upon addition of cyt c, so that its final concentration in solution was equal to 2.3, 3.9, and 5.4 μM, Δf decreased to reach plateaus after ca. 20, 25, and 30 min, respectively. These plateaus were established at Δf of −39, −84, and −173 Hz, which corresponds to the resonator mass increase, Δm, of 135, 291, and 600 ng, respectively, as determined by using the Sauerbrey equation [25]. Hence, the cyt c apparent surface coverage of the modified electrode, Γ, was estimated as 5.5×10^{-11}, 1.2×10^{-10}, and 2.5×10^{-10} mol cm^{-2}, respectively. In accordance with results of the SEM imaging [22], these values indicate remarkable surface roughness of the modified electrodes. This roughness can be expressed by relative surface area, $R_{sa}=A_{3D}/A_{2D}$, which, in the present case, exceeds 15, as anticipated by comparing the estimated maximum surface coverage and apparent surface coverage values.

Fig. 3 compares the UV–vis absorption spectrum of cyt c immobilized onto a C_{60}-Pd polymer film-modified ITO electrode, after subtraction of the background absorption of the C_{60}-Pd polymer film itself. For the film spectral measurement, the C_{60}-Pd modified ITO electrode was first soaked in a solution containing cyt c for about 10 min and then rinsed several times with the buffer solution in order to remove any unbound cyt c. In the optical spectrum of the resulting modified electrode (curve 2 in Fig. 3), both a broad Soret and Q band located at 407 and 520 nm, respectively, were observed. These bands compare with the corresponding bands at 409 and 530 nm, respectively, for cyt c dissolved in the buffer solution (curve 1 in Fig. 3). The blue shift and the broadness of the bands suggest that the cyt c molecules are tightly packed on the ITO electrode surface. We also attempted to characterize the immobilized cyt c by the XPS technique. However, the signals corresponding to iron and sulfur atoms of the heme moiety were very weak apparently indicating that the heme entity was buried too deep inside the polypeptide chain of cyt c. But the present QCM and optical absorption spectral studies clearly prove that cyt c is immobilized onto the surface of the C_{60}-Pd polymer film-modified electrode.

Fig. 4 compares CV behavior in phosphate buffer (pH = 7.4) of cyt c immobilized on the C_{60} drop-coated film GCE

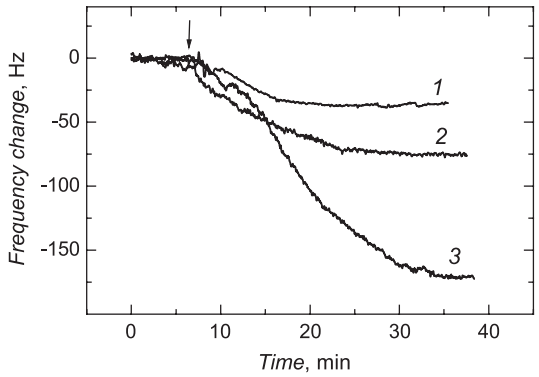


Fig. 2. Frequency change with time for the C_{60}-Pd polymer film-modified 5-MHz Au/Ti quartz crystal electrodes before and after addition of a cytochrome c solution (indicated with an arrow) to reach its final concentration of 2.3 μM (curve 1), 3.9 μM (curve 2), and 5.4 μM (curve 3) in 0.05 M phosphate buffer (pH=7.4). The C_{60}-Pd modified electrodes were prepared by performing 10 CV scans of electropolymerization at 100 mV/s.

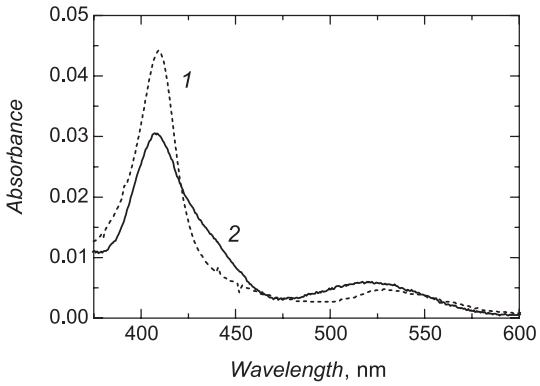


Fig. 3. UV–vis absorption spectrum for 3.8×10^{-7} M cytochrome *c* in phosphate buffer (pH=7.4)—curve 1, and for a cytochrome *c* film immobilized on the ITO electrode modified with the C_{60}-Pd polymer film (after background subtraction)—curve 2.

(Fig. 4a) and that on the C_{60}-Pd polymer film modified GCE (Fig. 4b). Addition of cyt *c* to the solution resulted in the appearance of a new cathodic peak at E_{pc}=−400 mV vs. Ag/AgCl. Upon the potential scan reversal, an anodic peak at E_{pa}=50 mV vs. Ag/AgCl was also observed for the C_{60}-Pd polymer film-modified electrode indicating the occurrence of a chemically reversible but kinetically slow electron transfer process. A plot of the cathodic or anodic peak currents (i_{pc} or i_{pa}) vs. potential scan rate, *v*, for CV data like that shown on curve 2′ in Fig. 4b yielded a straight line (not shown) characteristic of a surface-confined redox couple. However, a plot of log i_{pc} vs. log *v* yielded a straight line with a slope close to 0.8 (R^2=0.97). This slightly smaller slope value, as compared to the theoretically predicted value of 1.0 for surface-confined redox couple [26], may suggest the occurrence of competing semi-infinite diffusion controlled cyt *c* electroreduction.

In another experiment, the C_{60}-Pd polymer film-modified GCE was equilibrated in the cyt *c* buffer solution for 5 min, and then the electrode was removed, rinsed with distilled water, and a CV curve was recorded in a blank buffer solution. As shown in curve 3′ in Fig. 4b, the CV peaks were still present, indicating stable surface immobilization of cyt *c* onto the C_{60}-Pd polymer film-modified electrode. By integrating the current of the cathodic peak, we estimated apparent surface coverage of the modified electrode, as $\Gamma \approx 3.6\pm0.4\times10^{-10}$ mol cm^{-2}. This Γ value agrees well with that determined by piezoelectric microgravimetry from curves in Fig. 2. The CV peaks vanished after three to four potential cycles, most likely due to back diffusion of the reduced cyt *c* from the electrode surface to bulk solution. However, such experiments performed on pristine C_{60} film-modified electrodes revealed no CV response of cyt *c* reduction (curve 3 in Fig. 4a). These results indicate weaker adsorption of cyt *c* on the pristine C_{60} film-modified electrode than that on the C_{60}-Pd polymer film-modified electrodes.

The effect of the C_{60}-Pd polymer film thickness on CV properties of the immobilized cyt *c* was also examined. For this, the film thickness was controlled with the number of CV cycles executed during the film growth. Accordingly, the electrodes were modified by 5, 10, 15, and 20 cycles under CV conditions defined in Fig. 1. It appeared that the amount of immobilized cyt *c* increased with the increase of the C_{60}-Pd film thickness. That is, there was a gradual increase in the peak current of the cyt *c* reduction with the increase of the number of CV scans during the C_{60}-Pd film electrodeposition on the electrode surface. This effect might be due to the increase of the C_{60}-Pd film surface area during electropolymerization.

The mechanism of the investigated processes is proposed in Scheme 1 by way of example for cyt *c* immobilization onto the C_{60}-Pd polymer film-modified electrode. Conceivably, cyt *c* is immobilized by one or more of the following routes. (i) The electron-deficient C_{60} molecules in the films adsorbed on the electrodes may electrostatically bind to the cyt *c* molecules at their electron-rich aspartic–glutamic acid residue sites. (ii) It is known that the C_{60} films partially retain their negative charges either during the electrochemical conditioning of the drop-coated film electrode or during electropolymerization of the C_{60}-Pd film, behaving

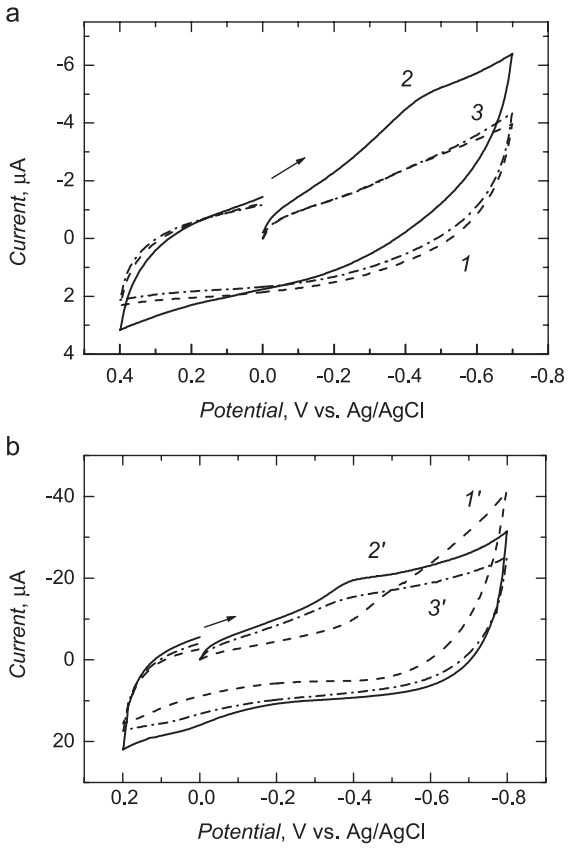
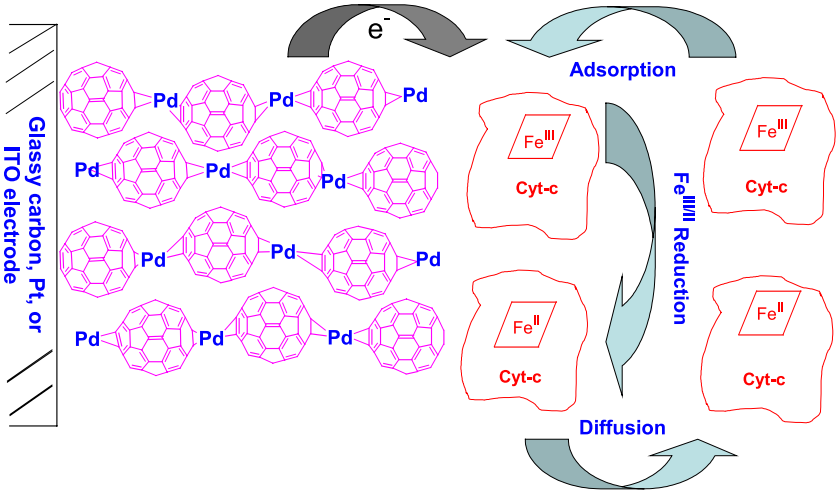


Fig. 4. Cyclic voltammograms for (a) pristine C_{60} film-modified glassy carbon electrode and (b) C_{60}-Pd polymer film-modified glassy carbon electrode (conditions as in Fig. 1b) in phosphate buffer (pH=7.4). Background for bare (unmodified) glassy carbon electrodes—curves 1 and 1′; fullerene film-modified glassy carbon electrodes in 0.05 mM cytochrome *c*—curves 2 and 2′; fullerene film-modified glassy carbon electrodes in blank phosphate buffer solutions—curves 3 and 3′. Potential scan rate, *v*=100 mV/s.



Scheme 1. Proposed mechanism of cytochrome *c* immobilization and electrochemical reduction by C_{60}-Pd polymer film modified electrode.

like electron accumulators [3,21,22]. As a result, the negative charges stored in the film may electrostatically bind, via ion pairing, positively charged parts of the cyt *c* protein. Owing to a high positive and negative charge on cyt *c* and C_{60}, respectively, the former might strongly adsorb. (iii) Surface morphology of the C_{60} or C_{60}-Pd film may affect immobilization of cyt *c*. From the SEM studies, it follows that structure of the electropolymerized C_{60}-Pd polymer films is porous featuring irregular grains [22]. Similarly, structure of the drop-coated C_{60} film electrodes is irregular and quasi-crystalline. Hence, cyt *c* molecules may be trapped in these pores or quasi-crystalline domains.

In summary, we have demonstrated immobilization of a redox-active protein, cyt *c*, onto two different fullerene film-coated electrodes. The resulting cyt *c* modified electrodes were characterized by piezoelectric microgravimetry, XPS, UV–vis spectroscopy, and CV. Direct electron transfer between the electrode and the immobilized cyt *c* was observed. While the fullerene film thickness plays an important role in the immobilization process, the experiments performed suggest that the C_{60}-Pd polymer film-modified electrodes bind cyt *c* more effectively than the pristine C_{60} drop-coated film-modified electrodes. A good agreement between the values of cyt *c* electrode surface coverage determined by piezoelectric microgravimetry and cyclic voltammetry was obtained. More extensive studies aiming at deeper understanding of the cyt *c* immobilization and accompanying redox processes as well as at seeking prospective bioanalytical applications of these novel modified electrodes for, e.g., mediating charge to cyt *c* oxidase, are being currently pursued in our laboratories.

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