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Immobilization of Adamantane-Modified Cytochrome *c* at Electrode Surfaces through Supramolecular Interactions

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Cytochrome *c* (Cyt *c*) was immobilized at the surface of a silver electrode through the formation of host−guest complexes between adamantane (A) units located at the protein surface (Cyt *c*−A) and chemisorbed thiolated *β*-cyclodextrin (CDSH). The voltammetric response of immobilized Cyt *c*−A is quasi-reversible and very stable with time. Addition of competitive guests, such as 1-adamantamol, results in the disappearance of the signal. In the presence of 4,4′-bipyridine, the quasi-reversible redox response changed to give the characteristic signal of a surface confined redox couple. Experiments in the presence of electroactive probes such as $Ru(NH_3)_6^{3+}$ and 1,2-naphthoquinone resulted in a decrease of the signal intensity of the probe and shifted the half-wave potential to more positive values. These results demonstrated the adsorption of Cyt *c*−A molecules at the electrode surface.

The immobilization of enzymes[1] on electrode surfaces constitutes an area of current interest and development due to their theoretical and practical applications, for instance, in the construction of biosensing devices.[2] Strategies for enzyme immobilization on electrodes include covalent attachment, physical adsorption, and film deposition.[3] Covalent attachment provides stable protein layers and requires chemical activation of the support before enzyme coupling. Physical adsorption is easy to accomplish and widely applicable but is greatly sensitive to ionic strength and substrate concentration. Finally, protein deposition on redox-, electroactive-, or ion-exchange polymers or on surfactant films to form bi- or multilayers is also a widely studied alternative. In the later two methods, the driving forces for protein immobilization are typically electrostatic and/or hydrophobic interactions, which often determine the orientation of the biomolecule toward the electrode surface. However, to the best of our knowledge, little or no attention has been paid to the use of supramolecular chemistry[4] (i.e., host−guest interactions) for this purpose. These interactions can be easily tuned by the appropriate selection of geometrically complementary host and guest molecules and can be studied by using a wide range of physicochemical techniques.[5] In the present work, we wish to report a novel strategy for protein immobilization on metal surfaces and a preliminary voltammetric evaluation of its effectiveness. As a model system we selected the redox heme-containing metalloprotein cytochrome *c* (Cyt *c*), which is among the most studied proteins from the electrochemical point of view.[3,6,7]

The strategy consists on the formation of supramolecular architectures between Cyt *c* modified with pending adamantane units and thiolated cyclodextrins chemisorbed on silver electrodes.[8] Cyclodextrins (CDs) are cyclic water-soluble D-glucopyranose oligomers featuring a central hydrophobic cavity capable to form stable inclusion complexes with organic molecules.[9] Among them, thiolated CDs are well-known electrode modifiers giving to the metal surface molecular recognition properties.[10] On the other hand, adamantane derivatives are one of the most studied types of organic guests for CDs since they form inclusion complexes with inclusion constants in the order of 10^4 M^{-1}.[11] For instance, 1-adamantanol is known to form interfacial inclusion complexes with CDSH immobilized

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(8) The CD modified silver electrode (Ag/CDSH) was prepared by immersing a bare polycrystalline silver electrode ($A = 0.125$ cm^2) into a 0.01 M DMSO solution of per-6-thio-6-deoxy-*β*-CD (CDSH)[10a] overnight, followed by rinsing with DMSO, EtOH, and doubly distilled water. The modified electrodes were voltammetrically tested in 0.1 M KH_2PO_4 and 0.1 M KCl, giving a flat background response between −0.3 and 0.7 V. The solutions were deoxygenated with argon before the measurements. CV were recorded in a Yanaco P-900 cyclic polarograph coupled to a Graphtec WX1000 X-Y recorder using a standard three cell holder (counter electrode, Pt wire; reference, Ag/AgCl).

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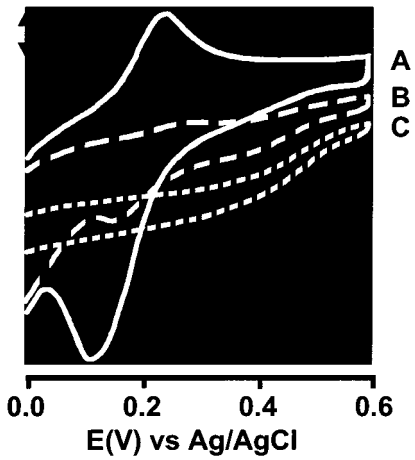

Figure 1. Cyclic voltammograms of Cyt *c* on the Ag/CDSH electrode recorded at different times. (A) Immediately after immersion of the electrode into the Cyt *c* solution. (B) After 5 min. (C) After 10 min. Conditions: [Cyt *c*] = 100 μM; supporting electrolyte: 0.1 M KH_2PO_4 (pH 7); scan rate: 50 mV/s.

on mercury electrodes with a binding constant of 1×10^4 M^{-1}, as determined by capacitance measurements.[11b] In other recent work, Galla and co-workers have determined the interfacial inclusion constants of 1-adamantanammonium and 1-adamantanecarboxylate ions at gold electrodes modified with a monolayer of mono-6-(3-mercaptopropionamide)-6-deoxy-β-cyclodextrin. They found K values of 1.3×10^4 and 2.4×10^3 M^{-1}, respectively, using impedance spectroscopy.[11c] Therefore, adamantane-appended Cyt *c* is expected to bind tightly to a CD-coated electrode thus giving rise to a monolayer of Cyt *c* molecules at the metal surface.

Cyt *c* modification with adamantane units was carried out as follows. Cyt *c* was allowed to react with excess of sodium 1-adamantanecarboxylate in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC) as a coupling agent in phosphate buffer pH = 6 at 4 °C followed of dialysis.[12] This method afforded a Cyt *c* modified through the lysine residues located at the protein surface (Cyt *c*−A). The average degree of substitution of Cyt *c* was estimated to be 8.9 by measuring the remaining number of free amino groups using the trinitrobenzenesulfonate method.[13] That means that Cyt *c*−A contains about 9 adamantane units per protein molecule (47% substitution of Lys residues). The UV−Vis spectrum of the conjugate in the porphyrin region shows two characteristic bands in positions similar to those of native Cyt *c*: an intense band at 409 nm (Soret band) and a weaker one centered at 527 nm (Q-band).

First, the cyclic voltammogram of native Cyt *c* was recorded at the Ag/CDSH electrode (Figure 1). When the electrode was immersed in a solution containing 100 μM Cyt *c* at pH = 7, a quasi-reversible signal was obtained with $E_{1/2} = 0.17$ V vs Ag/AgCl and $\Delta E_{ac} = 0.12$ V, which can be assigned to the Fe^{2+}/Fe^{3+} process (Figure 1A). This signal deteriorates rapidly and irreversibly with time, decreasing in intensity, and shifting $E_{1/2}$ to more positive values (Figure 1B). Then, after 10 min, no signal is observed (Figure 1C). The signal decrease is faster at higher Cyt *c* concentrations. These findings suggest that

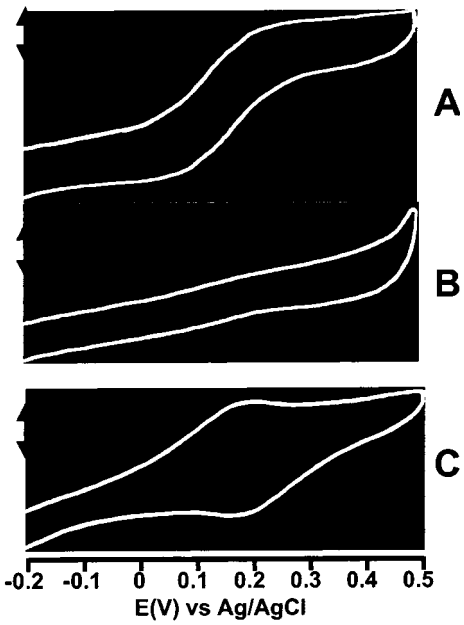

Figure 2. (A) Cyclic voltammograms of Cyt *c*−A recorded at the Ag/CDSH electrode. (B) Same as (A) but after overnight exposure to a saturated solution of 1-adamantanol. (C) Same as (A) but after addition of excess of 4,4′-bipyridine. Conditions: Supporting electrolyte: 0.1 M KH_2PO_4 (pH 7); scan rate: 50 mV/s.

the Cyt *c* molecule is decomposed at the electrode surface, similarly to what has been observed on bare electrodes,[14] although in our case this phenomenon is slower due to the protecting monolayer of the chemisorbed CDSH. However, Cyt *c* seems not to be adsorbed at the surface in the very first moments of the interaction with the Ag/CDSH electrode. The peak currents (measured immediately after immersion of the electrode into a Cyt *c* solution) increase linearly with Cyt *c* concentration in the range of 50−300 μM, and the anodic and cathodic peak currents increase almost linearly with the square root of potential scan rate at a fixed Cyt *c* concentration. This behavior is typical of a diffusion-controlled process. The lack of adsorption of Cyt *c* in the first moments of interaction with the electrode is not surprising since the outer Cyt *c* surface has no hydrophobic residues (i.e., from phenylalanine or tyrosine residues)[15] capable of interacting with the CD cavities. However, as time elapses, Cyt *c* is expected to interact not only with the hydrophilic surface provided by the chemisorbed CDs and but also with the uncovered surface. Nevertheless, no attempt was made to seal the uncovered electrode to avoid a reduction in the active electrode surface.

The voltammetric properties of Cyt *c*−A on the Ag/CDSH electrode came to be very different from those of native Cyt *c* (Figure 2A). The conjugate shows a quasi-reversible response with $E_{1/2} \sim 0.18$ V vs Ag/AgCl (Table 1). This value is close to that of native Cyt *c*, suggesting that the outer surface modification with hydrophobic moieties do not significantly alter the conformation of the protein and the iron coordination sphere and is in agreement with the UV−vis data. The signals are quite broadened with respect to Cyt *c*, which might be due to the polydispersion of Cyt *c*−A. Remarkably, the signals are reproducible and very stable with time for periods of 8−10 h if kept at room

(12) Synthetic procedure: A reaction mixture composed of 20 mg freeze-dried Cyt *c* (from horse heart, MW = 12500), sodium 1-adamantanecarboxylate (100 mg), and EDAC (50 mg) in deoxygenated 50 mM potassium phosphate buffer pH 6 (5 mL) was stirred overnight at 4 °C. The solution was dialyzed against phosphate buffer pH 6 several times and kept at 4 °C when not used for measurements.

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Table 1. Voltammetric Properties of Cyt *c* and Its Conjugates Recorded at the Ag/CDSH Electrode[a]

system	$E_{1/2}$ (V)	ΔE_{ac} (V)[b]
Cyt *c*	0.17	0.12
Cyt *c*−A	0.19	0.20
Cyt *c*−A + 4,4′-bipyridine	0.16	0.02

[a] All values are in volt vs Ag/AgCl. Scan rate: 50 mV/s. [b] $\Delta E_{ac} = E_{anodic\ peak} - E_{cathodic\ peak}$.

temperature and for a week if the solution is kept at 4 °C. However, we did not evaluate the electrochemical response under conditions of continuous cycling. The signal intensities do not depend on the bulk concentration of the conjugate present in solution for values higher than 100 μM. In fact, when the electrode was immersed in a buffered milimolar solution of the conjugate for several minutes, removed, rinsed with buffer, and tested for voltammetric responses in 0.1 M KH_2PO_4, these responses were identical to those of the electrode immersed in a 0.1 M KH_2PO_4 solution containing milimolar concentrations of the conjugate. The adsorption process seems to be very fast since no increase in the signal intensity was detected after 2 min of exposure to a ~100 μM solution of the conjugate.

In the presence of a saturated solution of 1-adamantanol (~1 mM) the signals disappear, but in contrast, this process is very slow and the response is completely suppressed only after an overnight exposure to 1-adamantanol (Figure 2B). Similar results were obtained with other competitive guests such as 1-adamantanecarboxylate, cyclohexanol, and litocholic acid. These findings suggest that Cyt *c*−A is strongly adsorbed at the electrode surface without losing its electroactive properties. One reason for this strong interaction could be that multiple adamantane moieties in the same Cyt *c*−A molecule are bounded to multiple CD cavities resulting in a multipoint interaction that reinforces the adsorption process. We can thus consider that the electrode is now doubly modified: (1) by a monolayer of chemisorbed CDSH acting as a support and (2) by a monolayer of Cyt *c*−A adsorbed though host−guest interactions with CDSH (from now on we will refer to this electrode as Ag/CDSH/Cyt *c*−A). Integration of the cathodic peak measured at low scan rates (<10 mV/s) and normalization to the electrode area gave a surface concentration of (9.5 ± 0.8) pmol/cm^2 for Cyt *c*−A. This value is lower than the theoretical value of 12−13 pmol/cm^2 calculated from geometrical considerations[7d,15] and reflects a somewhat irregular monolayer of adsorbed Cyt *c*−A. This is presumably due to the existence of defects in the chemisorbed monolayer of CDSH hosts[10a] and a reduction in lateral interactions in Cyt *c*−A due to the existence of pending adamantane units. Scheme 1 shows a schematic representation of the adsorption of Cyt *c*−A at the Ag/CDSH electrode through the inclusion of adamantane moieties in the CD cavity.

On the other hand, addition of 4,4-bipyridine (bipy) to a Ag/CDSH/Cyt *c*−A electrode resulted in a more reversible signal with $\Delta E_{ac} = 20$ mV (Figure 2C) and a 20 mV decrease in the $E_{1/2}$ value. This means that the presence of bipy facilitates the protein/electrode electron-transfer process and is in agreement with previous reports on the promotion of heterogeneous electron transfer in Cyt *c* using pyridine derivatives.[6,7] ΔE_{ac} is essentially scan rate independent in the range of 50−300 mV/s, and peak currents depend linearly with scan rate in the same range. The ΔE_{ac} value is slightly higher than the ideal value of 0 mV for a surface-confined reversible redox couple which might be the result of some dynamic contribution to the interaction between Cyt *c*−A molecules and CDSH, as expected for a host−guest complexation.

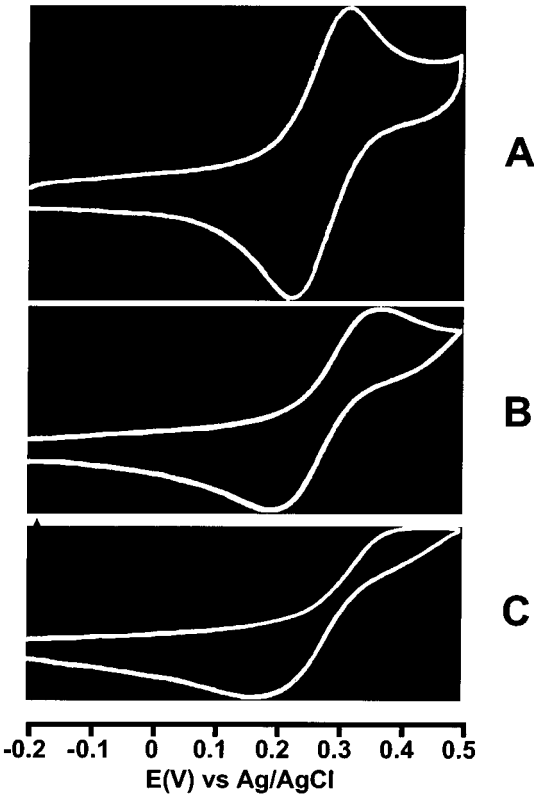
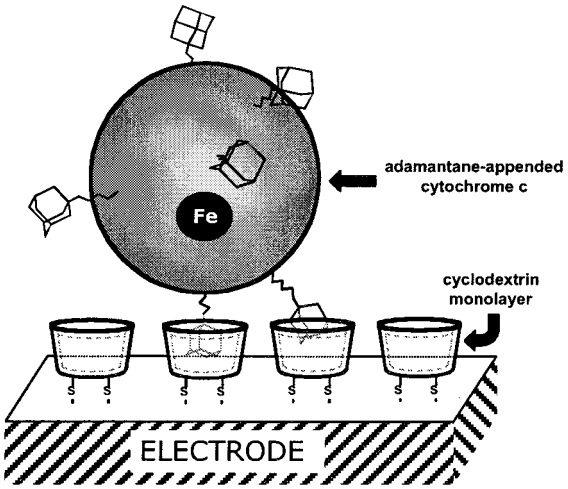


Figure 3. Cyclic voltammograms of a 0.01 M solution of Ru$(NH_3)_6^{3+}$ in the absence (A) and in the presence of 100 μM native Cyt *c* (B) and 100 μM Cyt *c*−A (C). Conditions: Supporting electrolyte: 0.1 M KCl; scan rate: 100 mV/s.

Scheme 1



Further evidence of the adsorption of Cyt *c*−A on the Ag/CDSH electrode comes from the study of its interaction with some electroactive probes. Figure 3 shows the CV of 10 mM Ru$(NH_3)_6^{3+}$ in the absence (Figure 3A) and in the presence of native Cyt *c* (Figure 3B) and Cyt *c*−A (Figure 3C). On the Ag/CDSH electrode this probe shows a reversible voltammetric signal with $\Delta E_{ac} = 90$ mV. This value is higher than the ideal value of 59 mV due to the presence of chemisorbed CDSH, which acts as a barrier to the electron-transfer process. In the presence of native Cyt *c*, ΔE_{ac} increases to 180 mV, while a maximum value of $\Delta E_{ac} = 280$ mV was observed at the Ag/CDSH/Cyt *c*−A electrode. The peak currents also decrease in these later cases with respect to the Ru$(NH_3)_6^{3+}$/Ru$(NH_3)_6^{2+}$ couple alone, which indicates a lower concentration of electro-

active species at the electrode/solution interface. These observations can be explained considering the repulsive interaction exerted by the monolayer of positively charged protein over the cationic probe.

The above-mentioned experiment has, however, the limitation that the $Ru(NH_3)_6^{3+}/Ru(NH_3)_6^{2+}$ couple exhibits a half-wave potential very close to those of Cyt *c* and Cyt *c*−A, thus preventing the direct observation of the signal associated to the protein Fe(II) center. To circumvent this limitation, we used a neutral electroactive probe, 1,2-naphthoquinone (NQ), which is reduced at more positive values than Cyt *c*. Quinones undergo a reversible two-electron reduction to give the corresponding hydroquinone and, due to their hydrophobic character, are expected to bind the CD cavities. For these reasons they have been used before as electroactive markers in CD-modified electrodes.[16] Figure 4 shows the CV of 1,2-naphthoquinone recorded at Ag/CDSH (Figure 4A) and Ag/CDSH/Cyt *c*−A electrodes (Figure 4B). As can be observed, the half-wave potential for the NQ reduction is displaced to more positive values, from 0.43 to 0.49 V, while ΔE_{ac} remains almost constant. The peak currents also decrease and the redox response of adsorbed Cyt *c* is clearly observed as a quasi-reversible signal centered at 0.20 V. These results complement those obtained with 1-adamantanol and the ruthenium complex, confirming the adsorption of Cyt *c*−A at the Ag/CDSH electrode.

In conclusion, we have reported a novel method to immobilize Cyt *c* at an electrode surface. The strategy combines the covalent attachment of a hydrophobic moiety to Cyt *c* followed by the formation of a supramolecular structure with a molecular receptor previously chemisorbed at a metallic surface. This strategy can be extended to some other proteins or enzymes and constitutes, in our opinion, an attractive approach, for instance, to the construction of novel biosensors. A disadvantage of the method is perhaps the necessity of a covalent modification of the protein (which is transformed in a non-native polydisperse structure). However, an appropriate selection of the host and the guest would permit to obtain a strong adsorption while allowing the SAM-modified electrode to be reused at will if it is treated with a competitive guest to remove the adsorbed protein. Further experiments in this direction are in progress in our laboratory.

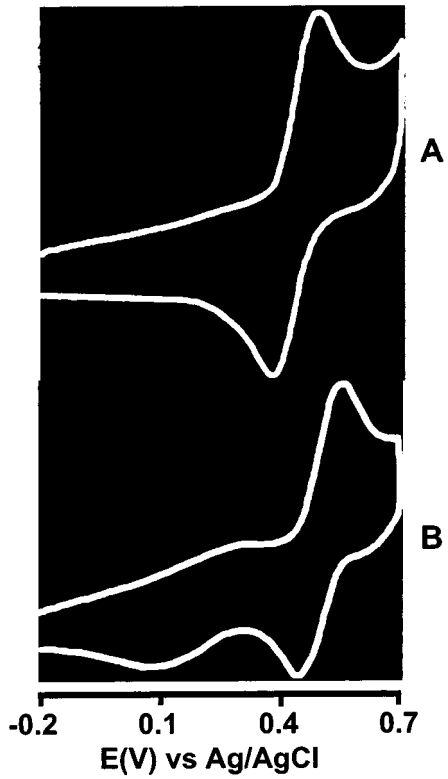


Figure 4. Cyclic voltammograms of a 10^{-4} M solution of 1,2-naphthoquinone in the absence (A) and in the presence of Cyt *c*−A (B). Conditions: supporting electrolyte: 0.1 M KCl; scan rate: 50 mV/s.

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