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Short Communication

A Biomimetic Electrode Platform for Cytochrome *c* Electrochemical Studies

María F. Cerdá[1], *Mariana Pereyra*[2], *Michael Wörner*[3], *Eduardo Méndez*[1,*]

[1]Laboratorio de Biomateriales, Instituto de Química Biológica, and [2]Unidad de Bioquímica Analítica, Centro de Investigaciones Nucleares. Facultad de Ciencias. Universidad de la República. Iguá 4225, 11400 Montevideo, Uruguay.
[3]Institut für Bio- und Lebensmitteltechnik, Bereich IV: Molekulare Aufarbeitung von Bioprodukten, Universität Karlsruhe. Engler Bunte Ring, PLZ 76131, Germany
[*]E-mail: emendez@fcien.edu.uy

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By employing a gold surface covered with the substituted mercaptopyrimidine 2-thiobarbituric acid (TBA), it was possible to prove the influence of the surface topography (through a fractal approach) and the formation of an H-bond network between the modified surface and the protein. The spontaneous adsorption of a dense monolayer of cytochrome *c* led to a quasi-reversible redox behavior with a high direct electron transfer rate constant (38 s^{-1}),with no measurable electrocatalytic effect. The redox potential for cytochrome *c* in the adsorbed state has the same value to that reported for the free protein in solution. Our results suggest that TBA-covered electrodes act as a biomimetic platform that mimic the natural environment to study redox proteins without interfering with their natural behavior.

Keywords: Cytochrome *c*,direct electron transfer, 2-thiobarbituric acid, topography

1. INTRODUCTION

The development of biomimetic protein-based electrode platforms which mimic the behaviour of the protein in solution is one of the most exciting areas in interfacial research [1-3], as it constitutes the basics of the protein monolayer electrochemistry (PME) [4], a well-known technique widely used for the study of the electrochemical properties of redox proteins and biosensors [5]. The behaviour of such electrode platforms is mainly dependent on two important interfacial characteristics: the amount of functional protein adsorbed on the surface, and the direct electron transfer (DET) process at the interface, which reversibility will determine, among others, the sensibility of the platform as biosensor. Ideally, the electrode platform should allow the protein to form a well oriented and compact monolayer, that renders a single DET path with a faradic current-potential profile with a width at half-

maximum (WHM) of 89 mV at 293 K [6], and with no alteration in the redox potential value. All these conditions are not always met with a single platform [7-9], and a surface modifier accomplishing all the desirable characteristics for PME measurements is still lacking.

In order to improve the adsorption/DET process of cytochrome *c* (a model redox protein) we tested two hypotheses. The first one, related to the amount of protein adsorbed, considers that a topographic complementation between the interacting surfaces should render the highest surface coverage. The second hypothesis, related to the DET process, considers the interaction guided by H-bonds, as the specific bio-recognizing agent. We will show that 2-thiobarbituric acid (TBA)-modified surface provides all the characteristics needed to prove both hypotheses, resulting in a biomimetic electrode platform onto which a dense protein monolayer with nearly null electrochemical dispersion is formed.

2. EXPERIMENTAL PART

Chemicals and solutions. All chemicals were used as received: 2-thiobarbituric acid (Sigma-Aldrich, >98%, p.a.), horse heart cytochrome *c* (Sigma-Aldrich, 12384 Da), sodium acetate (Fluka, > 99 %, p.a.). All solutions were prepared in Milli-Q water (0.055 μS cm^{-1}).

Electrodes. A polycrystalline gold wire (99.999%) of 0.106 cm^2 real area was employed as the working electrode for voltammetric measurements. The counter electrode was a Pt wire (Radiometer, Switzerland), and the reference electrode was a homemade Ag|AgCl electrode in saturated KCl solution (E = 0.204 V vs NHE, at 20 °C). All potentials in the text are referenced to this electrode. The working electrode surface was electrochemically polished between the potential onset for hydrogen and oxygen evolution at 0.10 V s^{-1}in H_2SO_4 0.5 M, until a reproducible voltammogram was obtained.

Quartz Crystal Microbalance measurements. The QCM cell consisted of three round Teflon pieces, 37 mm in total height and 35 mm in diameter. The exact gold surface exposed to the solution was calculated with the aid of image analysis (Rasband, W. *ImageJ*, version 1.30v; U. S. National Institute of Health: Bethesda, MD) after digitalization of the electrode. The corrected area (0.215cm^2) was considered as the actual geometric area in all calculations.

A CHI 440 potentiostat/galvanostat time-resolved quartz crystal microbalance was employed for QCM measurements. A piezoelectric quartz crystal electrode (13.7 mm in diameter) was employed, onto which a gold layer was deposited over a previously formed chromium layer. The electrochemical system was completed with a Pt wire counter electrode (Radiometer, Switzerland), and a saturated home-made Ag|AgCl reference electrode. All potentials in the text are referenced to this electrode. Measurements for QCM were made in time-resolved mode, thus the frequency difference of the working crystal and the reference crystal was measured. The reference crystal had an oscillation frequency of 8.000 MHz, so confident measurements correspond to the working crystal oscillation frequency interval between 7.995 and 7.950 MHz.

A TBA-modified gold surface obtained after 3 h adsorption in an aqueous solution, was Ar dried and let stabilize in 300 μL of 10 mM buffer acetate. Then, 50 μL of 1 mM cytochrome *c* solution

was added to the QCM cell (final concentration of the protein: 140 μM), and the mass was monitored until stabilization (Figure 1).

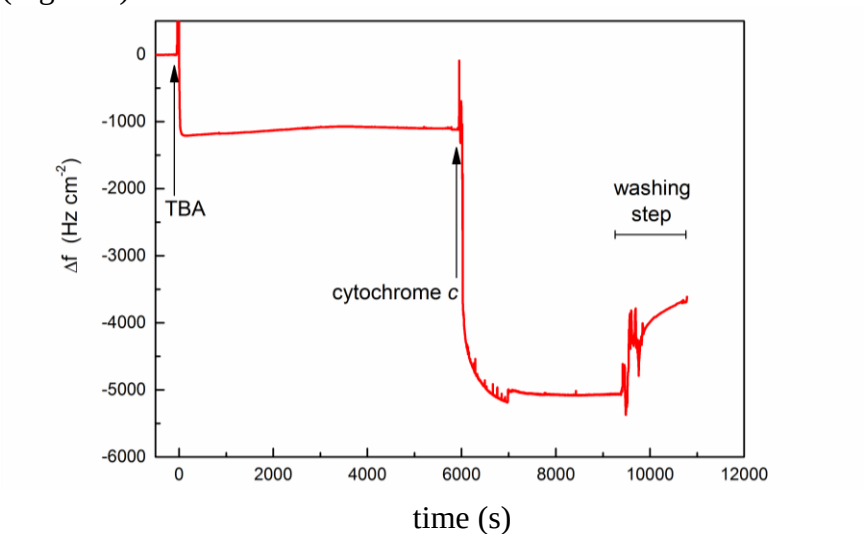

Figure 1. QCM profile for the successive spontaneous adsorption of TBA and cytochrome c on Au electrode in water. The frequency corresponding to the gold electrode was arbitrarily set to 0.

QCM data were sampled at 0.4 s intervals, and the experimental EQCM and QCM profiles were smoothed by averaging between 100 and 250 adjacent data points, taking care that no information was lost in the procedure. The measured change in the frequency produced by the adsorption of a foreign substance was related by the Sauerbrey equation with mass density change [10].

3. RESULTS AND DISCUSSION

To prove the first hypothesis, the surface dimension was taken as the parameter describing the surface roughness. By employing GEPOL [11] and Surf Race [12] algorithms, a value of 2.1 ± 0.1 was calculated for the surface dimension of cytochrome *c*, in agreement to the value reported for most proteins [13,14]. On the other hand, the surface dimension of TBA ad-layer was measured by the Stromme method employing hexaammine ruthenium (III) as the probe molecule, yielding a value of 2.17, in agreement to our previous report [15]. These values indicate that both the protein surface and the TBA-modified surface are slightly roughened and, according to their surface dimension values, both roughnesses are comparable, allowing for the test of the proposed hypothesis. The whole procedure from bare gold electrode to the final Au|TBA|cyt *c* electrode was followed by quartz crystal microbalance (QCM) measurements (Figure 1). After stabilization of the gold electrode in 300 μL of water, 50 μL of 0.15 M aqueous TBA solution was added, rendering a resonant frequency change corresponding to a mass density increase of 236 ng cm^{-2} after 50 min. The TBA solution was changed *in situ*, and the electrode was washed with 10 x 300 μL of water, with no appreciable change in the QCM profile. To this system, 50 μL of 1 mM aqueous cytochrome *c* solution was added, in one case without buffering and in the other case in acetate buffer. The net mass density increase calculated from

their respective resonant frequency changes after water/buffer washings were 852 and 557 ng cm^{-2}, respectively. These values correspond to surface concentrations of 45 and 30 pmol cm^{-2}, respectively, which compared to the theoretical maximum value deduced from the dimensions of cytochrome *c*, 25 x 25 x 37Å (27 pmol cm^{-2}) [16] suggest that a highly compact protein monolayer has been formed.

TBA ad-layer is also adequate to prove the second hypothesis, as on flat gold surfaces, it forms an hydrophilic ad-layer with contact angle of 46.1º [15], which remains electrically neutral in a wide pH range (1 – 13) [17], eliminating possible electrostatic interactions with charged lysine residues. In addition, the ability of TBA to produce large intermolecular interaction via H-bonds was demonstrated when adsorbed on gold nanoparticles [17]. Pseudocapacitance curves for cytochrome *c* ad-layer revealed a reversible nature of the anodic process in the sweep rate range 0.07 – 0.11 V s^{-1}, while some intermolecular interactions within the ad-layer is noticeable in the cathodic profile, in agreement with the slight departure from the langmuirian behaviour obtained after baseline correction (Figure 2).

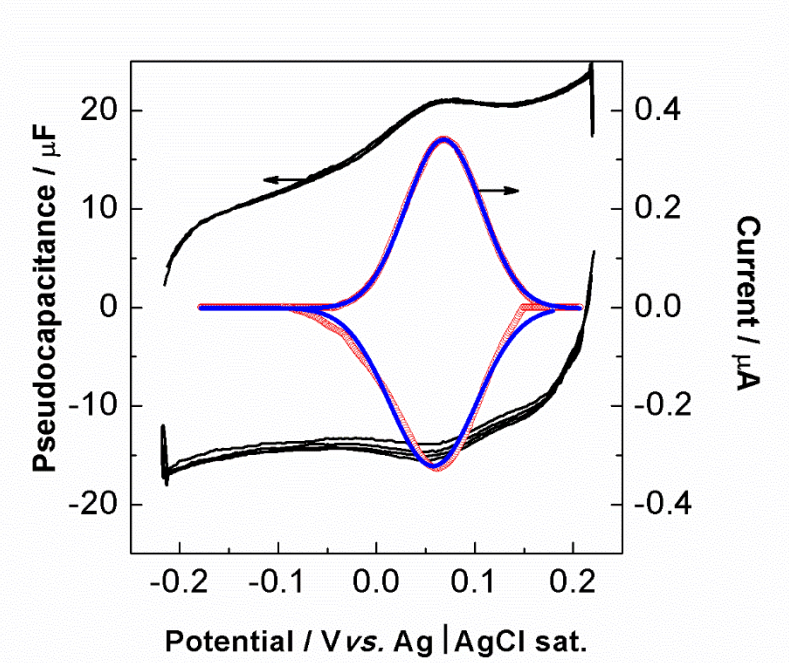

Figure 2. Pseudocapacitance (C) curves for adsorbed cytohrome c on Au|TBA electrode in 10 mM acetate buffer at sweep rates between 0.07 to 0.11 V s^{-1}; and Faradaic current (i) obtained at 0.11 V s^{-1} after baseline correction. Experimental data (red circles) and gaussian fit (blue line), T=293 K; electrode area = 0.106 cm^2.

Table 1. Peak parameters for the faradaic processes of adsorbed cytochrome c on Au|TBA electrode. Sweep rate = 0.11 V s^{-1}. Conditions as in Figure 2.

Peak parameter	Anodic	Cathodic
Potential (mV) [a]	68	63
Current density (μA cm^{-2})	3.23	3.32
WHM (mV)	90	103
Charge density (μC cm^{-2})	2.9	3.0
Surface concentration (pmol cm^{-2}) [b]	30	31

[a] Measured against a saturated Ag|AgCl electrode (0.204 V vs. NHE at 293 K).
[b] Calculated from the voltammetric charge density, considering a one-electron process.

Peak parameters obtained at 0.11 V s^{-1} are assembled in Table 1. The WHM for the anodic process is close to the theoretical value of 89 mV (293 K). In agreement with QCM results, the surface concentration is 30 pmol cm^{-2}, indicating that most of the adsorbed cytochrome *c* is electrochemically active. The peak potential separation, 5 mV, is equivalent to an apparent ET rate constant of 38 s^{-1} (293 K) [18], comparable to that obtained on PyC_{16}/C_{15}, 42 s^{-1} [9]. The redox potential, taken as the average between the anodic and cathodic peak potentials, is 65.5 mV (at 293 K, pH 4.6), close to that reported for the freely diffusing wild-type protein (68 mV at 293 K and pH 7) [19]. Despite the similarities of both systems in the effectiveness for the DET process, no electrocatalytic effect is observed on TBA-covered electrodes. The distance dependent equation for the DET process on Au|TBA can be formulated as $k_{ET} = k^o \exp(-\beta_{TBA} d_{TBA} - \beta_{prot} d_{prot})$, where $k^o = 10^{13}$ s^{-1} [20], β= 0.35 $Å^{-1}$ for TBA [17] and 1.4 $Å^{-1}$ for cytochrome *c* [19] is the tunnelling decay constant, and *d*= 5 Å for TBA [17] is the tunnelling distance. A value of d_{prot} = 18 Å is obtained for the tunnelling distance inside the protein. As electrostatic interactions at the interface can be ruled out, the DET pathway edge porphyrin-Cys17-Gln16-surface [21], where Gln16 interacts through H-bonds with TBA, can be considered as the probable electron pathway (Figure 3). The through-bond distance of this path, 16 Å, accounts for the experimental value. It is worth to mention that the total DET distance (including TBA) is close to the distance between heme centers in cytochrome *c*/cytochrome *c* oxidase complex [22,23].

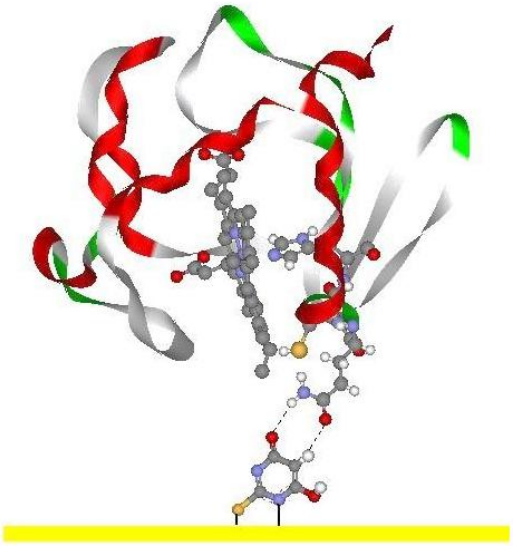

Figure 3. Proposed molecular model for the interaction between Gln16 residue of cytochrome c and TBA adsorbed on a gold surface.

Our results suggest that the topographic complementation between the interacting surfaces, together with the directional nature of H-bonds are the most important issues regarding the degree of docking, adsorption homogeneity, and effectiveness of the DET process, suggesting the following scenario for protein adsorption. The effect of the interfacial electric field on the permanent dipole of cytochrome *c* (325 D) [24] provides the means for a proper and homogeneous orientation of the protein approaching the surface, similarly as it takes place in physiological conditions between

cytochrome *c* and its redox partners [24]. The electroneutrality of Au|TBA surface prevents any pure electrostatic interaction [25], leaving H-bonds as the main biorecognition factor leading to adsorption. In addition, the similarity between the surface dimensions of the protein and Au|TBA electrode provides a sort of key-lock effect, achieving the highest possible surface concentration. The high DET rate constant obtained can be explained by the low decay constant for TBA ad-layer, and the relatively short path for the DET inside the protein. Finally, the lack of electrochemical dispersion deduced from WHM values is supported by the specificity of H-bonds in the biomolecular recognition towards a single aminoacidic residue.

In conclusion, we have described for the first time a SAM that induces all the desired characteristics for PME studies: a high surface coverage, a reversible electrochemical behaviour, and an effective control of electrochemical dispersion without shift in the protein redox potential. Such characteristics suggest that TBA modification of gold electrodes provide an adequate platform for biosensors and bioelectronic devices. In addition, the fractal description of the interacting surfaces at the interface provides a simple and reliable method to quantify the degree of roughness of real surfaces.

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