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Electrochemistry, Surface Plasmon Resonance, and Quartz Crystal Microbalance: An Associative Study on Cytochrome *c* Adsorption on Pyridine Tail-Group Monolayers on Gold

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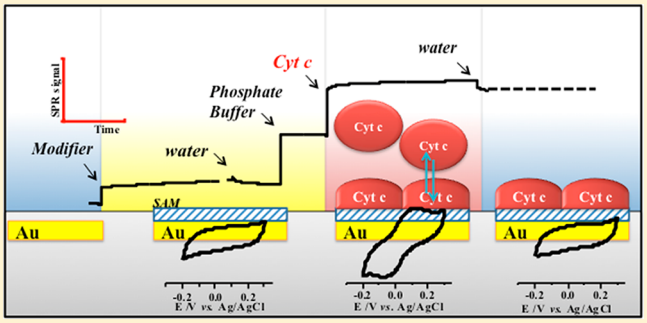
Supporting Information

ABSTRACT: Quartz crystal microbalance (QCM), surface plasmon resonance (SPR), and electrochemistry techniques were used to study the electron-transfer (ET) reaction of cytochrome *c* (Cyt *c*) on gold surfaces modified with thionicotinamide, thioisonicotinamide, 4-mercaptopyridine, 5-(4-pyridyl)-1,3,4-oxadiazole-2-thiol, 5-phenyl-1,3,4-oxadiazole-2-thiol, 4,4′-bipyridine, and 4,4′-dithiopyridine. The electrochemical results showed that the ET process is complex, being chiefly diffusional with steps depending on the orientation of the pyridine or phenyl tail group of the modifiers. The correlation between the electrochemical results and those acquired by SPR and QCM indicated the presence of an adlayer of Cyt *c* adsorbed on the thiolate SAMs. This adlayer, although being not electroactive, is essential to assess the ET reaction of Cyt *c* in solution. The results presented in this work are consistent with the statement (Feng, Z. Q.; Imabayashi, S.; Kakiuchi, T.; Niki, K. *J. Electroanal. Chem.* **1995**, *394*, 149−154) that the ET reaction of Cyt *c* can be explained in terms of the through-bond tunneling mechanism.



INTRODUCTION

Several experimental approaches in biological studies, clinical diagnosis, drug discovery, and proteomics require the immobilization of proteins on surfaces.[1−8] Moreover, redox proteins have been used as basis for analytic biosensors and in investigations on renewable energy technologies.[9,10] However, the immobilization of proteins on surfaces is often complicated due to the intrinsically low conformational stability. Upon immobilization, the protein can be denatured resulting in nonspecific protein binding. For mammalian cytochrome *c* (Cyt *c*) proteins, for instance, there are several papers showing the irreversible adsorption on metallic electrodes (Hg, Pt, Au) with consequent denaturation.[11−17] Cyt *c* is a redox protein that plays essential roles in bioenergetics, drug metabolism, and respiration and cell apoptosis of living organisms.[18−23] This metalloprotein belongs to the class of heme-containing proteins that are ubiquitous throughout living organisms since they carry out electron-transfer reactions in energy-related process. These reactions, in turn, take place along a potential gradient which are related to the reduction potential of the cofactors. Therefore, to study the electron transfer of Cyt *c* is crucial for the understanding of such mechanism and, in this sense, electrochemistry becomes a powerful tool. However, this technique requires the use of electrodes on which, as mentioned above, the protein may adsorb resulting in denaturation. Since the pioneering work of Hill[24] in 1977 on the assessment of the heterogeneous electron transfer (hET) reaction of Cyt *c* by using a SAM (self-assembled monolayer) formed with 4,4′-bipyridine (4,4′-bpy) on gold, several sulfur-containing molecules have been used as promoters.[25−39] The majority of the reported results are on diffusional redox processes with formal redox potentials (E') around 0.0 V vs Ag/AgCl which is consistent with the native form of the protein. Based on the reported data, a general conclusion was addressed; i.e., bifunctional molecules X−Y should be used where X represents a surface-active group that binds to the Au electrode surface and Y represents a group capable of interacting with the protein in solution. In addition, the chemical properties of the Y fragment should allow an optimum orientation of the protein in such a way that the heme group is close enough to the interface for the ET reaction to occur. Manipulation of the chemical properties of these groups can

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provide more information about the nature of the Cyt *c* ET process. If carboxylic acid terminated SAMs are used, for instance, strong adsorption of the highly positively charged protein on the SAM is found with a consequent observation of nondiffusional electrochemistry responses.[40−42] In such cases, it is suggested that the surface lysines of the protein mediate electrostatic interactions with the SAM.[43,44]

In modern bioelectrochemistry it is considered a primary goal to understand how and where exactly the interaction is established between the SAM and protein and, furthermore, how the electron travels from/to the heme group to/from the electrode. Despite the number of studies of Cyt *c* electrochemistry that have been devoted to understanding the mechanism of hET reaction, this process is not yet fully understood. In 2000, Fedurco[39] anticipated the necessity of systematic adsorption studies using techniques such as quartz crystal microbalance (QCM) to establish in which cases Cyt *c* undergoes adsorption on promoter-covered surfaces and, also, where such adsorption is completely absent. In addition, it has been pointed out that such studies would be especially helpful in the case of phenyl-terminated SAMs, where the lack of an electrochemical signal for the Cyt *c* could be due either to irreversible protein adsorption on the electrode surface (and denaturation), or to poor electronic coupling between the metalloprotein and the electrode covered by hydrophobic film with a low degree of wettability.

QCM and surface plasmon resonance (SPR) techniques are very effective due to the fact that they allow the real time monitoring of the mass variation of a given adsorbed species. We present in this article the results obtained by SPR and QCM for the adsorption of Cyt *c* on SAMs composed by thionicotinamide (TNA), thioisonicotinamide (iTNA), 4-mercaptopyridine (pyS), 5-(4-pyridyl)-1,3,4-oxadiazole-2-thiol (Hpyt), and 5-phenyl-1,3,4-oxadiazole-2-thiol (POT) on gold. We have used cyclic voltammetry with concomitant use of SPR and QCM in order to study the redox properties of Cyt *c*. In addition, an associative study of SPR and cyclic voltammetry was also carried out on some of the first molecules used as promoters by Hill[24] to assess the hET reaction of Cyt *c*: namely 4,4′-bipyridine (4,4′-bpy) and 4,4′-dithiodipyridine (pySSpy).

MATERIALS AND METHODS

Chemicals. Horse heart cytochrome *c* (type VI, 99%, Aldrich Co.) was purified as described elsewhere.[45] Thionicotinamide (TNA), thioisonicotinamide (iTNA), 4-mercaptopyridine (pyS), 5-(4-pyridyl)-1,3,4-oxadiazole-2-thiol (Hpyt), 5-phenyl-1,3,4-oxadiazole-2-thiol (POT), 4,4′-bipyridine (bpy), 4,4′-dithiopyridine (pySSpy), K_2HPO_4, KH_2PO_4, H_3PO_4, and KOH were purchased from the Aldrich Chemical Co. and used as received. Aqueous solutions were prepared using Millipore water of at least 18 MΩ cm^{-1} resistivity. Phosphate buffer solution (PBS) was used as supporting electrolyte at an ionic strength of μ = 0.1 mol L^{-1} prepared from K_2HPO_4 and KH_2PO_4. The pH was adjusted with either phosphoric acid or KOH and monitored with a pH meter.

Apparatus. AT-cut quartz crystals (5 MHz) of 24.5 mm diameter with gold electrodes deposited over a Ti adhesion layer (Maxtek Co.) were used for QCM measurements. An asymmetric keyhole electrode arrangement was used in which the circular electrode geometrical areas were 1.370 cm^2 (front side) and 0.317 cm^2 (back side). The quartz crystal resonator was set in a probe (TPS-550, Maxtek) made of Teflon in which the oscillator circuit was included. The electrolyte solutions were thermostatted at 25.0 °C by a water-jacketed beaker connected to a thermostatted bath (Digital Temperature Controller 9101, Fisher Scientific). Argon was used to degas the solutions before use and flowed over the solutions during experiments. The frequency was measured with a Research Quartz Crystal Microbalance (RQCM) from Maxtek, Inc. that was interfaced to a desktop computer. The mass changes (Δm) per unit area on the QCM sensor surface were calculated from the frequency shift (Δf) by using the Sauerbrey equation,[46] $\Delta m = C_f \Delta f$, where C_f (17.7 ng Hz^{1-} cm^{-2}) is the proportionality constant for the 5 MHz crystals used in this study.

Surface plasmon resonance measurements were acquired on a double channel Autolab ESPRIT instrument (Eco Chemie, The Netherlands) by using bare planar gold discs purchased from Eco Chemie. The rinsing and the sample solutions were injected in the cell using an autosampler equipped with a peristaltic pump. The pumping rate was set to 100 μL/min and the volume of the flow cell was 150 μL. The solution flow was programmed to stop during the adsorption steps. After the bare gold sensor disc was placed onto the prism of SPR system, its surface was cleaned with ethanol to remove all organic contaminants. The mass of the adsorbed species was calculated by SPR based on the relation[47,48] that a change of 122 mdeg (millidegrees) corresponds to 1.0 ng mm^{-2} at 25 °C.

Cyclic voltammetric measurements were carried out on a computer-controlled potentiostat PGSTAT 302N (Autolab, EcoChemie, Switzerland) by using QCM or SPR cells with QCM or SPR gold sensors and coiled platinum wires as working and auxiliary electrodes, respectively. Unless otherwise noted, all potentials are referenced versus a Ag/AgCl (3.5 mol L^{-1} KCl, Bioanalytical Systems Inc., BAS) electrode. The supporting electrolyte was purged with high-purity argon for 20 min prior to experiments, and the inert atmosphere was maintained during all the experiments with a blanket of argon gas delivered via a tube above the solution.

Electrode Modification. The modification of the gold quartz crystals with TNA, iTNA, Hpyt, and pyS species was carried out as described in the literature.[32,35,49−51] For the QCM measurements, first the gold sensors were modified by immersion in 1.0 × 10^{-4} mol L^{-1} aqueous solutions of the sulfur compounds for 30 min. After that, the modified gold sensors were washed, rinsed, and immersed in the cell containing 0.1 mol L^{-1} PBS. After stabilization of the signal (ca. 10 min), Cyt *c* dissolved in PBS was injected into the cell through a gastight syringe to a final concentration of 100 μmol L^{-1}. Although the injection sometimes caused a sudden frequency change, this parameter returned to its original value within a few seconds.

For the SPR measurements, water was injected into the cell until stabilization of the signal was achieved. After that, an aqueous solution of the modifier (1.0 × 10^{-4} mol L^{-1}) was injected and the signal was monitored until a steady state was reached whereupon water was injected for 5 min to remove loosely adsorbed molecules. This step was followed by injection of 0.1 mol L^{-1} PBS for stabilization of the baseline before the addition of 100 μmol L^{-1} Cyt *c* in this buffer solution.

RESULTS AND DISCUSSION

Electrochemistry. The redox behavior (rate and potential of electron transfer between the modified surface and the ferric/ferrous heme couple) of Cyt *c* has been used to provide qualitative information on conformation and/or orientation of the protein on the electrode surface.[15,38,39,52] Based on cyclic

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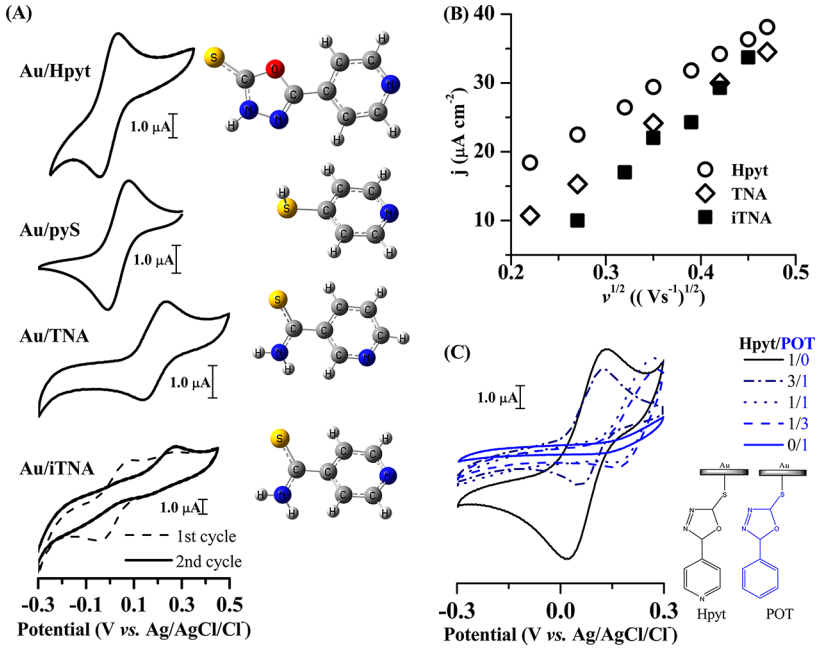

Figure 1. (A) CVs (0.1 V s^{-1} sweep rate) of the gold crystals modified with Hpyt, pyS, TNA, and iTNA; (B) plots of j vs $v^{1/2}$; and (C) CVs (0.1 V s^{-1} sweep rate) of the gold crystals modified with Hpyt and POT at different proportions. Electrolyte solution: 0.10 mol L^{-1} PBS (pH = 7) containing 100 μmol L^{-1} Cyt *c*.

voltammetry technique, the values of formal potentials (E'), which were calculated from the average between the anodic and cathodic peak potentials, that diverge from 0.0 V vs Ag/AgCl have been assigned to configurations that do not favor the electron transfer between the surface and the protein. In limiting situations, often caused by the irreversible adsorption of the protein on metallic surfaces, large changes in conformation have been reported leading, in most cases, to the denaturation of the protein.[11,15−19,28,29,51]

Figure 1 shows the cyclic voltammograms (CVs) of the modified electrodes in Cyt *c* solution as well as the structure of the surface-attached modifier molecules.

As can be ascertained from Figure 1, the redox behavior of Cyt *c* is dependent not only on the presence of the pyridine tail group of the SAMs but also on their orientation, which is slightly different in each molecule. For Hpyt and pyS SAMs, the E' values are observed at 0.008 and 0.03 V vs Ag/AgCl, respectively, indicating the native form of the metalloprotein is present.[24] In addition, the difference between the anodic and cathodic peak potentials (ΔE_p) were 62.4 and 83.0 mV for Hpyt and pyS SAMs, respectively, indicating a fast kinetics of electron transfer for the Cyt *c* protein. For TNA SAM, the $E_{1/2}$ value is observed at 0.16 V which, according to Whitesides et al.,[52] can be due to the presence of positive charges close to the heme moiety. In fact, recently reported results[50] indicated that the surface pK_a of TNA on gold is 5.0 and 8.5 for the N atom of the pyridine ring and the NH_2 group, respectively. This result indicates that, at physiological medium, the latter fragment is protonated. Therefore, in comparison to Hpyt and pyS SAMs, the positive charge density of the TNA SAM would explain the anodic shift of the E' value of Cyt *c*. For the iTNA SAM, the result does not allow a conclusive assignment since the voltammograms change from quasireversible at the first cycle to irreversible in subsequent cycles. A possible explanation for this result was recently raised based on the correlation between surface enhanced Raman scattering (SERS) spectroscopy and DFT calculations.[53] Accordingly, at physiological pH, both the ring N-atom and the NH_2 groups of iTNA are protonated on the surface. In such a situation, besides the increase in the positive charge density, the formation of hydrogen bonding between the adjacent molecules should be taken in account. Therefore, the more densely packed and charged SAM may be blocking the ET reaction of Cyt *c*, thus explaining the degradation of the voltammograms.

The plots of current density (j) as a function of the square root of the scan rate ($v^{1/2}$) were constructed from the CVs of the modified electrodes in solution containing Cyt *c* and are illustrated in Figure 1B. For the SAM formed with iTNA species, the anodic current was used since no cathodic wave is observed after the first cycle. Except for the iTNA SAM, the linear dependence of j on $v^{1/2}$ indicates a diffusion-controlled process for the electrode reaction of Cyt *c*. In addition, no redox process was observed when the modified electrodes were transferred to an electrochemical cell containing only the electrolyte solution, suggesting that the protein is not adsorbed to the SAMs. For the pyS SAM, this plot was not constructed due to the instability of this monolayer, which decomposes to give adsorbed elemental sulfur on gold thereby inactivating the surface for assessment of the protein hET reaction.[29,38,49,51,54]

In relation to the terminal (solution facing) end of the SAMs, the CVs obtained with mixed SAMs of Hpyt and POT (Figure 1C) clearly show that the presence of the pyridyl N atom is conditional for the ET reaction of Cyt *c* in solution since for the homogeneous SAM of POT (solid blue line) no peaks are observed in the CV. The mixed SAMs were prepared by immersing the gold electrodes in solutions containing various mixtures of Hpyt and POT assuming that the mole fraction on the surface correlates to that in solution. By increasing the composition of Hpyt on surface, a decrease in the cathodic-to-anodic peak separation is observed as well as an increase in the faradaic current, indicating an increase of electron-transfer rate.

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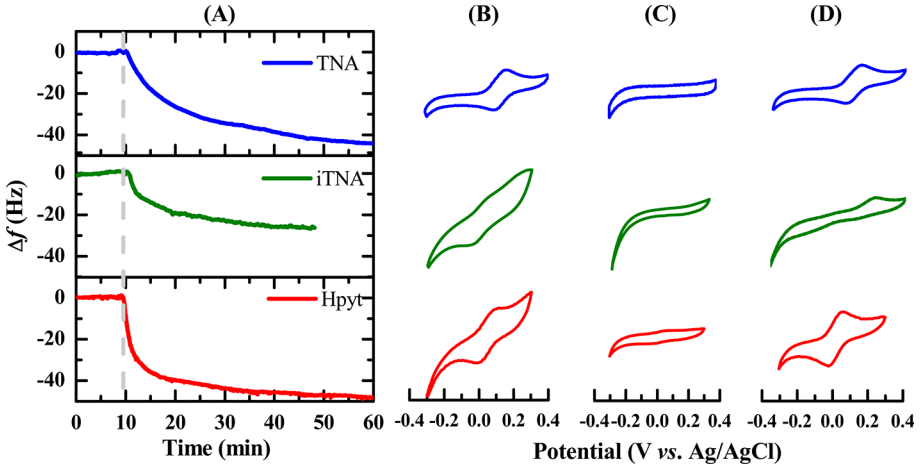

Figure 2. (A) Plots of frequency shifts (Δf, Hz) vs immersion time (min) for the adsorption of Cyt *c* on gold quartz crystals modified with TNA, iTNA, and Hpyt. CVs (sweep rate 0.1 V s^{-1}) of the gold crystals modified with TNA, iTNA, and Hpyt after reaching a steady state in (B) 0.10 mol L^{-1} PBS containing 100 μmol L^{-1} Cyt *c*, (C) 0.10 mol L^{-1} PBS alone, and (D) 0.10 mol L^{-1} PBS containing 100 μmol L^{-1} Cyt *c*.

Table 1. Values of Δm Calculated by QCM ($\Delta m_{[QCM]}$) and SPR ($\Delta m_{[SPR]}$) for the Adsorption of Cyt *c* on pyS, iTNA, TNA, and Hpyt SAMs on Gold

SAM	pyS	iTNA	TNA	Hpyt
$\Delta m_{[QCM]}$ (μg cm^{-2})[a]	0.430 ± 0.11	0.470 ± 0.07	0.710 ± 0.05	0.880 ± 0.05
$\Delta m_{[SPR]}$ (μg cm^{-2})[b]	0.175 ± 0.075	0.197 ± 0.003	0.340 ± 0.010	0.350 ± 0.090
$\Delta m_{[QCM]}/\Delta m_{[SPR]}$	2.46	2.39	2.09	2.51

[a] $\Delta m_{[QCM]}$: $\Delta m = C_f \Delta f$. [b] $\Delta m_{[SPR]}$: 122 mθ corresponds to 1.0 ng mm^{-2}.

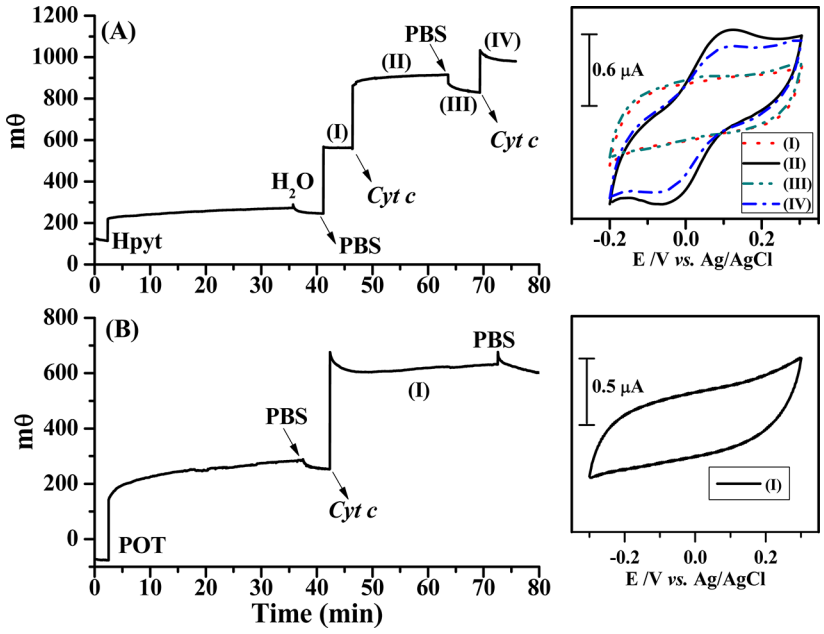

Figure 3. SPR responses as a function of time following injection of Hpyt (A) and POT (B), followed by sequential injections of solutions of PBS, Cyt *c*, PBS, and finally Cyt *c*. Insets: CVs at 0.1 V s^{-1} obtained according to the marked injection.

Quartz Crystal Microbalance (QCM). By accounting that the adsorbed mass is no greater than 2% of the mass of the quartz crystal, it is assumed a rigid model and, therefore, the Sauerbrey equation is applicable for the calculation of mass changes by means of QCM.[55,56] In addition, the systems studied in this work presented a behavior of a pure elastic film in which, as expected, no change in the resonant resistance is observed as can be seen in Figure S1 of the Supporting Information.

Once modified, the gold substrates were transferred into the QCM cell filled with PBS solution. After stabilization of the frequency, which takes ca. 10 min, Cyt *c* solution was injected to a final concentration of 100 μmol L^{-1}. Figure 2A shows a decrease in the frequency with time for the gold quartz crystals modified with TNA, iTNA, and Hpyt.

The decrease of the frequency upon the addition of Cyt *c* solution indicates the adsorption of this metalloprotein on the modified substrates. For all the modified electrodes, a rapid

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decrease is observed during the first 10 min followed by a gradual decrease that took about 30 min, after which a steady state was reached. Table 1 presents the values for the masses of Cyt *c* adsorbed on the modified gold crystals, which were calculated based on the Sauerbrey[46] equation.

The values of $\Delta m_{[QCM]}$ calculated for the adsorption of Cyt *c* in this work range from 0.43 to 0.88 μg cm^{-2} and are higher than the theoretical value reported for a full monolayer coverage,[48] based on the crystal structure of Cyt *c* (15 pmol cm^{-2} corresponding to 0.2 μg cm^{-2}). This excess of mass can be due to multilayers of Cyt *c* or, more likely, to the adsorption of water molecules and/or ions in solution.[57,58]

The CVs illustrated in Figure 2B were obtained in the QCM cell just after the frequency steady state was reached, ca. 50 min of immersion in PBS solution containing Cyt *c*. Then, the apparatus was washed and filled with PBS solution and the CVs illustrated in Figure 2C were acquired. In such case, no peaks assigned to Cyt *c* redox process were observed. Again, the QCM cell was filled with PBS solution containing Cyt *c* and the set of CVs showed in Figure 2D were obtained. As there was no increase in frequency that would indicate a loss of mass, these results suggest that there is an adlayer of Cyt *c* on the modified electrodes that mediate ET in the presence of Cyt *c* molecules in solution.

Surface Plasmon Resonance (SPR). The adsorption process of Cyt *c* on gold surfaces previously modified with pyS, iTNA, TNA, Hpyt, and POT was also followed by SPR. Figure 3 shows the SPR sensorgrams for the adsorption of Cyt *c* on Hpyt and POT SAMs. The CVs obtained during the monitoring are presented as insets. In comparison to the data obtained for the SAM formed with Hpyt, similar sensorgrams were observed for the SAMs formed with TNA, iTNA, and pyS and are not shown here.

The first part of the SPR sensorgram presents the variation of angle related to the adsorption of Hpyt and POT on gold (from 2 to 45 min) where the difference between the final and initial angles indicates the modification of the surface. The formation of the SAMs was followed by the injection of PBS (from 40 to 45 min) and then by the injection of Cyt *c*, where it can be observed as a significant perturbation on the SPR signal followed by a gradual increase during the incubation time (from 45 to 65 min for Hpyt and from 42 to 72 min for POT). After that, a slight decrease in the SPR signal is observed during the injection of the buffer solution. The signal, however, reaches a steady state at a plateau different from the one before Cyt *c* was injected indicating the adsorption of the protein on the studied SAMs. The mass values of Cyt *c* adsorbed on each SAM are displayed in Table 1. For the SAM formed with POT, the mass of Cyt *c* was only determined by SPR as 0.29 μg cm^{-2} (2.3×10^{-11} mol cm^{-2}). Comparing the data presented in Table 1, it can be ascertained that the values of Δm determined by QCM are ca. 2.4 times higher than those obtained by SPR. In fact, it is well-known that the mass change detected via SPR measurements is the molecular mass of the analyte while the mass change determined by QCM is the total mass of the system, i.e., all particles that are attached to the oscillator. Following this reasoning, one can conclude that the adsorption process of Cyt *c* is accompanied by a greater number of water molecules and/or electrolyte ions when pyS and Hpyt are the modifier species.

Based on the analysis of the CVs (insets in Figure 3) together with the SPR sensorgrams, three very important conclusions can be addressed: (1) the presence of the N atom as functional terminal end is conditional for the hET reaction since no redox peaks are observed for the POT SAM even after Cyt *c* adsorption; (2) the redox process of Cyt *c* is only that of the species in solution; (3) there is an adlayer of Cyt *c* on the SAMs that is electroinactive in the absence of Cyt *c* in solution.

These conclusions are in agreement with the electrochemical results in which the electrode reaction assigned to the $Fe^{III/II}$ redox couple of Cyt *c* was related to the electroactive species in solution (Figure 1). However, considering the results all together, one can conclude that there is an electroinactive adlayer of Cyt *c* on the SAMs and that the redox reaction of the protein in solution is only observed in the presence of this adlayer.

According to the literature,[15,59,60] two mechanisms are likely to be simultaneously operative for a heterogeneous electrode reaction involving metalloproteins: (1) "rapid and reversible binding (RRB)" mechanism where the adsorption of the protein molecules is weak. In such case, although both adsorption and desorption are rapid the protein molecule is oriented on surface in such a manner to allow the electron transfer to occur; (2) "adsorbed protein electron exchange (APEE)" mechanism, where the adsorption is so strong that the voltammetric response stems from molecules that do not exchange with those in the bulk solution. In such a mechanism, electrons are transferred indirectly between the electrode and the bulk protein molecules by passage through a layer of strongly adsorbed protein. Based on the results discussed in this paper, besides the *sine qua non* condition of a nonelectroactive adlayer of Cyt *c*, there is a dependence of the redox response of Cyt *c* not only on the existence of the N atom at the terminal end of the SAM but also on the configuration of the SAM on the surface. This experimental observation, therefore, strongly supports the through-bond-tunneling mechanism.

4,4′-Bpy and PySSpy. Aiming to get insights into the understanding of the redox process of Cyt *c*, SPR measurements were carried out using the SAMs formed with 4,4′-bipyridine (4,4′-bpy) and 4,4′-dithiodipyridine (pySSpy). These compounds were the very first species used to modify gold surfaces to assess the redox process of Cyt *c*.[24,25] Figure 4 shows the SPR sensorgrams, which were obtained by following the same protocols of those illustrated in Figure 3.

The SPR sensorgram obtained for the SAM formed with pySSpy was similar to those obtained for the SAMs formed with Hpyt (Figure 3). Adsorption of pySSpy on gold is suggested[61] to occur through pyS after the reduction of the disulfide bond by Au at the electrode surface, so the resulting CV naturally mirrors those obtained with pySH (cf. Figures 1 and 2). Accordingly, the ET reaction of Cyt *c* in solution is observed with an adlayer of Cyt *c* on the pySSpy SAM. For the SAM formed with 4,4′-bpy, the profile is apparently the same when PBS buffer and Cyt *c* are added (without washing), as can be seen in Figure 4b. However, after injection of water (Figure 4c), the SPR signal returned to its initial value, indicating that the 4,4′-bpy monolayer is destroyed by washing with pure water (but not with PBS) in accordance with previously reported data.[23] After that, the SPR signal increased after injection of PBS and Cyt *c* solutions. The fact that the SPR signal virtually does not change after the last buffer injection indicates that Cyt *c* is directly adsorbed on the bare gold electrode. In addition, no redox peaks attributable to Cyt *c* are observed even with the evidence of the adsorption of the protein on surface. Such a result corroborates the conclusion of Bond et al.[17] in the sense that the adsorption of Cyt *c* on the

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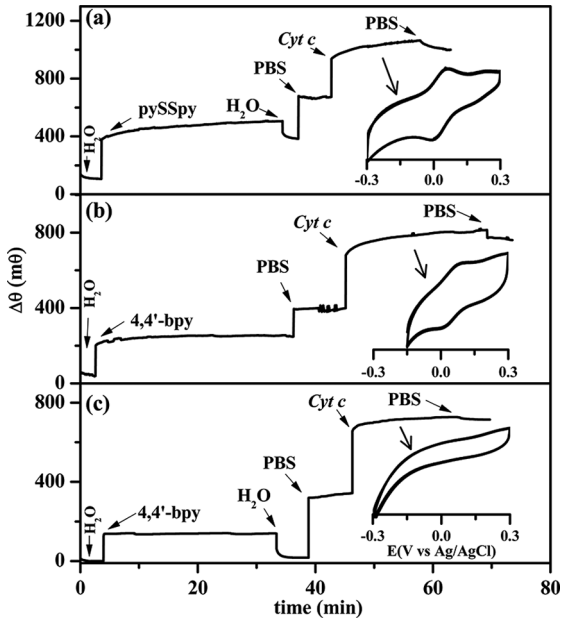

Figure 4. SPR responses in function of time following injection of (a) pySSpy and (b,c) 4,4′-bpy, followed by the injections of water and solutions of PBS and Cyt *c* as indicated by the arrows. Insets: CVs at 0.1 V s^{-1} obtained in PBS solution containing Cyt *c*.

bare metal surface inhibits the ET reaction due to the protein denaturation and fouling.

CONCLUSIONS

The redox process of the metalloprotein cytochrome *c* (Cyt *c*) was studied by using gold electrodes modified with 4-mercaptopyridine (pySH), thionicotinamide (TNA), thioisonicotinamide (iTNA), 5-(4-pyridyl)-1,3,4-oxadiazole-2-thiol (Hpyt), 5-phenyl-1,3,4-oxadiazole-2-thiol (POT), 4,4′-bipyridine (bpy), and 4,4′-dithiopyridine (pySSpy). The electrochemical results showed that the redox process is diffusional and that it is dependent on the orientation of the pyridine tail group of the modifiers. The correlation between the electrochemical results and those acquired by SPR and QCM indicated that there is an adlayer of Cyt *c* on the studied SAMs, which alone is electroinactive. However, to assess the redox process of this metalloprotein, the results all together suggest that it is imperative that this electroinactive adlayer of Cyt *c* is present. In addition, SPR and QCM data showed that all the SAMs, except 4,4′-bpy, are stable on surface during the time scale of the measurements. For the SAM formed with 4,4′-bpy, desorption from the surface is facile if the electrode is washed with water.

According to Bond et al.,[17] the adsorption of Cyt *c* on metal surfaces inhibits the ET reaction due to the protein denaturation. Therefore, the adsorption of Cyt *c* on the protein-friendly pyridyl-functionalized SAMs avoids conformational changes and also the protein adlayer does not inhibit the ET reaction. Our hypothesis is that the electron-transfer pathway from/to Cyt *c* to/from the gold surface involves not only the orbitals of the adsorbed protein but also those of the modifier species.

This statement reinforces the suggestion[16] that the redox reaction of Cyt *c* can be explained in terms of a through-bond tunneling mechanism.

ASSOCIATED CONTENT

Supporting Information

Figure S1 shows the plot of resonant resistance in function of resonant frequency for the Cyt *c* film formed on a quartz crystal coated with Hpyt SAM. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Zheng, G.; Patolsky, F.; Cui, Y.; Wang, W. U.; Lieber, C. M. Multiplexed Electrical Detection of Cancer Markers with Nanowire Sensor Arrays. *Nat. Biotechnol.* **2005**, *10*, 1294−1301.

(2) Aguey-Zinsou, K. F.; Bernhardt, P. V.; Kappler, U.; McEwan, A. G. Direct Electrochemistry of a Bacterial Sulfite Dehydrogenase. *J. Am. Chem. Soc.* **2003**, *125*, 530−535.

(3) Shukla, A.; Gillam, E. M.; Mitchell, D. J.; Bernhardt, P. V. Direct Electrochemistry of Enzymes from the Cytochrome P450 2C Family. *Electrochem. Commun.* **2005**, *7*, 437−442.

(4) Creevey, N. L.; McEwan, A. G.; Bernhardt, P. V. A Mechanistic and Electrochemical Study of the Interaction Between Dimethyl Sulfide Dehydrogenase and its Electron Transfer Partner Cytochrome c2. *J. Biol. Inorg. Chem.* **2008**, *13*, 1231−1238.

(5) Labaer, J.; Ramachandran, N. Protein Microarrays as Tools for Functional Proteomics. *Curr. Opin. Chem. Biol.* **2005**, *9*, 14−19.

(6) Kingsmore, F. S. Multiplexed Protein Measurement: Technologies and Applications of Protein and Antibody Arrays. *Nat. Rev. Drug Discov.* **2006**, *5*, 310−320.

(7) Lee, J. H.; Wark, A. W.; Corn, R. M. Microarray Methods for Protein Biomarker Detection. *Analyst* **2008**, *133*, 975−983.

(8) Weinrich, D.; Jonkheijm, P.; Niemeyer, C. M.; Waldmann, H. Applications of Protein Biochips in Biomedical and Biotechnological Research. *Angew. Chem., Int. Ed.* **2009**, *48*, 7744−7751.

(9) Léger, C.; Bertrand, P. Direct Electrochemistry of Redox Enzymes as a Tool for Mechanistic Studies. *Chem. Rev.* **2008**, *108*, 2379−2438.

(10) Armstrong, F. A.; Belsey, N. A.; Cracknell, J. A.; Goldet, G.; Parkin, A.; Reisner, E.; Vincent, K. A.; Wait, A. F. Dynamic Electrochemical Investigations of Hydrogen Oxidation and Production by Enzymes and Implications for Future Technology. *Chem. Soc. Rev.* **2009**, *38*, 36−51.

(11) Hinnen, C.; Parsons, R.; Niki, K. Electrochemical and Spectroreflectance Studies of the Adsorbed Horse Heart Cytochrome c and Cytochrome c_3 from D. Vulgaris, Miyazaki Strain, at Gold Electrode. *J. Electroanal. Chem.* **1983**, *147*, 329−337.

(12) Scheller, F. Functional Properties of Adsorbed Hemoproteins. *Bioelectrochem. Bioenerg.* **1977**, *4*, 490−499.

(13) Kuznetsov, B. A.; Shumakovich, G. P.; Mestechkina, N. M. The Reduction Mechanism of Cytochrome c and Methemoglobin on the Mercury Electrode. *Bioelectrochem. Bioenerg.* **1977**, *4*, 512−521.

(14) Haladjian, J.; Bianco, P.; Serre, P. -A. 296 - Role of Adsorption in the Electrochemical Behaviour of Cytochrome c at the Mercury Electrode. *Bioelectrochem. Bioenerg.* **1979**, *6*, 555−561.

(15) Armstrong, F. A. Probing Metalloproteins by Voltammetry. *Struct. Bonding (Berlin)* **1990**, *72*, 137−230.

(16) Feng, Z. Q.; Imabayashi, S.; Kakiuchi, T.; Niki, K. J. Electroreflectance Spectroscopic Study of the Electron Transfer Rate

of Cytochrome c Electrostatically Immobilized on the ω-carboxyl Alkanethiol Monolayer Modified Gold Electrode. *Electroanal. Chem.* **1995**, *394*, 149−154.
(17) Hill, H. A. O.; Hunt, N. H.; Bond, A. M. The Transient Nature of the Diffusion Controlled Component of the Electrochemistry of Cytochrome c at "bare" Gold Electrodes: an Explanation Based on a Self-blocking Mechanism. *J. Electroanal. Chem.* **1997**, *436*, 17−25.
(18) Reed, D. E.; Hawkridge, F. M. Direct Electron Transfer Reactions of Cytochrome c at Silver Electrodes. *Anal. Chem.* **1987**, *59*, 2334−2339.
(19) Hawkridge, F. M.; Taniguchi, I. The Direct Electron Transfer Reactions of Cytochrome c at Electrode Surfaces. *Comments Inorg. Chem.* **1995**, *17*, 163−187.
(20) Dickerson, R. E.; Timkovich, R. *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. *XIa*, pp 397−547.
(21) Lee, M. W.; Park, S. C.; Yang, Y. G.; Yim, S. O.; Chae, H. S.; Bach, J. H.; Lee, H. J.; Kim, K. Y.; Lee, W. B.; Kim, S. S. The Involvement of Reactive Oxygen Species (ROS) and p38mitogen-Activated Protein (MAP) Kinase in TRAIL/Apo2L-induced Apoptosis. *FEBS Lett.* **2002**, *512*, 313−318.
(22) Ferguson, H. A.; Marietta, P. M.; Van den Berg, C. L. UV-induced Apoptosis Is Mediated Independent of Caspase-9 in MCF-7 Cells: A Model for Cytochrome c Resistance. *J. Biol. Chem.* **2003**, *278*, 45793−58004.
(23) Hüttemann, M.; Pecina, P.; Rainbolt, M.; Sanderson, T. H.; Kagan, V. E.; Samavati, L.; Doan, J. W.; Lee, I. The multiple Functions of Cytochrome c and their Regulation in Life and Death Decisions of the Mammalian Cell: From Respiration to Apoptosis. *Mitochondrion* **2011**, *11*, 369−381.
(24) Eddowes, M. J.; Hill, H. A. O. Novel Method for the Investigation of the Electrochemistry of Metalloproteins: Cytochrome c. *J. Chem. Soc., Chem Commun.* **1977**, *21*, 771−772.
(25) Taniguchi, I.; Toyosawa, K.; Yamaguchi, H.; Yasukouchi, K. J. Voltammetric Response of Horse Heart Cytochrome c at a Gold Electrode in the Presence of Sulfur Bridged Bipyridines. *J. Electroanal. Chem.* **1982**, *140*, 187−193.
(26) Allen, P. M.; Hill, H. A. O.; Walton, N. J. Surface Modifiers for the Promotion of Direct Electrochemistry of Cytochrome c. *J. Electroanal. Chem.* **1984**, *178*, 69−86.
(27) Diógenes, I. C. N.; Nart, F. C.; Moreira, I. S. Back-bonding Manifestation on the Stability of a Pyridinethiolate Surface Modifier at a Gold Electrode. *Inorg. Chem.* **1999**, *38*, 1646−1647.
(28) Diógenes, I. C. N.; Nart, F. C.; Temperini, M. L. A.; Moreira, I. S. The $[Ru(CN)_5(pyS)]^{4+}$ Complex, an Efficient Self-Assembled Monolayer for the Cytochrome c Heterogeneous Electron Transfer Studies. *Inorg. Chem.* **2001**, *40*, 4884−4889.
(29) Diógenes, I. C. N.; Sousa, J. R.; Carvalho, I. M. M.; Temperini, M. L. A. Self-Assembled Monolayers Formed by $[M(CN)_5(pyS)]^{4+}$ (M = Fe, Ru) on Gold: a Comparative Study on Stability and Efficiency to Assess the Cyt c Heterogeneous Electron Transfer Reaction. *Dalton Trans.* **2003**, *11*, 2231−2236.
(30) Sousa, J. R.; Parente, M. M. V.; Diógenes, I. C. N.; Lopes, L. G.; Lima-Neto, P.; Temperinni, M. L. A.; Moreira, I. S. A Correlation Study Between the Conformation of the 1,4-dithiane SAM on Gold and its Performance to Assess the Heterogeneous Electron-Transfer Reactions. *J. Electroanal. Chem.* **2004**, *566*, 443−449.
(31) Sousa, J. R.; Batista, A. A.; Diógenes, I. C. N.; Andrade, G. F. S.; Temperini, M. L. A.; Lopes, L. G.; Moreira, I. S. Characterization of a 1,4-dithiane Gold Self-Assembled Monolayer: an Electrochemical Sensor for the Cyt-c Redox Process. *J. Electroanal. Chem.* **2003**, *543*, 93−99.
(32) Paulo, T. F.; Silva, M. A. S.; Pinheiro, S. O.; Meyer, E.; Pinheiro, L. S.; Freire, J. A.; Tanaka, A. A.; Lima-Neto, P.; Moreira, I. S.; Diógenes, I. C. N. 5-(4-pyridinyl)-1,3,4-oxadiazole-2-thiol on Gold: SAM Formation and Electroactivity. *J. Braz. Chem. Soc.* **2008**, *19*, 711−719.
(33) Diógenes, I. C. N.; Nart, F. C.; Barreto, M. B. P. M.; Temperinni, M. L. A.; Moreira, I. S. SERRS Study of $[Ru(CN)_5(pyS)]^{4+}$ SAM and Cytochrome c: A Suggestion Toward the Heterogeneous Molecular Recognition. *J. Solid State Electrochem.* **2007**, *11*, 1585−1590.
(34) Pinheiro, S. O.; Silva, F. O. N.; Carvalho, I. M. M.; Lopes, L. G.; Temperini, M. L. A.; Andrade, G. F. S.; Moreira, I. S.; Diógenes, I. C. N. Tetraammine Ruthenate Complexes: Cationic SAMs for Cytochrome c Recognition. *J. Braz. Chem. Soc.* **2006**, *17*, 1594−1599.
(35) Paulo, T. F.; Pinheiro, S. O.; Silva, M. A. S.; Lopes, L. G.; Aquino, G. F.; Pinheiro, L. S.; Temperini, M. L. A.; Lima-Neto, P.; Diógenes, I. C. N. Thionicotinamide SAM on Gold: Adsorption Studies and Electroactivity. *Electroanalysis* **2009**, *21*, 1081−1089.
(36) Sousa, J. R.; Diógenes, I. C. N.; Temperini, M. L. A.; Sales, F. A. M.; Pinheiro, S. O.; Costa- Filho, R. N.; Andrade, J. S., Jr.; Moreira, I. S. Synthesis, Characterization, and SAMs Electroactivity of Ruthenium Complexes with Sulfur Containing Ligands. *J. Organomet. Chem.* **2007**, *692*, 3691−3699.
(37) Pinheiro, S. O.; Sousa, J. R.; Santiago, M. O.; Carvalho, I. M. M.; Silva, A. L. R.; Batista, A. A.; Castellano, E. E.; Ellena, J.; Moreira, I. S.; Diógenes, I. C. N. Synthesis, Characterization and Structure of Ruthenium(II) Phosphine Complexes with N-heterocyclic Thiolate Ligands. *Inorg. Chim. Acta* **2006**, *359*, 391−400.
(38) Armstrong, F. A; Wilson, G. S. Recent Developments in Faradaic Bioelectrochemistry. *Electrochim. Acta* **2000**, *45*, 2623−2645.
(39) Fedurco, M. Redox Reactions of Heme-Containing Metalloproteins: Dynamic Effects of Self-Assembled Monolayers on Thermodynamics and Kinetics of Cytochrome c Electron-Transfer Reactions. *Coord. Chem. Rev.* **2000**, *209*, 263−331.
(40) Davis, K. L.; Drews, B. J.; Yue, H.; Waldeck, D. H.; Knorr, K.; Clark, R. A. Electron-Transfer Kinetics of Covalently Attached Cytochrome c/SAM/Au Electrode Assemblies. *J. Phys. Chem. C* **2008**, *112*, 6571−6576.
(41) Leopold, M. C.; Bowden, E. F. Influence of Gold Substrate Topography on the Voltammetry of Cytochrome *c* Adsorbed on Carboxylic Acid Terminated Self-Assembled Monolayers. *Langmuir* **2002**, *18*, 2239−2245.
(42) Ashur, I.; Schulz, O.; McIntosh, C. L.; Pinkas, I.; Ros, R.; Jones, A. K. Transparent Gold as a Platform for Adsorbed Protein Spectroelectrochemistry: Investigation of Cytochrome *c* and Azurin. *Langmuir* **2012**, *28*, 5861−5871.
(43) Avila, A.; Gregory, B. W.; Niki, K.; Cotton, T. M. An Electrochemical Approach to Investigate Gated Electron Transfer Using a Physiological Model System: Cytochrome *c* Immobilized on Carboxylic Acid-Terminated Alkanethiol Self-Assembled Monolayers on Gold Electrodes. *J. Phys. Chem. B* **2000**, *104*, 2759−2766.
(44) Song, S.; Clark, R. A.; Bowden, E. F.; Tarlov, M. J. Characterization of Cytochrome c/Alkanethiolate Structures Prepared by Self-Assembly on Gold. *J. Phys. Chem.* **1993**, *97*, 6564−6572.
(45) Brautigan, D. L.; Ferguson-Miller, S.; Margoliash, E. Mitochondrial Cytochrome c: Preparation and Activity of Native and Chemically Modified Cytochromes c. *Methods Enzymol.* **1978**, *53*, 128−164.
(46) Sauerbrey, G. Use of Quartz Oscillators for Weighing Thin Layers and for Microweighing. *Z. Phys.* **1959**, *155*, 206−222.
(47) Malmqvist, M. Biospecific Interaction Analysis using Biosensor Technology. *Nature* **1993**, *361*, 186−187.
(48) Zubritsky, E. Product Review: New Choice for SPR. *Anal. Chem.* **2000**, *72*, 289A−292A.
(49) Lima-Neto, P.; Parente, M. M. V.; Moreira, I. S.; Diógenes, I. C. N.; Mattos, O. R.; Barcia, O. E.; Santos, R. P.; Freire, V. N. J. The Influence of 4-mercaptopyridine Layer Instability on Rapid Electron Transfer Reaction. *Electroanal. Chem.* **2008**, *619*, 26−30.
(50) Paulo, T. F.; Abruña, H. D.; Diógenes, I. C. N. Thermodynamic, Kinetic, Surface pK(a), and Structural Aspects of Self-Assembled Monolayers of Thio Compounds on Gold. *Langmuir* **2012**, *28*, 17825−17831.
(51) Lamp, B. D.; Hobara, D.; Porter, M. D.; Niki, K.; Cotton, T. M. Research Article Correlation of the Structural Decomposition and Performance of Pyridinethiolate Surface Modifiers at Gold Electrodes for the Facilitation of Cytochrome c Heterogeneous Electron-Transfer Reactions. *Langmuir* **1997**, *13*, 736−741.

(52) Chen, X.; Ferrigno, R.; Yang, J.; Whitesides, G. M. Redox Properties of Cytochrome c Adsorbed on Self-Assembled Monolayers: A Probe for Protein Conformation and Orientation. *Langmuir* **2002**, *18*, 7009−7015.
(53) Paulo, T. F.; Ando, R. A.; Diógenes, I. C. N.; Temperini, M. L. A. Understanding the Equilibria of Thio Compounds Adsorbed on Gold by Surface-Enhanced Raman Scattering and Density Functional Theory Calculations. *J. Phys. Chem. C* **2013**, *117*, 6275−6283.
(54) Diógenes, I. C. N.; Nart, F. C.; Temperini, M. L. A.; Moreira, I. S. The Stability Study of Pentacyano(4-mercaptopyridine)ruthenate (II) Self-Assembled Monolayer onto Gold Surface. *Electroanalysis* **2002**, *14*, 153−147.
(55) Cheng, C. I.; Chang, Y.-P.; Chu, Y.-H. Biomolecular Interactions and Tools for their Recognition: Focus on the Quartz Crystal Microbalance and its Diverse Surface Chemistries and Applications. *Chem. Soc. Rev.* **2012**, *41*, 1947−1971.
(56) Marx, K. A. Quartz Crystal Microbalance: A Useful Tool for Studying Thin Polymer Films and Complex Biomolecular Systems at the Solution-Surface Interface. *Biomacromolecules* **2003**, *4*, 1099−1120.
(57) Paulo, T. F.; Diógenes, I. C. N.; Abruña, H. D. Direct Electrochemistry and Electrocatalysis of Myoglobin Immobilized on L-Cysteine Self-Assembled Gold Electrode. *Langmuir* **2011**, *27*, 2052−2057.
(58) Takada, K.; Abruña, H. D. *In Situ* Quartz Crystal Microbalance Study of Self-Assembly and Mass Transfer Processes of a Redox-Active Osmium Complex. *J. Phys. Chem.* **1996**, *100*, 17909−17914.
(59) Eddowes, M. J.; Hill, H. A. O. Electrochemical and Spectrochemical Studies of Biological Redox Components. *ACS Adv. Chem. Ser.* **1982**, *201*, 103.
(60) Scheller, F.; Strnad, G. Electrochemical and Spectrochemical Studies of Biological Redox Components. *ACS Adv. Chem. Ser.* **1982**, *201*, 219.
(61) Taniguchi, I.; Iseki, M.; Yamaguchi, H.; Yasukouchi, H. Surface Enhanced Raman Scattering from bis(4-pyridyl)-disulfide- and 4,4′-bipyridine-Modified Gold Electrodes. *J. Electroanal. Chem.* **1985**, *186*, 299−307.