The $[Ru(CN)_5(pyS)]^{4-}$ Complex, an Efficient Self-Assembled Monolayer for the Cytochrome *c* Heterogeneous Electron Transfer Studies

Izaura Cirino Nogueira Diógenes,[†] Francisco Carlos Nart,[‡] Márcia Laudelina Arruda Temperini,[§] and Ícaro de Sousa Moreira*,[†]

Departamento de Química Orgânica e Inorgânica, Universidade Federal do Ceará, Cx. Postal 12200, Campus do Pici s/n, 60455-760 Fortaleza-CE, Brazil, Instituto de Química de São Carlos-USP, Cx. Postal 369, São Carlos-SP, Brazil, and Instituto de Química da Universidade de São Paulo-USP, Cidade Universitária, Cx. Postal 26077, 05599-970, São Paulo-SP, Brazil

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The organothiol 4-mercaptopyridine (pyS) has been used extensively as facilitator for the assessment of heterogeneous electron transfer reaction of cytochrome *c* (cyt *c*). Its efficiency, however, is strongly affected by the instability of the adlayer due to the C−S bond cleavage. The $K_4[Ru(CN)_5(pyS)]\cdot 3H_2O$ complex was synthesized and characterized aiming its utilization as an inorganic self-assembled monolayer (SAM) that would enhance the gold adlayer stability. The SAM formed by this complex onto gold (RupySAu) was characterized by spectroscopic (FTIRRAS and SERS) and electrochemical (LSV) techniques. The ex situ vibrational SERS and FTIRRAS spectra data of this SAM formed onto gold suggest a σ interaction between the gold and sulfur atoms of the complex, inducing a perpendicular arrangement in relation to the surface normal. Additionally, SERS and FTIRRAS spectra performed for freshly prepared RupySAu adlayer and for large immersion times in the precursor solution have not shown any significant change that would reflect the degradation of the adlayer. The LSV desorption curves of this SAM indicate an enhancement in the C−S bond strength of the pyS ligand when coordinated to the $[Ru(CN)_5]^{3-}$ moiety. Comparatively to the data obtained for the desorption process of the pyS monolayer, the reductive desorption potential, E_{rd}, of the RupySAu presents a shift of −17 mV. This bond strength intensification leads to an increase in the stability of the monolayer. The voltammetric curves of cyt *c* carried out with the RupySAu electrode showed electrochemical parameters consistent with those reported for the *native* protein, as well as the maintenance of the electrochemical kinetic data after repetitive cycles. The results all together suggest that the π back-bonding effect from the $[Ru(CN)_5]^{3-}$ metal center plays an important role in the stability of the RupySAu adlayer, improving the assessment of the cyt *c* heterogeneous electron transfer reaction.

Introduction

It is well-known that surface chemical modification is an effective method for the promotion of direct electron transfer of redox metalloproteins. Since the first successful use of 4,4′-bipyridyl, as a gold electrode modifier to promote the direct electrochemistry of horse heart cytochrome *c* (cyt *c*) metalloprotein,[1] various bifunctional organic compounds have been investigated in this field.[2] For this purpose, the molecular bifunctionality of the promoter is required concerning both the attachment to the substrate through its headgroup and the interaction with the protein ambient phase through its terminal functional group. The geometrical arrangement of the promoters in the surface-bound adlayer depends on the bond strength of the substrate headgroup, the size of the headgroups, and the intermolecular forces. As the bonding of the thiolate group to the gold surface is very strong[3] (ca. ∼40 kcal mol^{-1}), the 4-mercaptopyridine (pyS) and 4,4′-dipyridyl disulfide (pySS) have been used successfully as facilitators for the assessment of heterogeneous electron transfer of cyt *c*.[4,5]

The in situ scanning tunneling microscopy (STM) imaging of these monolayers formed onto Au(111) have suggested a vertical orientation of the molecular plane of pyridine rings, with the molecular axis of the pyridinethiolate being considerably tilted with respect to the surface normal.[6] The great efficiency of these promoters toward cyt *c* electrochemistry is probable due to the high ordering of the adlayers onto gold electrodes, plus the basicity of the nitrogen atom of the pyridine rings.[5]

In spite of the successful use of the pyS as a gold electrode modifier in the study of the cyt *c* electrochemistry, this ligand suffers a structural conversion on the substrate yielding monolayers composed of atomic and/or oligomeric sulfur species due to the C−S bond cleavage.[7,8] These resulting monolayers have not presented a voltammetric response for cyt *c*.[7]

The coordination of pyS ligand to a π-donor anionic transition metal complex would enhance the adlayer stability, as well as the assessment of the inherent rate of the cyt *c* electron transfer

[†] Universidade Federal do Ceará.
[‡] Instituto de Química de São Carlos-USP.
[§] Instituto de Química de São Paulo-USP.

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reaction. The $[Ru(CN)_5]^{3-}$ metal center was strategically selected, mainly due to the following properties: (i) it reacts readily with pyridine derivative ligands, binding the nitrogen atom,[9−12] leaving the sulfur atom available for further gold surface interactions; (ii) the derivative complexes are kinetically inert toward substitution reactions;[9,10] (iii) its hydrophilicity and negative charge, as well as the cyanide environment, facilitate the electrostatic interaction[13,14] with the hydrophilic positively charged lysine groups of the cyt *c*. These properties, along with the Ru^{II} π-back-bonding capability[15−17] that would enhance the C−S bond strength with consequent stability gain of the adlayer, well elect the $[Ru(CN)_5(pyS)]^{4-}$ complex for designing an inorganic ordered monolayer on gold substrate to improve the assessment of the cyt *c* heterogeneous electron transfer reaction.

Experimental Section

The water used throughout was purified from a Milli-Q water system (Millipore Co.). Potassium hexacyanoruthenate(II) trihydrate, from Alfa Chemical Co., was used as received. The organothiol ligand, 4-mercaptopyridine (pyS), KOH, and KH_2PO_4, from Aldrich, were used without further purification. The high-purity H_2SO_4, from Merck, was used as received. Horse heart cytochrome *c* (type VI, 99%, Aldrich Co.) was purified as described elsewhere.[18]

Synthesis of $K_4[Ru(CN)_5(pyS)]\cdot 3H_2O$. The $[Ru(CN)_5(H_2O)]^{3-}$ complex was generated in situ from the reaction of $[Ru(CN)_6]^{4-}$ with Br_2, during the synthesis of the $K_4[Ru(CN)_5(pyS)]\cdot 3H_2O$ complex.[10] A 47 mg sample of $K_4[Ru(CN)_6]\cdot 3H_2O$ was dissolved in 3 mL of water followed by the addition of a Br_2 aqueous solution (0.10 mM; 1 mM KBr), dropwise with stirring. After 20 min of reaction, an almost 3-fold excess (36 mg) of the 4-mercaptopyridine ligand dissolved in 6 mL of water was added dropwise, under a stream of argon. The resulting solution, which developed an intense brown color, was allowed to stand for 1 h to ensure complete reaction and cooled in an ice bath. Upon the slow addition of cold acetone, a red-brown precipitate was obtained and collected by filtration, washed with acetone and ethyl ether, dried, and stored under vacuum, in the absence of light. Anal. Calcd: C, 21.75; H, 1.83; N, 15.23. Found: C, 21.44; H, 1.80; N, 15.19.

Kinetic Measurements. The aquation reaction of $[Ru(CN)_5(pyS)]^{4-}$ complex was studied in the presence of a large excess of dimethyl sulfoxide (dmso) as auxiliary ligand, at 25 ± 0.2 °C, as follows:

$$[Ru(CN)_5(pyS)]^{4-} + H_2O \underset{k_{pyS}}{\overset{k_{-pyS},\ slow}{\rightleftharpoons}} [Ru(CN)_5(H_2O)]^{3-} + pyS^-$$

$$[Ru(CN)_5(H_2O)]^{3-} + dmso \overset{fast}{\rightleftharpoons} [Ru(CN)_5(dmso)]^{3-} + H_2O$$

An aliquot of the $[Ru(CN)_5(pyS)]^{4-}$ complex solution was added to a solution containing dmso, both previously degassed and thermostated at 25 ± 0.2 °C. The disappearance of the $[Ru(CN)_5(pyS)]^{4-}$ MLCT band was monitored spectrophotometrically at $\lambda_{max} = 390$ nm. In order to avoid contributions of the back-reaction, k_{pyS}, the concentration of the $[Ru(CN)_5(pyS)]^{4-}$ complex was kept smaller than 1.0×10^{-4} M. Specific rate constant was calculated from the plots of $\log(A_\infty - A_t)$ versus time. These plots were linear for more than 3 half-lives.

Apparatus. The electronic spectra of aqueous solutions of complex and ligand were acquired with a Hitachi model U-2000 spectrophotometer. The transmission infrared spectra of the compounds dispersed in KBr were obtained by using a Perkin-Elmer instrument model Spectrum 1000. Electrochemical experiments were performed on a BAS 100W electrochemical analyzer (Bioanalytical Systems-BAS, West Lafayette, IN) at 25 ± 0.2 °C. A conventional three-electrode glass cell with glassy carbon of 0.1256 cm^2 geometrical area and a Pt foil were used as working and auxiliary electrodes, respectively, in the complex characterization.

The electrochemical experiments with cyt *c* were carried out by using a three-electrode configuration cell, using a 0.1 M buffer phosphate, pH = 7.0 as electrolyte, at 25 ± 0.2 °C. Before the experiments, the cyt *c* solutions were stored at 4 °C in order to avoid protein denaturation.[19] Gold surfaces modified with the promoters and Pt foil were used as working and auxiliary electrodes, respectively. The DIGISim 2.1 BAS software was used for the calculation of the heterogeneous electron transfer rate constant. Theoretical cyclic voltammograms with quasi-reversible kinetics were calculated by the Nicholson method.[20] For acquisition of the reductive desorption curves, a Teflon cell was used to prevent the KOH electrolyte chemical attack. A 0.0314 cm^2 polycrystalline gold surface and a gold flag were used as working and auxiliary electrodes, respectively.

For the ex situ Fourier transform infrared reflection−absorption spectra (FTIRRAS), 1024 scans were collected with a Bomem DA-8 spectrometer equipped with a liquid-nitrogen-cooled narrow band MCT detector. P-polarized light was employed as light incident on the sample at an 80° grazing angle at the vacuum/solid interface.

The gold film substrates for FTIRRAS measurements were prepared by vapor deposition of about 300 nm of gold (99.9% purity) onto glass substrate. To improve the adherence of the gold films, about 15 nm of chromium was deposited by evaporation onto glass substrate, before gold deposition.

The FTIRRAS data for the monolayers are presented in the form of $\%(R/R_0)$, where R and R_0 represent the reflectance of the adlayers and bare gold electrode, respectively.

The ex situ SERS (surface enhanced raman scattering) spectra of the monolayers formed by the pyS free ligand and coordinated to the $[Ru(CN)_5]^{3-}$ moiety were acquired by using a Renishaw Raman imaging microscope system 3000 equipped with a CCD (charge-coupled device) detector, and an Olympus (BTH2) with a 50× objective to focus the laser beam on the sample in a backscattering configuration. As exciting radiation, λ_0, the 632.8 nm line from a He−Ne (Spectra-Physics) laser was used. The gold substrates used for spectra SERS acquisition were activated by the ORC procedure in 0.1 M KCl as described by Gao et al.,[21] without the active species in solution. The activation of the gold surface for SERS spectra acquisition was made by using a PAR 273 potentiostat.

The polishing procedure of the gold surfaces was made as described by Qu et al.[22] These electrodes were mechanically polished with alumina paste of different grade to a mirror finish, rinsed and sonicated (10 min) in Milli-Q water. The electrode was then immersed in a freshly prepared "piranha solution" (3:1 concentrated H_2SO_4/30% H_2O_2; **CAUTION**: Piranha solution is a high oxidant solution that reacts violently with organic compounds), rinsed exhaustively with water, and sonicated again. The cleanness was evaluated by comparison of the $i-E$ curve obtained in a 0.5 M H_2SO_4 solution with the well-established one for a clean gold surface.[23] All potentials are quoted in reference to Ag/AgCl/3.5 M KCl (BAS).

The surface modification procedure was made by immersing the gold electrodes in a 20 mM aqueous solution of the pyS ligand or $[Ru(CN)_5(pyS)]^{4-}$ complex.

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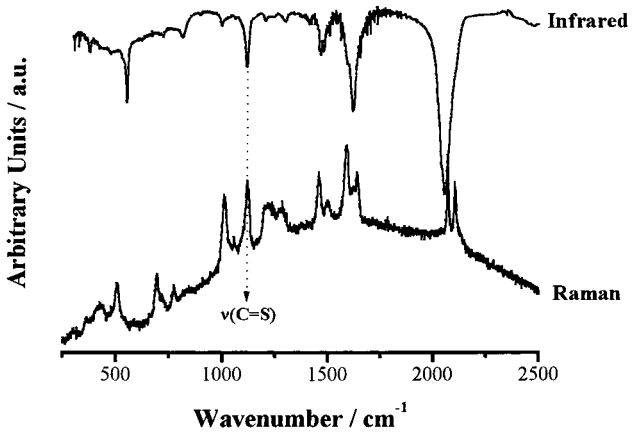

Figure 1. Raman and infrared spectra of the $K_4[Ru(CN)_5(pyS)]\cdot 3H_2O$ complex.

Results and Discussion

Complex Characterization. Prior to the study of the $[Ru(CN)_5(pyS)]-Au$ adlayer, the $K_4[Ru(CN)_5(pyS)]\cdot 3H_2O$ complex was characterized by elemental analysis, cyclic voltammetry, and infrared, Raman, and electronic spectroscopies.

The half-wave formal potential, $E_{1/2} = 780$ mV, for the $Ru^{III/II}$ redox process suggests a thermodynamic stability of the Ru^{II} toward its oxidized Ru^{III} state, as well as a stability gain comparatively to the electrochemical data, $E_{1/2} = 710$ mV, for the starting material $[Ru(CN)_6]^{3-}$. This potential value also indicates that the coordination site of the pyS ligand is the nitrogen atom. According to the previous results,[24] a shift up to 900 mV of the $Ru^{III/II}$ potential is observed when coordination of pyridine derivative moieties to the $[Ru(CN)_5L]^{3-}$ metal center occurs through a sulfur atom. This shift has been assigned to the interaction of the ruthenium $d\pi$ orbitals with the empty d orbitals of sulfur.

The intense band observed at $\lambda_{max} = 390$ nm ($\epsilon = 1.16 \times 10^3$ M^{-1} cm^{-1}) in the electronic spectrum of the $[Ru(CN)_5(pyS)]^{4-}$ complex was assigned to the metal to ligand charge transfer (MLCT), $p\pi^*(pyS) \leftarrow d\pi(Ru^{II})$ transition. The ligand bands were observed at $\lambda_{max} = 288$ nm ($\epsilon = 1.63 \times 10^3$ M^{-1} cm^{-1}) and $\lambda_{max} = 322$ nm ($\epsilon = 1.28 \times 10^3$ M^{-1} cm^{-1}).

The infrared and Raman vibrational spectra of the $K_4[Ru(CN)_5(pyS)]\cdot 3H_2O$ complex are shown in Figure 1. The main features of these spectra account for the cyanide axial ($\nu(CN_{ax})$) and equatorial ($\nu(CN_{eq})$) stretching frequencies in the range from 2050 to 2100 cm^{-1}, and for the $\nu(C{=}S)$ stretching mode at 1120 cm^{-1}. The $\nu(CN_{ax})$ and $\nu(CN_{eq})$ frequency values are typically assigned[25] to the presence of Ru^{II} reduced metal ion. A shift of about 70 cm^{-1} to higher frequency is expected for the Ru^{III} analogue complexes.[25,26]

The most prominent features of the spectra presented in Figure 1 are distinguished in Table 1, along with the vibrational data of the pyS free ligand for comparative purposes.

The signal observed at 1104 cm^{-1}, in the Raman spectrum of pyS, was assigned as X-sensitive bands with a large character of C═S stretching vibration.[29,30,34] Upon coordination, this vibrational mode shifts to 1120 cm^{-1}. The frequency of this mode is strongly affected by the nature of the trans substituent on the pyridine aromatic ring. Therefore, the high-frequency shift of this band in the Raman spectrum of the ruthenium complex, compared to that observed for the pyS free ligand spectrum, suggests that the coordination occurs through the nitrogen atom of the pyridine moiety. Additional evidence is the shift to higher wavenumber of the C═C pyridine ring stretching in the Raman spectrum[32,33] of the ruthenium complex (1640 cm^{-1}), compared with the pyS free ligand spectrum (1617 cm^{-1}). Therefore, upon coordination to the Ru metal center, the sulfur atom of the pyridine thiolate ligand is available for chemisorption interactions with the metallic surfaces.

A key point is that the introduction of a π-donor transition metal center coordinated to the nitrogen pyridine ring of the pyS would affect its electronic density[15−17] making the C−S bond strength stronger and increasing the stability of the adlayer.

Self-Assembled Monolayer Characterization. The gold surfaces were modified by immersing the electrode for 15 min in a 20 mM aqueous solution of pyS and $[Ru(CN)_5(pyS)]^{4-}$ (RupyS) compounds. The adlayers were characterized by SERS and FTIRRAS spectroscopies.

The SERS and FTIRRAS spectra of the adlayer formed by the RupyS onto gold (RupySAu) are shown in Figure 2. The spectral data clearly show an increase in the intensity of the modes assigned to the pyridine moiety, comparatively to the cyanide stretching frequencies. These results suggest, as expected by surface selection rules,[35,36] that the pyS coordinated ligand is closer to the electrode than the cyanides.

The band at 1120 cm^{-1} observed in both RupyS transmittance FTIR and normal Raman spectra, assigned[29] to $\nu(C{=}S)$, shifted to 1090 cm^{-1} and 1096 cm^{-1} in the adlayer FTIRRAS and SERS spectra, respectively. The $\nu(C{=}S)$ band shift, along with the intensity increase in the SERS spectrum, compared to the normal Raman spectrum, indicate a decrease of the double character of the CS bond, suggesting that the complex is adsorbed onto gold through the sulfur atom. The increase in intensity of the X-sensitive band also suggests a perpendicular arrangement of this adsorbate in relation to the surface normal.[30,37]

The RupySAu adlayer spectra are dominated by the in-plane vibrational modes of the pyS ligand. The bands in the range from 300 to 800 cm^{-1}, observed in the RupyS normal Raman and FTIR due to the out-of-plane vibrational modes of the pyridine ligand,[27−30] were absent in the RupySAu surface spectra. These results reinforce the assignment that the RupyS is chemisorbed on the gold surface in a perpendicular orientation. For this orientation, the adsorbate is expected to bind gold surface through sulfur σ interaction.[30] In fact, the strong affinity of thiol groups for gold surfaces is well documented in the literature.[38]

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Table 1. Vibrational IR and Raman frequencies, cm^{-1}, of the pyS and RupyS Compounds

pyS		RupyS		
IR	Raman	IR	Raman	assignment[25−33]
428	430		426	δ(C−S)/γ(CCC)
475	472		510	γ(CCC)
		555	545	ν(Ru−CN)
618, 699, 712	648		696	β(CCC)
	721		721	ν(C−S)/β(CC)
	790, 897		776	γ(CH)
988	988	1008	1011, 1063	ring breathing
1061	1046, 1078			β(CH)
1120	1104	1120	1120	ring breathing/ν(C−S)
1146, 1193, 1218	1200, 1247, 1287	1212	1208, 1220, 1288	β(CH)/δ(N−H)
1314, 1405, 1458, 1480	1395, 1457, 1476	1409, 1469, 1588	1458, 1496, 1588	ν(C═C/C═N)
1572, 1542				δ(N−H)
1614	1617	1619	1640	ν(C═C)
		2084, 2054, 2039	2068, 2100	ν(C≡N)

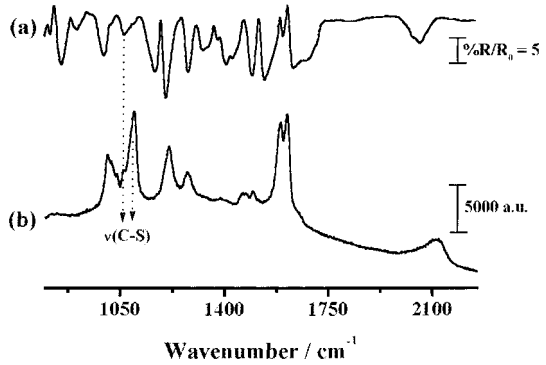

Figure 2. (a) FTIRRAS and (b) SERS spectra of the gold electrode modified after 15 min of immersion in a 20 mM aqueous solution of the $[Ru(CN)_5(pyS)]^{4-}$ ion complex.

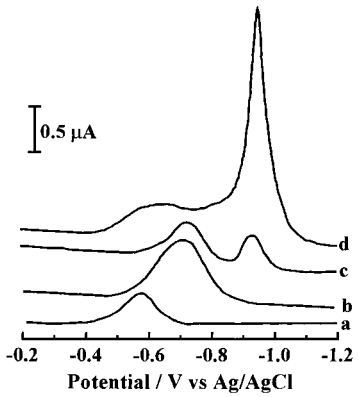

Figure 3. LSV desorption curves in 0.5 M KOH solution at 100 mV s^{-1} for monolayers formed after immersion of the gold electrode in 20 mM aqueous solution of pyS for 5 min (a) and 30 min (d) and RupyS for 5 min (b) and 30 min (c).

Contrary to that observed for the pySAu adlayer,[7] the SERS and FTIRRAS spectra performed for freshly prepared RupySAu and more extended immersion time (from 30 min to 24 h) of the electrode in the precursor solution have not shown any significant change indicative of the adlayer degradation.

Monolayer Electrochemical Stability. Porter and coworkers[39−43] have demonstrated, on the basis of a series of electrochemical data, that thiolate monolayers can be desorbed electrochemically from a gold electrode in alkaline medium. The desorption reaction can be written as

$$RSAu + e^- \rightarrow RS^- + Au \qquad (1)$$

For the pyS monolayer formed at a Au(111) electrode,[7] in a different immersion time of the electrode in an ethanolic solution of this ligand, the application of a linear voltage sweep originates one wave, $E_{rd} = -0.55$ V, for the reductive desorption of the thiolate adlayer. To further investigate the adlayer stability, the authors performed the voltammetric experiments of the samples prepared for more extended immersion times. A wave with $E_{rd} = -0.90$ appeared with a tendency to completely dominate the voltammogram as the immersion time increased.[7] This wave has been assigned[7,8] to an adsorption-induced activation of the oxidative cleavage of the C−S bond and the resulting formation of an adlayer composed of atomic and oligomeric forms of sulfur.

We have taken the RupyS complex along with the pyS promoter in order to modify a polycrystalline gold surface and perform similar reductive desorption experiments.

Figure 3 presents the reductive linear sweep voltammetric curves of the adlayers as a function of the immersion time of gold in 20 mM pyS and RupyS aqueous solutions.

The curve (Figure 3a) of the pyS adlayer for 5 min immersion time shows a cathodic wave with a peak centered at $E_{rd} = -0.56$ V. This peak was assigned to the reduction desorption (AuSpy + $e^- \rightarrow$ Au + pyS^-). As the immersion time increases, the typical wave of the oxidative cleavage of the C−S bond ($E_{rd} = -0.95$ V) is observed, and grows at the expense of the −0.56 V wave (Figure 3d).

The E_{rd} observed for freshly prepared RupySAu (Figure 3b) is significantly more negative (−0.73 V) than that for the pySAu surface. As long as the potentials for the reductive desorption processes are dependent on the strength of the Au−S interaction, the conclusion that the complex is more strongly bound to gold than the free ligand can be raised. This is consistent with the anticipated back-bonding effect, (Ru^{II}) $d\pi \rightarrow p\pi^*$ (pyS), that increases the electronic density of pyS improving the chemisorption process. This result also hints that the stability of the pyS adlayer on gold may be related to the electron density on the thiolate sulfur.

The wave assigned to desorption of sulfur forms, due to the RupyC−S bond cleavage, only appears after 30 min of immersion time (Figure 3c).

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The desorption curves can be useful to calculate the surface concentration of the adlayers[7] according to eq 2,

$$\sigma_{rd} = nFA\Gamma \quad (2)$$

where the electroactive surface coverage (Γ) of RupyS adsorbate was estimated by integrating the area under the reductive peaks of the Figure 3 curves. The σ_{rd} is the charge consumed in the process, F is Faraday's constant, n is the number of electrons evolved in the electrode reaction, and A is the geometrical electrode area.

Accounting for the gold polycrystalline surface, a little bigger charge consumed ($\sigma_{rd} = 193\ \mu C\ cm^{-2}$) by the desorption wave of sulfur forms (Figure 3d), due to the C−S bond cleavage of pyS−Au, is in good agreement with the value reported by the literature[7] (ca. 180 $\mu C\ cm^{-2}$). In a similar reductive desorption process observed for the RupyS adlayer (Figure 3c), very little charge is consumed ($\sigma_{rd} = 17\ \mu C\ cm^{-2}$), suggesting that the formation of this undesirable adlayer is minor compared to a similar process for pySAu.

Since the RupySAu SERS and FTIRRAS spectra performed for large immersion time preparation have indicated no adlayer degradation, attention must be addressed for the kinetic stability of the $[Ru(CN)_5(pyS)]^{4-}$ complex toward the aquation reaction. The pseudo-first-order rate constant for the aquation of this complex, $k_{obs} = 1.2 \times 10^{-4}\ s^{-1}$, indicates that the free ligand is present in solution, in the time scale of the modification procedure. Accounting for this aquation rate, $t_{1/2} = 96$ min, and for large immersion time preparation, the competitive adsorption of the dissociated free ligand (concentration of about 3.88 mM) must be considered for 30 min immersion time modification. Consequently, it would be reasonable to assume that the wave at −0.95 V (Figure 3c) derives from pySAu adlayer decomposition instead of RupySAu degradation.

Electroactivity of RupySAu Electrode. The protein degradative adsorption is the major problem concerning the electrochemical acquisition data of cyt *c* by the use of conventional metallic electrodes.[1] Conversely, rapid electron transfer reaction of cyt *c* has been observed at a pySAu-modified electrode with no evidence of protein unfolding.[37,44] The efficiency of RupyS adsorbate in the assessment of the heterogeneous electron transfer reaction of horse heart cytochrome *c* was first evaluated in relation to the protein denaturation process. Figure 4 presents the cyt *c* cyclic voltammetric curves obtained at a bare gold electrode and RupySAu adlayer prepared by 15 min of immersion time.

The cyclic voltammogram obtained with a bare gold electrode (Figure 4a) clearly shows an irreversible $Fe^{III/II}$ redox process of the heme group upon cyt *c* denaturation.[45] Once the sulfur atom of the amino acid residues presents a great affinity to the gold substrate, the protein unfolding has been assigned to the methionine-80 and gold surface interaction.[19] On the other hand, the cyclic voltammogram, obtained with the RupySAu adlayer (Figure 4b), presents a redox process with a typical half-wave potential of 20 mV for the heme-$Fe^{III/II}$ heterogeneous electron transfer process of the native cyt *c* protein.[1,19,46,47] This result indicates that the RupyS adsorbate blocks the cyt *c* degradative adsorption process at gold electrode. Additionally, the shape of the curve suggests[48,49] a rapid heterogeneous electron transfer kinetic.

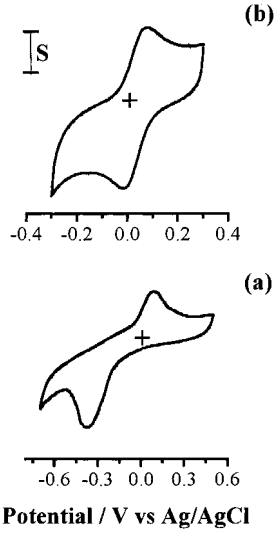

Figure 4. Cyclic voltammograms at 50 mV s^{-1} of 0.1 mM cyt *c* in 0.1 M KH_2PO_4, pH = 7, at (a) bare gold electrode, $S = 3.0\ \mu A$, and (b) RupySAu electrode after 15 min of immersion time in a 20 mM RupyS aqueous solution, $S = 0.3\ \mu A$.

Table 2. Values of ΔE_p and $E_{1/2}$ (V vs Ag/AgCl) and Cathodic Current (i_{pc}, μA) for Cyt *c* $Fe^{III/II}$ Redox Process

immersion time (min)	RupySAu			pySAu		
	ΔE_p	$E_{1/2}$	$-i_{pc} \pm 0.05$	ΔE_p	$E_{1/2}$	$-i_{pc} \pm 0.05$
15	0.07	0.03	0.50	0.08	0.03	0.44
30	0.08	0.03	0.50	0.12	0.06	0.27
60	0.08	0.03	0.50	n[a]	n	n
360	0.12	0.08	0.42	n	n	n
15[b]	0.07	0.03	0.50	n	n	n

[a] No faradaic current response. [b] 15 min immersion time, and adlayer exposed overnight to the atmosphere.

To further evaluate the electrode performance toward metalloprotein electrochemistry, the cyt *c* cyclic voltammograms were acquired in the range of scan rates 0.05−0.4 V s^{-1}. A linear dependence of peak current on $v^{1/2}$ was observed, and the peak-to-peak potential separation for the reduction and oxidation component of the experiment was found to change from 0.07 to 0.11 V, respectively.

The heterogeneous electron transfer rate constant, $k^0 = 8.0 \times 10^{-3}$ cm s^{-1}, was obtained by the Nicholson's method.[20] This value is slightly higher than that reported for the pySAu adlayer,[50] $k^0 = 6.0 \times 10^{-3}$ cm s^{-1}, due to the pyS monolayer structural conversion that yields sulfur species, making difficult the assessment of the electron transfer rate of cyt *c*.[7]

The effect of the immersion time of a gold electrode, in a 20 mM aqueous solution of RupyS and pyS promoters, on the adlayer's ability to assess the cyt *c* electrolysis was evaluated by a set of cyclic voltammetric experiments. The results taken in 0.1 mM cyt *c* and 100 mM phosphate buffer solution, pH = 7.0, are summarized in Table 2. For the experiments using the pySAu adlayer, the faradaic current response diminishes dramatically for immersion times longer than 15 min, while an increase in the difference between $E_{p,c}$ and $E_{p,a}$ (ΔE_p) is observed. At immersion times up to 30 min a complete loss in the capacity of this adlayer toward cyt *c* heterogeneous electron transfer reaction has been observed. Conversely, the cyt *c*

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electrochemical parameters have not change for RupySAu surfaces prepared by immersion times up to 60 min. The peak separation values, $\Delta E_{p,}$ are indicative of facile electron transfer kinetics on the voltammetric time scale.[20,48,49] Furthermore, the cyt *c* electrochemical response for RupySAu prepared within 15 min immersion time and kept out of the promoter solution overnight was about the same as for freshly prepared electrode.

On the basis of these results, we can conclude that the RupyS inorganic complex is an efficient self-assembled monolayer to assess the rapid electron transfer reaction of cyt *c*. Also, a greater stability of the gold substrate modified by the RupyS has been achieved, compared to the free ligand monolayer.

The structural stability of pyS monolayers is strongly affected by the electronic properties of the $[Ru(CN)_5]^{3-}$ substituent on the pyridine ring, particularly its π-electron-donating character. Also, the $[Ru(CN)_5(pyS)]^{4-}$ negative charge, as well as the cyanide environment, facilitates the electrostatic interaction[13] with the positive-charged lysine groups of cyt *c*. These properties all together well illustrate that the $[Ru(CN)_5(pyS)]^{4-}$ forms a very stable inorganic ordered monolayer on gold substrate, improving the assessment of the heterogeneous electron transfer reaction of cyt *c*.

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