J Solid State Electrochem (2007) 11:1585–1590
DOI 10.1007/s10008-007-0366-5

ORIGINAL PAPER

SERRS study of $[Ru(CN)_5(pyS)]^{4-}$ SAM and cytochrome c: A suggestion toward the heterogeneous molecular recognition

Izaura Cirino Nogueira Diógenes · Francisco Carlos Nart · Mônica B. P. Mello Barreto · Marcia Laudelina Arruda Temperini · Ícaro de Sousa Moreira

Received: 19 January 2007 / Revised: 14 February 2007 / Accepted: 22 May 2007 / Published online: 23 June 2007

Abstract Surface-Enhanced Raman Scattering (SERS) spectra of $[Ru(CN)_5(pyS)]^{4-}$ (RupyS) complex self-assembled monolayer (SAM) were obtained on gold and silver surfaces at 632.8 and 413.1 nm excitation radiations, respectively. The bands assigned to the heme iron of the cytochrome c (cyt c) metalloprotein group were observed by using the RupyS SAM on silver at 413.1 nm. The Surface-Enhanced Resonance Raman Scattering (SERRS) spectra of the RupyS SAM on silver in the cyt c solution obtained at −0.2 and +0.2 V present bands at 1,365 and 1,374 cm^{-1} characteristic of the heme group, indicating the reduced and oxidized states of this protein, respectively. The bands observed at 1,464, 1,504, and 1,638 cm^{-1} are used to confirm the redox state of cyt c. The presence of the oxidized and reduced bands in function of different applied potential is an evidence that the protein is interacting with the modifier.

Keywords SAM · SERS · SERRS · Cytochrome c · Ruthenium complexes

This paper is dedicated to Prof. Francisco Nart, in memoriam.

I. C. N. Diógenes · Í. 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
e-mail: icarosm@dqoi.ufc.br

I. C. N. Diógenes
e-mail: izaura@dqoi.ufc.br

F. C. Nart
Instituto de Química de São Carlos,
Universidade de São Paulo (USP), Cx,
Postal 369,
São Carlos, SP, Brazil

M. B. P. M. Barreto · M. L. A. Temperini
Instituto de Química, Universidade de São Paulo (USP),
Cidade Universitária, Cx,
Postal 26077,
05599-970 São Paulo, SP, Brazil

Introduction

Besides the inter-adsorbate interactions and the properties of the double layer at the interface, it is well known that the electroactivity of self-assembled nanoscale films depends on other several factors such as the chemical nature, molecular crystallinity, extent of planarity (effective conjugation length), and conformation and packing of the monolayer. The optimization of the surface modification procedure has a great potential for controlling the material properties by molecular engineering. In this sense, sensitive surface techniques have been extensively used to understand the molecular factors influencing the properties of nanoscale thin-film devices [1, 2]. Among these techniques, Surface-Enhanced Raman Scattering (SERS) spectroscopy is especially sensitive to local structure and interactions close to the substrate for molecules that present charge-transfer intramolecular processes [3–8]. The signal of this vibrational spectroscopic technique can be even amplified if an appropriate set of resonance conditions is achieved. For instance, when silver substrate is used with an excitation wavelength at 413.1 nm, the Raman signal is strongly intensified due to surface plasmon absorption. If the molecular adsorbed species presents an intrinsic electronic transition at this excitation radiation in such case, the technique is specifically named Surface-Enhanced Resonance Raman

Scattering (SERRS) [8]. This enhancement effect has been extensively used to study the electron transfer (ET) processes of heme-containing proteins because of an additional intensification furnished by the chromophore [9–11]. Considering that the heterogeneous electron transfer (hET) reaction between an electrode and a redox-active prosthetic group of this type of protein can serve as a model system for the understanding of the electron transport mechanisms in biological systems, we have been studying the cytochrome c (cyt c) metalloprotein by using gold surfaces modified with inorganic complexes of the type $[M(CN)_5(L)]^{4-}$, where M=Ru and Fe, and L=4-mercaptopyridine (pyS) and 1,4-dithiane (1,4-dt) [6, 7, 12, 13].

Aiming to understand the interaction between the inorganic monolayer formed by the $[Ru(CN)_5(pyS)]^{4-}$ complex (RupyS) and the cyt c metalloprotein in the electrolyte medium, SERS spectra were acquired in solution with and without this protein. To establish the SERS features of the RupyS modifier itself, the spectroscopic data are presented, at first, for this complex chemisorbed on silver and gold substrates in solution without the cyt c. Second, the SERRS spectra of this complex adsorbed on silver electrode in a solution containing the cyt c is studied to observe specific bands of this protein which are known as redox and spin state marker bands.

Materials and methods

Millipore water (18 MΩ cm) was used in all experiments. The $K_4[Ru(CN)_5(pyS)]\cdot 3H_2O$ (RupyS) complex was synthesized according to the literature [14]. KH_2PO_4 and $NaClO_4$, from Aldrich, were used without previous purification. Horse heart cytochrome c (type VI, 99%, Aldrich) was purified as described in the literature [15]. All other reagents are of analytical grade.

The electronic spectrum of the complex in aqueous solution was acquired with a Hitachi model U-2000 spectrophotometer.

The SERRS spectroelectrochemical experiments obtained for the silver substrate modified with the RupyS complex in solution with or without the cyt c protein were recorded in a Jobin-Yvon U1000 spectrometer, fitted with a photomultipliertube (RCA C31034-A02) coupled to a photon counter (EG&G PARC). The laser line 413.1 nm (from Ar^+ Coherent Innova 90-6) was used, and laser power at the sample has typically been 100 mW. A circular spinning cell was used to avoid laser degradation of the sample. For the spectra obtained with the cyt c protein, very dilute aqueous solution (10^{-8} M) was used to avoid the resonance Raman signals of the protein in the electrolyte medium (20 mM KH_2PO_4/0.1 M $NaClO_4$ buffered solution pH=7.0). Therefore, all signals assigned to the cyt c in the SERS spectra must be assigned to the molecules that are very near the RupyS modifier.

The SERS spectra of the gold surface modified by the RupyS complex were acquired by using a Renishaw Raman imaging microscope system 3000 equipped with a charge-coupled device (CCD) 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 activation of the silver and gold surfaces were made by oxidation-reduction cycles (ORC) procedure in 0.1 M KCl. For the silver polycrystalline electrode of 0.07 cm^2, the ORC were applied in the electrolyte solution without the RupyS modifier by scanning the potential range from −0.56 to 0.49 V vs Ag|AgCl|Cl^- until a charge of 25 mC. cm^{-2} in the anodic peak was achieved. For the gold surface, the potential was scanned from −0.2 to +1.3 V vs Ag|AgCl|Cl^-, as described in the literature [16]. Subsequent to the activation procedure, the Raman spectra of the surfaces were run to check the purity of both the electrolyte and the electrodes. Then, the substrate modifications were made by 15 min of immersion in a 20-mM aqueous solution of the RupyS complex. An EG&G (Princeton Applied Research) model 273 potentiostat/galvanostat was used for the activation procedure of the substrates for SERS spectra acquisition.

All experimental procedures were performed at room temperature, and the potentials cited throughout are quoted relative to an Ag|AgCl|Cl^- (3.5 M KCl) electrode.

Results and discussion

Before the discussion of the marker bands of the cyt c metalloprotein and the kind of interaction between this molecule and the RupyS modifier, the normal Raman of the RupyS complex in the solid state and the in situ SERS spectra RupyS-gold electrode at 632.8 nm are compared to the SERRS spectra of RupyS-silver electrode at 413.1 nm. In the last case, a multiplicative amplification is expected due to the resonance Raman effect of the RupyS moiety. A metal-to-ligand charge transfer (MLCT) transition of the RupyS adsorbate (Fig. 1a) is observed at 390 nm. The other absorptions observed in higher energies are assigned to the pyS intraligand transitions [17].

Figure 1b presents the normal Raman spectrum (from 950 to 1,800 cm^{-1}) of the RupyS complex in the solid state at 632.8 nm excitation radiation. The in situ SERRS spectra of the RupyS adlayer formed on silver electrode at 413.1 nm is illustrated in Fig. 1c, and the in situ SERS spectra of the RupyS on gold substrate at 632.8 nm is presented in Fig. 1d. The fully extended normal Raman or

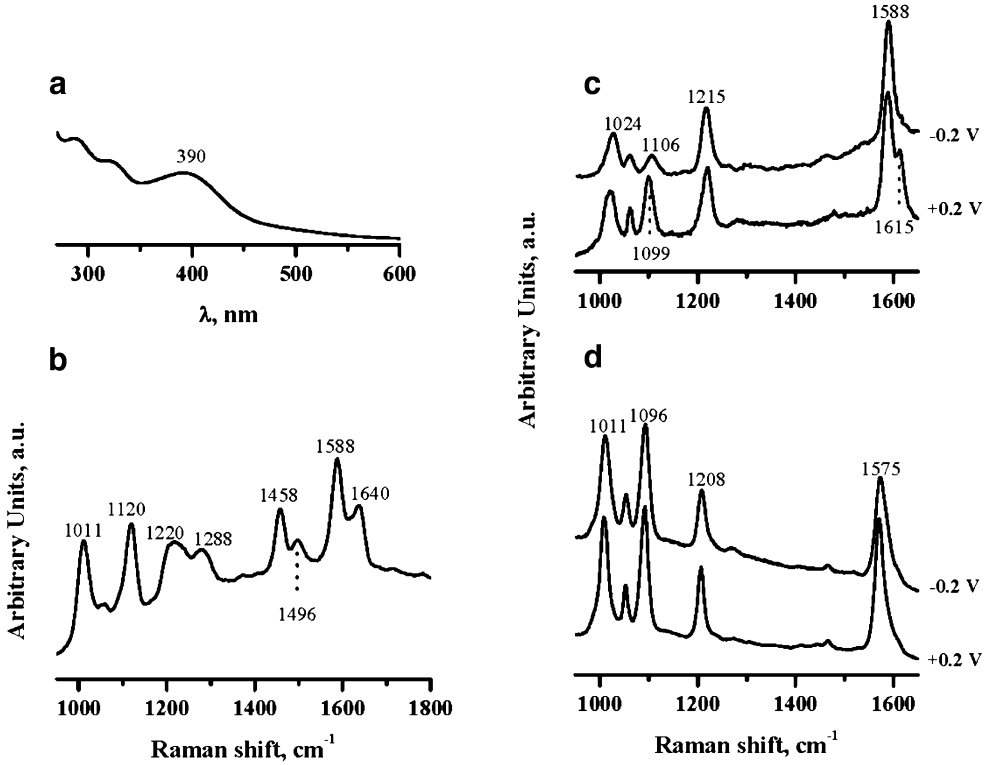

Fig. 1 **a** UV-Vis spectrum of the RupyS complex in aqueous solution, **b** normal Raman spectrum of RupyS complex in solid state (λ_0=632.8 nm), and SERS spectra at different potentials in a buffered solution (pH=7.0, 0.1 M $NaClO_4$, 20 mM KH_2PO_4) of the RupyS complex on **c** silver (λ_0=413.1 nm) and **d** gold (λ_0=632.8 nm)

SERS spectra of the RupyS complex with the frequency assignments and detailed discussion can be found elsewhere [6, 7]. For the sake of clarity, the most relevant signals related to the discussion of this work are assigned as follows. The pyS ring breathing mode is observed at 1,011 cm^{-1} [18–20]. The signal at 1,120 cm^{-1}, named as X-sensitive band, is assigned to the pyS ring breathing mode coupled with the C–S stretching vibration and is frequently used as a marker band for the assignment of the chemisorption mode of 4-mercaptopyridine derivative compounds on metallic surfaces because of its sensitiveness toward *trans* substituents on the pyridine ring [6, 7, 20–23]. The bands at 1,220 and 1,288 cm^{-1} are assigned to the CH-bending modes and those observed from 1,458 to 1,650 cm^{-1} to the C=C and C=N stretching modes [19–24]. The band at 1,588 cm^{-1} is strongly sensitive to the environmental change on the nitrogen atom [25].

SERS and SERRS spectra of the RupyS complex on gold and silver, respectively, in solution without cyt c

The incident radiation at 413.1 nm is within the MLCT transition of the RupyS complex (Fig. 1a), and it is expected to be an enhancement due to a resonance Raman effect for the vibrational modes that are affected by this electronic transition. Aiming to detect these modes, the in situ SERRS spectra of the RupyS adlayer formed on silver electrode (Fig. 1c) were compared to the in situ SERS spectra for this adlayer on gold substrate at 632.8 nm (Fig. 1d). Upon moving from a nonresonant lower energy (excitation wavelength 632.8 nm) toward higher energy (excitation wavelength 413.1 nm), some changes in the relative intensities within the pyS vibrational modes are observed. At 632.8 nm, the X-sensitive band (1,096 cm^{-1}) is the most intense signal and does not depend on the applied potentials. At this radiation, the spectra are dominated by the ring breathing modes (1,011 and 1,096 cm^{-1}), whereas at 413.1 nm, the C=C and C=N vibrations (1,588 and 1,615 cm^{-1}) are the most intense bands. By accounting for the maximum of the RupyS MLCT transition (Fig. 1), these results indicate that these modes are strongly involved in this charge-transfer process. Comparing the normal Raman spectrum in the solid state (Fig. 1b) to the SERS spectra presented in Fig. 1c and d, differences concerning the relative intensity of some bands are observed. The band at 1,640 cm^{-1} in the normal Raman spectrum is observed at 1,615 cm^{-1} in the in situ SERRS spectra (413.1 nm), reinforcing the assignment of the nitrogen atom as the pyS coordination site toward the ruthenium metal atom [7]. The Ru-N(pyS) bond results in a C=N bond-order decrease as consequence of the electronic density delocalization to antibonding orbitals that are directly involved in this interaction. The chemical adsorption via the pyS ligand is also shown by the significant shift of some modes, specially the one assigned to $\nu_{(C=S)}$ stretching [22]. This mode shifts from 1,120 cm^{-1} in the normal Raman spectrum (Fig. 1b) to 1,099 cm^{-1} in the SERRS of silver modified electrode (Fig. 1c) and to

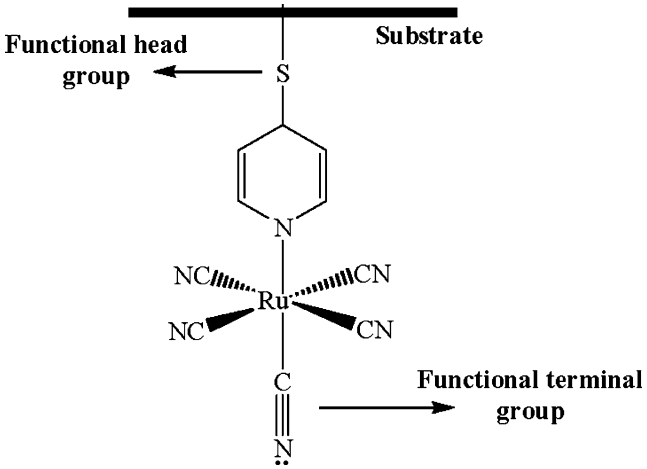

Fig. 2 Planar representation of the RupyS structure adsorbed on a metallic substrate

Similar observations were made by Hobara et. al [28] when studying the SERRS spectra of a silver surface modified with the pyS ligand free of coordination. These authors observed that some signals in the spectrum obtained at 413.1 nm had their relative intensities changed, comparatively to the spectrum obtained at 647 nm. These results were assigned to the charge-transfer contribution to the spectrum at the shorter excitation wavelength. For the RupyS modifier, beyond this contribution, the observation of the signal at 1,615 cm^{-1}, as well as some intensity differences between the spectra obtained at 413.1 and 632.8 nm, can be raised by the electronic delocalization involved in the MLCT transition.

1,096 cm^{-1} in the SERS spectra of gold modified electrode (Fig. 1d). This shift indicates a decrease in the double character of the C=S bond upon adsorption on gold or silver substrate. These observations indicate that the RupyS complex is adsorbed on the metallic surfaces by the sulfur atom of the pyS ligand functional head group, whereas the nitrogen atom of the axial cyanide moiety is the functional terminal group as illustrated in Fig. 2.

Considering the chemisorption as the dominating factor in determining the adsorption scheme, it is reasonable to ascribe that the RupyS complex chemisorbs on silver and on gold surfaces in a very similar way (via pyS ligand) [26].

As the excitation radiation at 413.1 nm is within the RupyS MLCT transition, the dependence on the applied potential observed in Fig. 1c (λ_0=413.1 nm) allows one to correlate some bands in the SERRS spectra with the back-bonding interaction. In positive potentials, the back-bonding interaction of the ruthenium atom and the surface atoms with the pyS ligand decreases, localizing the C=S bond and thus enhancing the signal at 1,099 cm^{-1}. Concomitant with this behavior, it is observed the appearance of a new band at 1,615 cm^{-1}, which is assigned to the C=C and C=N vibrational modes of the pyridine ring that are strongly affected by the neighboring nitrogen [25]. On the other hand, in negative potentials, the back-bonding interaction capability of the ruthenium and surface atoms is intensified implying in an aromaticity increment and, thus, in the intensity enhancement of the ring-breathing mode which is observed at 1,024 cm^{-1}. Considering that the electron transfer by space fillers is facilitated when these are in a linear conformation [27], the results obtained in the in situ SERRS spectra (413.1 nm) and the in situ SERS spectra (632.8 nm) reinforce the adsorbate configuration proposed in Fig. 2.

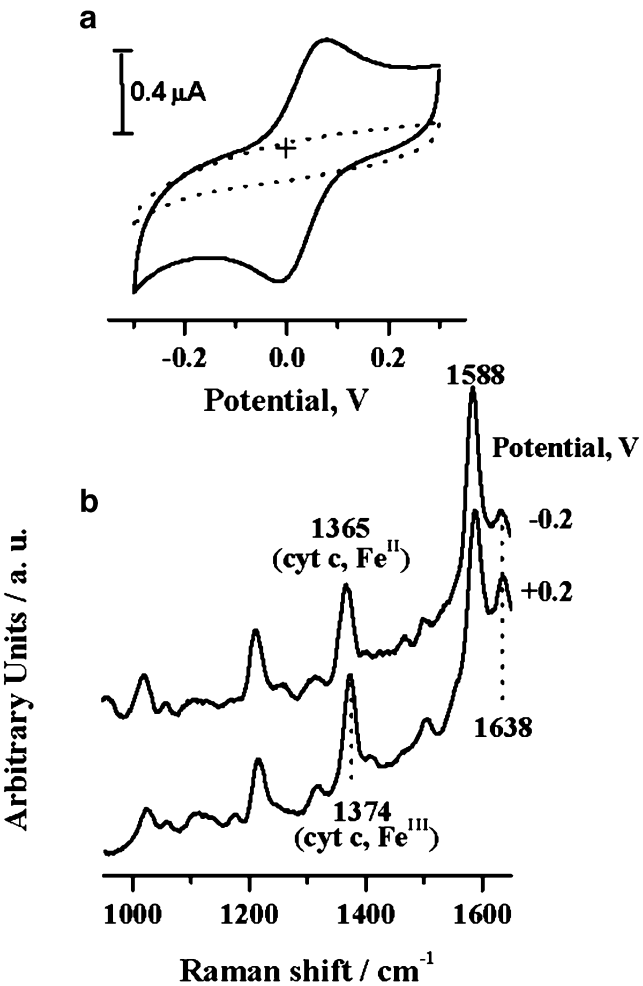

Fig. 3 **a** Cyclic voltammograms at 50 mV s^{-1} of 1.0 mM cyt c in 0.1 M KH_2PO_4 (pH=7.0) solution at RupyS SAM on gold (*solid line*) and on bare gold (*dot line*). + → i=0.0 μA. **b** In situ SERS spectra of the RupyS SAM on silver (λ_0=413.1 nm) in a buffered solution (pH=7.0, 0.1 M $NaClO_4$, 20 mM KH_2PO_4) containing cyt c, 10^{-8} M

SERRS spectra of the RupyS complex on silver in solution containing cyt c

The Raman spectra of heme-containing proteins such as cyt c present characteristic bands of the iron atom [29–31]. These bands can be observed if the protein is near the self-assembled monolayer (SAM), and the SERRS condition is achieved as cyt c bands due to the Soret transitions that occur at 400 nm [29]. Figure 3b shows the SERRS spectra of RupyS on silver electrode at 413.1 nm excitation wavelength in cyt c solution at potential range from −0.2 to +0.2 V as the redox process of this metalloprotein occurs around 0.0 V, as illustrated in Fig. 3a. Successive attempts to obtain similar profiles with gold modified surface were unsuccessful. For gold substrate, the spectral region where the plasmon surface is acquired is not coincident with the resonance Raman effect of the cyt c [27]. Hence, this is the most probable reason why we were not able to acquire the spectrum of the biomolecule.

The cyclic voltammogram of the cyt c solution at gold surface modified with the RupyS complex presents characteristics of a quasi-reversible process [32] indicating the high efficiency of this modifier in assessing the cyt c hET reaction. In addition, the value of the cyt c half-wave formal potential ($E_{1/2}$) close to 0.0 V indicates the integrity of the native structure for this protein [33]. For the silver modified surface, by considering the same modification conditions, the cyt c redox process was evaluated in an electrolyte solution containing $NaClO_4$ to avoid the silver oxidation. The cyclic voltammogram thus obtained (not shown in this paper) was similar to that acquired for the gold modified electrode. On the other hand, at gold or silver bare surfaces, no wave assigned to the cyt c redox reaction is observed within the potential range used.

Figure 3b shows the SERRS spectra of the silver substrate modified with the RupyS complex in an aqueous solution of the cyt c. These spectra, although presenting a lower signal-to-noise ratio comparatively to the spectra obtained in the absence of the cyt c in solution (Fig. 1c), the characteristic bands due to cyt c could be observed.

Most of the bands observed above 1,300 cm^{-1} in the SERS spectra presented in Fig. 3b are related to the oxidation and spin states as well as the coordination numbers of the heme iron [30, 31]. The oxidation state marker bands are clearly observed at 1,365 and 1,374 cm^{-1} in the SERS spectra obtained at −0.2 and +0.2 V indicating the reduced and oxidized states of the cyt c protein, respectively. Nevertheless, the presence of the band at 1,638 cm^{-1} as well as the broadening of the 1,365 cm^{-1} band indicate that oxidized cyt c is also present at −0.2 V. The relative intensities of the bands at 1,464 (reduced cyt c) and 1,504 cm^{-1} (ν3, oxidized cyt c) changes with the applied potential; at negative potential (−0.2 V), the ratio I_{1464}/I_{1504} is greater than at positive potential (+0.2 V), indicating that at −0.2 V, there are more reduced species at the electrode surface. Concerning the band at 1,588 cm^{-1}, no information about cyt c can be obtained as this signal is also observed in the SERS spectra of the RupyS complex without cyt c in solution (Fig. 1c). Hildebrandt et al. [11] observed similar results for the cyt c adsorbed on colloidal silver particles. The presence of the oxidized and reduced bands in function of different applied potential is an evidence that the protein is interacting with the modifier.

It is currently suggested, although without a direct evidence, that the external positive domains of the cyt c (lysine residues) may have an influence on the orientation and conformation of the cyt c on SAMs. Some authors claim that one must focus on the strength of a charged surface to ensure both the desired orientation and native conformation of the cyt c protein. In fact, modifiers that are adsorbed at a perpendicular orientation in relation to the metallic surface and that contain an anionic or weakly basic end were proposed to successfully assess the cyt c hET reaction [34]. Recently, however, the cyt c hET reaction was successfully assessed by SAMs composed of cationic ruthenium complexes [35], indicating that the charge of the modifier is not an excluding factor for the assessment of this heterogeneous reaction and reinforcing that hydrogen bonds are involved in the ET mechanism of the cyt c metalloprotein, as previously suggested by Allen et al. [34].

Although the experimental data in this study does not lead us to convict decidedly whether the interaction between the modifier and cyt c is electrostatic or through a hydrogen bond, these results show that the protein is adsorbed on the modifier or, at least, is very near to the RupyS SAM.

Conclusions

SERS spectra of RupyS complex SAM were obtained on gold and silver surfaces at 632.8 and 413.1 nm excitation radiations, respectively. For the cyt c SERRS study, the bands of this protein are only observed when the silver surface modified with the RupyS complex is employed. In this case, an additional effect is observed because of the cyt c Soret transitions (resonance Raman at 413.1 nm). The bands observed at 1,365 and 1,374 cm^{-1} in the SERS spectra obtained at −0.2 and 0.2 V indicate the reduced and oxidized states of the cyt c protein, respectively. The marker bands of the cyt c heme iron, observed at 1,464, 1,504, and 1,638 cm^{-1}, are used to confirm the oxidized state of cyt c.

Acknowledgments The authors I. C. N. Diógenes, I. S. Moreira, and M. L. A. Temperini would like to express their thankfulness to "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) for grants.

References

1. Malandrinos G, Dodi K, Louloudi M, Hadjiliadis N (2000) J Inorg Chem 79:21
2. Louludi M, Hadjiliadis N (1994) Coord Chem Rev 135/136:429
3. Cotton TM, Clark RJH, Hester RE (1988) Spectroscopy of surfaces. Wiley, New York
4. Kneipp K, Kneipp H, Itzkan I, Dasari RR, Feld MS (1999) Chem Rev 99:2957
5. Nie S, Emory SR (1997) Science 275:1102
6. Diógenes ICN, de Sousa JR, de Carvalho IMM, Temperini MLA, Tanaka AA, Moreira IS (2003) Dalton Trans 11:2231
7. Diógenes ICN, Nart FC, Temperini MLA, Moreira IS (2001) Inorg Chem 40:4884
8. Creighton JA, Clark RJH, Hester RE (eds) (1988) Selection rules for surface-enhanced raman spectroscopy. In: Spectroscopy of surfaces. Wiley, New York
9. Hildebrandt P, Stockburger M (1989) Biochemistry 28:6710
10. Lu T, Yu X, Dong S, Zhou C, Ye S, Cotton TM (1994) J Electroanal Chem 369:79
11. Hildebrandt P, Stockburger M (1986) J Phys Chem 90:6017
12. Sousa JR, Batista AA, Diógenes ICN, Andrade GFS, Temperini MLA, Lopes LGF, Moreira IS (2003) J Electroanal Chem 543:93
13. Sousa JR, Parente MMV, Diógenes ICN, Lopes LGF, Neto PL, Temperini MLA, Batista AA, Moreira IS (2004) J Electroanal Chem 566:443
14. Moreira IS, Franco DW (1994) Inorg Chem 33:1607
15. Brautigan DL, Fergusson MS, Margoliash E (1978) Methods Enzymol 53:128
16. Gao P, Gosztola D, Leung LWH, Weaver MJ (1987) J Electroanal Chem 233:211
17. Albert A, Barlin GB (1959) J Chem Soc 2384
18. Hexter RM, Albrecht MG (1979) Spectrochim Acta, Part A 35:233
19. Cook D (1961) Can J Chem 39:2009
20. Green JHS, Kynaston W, Paisley HM (1963) Spectrochim Acta 19:549
21. Spinner E (1960) J Chem Soc 1237
22. Baldwin JA, Schuhler N, Butler IS, Andrews MP (1996) Langmuir 12:6389
23. Baldwin JA, Vlckova B, Andrews MP, Butler IS (1997) Langmuir 13:3744
24. Yu H-Z, Xia N, Liu Z-F (1999) Anal Chem 71:1354
25. Hu JW, Zhao B, Xu WQ, Li BF, Fan YG (2002) Spectrochim Acta, Part A 58:2827
26. Corio P, Andrade GFS, Diógenes ICN, Moreira IS, Nart FC, Temperini MLA (2002) J Electroanal Chem 520:40
27. Larsson S (1983) J Chem Soc, Faraday Trans 79:1375
28. Hobara D, Niki K, Zhou C, Chumanov G, Cotton TM (1994) Colloids Surf, A Physicochem Eng Asp 93:241
29. Gouterman M, Dolphin D (ed) (1978) The porphyrins. Academic, New York
30. Hildebrandt P, Scott RA, Mauk AG (eds) (1996) Resonance spectroscopy of cytochrome c. University Science, Mill Valley, CA
31. Spiro TG, Lever AB, Gray HB (eds) (1982) Iron porphyrins, Part II. Physical Bioinorganic Chemistry Series. Addison-Wesley, Reading, MA
32. Bard AJ, Faulkner LR (1980) Electrochemical methods, fundamentals and applications. Wiley, New York
33. Armstrong FA (1990) Struct Bond 72:137
34. Allen PM, Hill HAO, Walton NJ (1984) J Electroanal Chem 178:69
35. Pinheiro SO, Silva FON, Carvalho IMM, Lopes LGF, Temperini MLA, Andrade GFS, Moreira IS, Diógenes ICN (2006) J Braz Chem Soc 17:1594
36. Hildebrandt P, Stockburger M, Alix AJP, Bernard L, Manfait M (eds) (1985) Spectroscopy of biological molecules. Wiley, New York