Total Internal Reflection Tip-Enhanced Raman Spectroscopy of Cytochrome C

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ABSTRACT. Surface and tip-enhanced Raman spectroscopies in total internal reflection (TIR-SERS and TIR-TERS) are used to characterize the oxidation, spin and ligation state of cytochrome C (Cc) molecules electrostatically bound on hydrophilic thiol self-assembled monolayer. TIR-SERS spectra of this model hemoprotein show marker bands typical of the 6cLS ferric state of Cc. The performances of the TIR-TERS technique as a function of the incidence

angle of the incoming light excitation are described by comparison with conventional Raman spectroscopy in TIR and bottom-illumination TERS with radially polarized light irradiation. They reveal a significant advantage of the TIR-TERS configuration in terms of electromagnetic enhancement of the Raman signal (increased by a factor ~ 25 with respect to the traditional bottom-illumination geometry). Under optimized light excitation, TIR-TERS spectra of Cc are obtained and confirm the 6cLS ferric state assignment deduced from TIR-SERS spectra. This study thus demonstrates the possibility to probe with nanoscale spatial resolution Cc 6cLC ferric forms that are potentially implicated in the cell apoptotic processes, with minimal far-field contribution of the sample (the analyte and its environment) due to the evanescent nature of the light excitation in TIR-TERS, a property of probably high interest for nanoscale chemical characterizations in complex biological media.

TOC GRAPHICS

KEYWORDS Tip-enhanced Raman spectroscopy, Surface-enhanced Raman spectroscopy, Total internal reflection, Finite difference time domain, Cytochrome C.

TEXT. Tip-enhanced Raman spectroscopy (TERS) established itself as a powerful technique to characterize many biomolecules, such as nucleic acids, peptides/proteins and complex biological systems (cells, viruses, bacteria...).¹ In TERS, a monochromatic propagative wave excites resonantly a tapered noble metal probe in order to generate an enhanced and confined electromagnetic field at the really end of this tip. The produced intense and localized evanescent field results from two enhancement mechanisms, namely the resonant plasmonic effect that is highly dependent on the material, the size, the shape and the surrounding medium of the TERS probe, and the non-resonant lightning-rod effect increasing the electron density at the tip apex. In addition, the resonant nanoantenna effect allows for the conversion of the evanescent (near) field into propagative (far) field, at the origin of the detected TERS signal. In traditional bottom, side or top-illumination configurations,¹ a propagative-wave excitation lead to the irradiation of a tip portion with diffraction-limited dimensions much larger than the nanoscale apex size, which may induce the detection of parasitic signals generated by the tip sides. Furthermore, photosensitive molecules, such as hemoproteins, may be affected when they are resonantly excited by direct light irradiation at the same time as the TERS tip, especially in bottom-illumination configuration. In order to limit these issues, an incident excitation in total internal reflection (TIR) geometry can be proposed. TIR-TERS has been recently applied to characterize a thin layer of copper phthalocyanine.² Not only did the authors demonstrate the feasibility of TIR-TERS measurements with nanoscale (better than 40 nm) spatial resolution but they also revealed that the signal enhancement is more than 20 times over side-illumination configuration. However, in this case, TIR was realized through a prism instead of a high numerical aperture objective currently used in TERS instruments. TIR-TERS performances were not compared with

those obtained in bottom-illumination configuration that is often employed to probe biological specimens.¹

Herein, we present a TIR-TERS technique implemented in a conventional bottom-illumination AFM-TERS instrument, by probing cytochrome C (Cc) biomolecules under resonant excitations. Cc is a globular monoheme protein of relatively large size (ca. 3.4 nm in diameter) which acts as an electron carrier in the respiratory chain of aerobic organisms.³ It transfers electrons between the Cc reductase and Cc oxidase enzyme complexes which are both embedded in the mitochondrial membrane. It was shown to be implicated in apoptotic processes via its transfer from the intermembrane space to the cytosol, one of the main enzymes acting in the reaction cascades of apoptosis.^{4,5} Apoptosis or programmed cell death is one of the mechanisms that may induce neuronal loss in the brain of persons suffering from Alzheimer's disease (AD). Cc exists in three ferric and two ferrous states, namely the six-coordinated low-spin (6cLS), six-coordinated high-spin (6cHS), five-coordinated high-spin (5cHS) ferric states, the six-coordinated low-spin (6cLS) and five-coordinated high-spin (5cHS) ferrous states. However, only the 6cLS state was suggested to characterize the active form implicated in the apoptotic processes.⁶ In order to discriminate the oxidation, spin and ligation state of the Cc heme iron, many Raman and surface-enhanced Raman scattering (SERS) experiments under resonant excitation of Cc were achieved.⁶⁻¹⁴ They showed that each Cc state was related to specific Raman bands in the 1300-1700 cm^{-1} ,^{6,9-14} and may present an additional relevant feature at $\sim 750 \text{ cm}^{-1}$.¹⁰⁻¹² SERS experiments on Cc molecules electrostatically bound to thiol self-assembled monolayers (SAMs) on Ag and Au electrodes (see Supporting Information for explanations about the interest of Cc attachment on alkanethiol SAMs) revealed that the 6cLS state of the adsorbed Cc prevails in the oxidized form while the 5cHS state seems to be predominant in the

reduced form.^{7,8,10} All the observed bands were assigned to specific heme modes. However, amino acid bands were observed neither in RR nor in SERS.^{6,12} Only tip-enhanced resonance Raman spectroscopy (TERRS) allowed vibration modes assigned to Histidine (His), Tyrosine (Tyr) and Phenylalanine (Phe) residues at 837, 932 and 1002 cm^{-1} respectively,¹² and those characterizing the Cc oxidation and spin states,¹²⁻¹⁴ to be discerned. Nevertheless, very few TERS data on Cc molecules in the pure ferric 6cLS state were reported so far.¹⁴

Prior to the description of TIR-TERS, resonance TIR-SERS measurements on Cc molecules are presented to provide benchmark spectra for subsequent comparison with their TIR-TERS counterparts. The advantages of TIR-TERS are then emphasized in the light of the performances of TIR-Raman and bottom-illumination TERS, using an experimental/theoretical approach. Finally, TIR-TERS is employed to probe Cc and confirm its oxidation, spin and ligation state.

Determination of the Cc state by TIR-SERS. TIR-SERS measurements of Cc molecules electrostatically bound to a SAM of 11-mercaptoundecanoic acid (MUA) covering a rough Ag film (see experimental details in Supporting Information) were carried out at 532nm irradiation wavelength. This allows for the resonant excitation of both Cc molecules and the localized surface plasmon resonance (LSPR) of the SERS-active Ag substrate. The UV-visible absorption spectrum of Cc in 0.2mM aqueous solution displays indeed a Soret band at 409nm, and a weaker Q band at 530nm, both originating from the porphyrin chromophore of the heme group (Figure 1A). Moreover, dark-field Rayleigh scattering microscopy (DFRSM) spectra of uncoated SERS substrates show LSPRs at 570 nm that red shifted by 60 nm upon MUA coating (Figure 1B). The addition of Cc on the thiol SAM does not affect further LSPRs. Only the scattering intensity is progressively decreased upon increasing the thickness of the dielectric layer (Cc and thiols) on top of the metal substrate (Figure 1B). For 532 nm excitation, the investigated 500-1800 cm^{-1}

spectral range corresponds to the 546-588 nm region located near the maximum of the LSPR band of SERS substrates. This irradiation wavelength is thus perfectly suited to achieve resonance SERS measurements of Cc. In contrast, the excitation of the Soret band could lead to very intense resonance Raman signals but the SERS enhancement of Raman signatures associated with both Cc and the underlying thiols would be quite weak, and inappropriate for the forthcoming comparison with TERS.

Figure 1. A) UV-visible absorption spectrum of an 0.2 mM aqueous solution of Cc. Laser wavelengths used to excite the LSPR of the SERS substrates and the TERS tip are indicated. B) From top to bottom, DFRSM spectra of uncoated and MUA-coated silver SERS substrates as well as DFRSM spectrum of silver SERS substrate coated with MUA and Cc molecules. The laser wavelength allowing for the excitation of these substrates is mentioned. C) Resonance TIR-SERS spectra of MUA (bottom) and Cc deposited on a MUA SAM (top) for a 532 nm irradiation. The dashed lines indicate the wavenumber of expected Cc marker bands.

Figure 1C presents resonance TIR-SERS spectra of pristine and Cc-covered MUA SAMs. MUA molecules have been selected to functionalize the SERS substrate because of the high affinity of Cc for hydrophilic surfaces. This originates from the fact that polar (hydrophilic) amino acid residues of Cc mainly reside on the outer surface of the protein, whereas hydrophobic amino acids are essentially located inside the protein. For this reason, replacing MUA by a mixture of MUA and hydrophobic undecanethiol molecules to better mimic the heterogeneity of real membranes reduces the SERS signal intensity, but the SERS fingerprint itself with specific wavenumbers is not affected (Figure S1 in Supporting Information). In the absence of Cc, the successful functionalization with MUA molecules is revealed by the intense band centered at 709 cm^{-1} assigned to C-S stretching vibrations as well as the band at 1099 cm^{-1} assigned to the C-O stretching vibration of the MUA terminal -COOH/-COO- group.¹⁵ Carbonyl bands of MUA molecules expected in the 1700-1740 cm^{-1} were not observed. This may be related to their low Raman cross-section and to the presence of negatively charged terminal carboxylate groups instead their hydrogenated counterparts. After deposition of Cc molecules, many new SERS bands centered at 750, 1373, 1585 and 1636 cm^{-1} and assigned to the porphyrin ring appear. These four marker bands are assigned to the so-called ν_{15} , ν_4 , ν_2 and ν_{10} vibration modes of the heme group respectively, and are fully consistent with the presence of Cc in the ferric 6cLS state,^{6,12} as expected in the deposited neutral-pH Cc aqueous solution and on SERS-active substrates at 0 V electrical potential.⁶ Previous Raman measurements reported also a ν_3 band at 1502 cm^{-1} typical of this state,⁶ but it is hardly discernable in our SERS spectra. It is important to point out that, native and non-native forms of Cc exhibiting different axial ligands and biological activities may both be 6cLS species. Thus, only very subtle Raman shifts, requiring a very high spectral resolution to be identified, allow them to be distinguished from each other.⁶ However,

conventional $1\text{-}3\text{cm}^{-1}$ spectral resolutions are sufficient to exclude indubitably the presence of 6cHS ferric, 5cHS ferric, 6cLS ferrous and 5cHS ferrous states of Cc.

TIR-Raman characterization of Cc. For the study of Cc, a glass/Au (10 nm)/MUA/Cc (20 nm) multilayer sample has been selected for three reasons. As explained in the preceding section, Cc molecules have a good affinity for hydrophilic substrates such as MUA-coated Au films. Furthermore, in order to work in TIR, the critical angle must be precisely determined. However, as the refractive index of Cc on glass is very close to that of borosilicate glass alone (1.44 vs. 1.518),^{16,17} it turns out to be extremely difficult to identify the glass/Cc interface in the absence of Au interlayer, and thus to identify the TIR regime. In practice, the intensity of the Raman signal associated with a glass/Cc sample was found to be almost constant irrespective of the incidence angle, albeit slightly higher (by 30% at most) under p-polarized than s-polarized light excitation. Finally, the thickness of the Au interlayer was optimized to lead to the strongest Raman signal in TIR configuration. The determination of the ideal thickness not only requires the calculation of mean-square electric fields within the 20 nm-thick Cc layer but also that of the transmittance of the Au interlayer, since the collected Raman signal must traverse the Au film to be detected. Considering these two quantities, the strongest TIR-Raman signal is found for 10-20 nm Au thicknesses under p-polarized 561 nm excitation and 5 nm Au thickness under s-polarized 561 nm excitation (Figure S2). Note that the 561 nm wavelength has been preferred to the 532 nm one for reference TIR-Raman characterizations because it allows for the excitation of the LSPR of our Ag/Au TERS probes¹⁸ and of Cc (Figure 1A), while the resonant excitation of our TERS probes is impossible at 532 nm (vide infra). Figure 2A presents mean-square electric fields inside a 20 nm-thick Cc layer covering a 10 nm-thick Au layer under p-polarized and s-polarized light irradiation. Figure 2B shows the corresponding transmittance of the same Au

layer. The maximal mean-square electric field is found at 48° and 41° incidence angles for p-polarized and s-polarized excitations, respectively, considering the 41° critical angle in our configuration. On the experimental standpoint, the dependence of the Raman intensity associated with a glass/Au (10 nm)/MUA/Cc multilayer sample as a function of the incidence angle can be estimated either by integrating the spectral region centered at 750 cm^{-1} (Figures S3 and S4) and at $\sim 1600\text{ cm}^{-1}$ (Figures 2C and S4) that are typical of Cc molecules. Incidence angles leading to maximal Raman intensities appear to be $46\text{--}47^\circ$ and $42\text{--}43^\circ$ under p-polarized and s-polarized 561 nm laser light, which is very close to aforementioned theoretical values. Figure 2C also shows that the Raman intensity under p-polarized excitation is more than twice as high as its counterpart under s-polarized excitation. As p polarization (with dominant component in the z-direction perpendicular to the xy sample plane) is particularly convenient to excite TERS tips in bottom-illumination configuration,¹⁹ the high mean-square electric field under p-polarized excitation constitutes an obvious advantage to probe Cc in TIR-TERS.

Figure 2. A) Calculated mean-square electric fields in a 20 nm-thick Cc layer on top of a 10 nm-thick Au film for p-polarized (straight line) and s-polarized (dashed line) incident light excitations and different incidence angles. B) Calculated transmittance of a 10 nm-thick gold film for p-polarized (straight line) and s-polarized (dashed line) incident light excitations and different incidence angles. C) Raman intensity of the spectral region centered at 1600 cm^{-1} for a 20 nm-thick Cc layer on a 10 nm-thick Au film excited using p-polarized (straight line with full circles) and s-polarized (dashed line with open circles) 561 nm incident laser light and different incidence angles.

Performances of TIR-TERS. In order to optimize the intensity of the TERS signal by benefiting especially from any potential gap mode,²⁰ a noble metal tip must be placed a few nanometers above the glass/Au/MUA/Cc multilayer sample, where the Cc coating is limited to a monolayer.

The distance between the tip end and the Au layer is then ~ 6 nm, owing to the ~ 5 nm MUA/Cc layer and the 1 nm tip-surface distance. In this configuration, the theoretical estimate of the electric field enhancement in the 60nm-broad portion of the MUA/Cc molecular layer below the tip end highlights the interest of TIR-TERS (Figure 3). Experimental Ag(30nm)/Au(5nm) silicon tips are then modelled by Ag tips showing a LSPR at 561 nm, as observed in practice.¹⁸ In the traditional bottom-illumination excitation, the tight focusing of the radially polarized light leads to a strong electric field component along the tip axis, i.e. a p-polarized electric field at the tip. In this case, a moderate average enhancement of the electric field by a factor 2.5 can be directly linked to the presence of the Ag tip (Figure 3A, top panel of Figure 3C). This leads a total electromagnetic enhancement of ~ 40 , which is obviously much lower than the value $\sim 10^4$ values reachable in the absence of the 5nm-thick dielectric molecular layer.²¹ In contrast, the enhancement of the electric field can be much higher if the incident light is injected in TIR (Figure 3B, bottom panel of Figure 3C). The highest value of the average electromagnetic enhancement induced by the Ag tip (~ 5.6) is obtained for a 42° incidence angle (Figures 3D and 3E), and corresponds to a total electromagnetic enhancement of $\sim 1.0 \times 10^3$. This 42° angle is very close to the 41° critical angle. At that 42° value, a 250nm-long portion of the Ag tip should be excited by the evanescent field (Figure S5). The resulting lightning-rod effect should thus be increased with respect to that arising from TIR excitations at wider angles of incidence, for which shorter tip portions are excited. This could explain the strong total field enhancement at 42° in TIR configuration. Finally, we can conclude that TIR-TERS provides an additional amplification of the TERS signal by a factor 25 with respect to the traditional bottom-illumination excitation with radially-polarized light. This value is consistent with the factor 20 determined by other authors by comparing TIR-TERS (in ATR mode) and side-illumination

TERS measurements.² This original property is highly advantageous for TERS. As the laser power density is ~ 560 times lower for TIR irradiation than for classical direct illumination (see experimental details in SI), the far-field contribution is strongly reduced in TIR and can even be undetectable, as it is the case for the present glass/Au/MUA/Cc multilayer sample (vide infra). This low fluence avoids any degradation of light sensitive molecules, such as Cc that has to be resonantly excited, but the additional enhancement of the electric field allows intense enough TERS signatures to be collected (Figure 4).

Figure 3. FDTD spatial distribution of the electric field magnitude $|E|/|E_0|$ when a silver tip is positioned 1 nm above a 5 nm-thick molecular layer deposited on a 10 nm-thick Au layer, for A)

a 561 nm radially-polarized light excitation in bottom-illumination configuration, and B) a 561 nm light excitation in TIR (with a 46° angle of incidence). C) Electric field magnitudes in a 60 nm-broad portion of the molecular layer for the radially polarized (top) and TIR (bottom) light excitations. D) Variation of the $|E|/|E_0|$ value integrated over the 60nm-broad region plotted in C (bottom) as a function of the angle of incidence in the presence (full circles) and in the absence (open circles) of Ag tip. E) Ratio $R=(|E|/|E_0|)_{\text{tip engaged}}/(|E|/|E_0|)_{\text{tip withdrawn}}$ as a function of the angle of incidence calculated from data plotted in D.

TIR-TERS characterization of Cc. On the experimental standpoint, the maximal TERS signal is found just above the 41° critical angle (~42°), in excellent agreement with FDTD calculations. Figure 5A presents five TIR-TERS spectra obtained with different TERS probes. The first striking difference from one spectrum to another relates to relative intensities of TERS bands. This is a common behavior in TERS due in particular to the nanoscale confinement of the electromagnetic field at the tip apex.¹⁹ Such intensity fluctuations are consistent with the distinct relative intensities observed in TERS spectra reported in the literature for Cc molecules.¹²⁻¹⁴ Despite this drawback, the ν_{15} , ν_4 , ν_3 , ν_2 and ν_{10} vibration modes can be discerned in most TERS spectra. Considering our 3 cm⁻¹ spectral resolution, the Raman shifts of these Cc marker bands does not seem to be rigorously constant, but they are very close to the benchmark values deduced from SERRS measurements (751 cm⁻¹, 1375 cm⁻¹, 1497 cm⁻¹, 1588 cm⁻¹ and 1629 cm⁻¹ instead of 750 cm⁻¹, 1373 cm⁻¹, 1502 cm⁻¹, 1585 cm⁻¹ and 1636 cm⁻¹ for ν_{15} , ν_4 , ν_3 , ν_2 and ν_{10} vibration modes, respectively). Therefore, a single marker band is not specific enough to characterize the 6cLS ferric state of Cc. For example, the TIR-TERS band at 1497 cm⁻¹ and 1588 cm⁻¹ are very close to the 1497 cm⁻¹ and 1591 cm⁻¹ bands expected for the 5cHS ferric and 6cLS ferrous state of Cc, respectively.⁶ However, the presence of three to five TIR-TERS bands at the expected

wavenumbers for ν_{15} , ν_4 , ν_3 , ν_2 and/or ν_{10} vibration modes of the 6cLS ferric state of Cc clearly characterize this state. Figure 5 thus demonstrates that SERS and TERS spectra of Cc in its 6cLS state are consistent to each other, and confirm the often admitted law that SERS vibration modes can be considered as good references to assign TERS bands of biomolecules. Finally, this study also reveals the possibility to specifically identify the 6cLS ferric state of Cc from other states with the nanoscale spatial resolution of TERS and the low-fluence excitation in TIR, which is of high interest for future investigations on biological tissues.

Figure 5. A) TIR-TERS spectra of a glass/Au/MUA/Cc sample obtained by using different metal-coated tapping mode AFM tips, for an incidence angle just above the critical angle. Dashed lines indicate the wavenumbers of ν_{15} , ν_4 , ν_3 , ν_2 and ν_{10} vibration modes. B) Variation of

the Raman shifts associated with ν_{15} (full squares), ν_4 (open triangles), ν_3 (full triangles), ν_2 (open circles) and ν_{10} (full circles) vibration modes for the five spectra plotted in A.

In summary, we have demonstrated that performing TERS in TIR configuration may be an efficient way to probe biomolecules with minimal incident laser power densities to avoid any sample degradation. In the case of Cc, the 6cLS could be nicely characterized and its spectral signature appeared to closely match that found in TIR-SERRS experiments, performed for the first on a biomolecule as well. Our results thus suggest that 6cLS species of Cc, potentially implicated in apoptotic processes, could be detected with nanoscale spatial resolution. Finally, as TIR-SERS has been recently proven to be a particularly promising technique for investigations in liquid medium,²² the next challenge would be to develop the TIR-TERS technology for studies in this biologically relevant environment.

ASSOCIATED CONTENT

Supporting Information.

Experimental and theoretical details, Interactions between Cc and thiol SAMs linked to electron transfer, SERRS spectra of Cc deposited on mixed MUA/undecanethiol (UT) SAMs, Theoretical calculations of mean-square electric fields in a Cc layer and transmittances in Au layers, Conventional Raman measurements in TIR geometry under p-polarized and s-polarized light excitations, Calculation of the power density in TIR and classical Raman configurations, Near-field nature of the excitation of the TERS probe showing the spatial extent of the evanescent field as a function of the incidence angle.

The following files are available free of charge.

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Notes

The authors declare no competing financial interests.

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