

Tip-Enhanced Raman Spectroscopy Can See More: The Case of Cytochrome c

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The heme protein, cytochrome c (Cc) has been studied using tip-enhanced Raman spectroscopy (TERS). By virtue of its sensitivity and superior spatial resolution, TERS detected both the heme and amino acid vibrational bands of Cc using resonance excitation at 532 nm. This is in contrast to conventional surface-enhanced Raman spectroscopy (SERS) where ensemble information is obtained, leading to the strongest Raman bands obscuring the weaker ones; i.e., only the resonantly enhanced heme bands are observed. Matrix-assisted laser desorption/ionization mass spectrometry supported the interpretation of the Raman data by showing that Cc remains intact on Ag surfaces. This work demonstrates the sensitivity of TERS for analyzing proteins (complete with band assignments) and that a collection of TER spectra gives a more complete description of large biomolecules. The latter is an important advantage usually not found in SERS measurements.

1. Introduction

Intriguing questions about the numerous biological processes occurring at the lipid membrane of a living cell have been raised, including the chemical identity and dynamics of membrane proteins diffusing in the lipid bilayer.^{1,2} These issues are intimately connected to how a cell functions and its vulnerability to invasion from viruses. To understand them, spectroscopic instrumentations that can give molecular fingerprints of biological molecules in situ with nanometer lateral resolution are required. This cannot be achieved by conventional optical techniques due to their inadequate spatial resolution or inability to give chemical information unless the analyte is artificially tagged with fluorophores or has an intrinsically high fluorescence quantum yield. To overcome these disadvantages, tip-enhanced Raman spectroscopy (TERS), a label-free nanoanalytical method, was developed in 2000.^{3–6} TERS can be considered as the high lateral resolution counterpart of surface-enhanced Raman spectroscopy (SERS). It makes use of a metal-nanoparticle-coated tip to enhance the intrinsically weak Raman signals (the chemical signature) of the analyte. The spatial resolution is given by the sizes of the enhancing nanoparticle sites located at the tip apex, and values down to several nanometers have been reported.⁷ To date, TERS has mostly been applied in proof-of-principle studies. An extension of this technique to the study of biologically important molecules with accurate vibrational band assignments is necessary and will lead to significant advances in the biomedical field.

The better resolution afforded by TERS as compared with Raman/SERS can also yield improved specificity of information. TER and SER spectra are expected to differ in some ways, and this difference can be put to good use. For Raman/SERS, the resultant vibrational spectrum is always dominated by features from the strongest Raman scattering bands or from the major analyte present. A good example is the heme protein, cytochrome c (Cc), found in the inner membrane of mitochondria. It contains a heme c, covalently bound to the protein through thioether linkages and Fe–amino acid coordination (Figure

1a): its SER spectrum (using resonance excitation at 400–550 nm) is “incomplete”, i.e., bands from the amino acids are not observed, despite their larger abundance as compared with the heme. This is attributed to the greater resonance enhancement of the heme bands (due to electronic transitions in the Fe–porphyrin) that will overwhelm the weaker Raman scattering amino acid bands (which absorb in the UV range).^{8,9}

If TERS can be considered as a locally resolved SERS experiment as illustrated in Figure 2, it should be possible to probe individual segments of a protein (e.g., Cc) using its superior spatial resolution. This could be done by placing the enhancing tip on different parts of the protein structure. The problem of the highly intense heme signals overwhelming the weaker protein ones can then be overcome. The TER spectra (i) can be “added up” to give a more complete physical representation of the structure of the analyte and (ii) be used to deduce the mode of adsorption of Cc (and other proteins) on surfaces; i.e., if the heme center of Cc is detected by chemical enhancement using the TER tip, it is probably not chemically bound to the metal surface. An understanding of protein orientation on surfaces is essential for the construction of chemical- and biosensors.^{10,11}

In this work, TERS was employed to detect and determine the behavior of Cc under the influence of Ag-coated tips. These experiments were complemented by normal Raman spectroscopy, SERS, and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) measurements. The detection of intact Cc was achieved with TERS. Due to the stronger and more local enhancement afforded by the enhancing tip, both the heme as well as the amino acids of Cc were identified. The observed spectroscopic features were assigned to their corresponding molecular adsorbates. This work demonstrates the ability of TERS to detect and to give more complete structural information for Cc as compared with conventional SERS and its use in the study of Cc orientation on surfaces. It paves the way for detailed nanometer-scale spatial resolution studies of proteins in lipid membranes.

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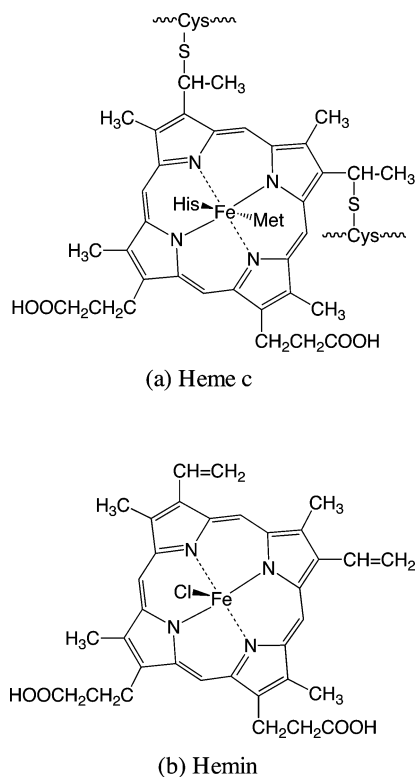


Figure 1. Molecular structures of (a) heme c and (b) hemin. In Cc, the heme c is bonded to a polypeptide chain through the four amino acids, as shown in the figure.

2. Experimental Section

Chemicals. Cc (bovine heart, $\geq 95\%$, Fluka) and hemoglobin (Hb, bovine blood, $\geq 90\%$, Fluka) were used as received and dissolved in H_2O . A mass of 10 mg of sinapinic acid (SA, $\geq 99\%$, Fluka) dissolved in a 1 mL acetonitrile/ H_2O /trifluoroacetic acid (1:1:0.01) mixture was used as the MALDI matrix.¹² Hemin (porcine, 99%, Acros, Figure 1b) and aromatic L-amino acids ($\geq 90\%$, Fluka) phenylalanine (Phe), histidine (His), tyrosine (Tyr), and tryptophan (Trp) were used as reference compounds.

Raman Spectroscopy Sample Preparation. Several microliters of the solution were deposited on clean glass surfaces by either drop- (normal Raman) or spin- (TERS) coating and allowed to dry. The Cc is expected to be randomly oriented.

SERS and MALDI Sample Preparation. A 6 nm layer of Ag was vapor-coated on clean glass slides.¹³ Submonolayers of Cc were then deposited on these surfaces by spin coating. Prior to the MALDI experiment, 1 μL of the matrix solution was spotted on top of the samples and allowed to dry at room temperature.

Raman Spectroscopy Experiments. The Raman setup consists of a confocal microscope (IX70, Olympus, Japan), an atomic force microscope (Explorer AFM, Veeco, U.S.A.), and a Raman spectrograph (Kaiser Holopsec, U.S.A.).¹⁴ A 532 nm diode-pumped solid-state laser (Ventus, Laser Quantum, U.K.) was used as the excitation source. For the normal Raman and SERS experiments, $\sim 2\text{--}80\ \mu\text{W}$ light was focused onto the sample using a 1.4 N.A., 60 \times oil-immersion objective lens. The same parameters were used for the TERS experiment with the addition of the Ag-coated tip in the laser focus. SiN AFM contact tips (RC800PSA, Olympus) coated with 20 nm of AlF_3 , followed by 30 nm of Ag were used.¹³

MALDI Experiments. The samples were mounted onto a stainless-steel plate by copper adhesive tape. MALDI measure-

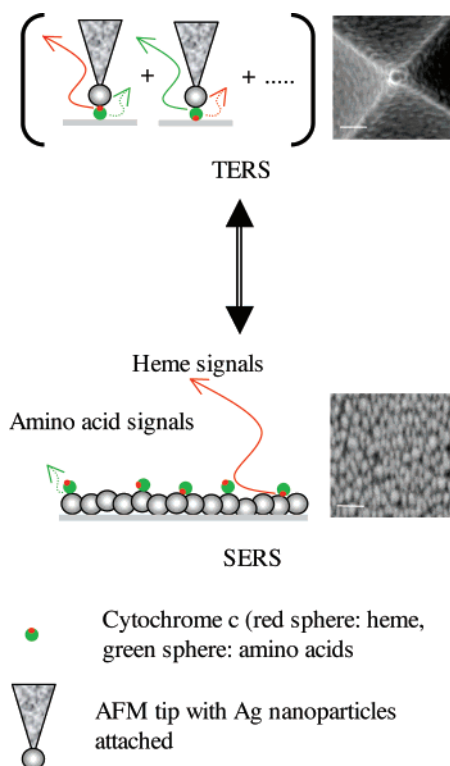


Figure 2. Schematic depicting a SERS experiment as an ensemble of TERS experiments. The roughened Ag metal film is replaced with a metallized AFM tip that is scanned over the analyte. The tips are shown in contact with various parts (heme, amino acids) of Cc. The heme is located at the surface of the protein.²⁵ SEM images (scale bar: 100 nm) of a Ag-coated AFM tip and glass slide are shown next to the cartoons. The apex of the TERS tip is in the center of its image. The solid and dashed curly arrows represent the stronger and weaker signals, respectively, as a consequence of metal nanoparticles–molecule interaction. Unlike TERS, the SER signals from the heme (red trace) always overwhelm completely those from the amino acids (green trace).

ments were performed on a time-of-flight mass spectrometer (Axima CFR, Shimadzu, U.K.) with a 337 nm N_2 laser as the ionization source. The instrument was operated in the positive linear mode, with delayed extraction and an acceleration voltage of 20 kV. To minimize a possible dependence on surface morphology or inhomogeneous analyte distribution, each MALDI spectrum presented is an average of 150 measurements taken at various locations of the sample.

Data Processing. No background subtraction or smoothing of the spectra was made. The data points were processed using Igor Pro, version 4.09A Carbon (WaveMetrics, U.S.A.).

3. Results

The results are organized in the following order: the resonance Raman (RR) and SER spectra of Cc are first presented. These are followed by the TERS detection of Cc. Finally, MALDI-MS experiments to corroborate the vibrational data are shown. All measurements were repeated 2–3 times, and similar spectra profiles were obtained.

3.1. Resonance Raman Spectroscopy (RRS) of Cc and Hemin. Figure 3a shows the RR spectrum of Cc collected with 532 nm excitation. It consists only of bands from the heme. The ν_{15} peak, one of the most easily resolved bands of Cc, can be observed at $748\ \text{cm}^{-1}$. The absence of other vibrational features in the region makes this peak a convenient marker for the presence of Cc. The marker bands ν_{10} , ν_{19} , and ν_4 can be

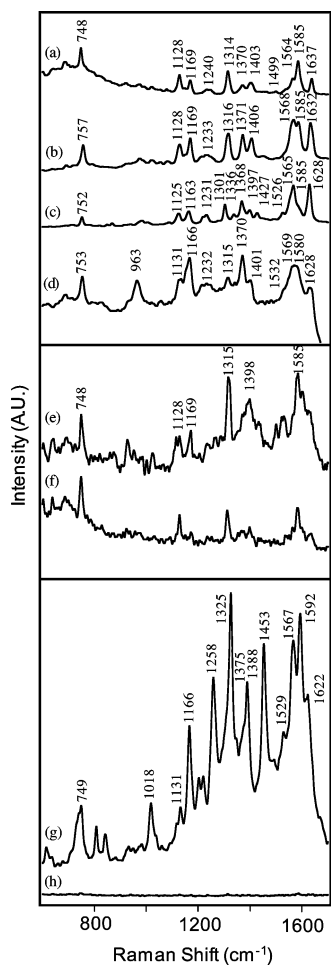


Figure 3. Raman spectra of (a) LS Cc³⁺, (b) HS Cc³⁺, and (c) hemin. (d) SER spectrum of submonolayers of Cc on Ag. Raman spectra of Cc acquired with Ag-coated tip in contact (e and g) and retracted (f and h). For easier comparison, the spectrum in Figure 3a has been rescaled.

used to determine the spin and oxidation states of the Cc.¹⁵ The Raman shifts of these peaks at 1637 (ν_{10}), 1585 (ν_{19}), and 1370 cm⁻¹ (ν_4) confirm the presence of low-spin (LS) Cc³⁺. These are its expected spin and oxidation states at room temperature and are also consistent with the supplier's specifications.¹⁶ Since 532 nm falls within the α - β absorption band of Cc, the totally symmetric A_{1g} modes, which have been found to be ineffective in vibronic mixing, are expected to be weak in intensity. This is the case, as evident from the weak 1499 (ν_3) and 1370 (ν_4) cm⁻¹ peaks.

High-spin (HS) Cc³⁺ was prepared by dissolving the LS species into H₂O acidified by HCl to pH 2. The change in spin was verified by UV-vis absorption spectroscopy, and its structural intactness was determined by MALDI-MS (data not shown). HS Cc³⁺ was identified by its bands at 1632 (ν_{10}) and 1568 cm⁻¹ (ν_{19}) (Figure 3b).¹⁷ Preservation of the ν_4 frequency at 1371 cm⁻¹ demonstrates that its +3 oxidation state has been maintained. Corroborative RR data from hemin (HS Fe³⁺ heme) was also recorded, and the band frequencies were found to be matching, thereby confirming spin conversion (Figure 3c). All the observed frequencies agree well with those from previous experiments, and their band assignments are presented in Table 1.¹⁶⁻¹⁹

3.2. SERS of Cc. The SER spectrum of a submonolayer Cc spin-coated onto a Ag film is shown in Figure 3d. Broadening

of the peaks in the region 1560–1640 cm⁻¹ is observed. The growth of the ν_{10} and ν_{19} bands at 1628 and 1569 cm⁻¹, respectively, with the frequency conservation of the oxidation state marker peak ν_4 at 1370 cm⁻¹, demonstrates a LS to HS conversion of Cc³⁺. The $\nu(\text{CC})_{\text{propionate}}$ vibration at 963 cm⁻¹ is strongly enhanced in the SER spectrum (relative to its RR spectrum), which suggests that the propionate substituent of the heme is bonded to the Ag surface. Such a binding mode has been postulated for Cc and Hb adsorbed on Ag colloids and is consistent with the Fe heme–Ag surface contact, a requisite for spin conversion.^{16,20}

The SER bands can only be attributed to the heme of the Cc. No signs of amino acid bands were found in the SER spectrum, consistent with previous work.^{8,10,16} This should not suggest that the Cc is adsorbed in a fashion such that in each case, the heme is bound to the Ag surface. A SER spectrum consisting of only heme bands can arise from a minority of such adsorbed species. This is because even after considering the chemical enhancements for both the heme and amino acids, the former would still have a larger Raman scattering cross section due to resonance effects at 532 nm excitation, and the dynamic range afforded by conventional apparatus would never be sufficient to allow the detection of very weak amino acid bands. The HS Cc³⁺ frequencies in Figure 3d are summarized in Table 1.

3.3. TERS of Cc: Identifying its Various Facets. With a firm foundation for the vibrational characteristics of Cc laid, TERS spectra were then measured. It will be shown here that these spectra can be unambiguously assigned to the heme and amino acids of Cc, demonstrating both the sensitivity and power of TERS to detect and to give local information about this analyte.

A. Type I. The TER spectrum of Cc (acquisition time: 30 s) is shown in Figure 3e. When the Ag-coated AFM tip contacts the sample surface, the peak intensities increase by 2 times relative to those from the far-field spectrum (Figure 3f). The frequencies of the TER bands agree well with those from the RRS (Figure 3a), thereby demonstrating the presence of LS Cc³⁺. Although there are some features at \sim 1625 and \sim 1570 cm⁻¹ that might be assignable to HS Cc³⁺, these are weak shoulders in the TER spectrum. We conclude that there are no clear indications of the presence of HS Cc³⁺, and if it is present at all, it would be a minor species. These observations are evidently a result of the Ag nanoparticles on the tip not being in direct contact with the Cc. Without direct contact to the Ag metal, neither LS to HS Cc³⁺ conversion nor chemical enhancement of the amino acid bands are possible. It should be noted that chemical enhancement is a known prerequisite for the observation of amino acid bands in SERS.²¹⁻²³ The enhancement contribution here must then be purely electromagnetic in nature. A similar observation has also been reported in the TERS of DNA bases.²⁴

B. Type II. Figure 3g shows another TER spectrum (acquisition time: 10 s). The bands therein show substantial intensity and frequency differences when compared with the RR or TER spectrum of LS Cc³⁺. The ν_{15} band at 749 cm⁻¹ can still be easily identified, thereby confirming the TERS enhancement of the Cc. The bands are assigned to HS Cc³⁺. The presence of this species is demonstrated by the ν_{10} and ν_{19} bands at 1622 and 1567 cm⁻¹, respectively. The band position of the oxidation state marker, ν_4 at 1375 cm⁻¹, indicates that the Cc retains its oxidation state of +3. The frequency increase of the ν_{21} band to 1325 cm⁻¹ has also been similarly reported during LS to HS conversion of Cc.¹⁷

TABLE 1: Mode Assignments and Observed Raman Shifts (cm⁻¹) for Cc

modes (symmetry)	LS Cc ³⁺			HS Cc ³⁺		HS Cc ³⁺			Hemin	
	RR Fig. 3a ^a	RR ^b	RR ^c	SERS Fig. 3d ^a	TERS Fig. 3g ^a	RR Fig. 3b ^a	RR ^d	SERS ^e	RR Fig. 3c ^a	RR ^e
ν_{10} (B _{1g})	1637	1635	1634	1628	1622	1632	1623	1626	1628	1626
ν (CC) _{vinyl}										1626
ν_{37} (E _u)					1592	1585			1585	1591
ν_2 (A _{1g})							1575	1570		1570
ν_{19} (A _{2g})	1585	1584	1584	1569 (HS) 1580 (LS)	1567	1568	1569	1568	1565	1571
ν_{11} (B _{1g})	1564	1561	1561				1556			1553
ν_{38} (E _u)				1532	1529				1526	1533
ν_3 (A _{1g})	1499						1490	1492		1491
δ (CH ₂) ⁽¹⁾					1453				1427	1435
ν_{20} (B _{2g})	1403	1402	1405	1401	1388	1406	1410		1397	1403
ν_4 (A _{1g})	1370	1371	1373	1370	1375	1371	1376	1373	1368	1373
δ (CH ₂)									1336	1340
ν_{21} (A _{2g})	1314	1315	1315	1315	1325	1316	1324			1309
δ (CH ₂) ⁽²⁾									1301	1308
ν_{13} (B _{1g})	1240	1246	1230	1232	1258	1233	1235		1231	1228
ν_{30} (B _{2g})	1169	1168	1171	1166	1166	1169	1172		1163	1170
ν_{22} (A _{2g})	1128	1127	1129	1131	1131	1128	1144		1125	1127
ν_{31} (B _{2g})		1021			1018					
ν (CC) _{propionate}		974		963						
ν_{15} (B _{1g})	748	748	753	753	749	757			752	

^a This work. ^b Reference 18. ^c Reference 16. ^d Reference 17. ^e Reference 19.

The peak at 1018 cm⁻¹ is assigned to ν_{31} , the ν (CC) vibration between the porphyrin ring and its peripheral substituent.⁹ The enhancement of this band suggests an edge-on interaction of the heme with the tip, consistent with the former's semi-exposed position in Cc as found from crystallographic studies.²⁵ This contrasts with the SERS of Cc where the ν (CC)_{propionate} at 963 cm⁻¹ was enhanced (Figure 3d). It therefore suggests that the Cc is immobilized on the glass slide and has no possibility of reorienting or conformationally adjusting itself when the TERS tip is in contact. Fe heme–Ag tip chemical contact is consistent with the requirement for LS to HS conversion of the Fe center and has been observed in SERS experiments with intact Cc adsorbed on Ag colloids.¹⁶ Enhancement of the ν_{31} band in the SER spectrum (which was not observed in the RR spectrum) has also been reported for Cc²⁺ adsorption on electrochemically roughened Ag electrodes.¹⁰ Note the absence of amino acid bands: this is attributed to obscuring effects by the highly enhanced heme bands, analogous to findings from SERS studies.

C. Type III. Bands that cannot be easily assigned to the heme of Cc (after comparison with the literature and the preceding data shown in this work) were periodically observed in the TER spectra and are represented in Figure 4a (acquisition time: 10 s). These can be assigned to the amino acids. The region at 800–1100 cm⁻¹ is presented because it is relatively free from strong heme bands (see Figure 3a). The presence of Phe (four in a Cc), His (three), and Tyr (four) could be discerned. In particular, the band at 1002 cm⁻¹ can be ascribed to the aromatic ring-breathing mode of Phe. It is an outstanding line in the SER and Raman spectra of many proteins such as bovine serum albumin (BSA) and lysozyme.^{22,23,26} Having a characteristic signature at \sim 1000 cm⁻¹ and being remote from the dense spectral region at 1300–1800 cm⁻¹, this band can be unambiguously assigned and has been used as a reliable marker for the presence of proteins.²⁷ The bands centered at 837 and 932 cm⁻¹ can also be ascribed to His and Tyr, respectively. The indole ring-breathing mode of Trp which lies at 1007 cm⁻¹ was not found, which may be attributed to the fact that it is a rare

amino acid in Cc (one).²⁸ A similar observation has been reported for the SERS of BSA, which also has relatively few Trp in its sequence.^{22,23}

Corroborative TERS experiments have been performed on Hb (another heme protein) in order to verify the identity of these bands (Figure 4b). As expected, the amino acid TER frequencies from Cc match well with those from Hb. Their vibrational features are also in good agreement with Raman studies of the same amino acids (Figure 4c–f) and homodipeptides.^{29,30}

It ought to be mentioned that judicious control of the incident laser power in the TERS experiments prevented the production of amorphous carbon bands. These can be easily identified based on their well-known spectroscopic behavior and are presumed to originate from carbonaceous materials already attached on the Ag of the tip rather than from dissociation of the Cc.³¹

3.4. Is Cc Intact on Ag? To further corroborate the vibrational data that Cc was detected intact by TERS and SERS, MALDI-MS was performed on submonolayers of Cc spin-deposited onto Ag surfaces. The low analyte coverage ensured that all the molecules had a chance of interaction with the Ag atoms. Some of these samples were also annealed in an oven at 150 °C for 10 min to facilitate any chemical reactions. Figure 5a–c shows the MALDI spectra collected from Cc deposited on glass (reference) and on Ag (with and without heating). The signals at $m/z \sim$ 12 300 and \sim 6200 were assigned to the singly ((Cc + H)⁺) and doubly ((Cc + 2H)²⁺) charged molecular ions of intact Cc (both heme and protein complement) respectively. No peaks belonging to the apoprotein were discernible at $m/z \sim$ 11700. The MALDI-MS spectra of Cc mixed with different matrices have also exhibited similar spectra.³²

Numerous peaks below m/z 5000 can be observed in the spectra presented in parts b and c of Figure 5. Most of these peaks have a regular mass difference of 108 Da and are assigned to Ag_{*n*} cluster ions sputtered off the sample. The production of metallic clusters has been reported in MALDI-MS investigations of matrices mixed with Ag salts and laser-induced desorption from Au targets.^{33,34}

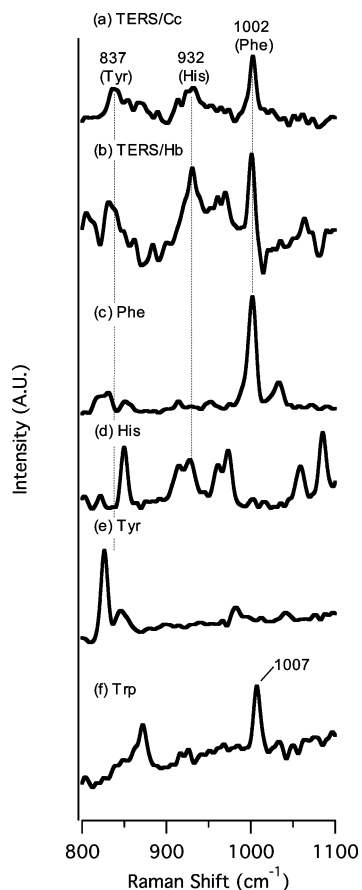


Figure 4. TER spectra of (a) Cc and (b) Hb. Reference Raman spectra of L-amino acids: (c) Phe, (d) His, (e) Tyr, and (f) Trp. For easier comparison, the spectra in parts b–f of Figure 4 have been rescaled.

The spectra in parts b and c of Figure 5 were also compared with one collected from a clean Ag surface with only SA matrix (control experiment). This is to ensure that all the peaks in the entire m/z range (e.g., from SA, $(SA + Ag_n)^{m+}$, etc.) could be accounted for. Only the value of $m/z \leq 2000$ is presented here as some fragments (e.g., the heme) of Cc will surely be within this window. Parts e and f of Figure 5 correspond to the low m/z regions of the spectra in parts b and c of Figure 5, respectively. It was found that all the peaks in Figures 5b and c (excluding $m/z \sim 12\,300$ and ~ 6200) are also present in the control spectra (Figure 5d); i.e., no extra ions which may correspond to peptide fragments or heme (from dissociated Cc) can be found. Using MALDI, it is hence directly demonstrated that submonolayers of Cc do stay intact on Ag surfaces even after heat treatment.

4. Discussion

4.1. Probing Cc with TERS. The detection of Cc, an important membrane protein, has been achieved in this work using TERS. This paves the way for detailed nanometer-scale spatial resolution studies of proteins in lipid membranes. Central to this work is the assignment of the observed TERS bands, a critical point that ought to be satisfied so that the technique can be useful for chemical analysis.³¹

We will now discuss the reasons why TERS can yield such rich spectra that are sometimes quite different from RR and SERS data. (i) The spatial resolution afforded by the Ag-coated tip is very high, in the low nanometer range.^{7,35} Hence, small changes in the type of oscillators present in the nanometer-sized enhancement site can be captured in the TER spectrum. This

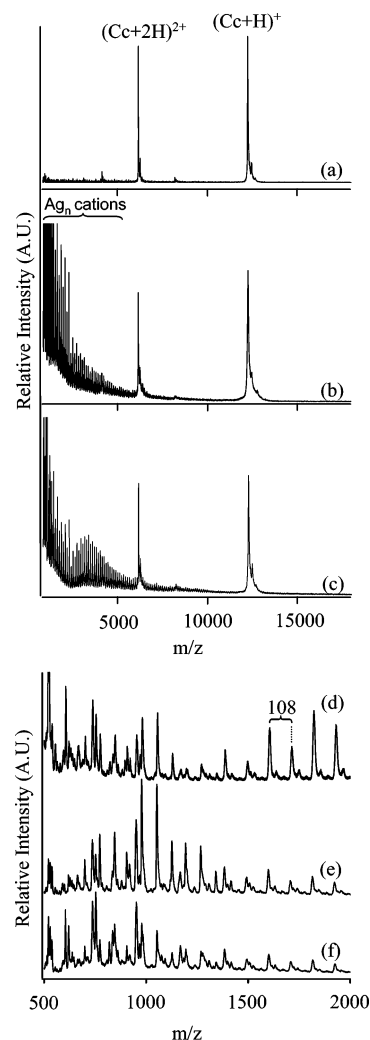


Figure 5. MALDI-MS spectra of a submonolayer of Cc deposited (a) on glass, (b) on Ag, and (c) on Ag followed by thermal annealing at 150 °C for 10 min. (d) Control experiment of Ag surface with only SA added. (e and f) Low m/z value regions of b and c, respectively.

contrasts with the low spatial resolution SER spectrum of Cc that is dominated by heme bands. (ii) TER bands not resembling the RR bands of Cc are observed because of Ag tip–molecule interactions that cause specific enhancements or frequency shifts. For randomly oriented Cc on the surface, a close Ag tip–heme contact will lead to the observation of HS Cc^{3+} , whereas a close Ag tip–amino acid contact will lead to the observation of amino acid bands that have been additionally amplified by chemical enhancement. The latter requires direct contact of the amino acids with the Ag tip and is essential for the observation of their TER signals.^{21–23} This interpretation is corroborated by a SERS experiment in which no signals could be observed from BSA adsorbed on Ag colloids in H_2O because of the presence of a hydration layer between the metal and protein that prevented a direct contact.³⁶ (iii) Chemical changes to Cc introduced by the preparation method could also have been suspected to contribute to the spectral differences. This can be ruled out. Cc is a structurally robust molecule and has generally been observed to remain intact in SERS experiments using Ag colloids.^{8,16} Furthermore, both the RRS (Figure 3a) and the MALDI-MS data (Figure 5) agree with those of intact Cc.^{16,18,32}

It is thus difficult to predict the type of TER spectrum (I, II, or III) and the relative intensities that will be recorded in a measurement. Likewise, the amino acids are also not always observed, although these are clearly present at each contact

between the tip and Cc. This observation is attributed to the nature of the tip as well as the number and orientation of the Cc molecules in its enhancement region. The former determines the detection sensitivity of the measurement and may not be consistently the same. Precise control of these experimental parameters is difficult at present.

Experiments comparing the TER and Raman spectra of a single-walled carbon nanotube have shown that local variations of the molecule could only be distinguished under the enhancement of the Ag tip.^{37,38} Similarly, the interaction of a Ag tip to the different N atoms of adenine has been studied. The presence of various Ag–adenine isomers with unique vibrational bands were found, indicating atomic site selectivity in TERS.³⁹ The present study demonstrates, in addition, that TERS can detect molecular bonds hitherto “invisible” to SERS. In the absence of the tip, all the Raman/SER bands of an analyte such as dye molecules would still be present although convoluted due to the inadequate spatial resolution. In the case of Cc, due to the dominance of the heme bands at 532 nm excitation, i.e., the amino acids bands are not observed, unless TERS is applied.

The present findings can also be used as an approach to deduce the orientation of Cc on the surface: the LS to HS conversion that is sometimes observed in TERS experiments would suggest that a larger fraction of the probed Cc molecules have their heme facing the tip. In other cases, various amino acid bands were observed by TERS, suggesting a different orientation. Overall, the Cc must therefore be randomly oriented on the surface; otherwise, such spectroscopically diverse TER spectra would not be obtained. A random orientation is of course expected when depositing Cc on a glass surface by spin coating. Knowing the orientation of Cc on metal surfaces is essential for the construction of nanoscale redox reaction chemical sensors: the electron-transfer dynamics are determined by the distance between the heme and the electrode.¹⁰

4.2. Determining the Structural Intactness of Cc with MALDI-MS. Cc has been shown by MALDI-MS to stay molecularly intact on Ag films under the present experimental conditions. This work emphasizes the usefulness of having a complementary technique to corroborate the vibrational data. In a study of Cc adsorption on borohydride-reduced Ag colloids, vibrational features assignable to HS Fe³⁺ heme dimers were observed. This led to the postulation that Cc had undergone deproteination.²⁰ However, HS Cc³⁺ formed from the interaction of the LS Cc³⁺, and Ag metal would also give a similar SER spectrum.¹⁶ Since the observed frequencies originate from the heme and tell little about the protein chain, it is difficult to clearly distinguish even the actual state of the analyte. MALDI-MS is the ideal technique to confirm the intactness of an analyte by analyzing its *m/z* ratio.

The usefulness of MALDI-MS to complement the vibrational data becomes more apparent as the size of the analyte increases. This is because the ability of SERS to collect the complete spectra of large molecules (“molecular fingerprinting”) begins to be limited due to the rapid decrease of the enhancement with distance from the metal surface.²² For example, all the vibrations of a small molecule such as benzene will be enhanced, while a large polynucleotide has only parts of its structure enhanced, depending on their proximity to the metal surface.

5. Conclusion

In this work, the membrane protein Cc has been studied using TERS, SERS, RRS, and MALDI-MS. By virtue of its superior

spatial resolution and with both chemical and electromagnetic enhancements, TERS detected both the heme and amino acids of randomly oriented Cc using resonance excitation with a 532 nm laser. This is in contrast to SERS where only ensemble information is obtained, leading to the strongest Raman bands obscuring the weaker ones; i.e., only the resonantly enhanced heme bands are observed. MALDI-MS has aided the interpretation of the Raman data by demonstrating that Cc stays intact on Ag surfaces, even after thermal annealing at 150 °C. This study demonstrates the sensitivity of TERS for Cc detection, with accurate band assignments. It also shows that TERS can give more structural information about large biomolecules as compared with SERS. The present findings are crucial steps toward the use of TERS as a technique for visualizing proteins in lipid membranes.

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