Tip-Enhanced Raman Spectroscopy
Finds a Growing Number of Applications in Biology

Marc CHAIGNEAU
Maruda SHANMUGASUNDARAM
Fran ADAR

Tip-enhanced Raman spectroscopy (TERS) combines the high chemical specificity of Raman scattering with the nanoscale spatial resolution of Scanning Probe Microscopy (SPM). It enables label-free detection of surface components in multi-component samples with ultra-high spatial resolution (better than 20 nm). This article describes how TERS can be applied to study biological systems ranging from pure components like amino acids to membrane receptors found in complex environments such as cell surfaces.

Introduction

Despite being able to provide fingerprint information in biology, medicine and other fields, Raman spectroscopy suffers from a signal-to-noise ratio that is limited by the inherent weakness of Raman scattering and a spatial resolution that is diffraction-limited (~0.5λ[*1]). It is often desirable to advance spatial resolution beyond the diffraction-limit into the nanoscale dimension, which can provide chemical information with potentially single molecule resolution. This spatial resolution enhancement is achievable through a technique called Tip-Enhanced Raman Spectroscopy (TERS).[1]

*1: 0.5λ: Half wavelength of radiation

TERS combines the high chemical specificity of Raman scattering with the nanoscale spatial resolution of Scanning Probe Microscopy (SPM). It enables detection of surface components in samples with ultra-high spatial resolution (down to 10 nm), making it suitable for studying nanomaterials and nano-objects.[2-6] TERS also offers several benefits in studying biomolecules compared to other spectroscopic and microscopic techniques. For example, it can overcome low signal-to-noise that hampers some bioapplications and thus avoid the need to use larger sample volumes. This is because TERS offers enhanced Raman signal similar to Surface Enhanced Raman Spectroscopy (SERS) with the added benefit of nanometer resolution; hence it can target potentially single molecules.

Alternative methods to study nanoscale chemical composition such as super-resolution fluorescence based techniques or methods to study morphology are available, and conventionally electron microscopy (EM) is used along with SPM to study particle dimensions on the nanoscale. However, EM, like SPM, gives limited chemical information and may require heavy metal staining of biological samples. Alternatively, super-resolution fluorescence microscopy techniques have been developed to obtain chemical information beyond the diffraction limit. Such techniques still use fluorescent labels whose broadband signal does not provide detailed molecular information similar to that of Raman spectroscopy. A label-free method can avoid unwanted interaction between the label and the analyte, preserve the analyte's function and avoid signal from the label itself, thus achieving a high degree of specificity with regard to chemical identity or characterization. TERS has this capability to provide nanoscale chemical composition in a label-free manner.

This article describes how TERS can be applied to study a number of biological systems such as amino acid and nucleobase monolayers, proteins, macromolecular protein assemblies, nucleic acids, cell surfaces and cell surface interactions.

Bringing Raman and AFM Together

Raman and AFM (Atomic Force Microscope from the SPM family) analysis can be combined on a single microscope system; it opens interesting new capabilities and provides new information on sample composition and structure by adding together physical and chemical information from a sample surface. Indeed, co-localized AFM/Raman measurement is the sequential or simultaneous acquisition of overlapped SPM (Scanning Probe Microscope) and Raman maps. On the one hand, the AFM and other SPM techniques provide topographic, mechanical, thermal, electrical, and magnetic properties down to the molecular resolution (~ nm, over μm^2 area),

on the other hand the confocal Raman spectroscopy and imaging provides specific chemical information about the material, with a diffraction limited spatial resolution (sub-micron).

With such so-called AFM/Raman system, it's also possible to bring Raman spectroscopy into nanoscale resolution imaging with Tip Enhanced Raman Spectroscopy (TERS). This technique is based on a metallic tip (generally made of gold or silver) employed to concentrate the incident light field at the apex of the tip, which acts as a nano-source of light and a local field enhancer, greatly improving the Raman sensitivity (factor of 10^3–10^7) and reducing the observation area to that localized at the tip.

Two different configurations exist for this coupling: one in transmission and one in reflection, having their own advantages and drawbacks (Figure 1).

The reflection configuration in oblique illumination is designed for TERS experiments for use with a large variety of SPM probes on many kinds of samples (opaque and transparent). The setup integrates a high NA long-working distance objective (up to 0.7 NA) at an optimum angle (60°). It brings the laser beam on the tip with ideal polarization orientation for TERS amplification (p-polarization) and ensures maximum collection efficiency by minimizing shadowing from the scanning probe-tip.

The inverted configuration which provides transmission measurements is ideal for transparent samples, which, of course, includes many biological applications: it allows the use of very high numerical aperture (NA) objectives, including oil immersion objectives (with NA up to 1.45), giving high power density at the focal point and enabling the collection of a high Raman signal level. This configuration in transmission also requires an appropriate polarization orientation. Radially polarized laser beam is thus mandatory for the plasmonic excitation and the induced enhancement of the electromagnetic field at the tip's vicinity.

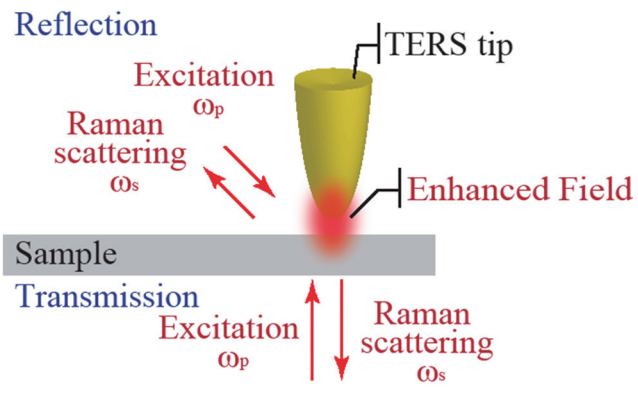

Figure 1 Reflection and transmission TERS configurations.

The TERS effect is produced by the strong local enhancement of the electromagnetic field at the apex of a sharp noble-metal tip when illuminated with a focused laser beam. The manufacturing of the TERS tips is currently based either on coating standard AFM tips (fully or partially) with a metallic layer, or etching all-metal tips (bulky metallic wires) by electrochemistry.[7] These wire tips are used for STM regulation or Normal/Shear-force microscopy when mounted on the fork of a quartz resonator. The most common method today is the metallization of conventional AFM cantilevers with a thin layer of a few tens of nanometers of gold or silver. The coating is usually done by evaporation,[8, 9] sputtering,[10] or electroplating.[11, 12] Etching this layer by focused ion beam (FIB) right after coating could also be used to increase the enhancement of the electromagnetic field.[13] In addition, the tunability of the resonance wavelength plasmon can then be obtained by varying the dimensions of the final apex using FIB nano-machining,[14] which can be also used for etched bulky metallic wires.[15]

Spatial Resolution Down to the Nanometer

TERS is a near-field spectroscopic imaging technique, so in this sense it belongs to the family of scanning probe microscopes by combining the scattering-SNOM technique with a Raman spectrometer. It is nonetheless an optical imaging technique and the concept of resolution must follow the general definition in optics.

Thus, in optics, the strict definition of the resolution is the minimum distance in between two objects that can be imaged; this distance is governed by the Rayleigh criterion which states that the resolution can't be smaller than half the wavelength of the radiation used. In near field optics and consequently with TERS, the resolution must follow that definition. However, the required condition is very rarely satisfied (i. e. the presence of two nanoscale objects close enough to each other on the sample surface) and it is commonly accepted that the spatial resolution in TERS (and other near-field optical imaging techniques) can be quantified via the optical section analysis through a single nano-object.

In the example shown in Figure 2, the TERS image (100 × 100 nm scanning area, acquired with a pixel step size of 1.3 nm, total acquisition time < 9 min, 100 ms integration time per pixel), showed nanoscale chemical imaging of a single carbon nanotube (CNT) with a spatial resolution of 8 nm, confirmed from the section analysis of the intensity of the D band at 1360 cm^{-1}. As a first approximation, this resolution is dependent on the radius of curvature of the TERS tip, i. e. the "8 nm" estimation is a convolution of the actual size with the tip radius and the nanotube. Thus,

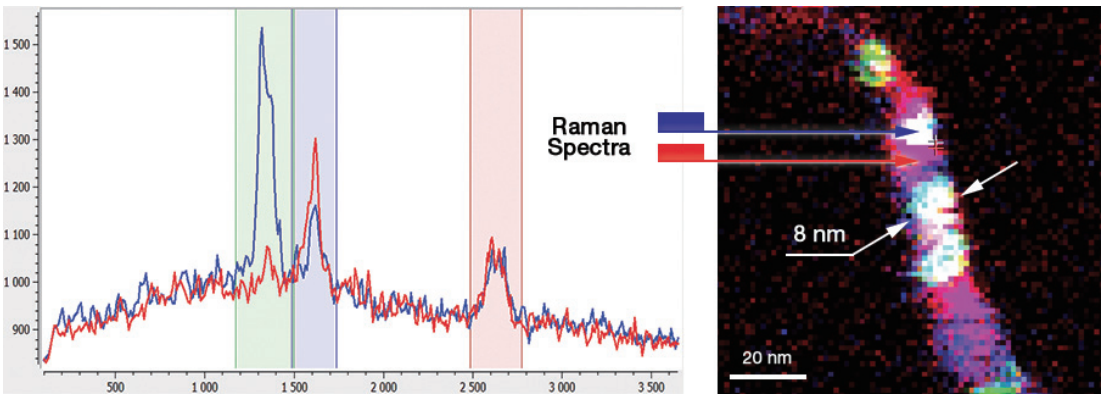

Figure 2 TERS image of 100 nm × 100 nm (75 × 75 pixels) of a carbon nanotube showing an optical spatial resolution down to 8 nm; total map acquisition time < 9 minutes.

the achievable TERS resolution can be approximated and considered equal to half the radius of curvature of the TERS tip. To go further with the present example in Figure 2, in this TERS map, the intensity of the D band

(white and green pixels) shows the imperfection in the structure of the lattice; in contrast, the areas in red correspond to the pure graphitic arrangement of the CNT through the intensity of the 2D band. Close to the local lattice defects, a single step gives rise to noticeable intensity of the D peak, showing a chemical sensitivity down to 1.3 nm along the tube.

Applications of TERS in Biology

Initial TERS studies in biology focused on pure components like nucleobases and amino acids. TER spectra have been reported for all normal nucleobases, showing characteristic peaks with enhanced signal compared to the far-field spectrum. The plasmonic enhancement of the Raman signal allows label-free detection of nucleobases in picomolar quantities.[16] TERS studies also showed that distinct signatures of different nucleobases can be detected in RNA and DNA, thereby opening up opportunities for a direct, label-free method for nucleic acid sequencing (Figure 3). In principle, this can be used to detect chemical modification in DNA, which may result from environmental stress such as UV rays or from a byproduct of metabolism such as free radicals, and hence can provide valuable understanding of DNA repair mechanisms. TER spectra have been obtained from cystine and histidine monolayers, which revealed different ionization states adsorbed on the surface[17] which is relevant to histone formation. A similar degree of chemical specificity has also been demonstrated in proteins. TER spectra obtained from cytochrome c showed not only distinct spectral features of amino acid and the associated heme moiety but also variations due to different orientations of the molecule.[18] This was in contrast to SERS which, despite its high sensitivity, provided ensemble information of the analyte.

Additional studies have applied TERS to surface characterization of amyloid fibrils, which are macromolecular protein assemblies implicated in neurodegenerative diseases. They have elucidated the fibril surface composition

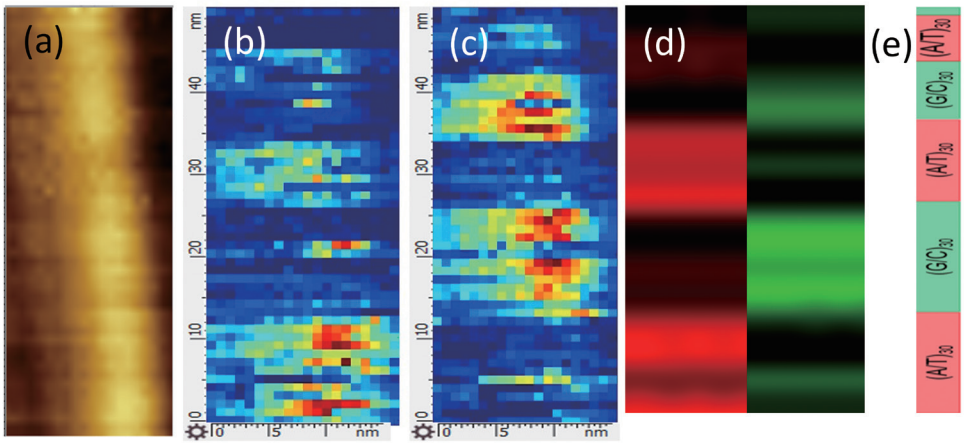

Figure 3 (a) AFM topography of engineered DNA, (b)-(c) corresponding TERS spectral mapping of over 50 × 20 nm^2 showing clear differentiation of spectral regions of pattern and size consistent with the expected (b) A/T and (c) G/C homopolymeric blocks, (d) horizontally averaged spectral map from the previous TERS maps, showing a good agreement with the (e) original sequence. (Data courtesy of Dr Noah Kolodziejski, Radiation Monitoring Devices.)

of amino acids and secondary structural conformations. This showcases the potential of TERS to study fibril surface structure, which is poorly understood in general compared to overall fibril structure. More recently, TERS studies were extended to discriminating polymorphs of insulin fibrils, also based on surface composition. Because different fibril polymorphs are associated with different levels of toxicity, an understanding of their propagation mechanisms may enable the design of suitable inhibitors. Based on correlation between the surface chemical compositions of two insulin fibril polymorphs ('flat' and 'twisted') to that of insulin protofilament, distinct propagation mechanisms followed by the polymorphs during fibril growth have been proposed.[19]

TERS Applications for Complex Biological Systems

In addition to studying multicomponent samples like nucleic acids and amyloid fibrils, TERS has been used to study the biochemical composition of other complex biological systems, such as the surface of a virus, bacterium or a human cell. TER spectra have been obtained from the surface of a tobacco mosaic virus that shows specific chemical signals from viral coat proteins and RNA. More recently, TERS was used to distinguish between Varicella-zoster virus and Porcine teschovirus based on differences in their surface protein and lipid composition.[20]

Of particular note is the detection of membrane protein from a human erythrocyte under aqueous conditions, with both tip and analyte immersed.[21] This approach, which remains to be explored further, offers the advantages of studying biomaterials in their native environment and minimizing sample decomposition due to laser-induced heating and oxygen-mediated photobleaching. One interested in studying large, complex heterogeneous cell surfaces might face the problem of locating nanoscale features of interest. To address this, a targeted approach of using antibody-conjugated nanoparticles that are detectable by dark-field microscopy has been proposed[22]; the specific interaction between antibody and cell surface antigens can then be probed by TERS. This approach has enabled the detection of integrin receptors on intact cell membranes.[23]

Summary

TERS is a new technique that provides a specific chemical signature with nanoscale spatial resolution, making it a suitable technique to study surface chemistry and composition. TERS is therefore a super-resolution chemical imaging technique. Better yet, it is a label-free super-resolution imaging technique. Simultaneous with advances in biological applications described here, advances in instrument design have been made. Correlated topographic and TER hyperspectral imaging capabilities, as opposed to single point measurements, are commercially available now. Spatial resolution has been improved from 50 nm in earlier studies to ~10 nm currently achievable. Measurements in liquid have also been reported recently, which would enable the potential of TERS for label-free detection of biomaterials under native conditions.

References

[1] "Nanoscale chemical analysis by tip-enhanced Raman spectroscopy", R. M. Stöckle, Y. D. Suh, V. Deckert, and R. Zenobi, Chem. Phys. Lett., **318**, 131 (2000).

[2] "Improving resolution in quantum subnanometre-gap tip-enhanced Raman nanoimaging", Y. Zhang, D. V. Voronine, S. Qiu, A. M. Sinyukov, M. Hamilton, Z. Liege, A. V. Sokolov, Z. Zhang and M. O. Scully, *Scientific Reports*, **6**, 25788 (2016).

[3] "Nanoscale imaging and identification of a four-component carbon sample" E. Sheremet, R. D. Rodriguez, A. L. Agapov, A. P. Sokolov, M. Hietschold, D. R.T. Zahn, *Carbon*, **96** (2016) 588-593.

[4] "20-nm-Resolved Stress Profile in SiGe Nano-stripes Obtained by Tip-Enhanced Raman Spectroscopy", M. Chaigneau, G. M. Vanacore, M. Bollani, G. Picardi, A. Tagliaferri and R. Ossikovski, Handobook of Enhanced Spectroscopy, Chapter 13, (Pan Stanford Publishing 2014).

[5] "Simultaneous topographical, electrical and optical microscopy of optoelectronic devices at the nanoscale", N. Kumar, A. Zoladek-Lemanczyk, A. A. Y. Guilbert, W. Su, S. M. Tuladhar, T. Kirchartz, B. C. Schroeder, I. McCulloch, J. Nelson, D. Roy and F. A. Castro, *Nanoscale*, **9**(8), 2723-2731 (2017).

[6] "Side-illuminated tip-enhanced Raman study of edge phonon in graphene at the electrical breakdown limit", Y. Okuno, S. Vantasin, I. S. Yang, J. Son, J. Hong, Y. Y. Tanaka, Y. Nakata, Y. Ozaki, N. Naka, *Appl. Phys. Lett.*, **108**, 163110 (2016).

[7] "Preparation of gold tips suitable for tip-enhanced Raman spectroscopy and light emission by electrochemical etching", B. Ren, G. Picardi, B. Pettinger, *Rev. Sci. Instruments.*, **75**, 837-841 (2004).

[8] "Enhancement of Raman signals with silver-coated tips.", B. S. Yeo, W. Zhang, C. Vannier, R. Zenobi, *Appl. Spectrosc.*, **60**(10), 1142-1147 (2006).

[9] "Highly reproducible tip-enhanced Raman scattering using an oxidized and metallized silicon cantilever tip as a tool for everyone", N. Hayazawa, T. Yano, S. Kawata, *Journal of Raman Spectroscopy*, **43**(9), 1177-1182 (2012).

[10] "The controlled fabrication of "Tip-On-Tip" TERS probes", Y. Yang, Z. Y. Li, M. Nogami, M. Tanemura, Z. Huang, *RSC Advances*, **4**(9), 4718-4722 (2014).

[11] "Rational fabrication of a gold-coated AFM TERS tip by pulsed electrodeposition", L. K. Yang, T. X. Huang, Z. C. Zeng, M. H. Li, X. Wang, F. Z. Yang, B. Ren, *Nanoscale*, **7**(43), 18225-18231 (2015).

[12] "Facile Fabrication of Spherical Nanoparticle-Tipped AFM Probes for Plasmonic Applications", A. Sanders, L. Zhang, R. W. Bowman, L. O. Herrmann, J. J. Baumberg, *Particle & Particle Systems Characterization*, **32**(2), 182-187 (2015).

[13] "Nano-Raman spectroscopy with metallized atomic force microscopy tips on strained silicon structures", L. Zhu, C. Georgi, M. Hecker, J. Rinderknecht, A. Mai, Y. Ritz and E. Zschech, *J. Appl. Phys.*, **101**(10), 104305 (2007).

[14] "Optical antennas for tunable enhancement in tip-enhanced Raman spectroscopy imaging", I. Maouli, A. Taguchi, Y. Saito, S. Kawata, P. Verma, *Applied Physics Express*, **8**(3), 032401 (2015).

[15] "Tuning Localized Surface Plasmon Resonance in Scanning Near-Field Optical Microscopy Probes", T. L. Vasconcelos, B. S. Archanjo, B. Fragneaud, B. S. Oliveira, J. Riikonen, C. Li, D. S. Ribeiro, C. Rabelo, W. N. Rodrigues, A. Jorio, C. A. Achete, L. G. Cancado, *ACS Nano*, **9**(6), 6297-6304 (2015).

[16] "Tip-enhanced Raman spectra of picomole quantities of DNA nucleobases at Au(111)", K. F. Domke, D. Zhang, and B. Pettinger, *J. Am. Chem. Soc.* **129**, 6708 (2007).

[17] "Spectroscopic imaging of biological samples using near-field methods", L. Langelüddecke, T. Deckert-Gaudig, and V. Deckert, Infrared and Raman Spectroscopic Imaging, Chapter **11**, Wiley-VCH, (2014).

[18] "Tip-enhanced Raman spectroscopy can see more: The case of cytochrome C", B-S. Yeo, S. Mädler, T. Schmid, W. Zhang, and R. Zenobi, *J. Phys. Chem. C* **112**, 4867 (2008).

[19] "Surface characterization of insulin protofilaments and fibril polymorphs using tip-enhanced Raman spectroscopy (TERS)", D. Kurouski, T. Deckert-Gaudig, V. Deckert, and I. K. Lednev, *Biophys. J.* **106**, 263 (2014).

[20] "A manual and an automatic TERS based virus discrimination", K. Olschewski, E. Kämmer, S. Stöckel, T. Bocklitz, T. Deckert-Gaudig, R. Zell, D. Cialla-May, K. Weber, V. Deckert, and J. Popp, *Nanoscale*, **7**, 4545 (2015).

[21] "Tip-enhanced Raman spectroscopy – Its status, challenges and future directions", B-S. Yeo, J. Stadler, T. Schmid, R. Zenobi, and W. Zhang, *Chem. Phys. Lett.* **472**, 1 (2009).

[22] "Tip-enhanced Raman detection of antibody conjugated nanoparticles on cellular membranes", K. D. Alexander and Z. D. Schultz, *Anal. Chem.* **84**, 7408 (2012).

[23] "TERS detection of $\alpha_v\beta_3$ integrins in intact cell membranes", H. Wang and Z. D. Schultz, *Chem. Phys. Chem.* **15**, 3944 (2014).

Marc CHAIGNEAU, Ph.D.
AFM/Raman Product Manager
Sales & Marketing Division
HORIBA FRANCE SAS

Maruda SHANMUGASUNDARAM
Application Scientist
HORIBA Instruments Inc.

Fran ADAR, Ph.D.
Raman Principal Scientist
HORIBA Instruments Inc.