Advances in TERS (tip-enhanced Raman scattering) for biochemical applications

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Abstract

TERS (tip-enhanced Raman scattering) provides exceptional spatial resolution without any need for labelling and has become a versatile tool for biochemical analysis. Two examples will be highlighted here. On the one hand, TERS measurements on a single mitochondrion are discussed, monitoring the oxidation state of the central iron ion of cytochrome c, leading towards a single protein characterization scheme in a natural environment. On the other hand, a novel approach of single molecule analysis is discussed, again based on TERS experiments on DNA and RNA, further highlighting the resolution capabilities of this method.

Introduction

TERS (tip-enhanced Raman scattering) is based on the intrinsic properties of plasmonic structures to enhance the generally weak Raman signal and simultaneously achieve high lateral resolution [1,2]. A combination of scanning probe techniques [AFM (atomic force microscopy) and/or STM (scanning tunnelling microscopy)] [3] with a conventional Raman microscope is used to establish the system as an analytical tool. SPM (scanning probe microscopy) alone potentially allows imaging with single-atom spatial resolution, but the main information is the morphology of the sample. Vibrational spectroscopy generally provides molecular information and/or serves as a unique fingerprint for identification. To enhance the inherently weak Raman signal, rough or periodically structured metallic surfaces or colloidal solutions serve as plasmonic field enhancers and result in the so-called SERS (surface-enhanced Raman scattering) effect [4]. If the roughness is reduced to a single feature, as it is the case for a metal-coated or metallic SPM tip, the plasmonic structure consequently increases the spatial resolution related to the feature size, which means down to the nanometre scale.

Several approaches are used to produce TERS active probes; one way is the evaporation of silver or gold onto conventional AFM tips [1]. This method results in isolated metal nanoparticles on the tip and regularly at the tip apex. In a more elaborate approach, the attachment of individual single-crystalline silver nanowires to tungsten wires using alternating current dielectrophoresis has also been reported [5]. Closely related is the growth of hemispherical gold droplets on top of silicon nanowires, which are subsequently attached to an AFM tip [6,7]. A promising method is the application of adiabatic plasmon focusing tips to concentrate the electromagnetic field on to the apex of an SPM probe. This can be realized by etching a tip-shaft grating into metal scanning probe tips, resulting in an optical antenna that allows the coupling of an external beam into the grating and the adiabatic propagating SPP (surface plasmon polariton) conversion into a localized SPP at the tip apex [8]. Similar probes result from a combination of standard AFM cantilevers with a photonic crystal and a plasmonic waveguide. This enables focusing of the excitation laser to the apex of the waveguide and a photon confinement corresponding to the tip dimensions [9].

For the actual TERS measurements, several excitation and collection geometries have been implemented so far. The back-reflection or transmission mode requires an inverted Raman microscope in combination with an SPM. The TERS tip is illuminated from below through substrate and sample, and the back-scattered Raman signal is collected through the same objective in epi geometry [1]. Obviously, this method is limited to transparent samples. Non-transparent samples can be studied using a side-illumination [8,10] or top-illumination [11] mode. However, this flexibility with respect to the sample comes at the expense of a reduced collection angle, resulting in a lower contrast between near-field and far-field signals. Hence A TERS geometry using a parabolic mirror provides the most efficient alternative for opaque samples [12,13].

The spatial resolution of TERS can be currently estimated to be better than 10 nm [14] and models even predict resolutions on a molecular level (<1 nm) [15]. A comparable resolution with molecular specificity at present can only be achieved by label-based methods such as PALM (photo-activated localization microscopy) [16], STORM (stochastic optical reconstruction microscopy) [17] and STED (stimulated emission depletion) microscopy [18].
With respect to applications, TERS has already been applied to numerous samples. Inorganic materials, such as strained silicon [19] and carbon nanotubes [20,21], and organic materials such as calcium alginate fibres [22] were investigated. The sub-diffraction limit resolution capabilities are well suited for the characterization of single viruses [23] or identification of different virus particles [24]. Also cell wall domains can be well characterized with nanometre resolution and one example will be discussed here in more detail. Specifically TERS is useful for single molecule or single particle investigations. In particular, experiments on fibrils [25–27] and DNA and RNA strands are noteworthy, hence TERS DNA experiments are discussed as well.

TERS on biomembranes

Using the outstanding spatial resolution of TERS, objects much smaller than the $\lambda_{ex}$ can be investigated. Specifically the analysis of nanoscale components within a complex environment is a major strength of the technique. Biomembranes, surrounding whole cellular and bacterial systems, are typical examples. These lipid-based matrices are only a few nanometres thick and many different proteins are embedded in this matrix on a similar length scale [28,29]. The high spatial resolution of TERS results in a clear discrimination of the investigated volume and the surrounding. Consequently, the information stems from fewer molecules compared with standard microscopy techniques and the usual interference of other compounds is greatly diminished. This property distinguishes the method from conventional vibrational spectroscopy (Raman/IR). Here, the resolution is defined by the size of the laser spot used for excitation and accordingly investigations aim at characterizing complete cells or tissue sections and consequently many more molecules contribute to the signal and result in a broadening of the spectra [30,31].

Figure 1 indicates the scaling characteristics of Raman and TERS results of K15 (cytokeratin 15)-positive human hair follicle ePCs (epithelial progenitor cells) [32]. Figure 1(a) shows the intensity ratio of $I(2770–3110 \text{ cm}^{-1})/I(1205–1290 \text{ cm}^{-1})$ measured on a single cell using Raman imaging, which characterizes the lipid-high and protein-poor regions. The lipid distribution can be used to specify stem cells and their progeny via monitoring differentiation [33]. However, the local resolution does not enable the investigation of single membrane components on the nanoscale. This information can be obtained by TERS, as shown in Figure 1(b) for the same ePC. Using the lateral and vertical resolution, specific membrane areas are characterized instead of the whole cell. This is indicated by the bar code shown in Figure 1(c), which represents the distribution of lipid (red), protein (blue) and mixed (light green) domains within a 4 $\mu m$ line scan at 200 consecutive positions. Obviously, this part of the cell is dominated by lipids. In the following section, the main advances in biomembrane research applying TERS are presented.

The first experimental proof demonstrating the surface sensitivity of TERS on complex biological samples was provided in 2006 by Neugebauer et al. [34], measuring single bacterial cells of Staphylococcus epidermidis. The spectral results point towards diverse polysaccharides and proteins, reflecting the surface composition of a Gram-positive bacterium. These studies were completed with TERS investigations of surface dynamics on the same bacterium [35].

In 2009, TERS was applied for combined measurements on human cells (human dermal derived keratinocyte, HaCaT) and artificial lipid membranes [36]. Böhme et al. [36] demonstrated the surface sensitivity by the detection of selective lipid contributions on the cells. The results were verified using supported lipid structures as model systems. As the composition and stability of such artificial systems...
can be selected from a wide variety of compounds, they are well suited for basic investigations of biomembranes. The inclusion of distinct protein structures provides an elegant way to increase the complexity under controlled conditions. This was demonstrated with TERS investigations of SLBs (supported lipid bilayers) with embedded streptavidin molecules [37]. The spectral features of lipids and proteins were detected in the optical near field within a nanoscale grid and consequently a molecular distinction far below the diffraction limit could be achieved. Employing several specific Raman marker bands, characteristic TERS spectra of both domains as well as mixed contributions were identified, depending on the specific position of the TERS probe. This assignment procedure was also used to investigate the arrangement of lipids and proteins within a TERS grid on human colon cancer cells (HT 29) [38]. These investigations utilized statistical methods to classify the spectra. Eventually, both TERS studies establish a lipid/protein differentiation on the nanoscale. A further specification of the proteins for instance is currently extremely difficult, as the spectra of many components of the membrane are yet unknown.

It is well known that the organization of biomembranes is mainly influenced by the so-called lipid rafts. Opilik et al. [39] investigated a binary lipid mixture of separated domains by tip-enhanced Raman spectroscopy to model those. The lipid molecules used for their studies differ between deuterated and non-deuterated. Hence the intensity ratio of the C–2H and the C–1H stretching region was used for compositional differentiation. The molecular distribution, deduced from the spectral information, matches nicely the related AFM phase image.

Ideally an analytical technique would allow the specification of proteins in their native environment and their reactivity. TERS experiments on isolated mitochondria were able to distinguish between different cytochrome c oxidation states [40]. Using a resonant excitation, the Raman spectroscopic features of this protein are mainly defined by the central haem moiety. The porphyrin ring co-ordinates to a central iron ion that can switch between different oxidation states [Fe(II)/Fe(III)]. The redox sensitivity of cytochrome c Raman signals according to oxidation and spin marker modes (see, for instance, [41]) enables the characterization of the electronic states of cytochrome c.

Figure 2 shows the AFM topography image of a mitochondrion and the selected positions for spectral measurement. The TERS probe was moved along a line with a step size of 8 nm. Spectral positions of the haem oxidation Raman marker are shown for each position. This characteristic spectral band appears at 1375 cm\(^{-1}\) for Fe(III) and at 1360 cm\(^{-1}\) for Fe(II) respectively, and highlights an effective shift from an oxidized to a reduced protein domain. The distribution of the spin marker bands, which is not shown here, further supports the assignment. Regarding the relative occurrence of the cytochrome c within the mitochondrial double membrane structure and the lateral resolution potential of the applied setup, conditions close to single protein detection were reached. The detection of mixed states points towards the detection of at least two proteins with different oxidation states within the near-field region of the tip. Interestingly, a very high vertical resolution is also indicated by the relative intensity of lipid matrix signals compared with the resonantly enhanced haem group. This behaviour can be only explained by the fast decay of the evanescent field within the very first nanometres of the tip and is different from standard Raman experiments of whole mitochondria where the whole spectrum is dominated by the resonant cytochrome c signals. As the protein lies just underneath the lipid bilayer (~6 nm), the competition between lipid Raman and cytochrome c resonance Raman signals can be explained by rapid field decay.

In conclusion, resonantly enhanced proteins containing prosthetic groups can be easily addressed by TERS. Compared with the majority of proteins, these are much better characterized by standard methods and also allow sub-membrane investigations. Further studies of proteins, resonant or non-resonant, will extend the applications of TERS in biochemical research. Technical improvements of the method, such as the transfer of the measurement scheme into an aqueous environment will also be an important step towards investigations under native conditions. Schmid et al. [42] already addressed this topic in 2009 by a TERS
analysis of thiophenolate on a gold surface in water. The authors also highlighted essential developments concerning a suitable TERS probe protection procedure. Short-chained hydrocarbon-thiolate layers as a thin protective surface enabled the sample to approach the plasmonic particle very closely, and also prevented interactions with other parts of the probe. This study demonstrates a potential route for the investigation of biological systems in an aqueous environment. In such a system, a broad variety of molecular processes can be controlled and detected simultaneously by TERS.

TERS on DNA

The high resolution of TERS not only enables investigation of single proteins, but also exhibits a tendency to explore the blueprint of life itself: DNA. First experiments addressed single nanocrystals of the DNA base adenine, also with regard to tip-pressurization effects [43]. The normal modes of eight Raman bands were identified using quantum chemical modelling. In particular, potential adenine–silver complexes were investigated and specific interactions of the molecule with the silver metal surface and tip were considered. Shifts of Raman bands were attributed to a deformation of adenine by the metal tip and even the possibility of molecular resolution with vibrational spectroscopy was suggested. Subsequently TERS spectra of nanometre-sized crystals of the DNA pyrimidine bases cytosine and thymine were collected, comparing near-field and far-field contributions as well as TERS, SERS and Raman spectra [44]. In further experiments, TERS spectra were recorded on (sub)monolayers of all four DNA bases, adenine, guanine, thymine and cytosine [45]. The results emphasize the extraordinary sensitivity of the method and also point towards the feasibility of TERS for single molecule detection.

To increase the DNA Raman signals, experiments utilizing resonant excitation similar to the previously mentioned experiments on cytochrome c are an alternative. TERRS (tip-enhanced resonance Raman spectroscopy) measurements on solid adenine nanocrystals have been reported [46]. Since adenine exhibits an absorption maximum at approximately 260 nm, an \( \lambda_{\text{ex}} \) of 266 nm was chosen and demonstrated that it is possible to utilize single particle plasmon enhanced deep-UV resonance Raman scattering from a biologically relevant molecule. Potential sample damage could be minimized by reduced laser power.

A crucial point regarding the base-pairing process in the formation of a DNA double helix are hydrogen bonds. TERS enables a sensitive study of hydrogen bonding at single crystalline surfaces as has been illustrated for adenine and thymine on gold(III) [47]. The method allows the estimation of the interaction geometry of the molecules with respect to the metal surface as well as the respective strength of the bonds.

After analysis of the isolated nucleobases, TERS experiments were carried out on single DNA and RNA strands. A TERS measurement on a single-stranded RNA homopolymer of cytosine was the initial experiment [48].
Spectra were collected on several adjacent spots on the strand, demonstrating the stability of the setup and the reproducibility of the spectral features, exhibiting only minute changes due to nanoscale effects. Furthermore, considering the signal-to-noise ratio in the TERS spectra, single-base sensitivity was achieved. Consequently, TERS experiments on poly-adenine and a single strand of poly-uracil followed, further emphasizing the reproducibility, spectral information with high lateral resolution and single-molecule sensitivity of this technique [49]. Moving towards a tip-enhanced Raman-based sequencing method, experiments on calf thymus DNA, either adsorbed as a (sub)monolayer on a gold(III) crystal [50] or on a mica substrate [49] further proved the reliability of the method. An important result was that spectral contributions from all nucleobases could be detected and distinguished on a strand. Hence the different scattering cross-sections of the distinct bases do not result in obscuration of the spectra.

Consequentially a TERS experiment on an artificial DNA single strand with the known sequence of alternating blocks of adenine and cytosine, \((\text{A}_{15}\text{C}_{15})_8\), was carried out. The motivation was the identification of the two distinct bases on one strand by means of their spectral marker bands. After immobilization of the strands on a mica substrate (Figure 3a), TERS spectra were recorded. Two selected spectra measured at a distance of 2.1 nm are shown in Figure 3(b). Figure 3(a) shows the respective AFM topography of the single DNA strand and indicates the positions of the TERS measurements. The lower-wavenumber region (<1100 cm\(^{-1}\)) mostly contains spectral contributions originating from the substrate (∼704 cm\(^{-1}\)), deoxyribose (∼899 cm\(^{-1}\)) and phosphate backbone (∼1089 cm\(^{-1}\)), which are relatively constant. The remaining unmarked bands can be assigned to vibrational modes of both adenine and cytosine. Bands corresponding to adenine are highlighted green and clearly dominate emissions of both adenine and cytosine, \((\text{A}_{15}\text{C}_{15})_8\), was carried out. The measurement points were separated by 2.1 nm and clearly show different spectral characteristics that can be well explained by a transition from adenine to cytosine. The result matches nicely the resolution discussed for the haem-containing proteins insofar as the former were further away from the tip region. Future experiments will cover an increasing length of the DNA strand with accordingly higher numbers of spectra to examine sequence changes and in combination with spectral deconvolution methods will eventually lead to a direct sequencing of chain-like molecules.

In conclusion, TERS provides a tool to address nanoscale domains of biological interest. Complex sample compositions can be deconvoluted by the sheer resolution capabilities of the method and consequently new challenges can be addressed. First steps towards TERS experiments in a liquid environment are very encouraging and will also enable the investigation of biological samples under in vitro conditions, while maintaining the advantages with respect to resolution, sensitivity and specificity.

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### References
