Intracellular applications of analytical SERS spectroscopy and multispectral imaging

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The aim of this *tutorial review* is to give an overview of the state of the art of intracellular applications of analytical SERS spectroscopy. We pay particular attention to nanoparticle-based SERS spectroscopy since this currently dominates the published literature on non-disturbing analysis of live cells. We describe recent advances in this domain due to the development of multispectral imaging and to the combined use of SERRS (surface-enhanced resonance Raman scattering) and fluorescence spectroscopy. Finally, a perspective view is given on the tip-based approaches like tip-enhanced Raman spectroscopy (TERS) which allow micrometric and nanometric resolution.

1. Introduction

Due to its advantageous characteristics in terms of specificity, selectivity, and sensitivity, surface-enhanced Raman scattering (SERS) spectroscopy has become one of the most incisive analytical methods for chemical and biochemical detection and analysis. SERS spectroscopy provides structure-specific vibrational spectra of analytes with extremely high sensitivity comparable to or even better than fluorescence. High selectivity of the technique is ensured by enhanced detection of only species adsorbed on noble metal surfaces. One of the most exciting domains of SERS application, which became possible with the advent of scanning probe microscopy, is the investigation of molecular phenomena inside living cells. The value and the interest of this analytical challenge are evident if one takes into account cell fragility, small size, and the complex molecular composition of sub-cellular compartments. In practice, it is necessary to distinguish two types of intracellular SERS applications: (a) sensing applications, where SERS nanoparticulate substrates are embedded in highly active SERS molecules (reporters) and in silica or polymer coatings to label cells and tissues and (b) analytical SERS spectroscopy, where SERS substrates (nanoparticles or micro-tips) are used to obtain spectra from target analytes present in

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sub-cellular compartments. Versatile optical sensing labels suitable for both one- and two-photon-excited probing in cells are being developed on the basis of gold and silver nanoclusters.[1–8]

It has been reported that successive SERS tags have been made by binding the surface of silver or gold nanoparticles to some reporter chromophores, *i.e.*, Raman-active dyes with functional groups ensuring strong dye–metal interaction like thiol (4-methylbenzenethiol, 2-naphthalenethiol, benzenethiol[6]).

For stability and biocompatibility, these particles have to be coated with a protective layer of a polymer like polyethylene glycol (PEG) that is known to improve nanoparticle biodistribution and pharmacokinetics properties *in vivo*. The polymer should be conjugated to a biological targeting ligand like the ScFv antibody that recognizes the epidermal growth factor receptor (EGFR) overexpressed in human tumours. Qian *et al.*[7] described successful labelling of tumour cells *in vitro* and *in vivo* (nude mouse) using colloids carrying the near-infrared dye diethylthiatricarbocyanine (DTTC), coated with thiol-modified PEG (PEG-SH) and ScFv antibody. In comparison with near-infrared-emitting quantum dots,[9] these SERS tags were non-toxic, more than 200 times brighter, and provided advantageous molecular signatures for spectral encoding and multiplexing. Furthermore, the plasmonic light-absorbing properties could be useful for tumour imaging and treatment using the photothermal effect. Core–shell nanoparticles functionalized with pH-sensitive reporter molecules have been described as nano-sensors of intracellular pH.[8] The intense research in the domain of SERS-based tags for sensing applications is reviewed elsewhere[10,11] and will not be discussed in detail here. The present review intends to summarise the state of the art in the domain of intracellular SERS with a particular emphasis on analytical spectroscopy and spectral imaging.

2. Substrates for intracellular SERS

There are two enhancement mechanisms assigned to the SERS effect: electromagnetic and chemical. According to the electromagnetic enhancement mechanism, the SERS effect is related to an enormous electromagnetic field created in the vicinity of the metal surface due to resonant excitation of metal plasmons. By contrast, the chemical mechanism implies an increase of the molecular polarisability of the molecules chemically bound to the metal surface. For these molecules strong electronic coupling with metal results in new charge-transfer states.[12] For both mechanisms, adsorption of the analyte on the enhancing surface (SERS substrate) is required for SERS applicability. This means that intracellular SERS analysis is restricted to the regions in the close vicinity of the SERS substrates and to the molecular species able to adsorb to the metal surface.

Among a large variety of SERS substrates including metal island films, electrodes, and colloidal nanoparticles, the intracellular applications have mainly involved internalised colloidal nanoparticles and micromanipulated micro-electrodes or tips.

2.1 Colloidal nanoparticles

The expanding nanoparticle-based SERS (NP-SERS) has been reviewed recently by Aroca *et al.*[13]

Since the first SERS on silver and gold sols was reported in 1979 by Creighton *et al.*,[14] metal colloids have become the most commonly used nanostructures for SERS. More recently, NP-substrates have become central to single-particle, single-molecule Raman spectroscopy with the achievement of single molecule detection (SMD).[15] Particles of a variety of shapes, nanowires, and aggregates are being developed using a number of methods, of which the most universally used one is chemical reduction. Nanoparticle fabrication by wet chemistry (chemical reduction, photoreduction, laser ablation *etc.*) is inexpensive and versatile. It is usually performed by using a starting metal salt, which is reduced by a chemical agent to produce colloidal suspensions containing nanoparticles with variable sizes (generally 10–80 nm) depending on the method of production. These particles will thus exhibit different plasmon resonances depending on the size, shape, and the dielectric constant of the metal.[11]

Single particles generally do not yield sufficient enhancement and most of the SERS applications are made with aggregated nanoparticles where large enhancement is associated with interparticle junctions.[16] In fact, the surface of initial NP in colloidal suspensions is encountered with Debye–Hückel ionic layers. For instance, citrates present on the surface of silver colloids reduced according to Lee and Meisel[17] are known to stabilize the colloidal suspension by electrostatic repulsion. Once in an aqueous buffer like PBS (phosphate buffered saline) that has high ionic strength and high chloride ion content, the ionic exchange on the NP surface leads to particle aggregation. Treatment of NP with halide ions (Cl^-, Br^-, I^-) is suggested to create highly specific SERS-active sites at the metal surface[18] that can account for the major enhancement effect. However, at high Cl^- concentration, quenching SERS intensity takes effect.[19]

Aggregation of silver and gold nanocrystals results in broadening and the red-shift of their plasmon resonance spectra. For instance, plasmon resonance of isolated citrate-reduced silver nanoparticles is observed at *ca.* 410 nm, while their aggregates exhibit plasmon absorbancies up to near-infrared (NIR). As a result, the wavelength range allowing efficient SERS excitation is extended from blue to NIR laser lines. The highest SERS intensities are observable when the excitation wavelength is in resonance with both the substrate plasmons and the analyte electronic absorption bands.

Nanoparticulate substrates have been demonstrated to provide enhancement factors from 10^6 (average contribution from numerous molecules) to 10^{15} (small number of molecules sitting in highly enhancing junctions of nanoparticles called 'hot spots'[20]). The latter case corresponds to a single-molecule spectroscopy that is not a subject of the present review. The former case is more interesting from the point of view of analytical spectroscopy, since the SERS signal is more reliable and stable.

For intracellular applications, the NP substrates have both advantages and limitations. Among the advantages of NPs, the main one is the small size (below 150 nm for isolated NPs

and below 1–2 μm for aggregates) compared to cell dimensions (typically 10–20 μm). NPs have been used to analyse drug adsorption on the plasma membrane microenvironment of sensitive and resistant cancer cells.[21] The small nanoparticles or aggregates can also be introduced into a cell with minimal damage to the integrity of the cellular membrane and then distributed within the cell with minimal perturbation of the sub-cellular environment. The principal limitations of nanoparticulate SERS substrates come from the difficulty of delivering them into cells and the control of their intracellular aggregation and distribution. SERS sensing applications have demonstrated that the surface of the noble metal-NP can be functionalized with biological ligands or antibodies in order to favour their internalization by living cells and to target them to certain cellular compartments.[7,22] As an alternative to delivered colloids, Shamsaie *et al.*[23] have provided a protocol for using intracellularly grown gold nanoparticles (IGAuNs) to obtain SERS spectra from cytoplasm and/or the nucleus of a single cell. These authors report that MCF10 epithelial cells with IGAuNs show a large amount of gold nanoparticles inside the nucleus and cytosol. The presence of large nanoparticles in the nucleus was most surprising and could not be explained by known mechanisms. The authors of this very interesting work hypothesize that IGAuNs could potentially detect the nuclear and/or cytoplasmic molecules. The inconvenience of this study was, according to the authors, that the majority of Raman spectra collected on IGAuNs were of a non-SERS origin (laser power was quite high, 10 mW of a 785 nm laser line).

For intracellular SERS spectroscopy, most of the known applications are based on the use of non-functionalized NP substrates. In fact, a simple transfer of silver or gold NPs into cell culture medium should result in their partial aggregation and coating with proteins like BSA (bovine serum albumin) that adsorb to the particle surface. This coating should then favour the internalization of the NP by living cells through phagocytosis and/or endocytosis. Tang *et al.*[24] reported that gold nanoparticles of 60 nm in size can be introduced into living human osteosarcoma cells (G292 line) by adding them to the growth medium. Most of the gold NPs were found to reside in the cytoplasm and around the nucleus, and a small number of them were observed in the nucleus. In order to confirm that some gold nanoparticles were able to enter the nucleus and form SERS-active clusters despite their size, Tang *et al.* recorded Raman lines assigned to vibrations of the DNA backbone. For macrophages a simple incubation with silver colloids just transferred to the cell culture medium is also known to provide a significant internalization of the substrates to the cytoplasm and to the nuclear membrane. However, the silver nanoparticles introduced into the culture medium provided little SERS effect and the spectra were representative of interfering molecules rather than intracellular target analytes. This problem was overcome when the cells were placed in PBS buffer instead of culture medium. To stimulate the NP internalization by non-macrophage cells, the cell–colloid mixture can undergo mild centrifugation (100–200 *g* for 2–5 min) in PBS buffer.[25] After that, the excess of non-internalized colloids can be removed by additional washing with PBS.

Among the molecular species intrinsically present within cellular compartments, aromatic amino acids and their derivatives are particularly SERS-active, possibly due to their high affinity to silver and gold surfaces.[26] Several authors[24,27] have found SERS signals of the native chemical constituents of cells such as DNA backbone in the nucleus and proteins (namely amino acids like phenylalanine) in the cytoplasm from deposited clusters of gold colloidal particles. However, these authors comment on the very inhomogeneous chemical constitution of the cells. In these studies, the data showed a particularly high number of gold particles per cell that could negatively affect the cell's integrity. In our experience with a few silver NP delivered into intact living cells, there was no analytically significant SERS response of intrinsic molecules. By contrast, a strong SERS signal of adenosine-like species indicated damage and that the cell integrity was compromised by exposure to intracellular silver and/or with the time the cells persisted in PBS. Tang *et al.*[24] also observed that the SERS spectra of the cells increased and changed dramatically when the cells were air dried at room temperature.

The analytical interest of subcellular SERS spectroscopy has been more often confirmed with the detection of exogenous chromophores like drugs and tag molecules. In contrast to most of the tags or non-toxic drugs, anticancer agents are highly cytotoxic and cannot be accumulated in the cells at high concentrations. Typical LC_{50} concentrations (corresponding to 50% of killed cells) of anticancer drugs are of nanomolar scale, and micromolar concentrations are already an upper limit to exploration within viable cells. Therefore, the study of intracellular anticancer agents is an exciting challenge for SERS spectroscopy, since this application requires high detection sensitivity.

The SERS spectra of the antitumour drug doxorubicin recorded from treated K562 cancer cells incubated with citrate-reduced silver colloids were reported in 1991.[28] These spectra were the very first feasibility data demonstrating the promising perspectives of SERS spectroscopy at a single-cell level. Later, the protocols based on citrate-stabilized silver colloids were improved and applied to study living cells of other cancer cell lines (HCT-116, MCF-7, HL-60) and treated with other anticancer drugs (amsacrine,[25] mitoxantrone[29,30]). These small aromatic chromophores appear to have high enough affinity to the silver NP surface to win the adsorption competition and provided reproducible and stable SERS spectra, even when the cells were treated with sub-micromolar drug concentrations. The interest of such analytical SERS spectroscopy is the possibility to follow molecular interactions of the drug in complexes with intracellular biological molecules such as DNA and proteins.

For mitoxantrone[31] and amsacrine,[25] it was possible to obtain experimental evidence that the adsorption on silver colloids was not perturbing the drug–DNA interactions in solution. The SERS results for complexes of these drugs with DNA and nuclear enzyme topoisomerase II were in good agreement with the suggested action mechanisms. Based on these important statements, it was possible to interpret the intracellular SERS spectra of anticancer drugs.[25,30]

Nevertheless, several important problems persisted with intracellular SERS and these were related to the use of the

small nanoparticles. The very first studies described spectra recording by manual focusing of the laser on colloidal aggregates roughly situated in/on the cell. Visual detection of the aggregates means that aggregates have to be quite large (1–2 μm) and manual focusing means that the precise origin of the spectrum is not guaranteed.

The experimental feasibility of intracellular SERS spectroscopy was considerably facilitated with the advent of confocal scanning microspectrometers and with the recent development of multispectral imaging approaches. The first step of multispectral imaging consists in recording of hundreds to thousands of spectra by scanning a cell. The multispectral map is then processed to generate images corresponding to characteristic spectral changes.

Although most of the above difficulties could be overcome, the origin of the signal remains uncertain. Recently, we proposed an advanced multispectral imaging approach using a combination of SERRS (surface-enhanced resonance Raman scattering) with fluorescence techniques (briefly presented in Adar *et al.*[32]). The fluorescence emission and SERRS spectra of the same anticancer drug, mitoxantrone, were recorded simultaneously over an optical section of the cell (Fig. 1). The cells were incubated for 1 h in RPMI medium containing 0.5 μM mitoxantrone and washed twice: in fresh RPMI then in PBS. Then, nanoparticle-SERS substrates (citrate-reduced silver colloids) were pre-aggregated in PBS and incorporated in the cells under mechanical stimulation, *i.e.* through a mild centrifugation (2 min, 100 *g*) of the cell–colloid mixed suspension. The excess colloids were removed by washing the cells in PBS. A multispectral map of 30 × 30 spectra (900 mapping points) was recorded with a 0.7 μm scanning step and 0.05 s collection time per point. Reduced exposure of the cells to both drug concentration and laser radiation (30 μW and 0.05 s per spectrum) was considered better for maintaining cellular physiology.

As can be seen in Fig. 1A, the silver colloid aggregates used in this application are so tiny and few that their presence is difficult or impossible to localise on the non-confocal white light image of the cell. Nevertheless, these aggregates can be revealed on the confocal microspectroscopy map due to the characteristic SERRS spectra of mitoxantrone they provide. The spectral intensity map in Fig. 1B (average intensity in the spectral region including both the fluorescence and SERRS maxima) shows a large zone of high intensity (presumably nuclear and peri-nuclear drug fluorescence) and at least four particularly bright spots localised in low-intensity zones (cytosol and membrane) of the cell. The spectra corresponding to these spots (Fig. 1C) confirm the presence of tiny silver aggregates, since they contain fluorescence background superimposed with SERRS signal of mitoxantrone, noticeable due to the most intense band at *ca.* 1300 cm^{-1}. In fact, each spectrum corresponds to the volume of *ca.* 1 μm diameter and about 3 μm in depth. Therefore, the SERRS spectral contribution from tiny silver aggregates is combined with the fluorescence from the surrounding volume. The SERRS intensity appears at least as high as that of fluorescence, thus indicating that in terms of sensitivity of subcellular analysis these two techniques are comparable. Fig. 1C illustrates the semi-quantitative analysis of the intracellular spectra, since

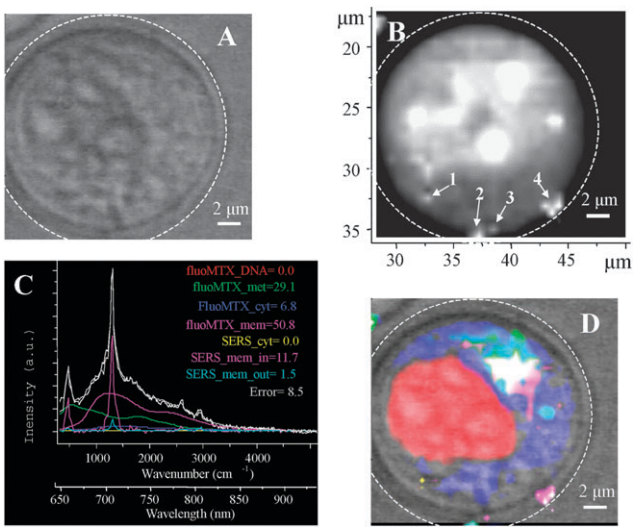

Fig. 1 Combined fluorescence and SERRS confocal spectral imaging of a fluorescent anticancer drug mitoxantrone (MTX) within an MCF-7 cancer cell. (A) White light microscope image of the cell, dashed line shows the outer membrane limits. (B) Spectral intensity distribution map (average intensity in the region of 1200–1500 cm^{-1} or 685–699 nm) reveals four silver colloid aggregates (bright spots 1–4). (C) Intracellular spectrum (inner part of the aggregate 4 located in the membrane of the cell) fitted with a sum of proportional contribution of characteristic fluorescence and SERRS spectra: fluorescence of drug–DNA complexes in nucleus (red, fluoMTX_DNA = 0.0%); fluorescence of an oxidative metabolite (green, fluoMTX_met = 29.1%); fluorescence of MTX in respectively hydrophilic and hydrophobic complexes in cytosol and membrane (blue and magenta, fluoMTX_cyt = 6.8%, and fluoMTX_mem = 50.8%); SERRS on the aggregate 1 showing MTX interacting in cytosol (yellow: SERS_cyt = 0.0%); SERRS on the aggregates 2–4 corresponding to MTX interacting, respectively, in inner and outer layers of cytosol membrane (magenta and cyan, SERS_mem_in = 11.7% and SERS_mem_out = 1.5%). (D) Overlay of the microscopic image of the cell with the fluorescence and SERRS spectral maps (encoded with the same colours as in window C) showing mitoxantrone subcellular distribution and interaction. Experimental details are given in the text.

each of them can be deconvoluted into a proportional addition of characteristic fluorescence and SERRS spectra. For instance, the spectrum in Fig. 1C indicates that the SERRS signal in the given location (inner part of the aggregate 4 located in the membrane of the cell) is co-localised with the fluorescence characteristics of mitoxantrone[33] in a low-polarity environment of membranes (contribution of 50.8%), with that of an oxidative metabolite (contribution of 29.1%). These data, together with 6.8% contribution of the fluorescence characteristic of hydrophilic cytosolic complex of the drug, are in agreement with the assignment of the main SERS pattern to the inner cellular membrane (contribution of 11.7%). These devonvolution coefficients are used to generate specific fluorescence and SERRS maps that can be superimposed with the white light image of the cell. Therefore, the combination of SERRS and fluorescence multispectral imaging is a real opportunity to co-localise nanoparticulate SERS substrates with sub-cellular compartments. Analysis of such SERRS–fluorescence multispectral maps provides multiple information about the drug molecular contacts in a given

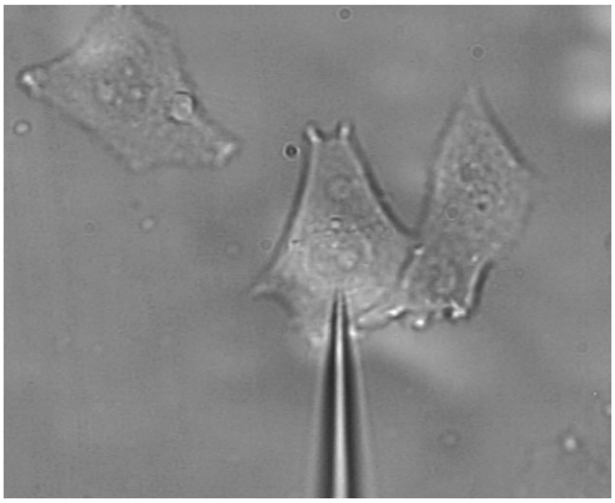

Fig. 2 Intracellular injection with a micromanipulated femtotip needle allows delivery of chemicals and colloids into cell compartments.

sub-cellular compartment. It becomes possible to complete the fluorescence data on the drug environment (polarity, pH, DNA intercalation) with SERRS data on vibrations (chemical groups) implicated in the interactions of the drug with intracellular DNA and proteins. Such data contribute to a deeper insight into the therapeutic efficacy of MTX and the design of new DNA targeted drugs.

Following recent developments in femto-injection devices, the introduction of colloids in selected cellular regions by micromanipulation is also being studied. The outer/inner diameters of a commercially available femtotip needle can be as small as 0.7/0.5 μm (FemtotipII, Eppendorf GA, Hamburg, Germany), thus allowing the injections to be made with minimal mechanical damage and preserving cell integrity (Fig. 2).

Although the injection can ensure NP delivery to certain cytosolic regions, final intracellular distribution of the injected particles remains uncontrollable. Once internalized, the NPs are distributed according to their affinity to cellular compartments. For SERS sensing applications, nanoparticles conjugated to either biomolecules or biotin were shown to target cellular enzymes and receptors.[34] In view of this, it should be interesting to study the possibility of using specifically functionalized NP substrates for subcellular SERS spectroscopy.

In fact, the surface functionalization could contribute not only to NP delivery to the target sites, but also to the selective adsorption of molecular species to be detected.

The competitive adsorption of multiple molecules at the SERS-active surface is dependent on their concentration and molecular structure, as well as on the medium pH and ionic strength.[18,35,36] The adsorption/desorption equilibria are governed by equilibria between chemical affinity/steric hindrance or electrostatic attraction/repulsion. Negative potential of chemically reduced and halide-activated colloids favours SERS detection of cationic dyes. To attract anionic chromophores, the substrate surface was coordinated with cationic polymers such as poly-L-lysine.[37] Unfortunately, the latter gave rise to an interfering SERS signal.

It is well known that molecules like thiols form a strong covalent bond with silver and gold surfaces and can be used to promote the capture of multiple charged or neutral analytes. Gold colloids were coated with SH-PEG and conjugated with a variety of biologically relevant ligands.[7] The SH-PEGylated gold nanoparticles were efficiently internalized in the cells within 1 h of incubation and localized in peri-nuclear regions. The coating did not displace reporter molecules, including non-sulfur containing ones. However, even after the surface modification, the problem of binding and detection of interfering molecules instead of analytes may persist in subcellular SERS studies. The most critical limitation of the SH-PEG coating for target analyte detection is that it prevents free reporter molecules in solution from accessing a gold colloid surface. For analytical spectroscopy, the coating should be less protective and more reactive and permeable than for the sensing applications. Since PEG-like coatings hinder further particle aggregation, it seems more appropriate to develop coated SERS substrates on the basis of newly engineered nanoshell structures like hollow gold nanospheres[3,8] that are capable of providing sufficient SERS intensity from individual particles.[38] The nanoshell systems can be physically analogous to the particle-pair system, since the nanoshell plasmon resonance results from the coupling of the inner shell surface (cavity) and the outer shell surface (sphere) plasmons over a separation distance essentially given by the metal shell thickness.[39] Depending on the size and composition of each layer of the nanoshell, such particles can be designed to either absorb or scatter light over much of the visible and infrared regions of the electromagnetic spectrum. Bimetallic Au/Ag core–shell nanoparticles, conjugated with monoclonal antibodies, were also prepared and used for highly sensitive SERS imaging of live cells of an HEK293 line.[22]

2.2 Microelectrodes and microtips

Manipulated microelectrodes and -tips are the SERS substrates actively developed in the last decade.

Stokes *et al.*[40] have recently fabricated a SERS-inducing probe from an optical fibre tapered to a tip 100 nm in diameter and covered with a thin layer of silver islands *via* thermal evaporation. The SERS capability of the probe was demonstrated on glass surfaces coated with brilliant cresyl blue and *p*-aminobenzoic acid. For five different probe tips, a 25% relative standard deviation in SERS signal was observed. The small scale of the tip may be amenable to localized, nondestructive SERS-based analysis of intracellular regions with submicrometre spatial selectivity.

Hankus *et al.*[41] have developed a non-scanning chemical imaging probe based on SERS arrays used to measure simultaneously 30 000 individual sub-diffraction-limited locations on a sample's surface. Fabricated from coherent fibre-optic imaging bundles, these arrays formed a highly ordered roughened metal surface providing uniform SERS enhancement ($<2\%$ relative standard deviation). Tapered tips of the collection probes allowed them to acquire imaging with spatial resolution below the diffraction limit of light. The chemical differentiation capability of this system associated with a multispectral imaging system was tested on a composite

gelatin sample doped with several dyes. Nevertheless, such a tip can be applied only to an outer cell membrane.

In contrast to eukaryotes, small bacteria and yeast cells are accessible for SERS measurements only at their surface. To immobilise them during SERS measurements, an approach using optical tweezers has been demonstrated.[42]

Recently, Popp's research team[43] developed and evaluated the applicability of an etched silver or gold coated SERS fibre probe to perform high spatial resolution micro-Raman measurements on different biological samples. This kind of setup appears applicable to intracellular SERS exploration.

New silicon nanowires decorated by chemical assembly of closely spaced Ag nanocrystals and modified with the well-known 'silver mirror' reaction[44] could be potentially suitable for intracellular SERS spectroscopy. These substrates were used for SERS and SEHRS (hyper-Raman) spectroscopy of four analytes (Rhodamine 6G, crystal violet, a cyanine dye, and a cationic donor–acceptor substituted stilbene). Although good quality SERS spectra were obtained using <1 μW of 458-nm cw laser power, the conditions were not really applicable to live cell analysis because the protocol involved soaking the substrates overnight in 10^{-4} M analyte solutions and involved a lengthy (60 s) acquisition time.

2.3 Tip enhanced Raman spectroscopy (TERS)

In the numerous studies of biological systems, two major parameters were shown to influence the meaning and usefulness of the vibrational information: (1) the lateral resolution and hence the size, number, amount, and heterogeneity of biological object(s), from which the information is obtained; and (2) the detection sensitivity, which is of consequence to the duration of the measurements and therefore to sample integrity, time resolution, and *in situ* applicability. SERS has a spatial resolution in the micron range. It is desirable to extend the sampling range in which this measurement can be used for purposes of examining nano-structures with single molecules sensitivity of biological samples. To this end, nano-Raman spectroscopic techniques that permit the characterization of buried nanoscale features in a non-destructive way are central for understanding of biological processes.

In the last decade, a powerful concept was proposed for improving the spatial resolution of Raman spectroscopy: the concept of a few 'hot spots' making up most of the SER signal while the rest of the surface is comparatively SERS inactive.[20] Experiments showed that even a single metallic nanostructure, for example a nanoparticle[45] or a metallic nano-tip,[46] can induce SERS. Precise positioning of such a nano-probe allows localized enhanced Raman spectroscopy with nanometric spatial resolution beyond the diffraction limits of light (nano-Raman). Different methods and configurations are applicable to accomplish near-field Raman spectroscopy. One commonly used technique is tip enhanced Raman spectroscopy (TERS) based on metallized tips of scanning probe microscopy (SPM), such as scanning near-field optical microscopy (SNOM),[47,48] atomic force microscopy (AFM)[49] and scanning tunnelling microscopy (STM).[50] The fabrication of nano-tips in SPM has been sufficiently developed.[51] The advantages of the combination SERS/SPM are its capacities to accomplish nanometric positioning and scanning of a SERS probe on sample surfaces through three-dimensional displacement control of SPM, allowing single molecular measurements and imaging with a resolution better than $\lambda/20$. The interpretation of the nano-Raman spectroscopy is based on both near-field optics and the SERS mechanism.[52] A full explanation of this interpretation is beyond the scope of this work. It is usually understood that the nano-probe interacts with the sample molecules immersed in the evanescent field in at least two different ways—electromagnetically and chemically. Since this enhancement is extremely localized at the nano-probe, it provides high spatial resolution without the diffraction limits. Nano-Raman spectroscopy techniques such as TERS are still in evolution. Compared to theoretically predicted enhancement factors of more than 10^{10} and in view of giant enhancements reported for single-molecule SERS ($\sim 10^{14}$), up until now TERS has shown a rather moderate enhancement range, typically in the range from 10^4 to 10^6. Obviously, one can expect much higher TERS signals if the experimental conditions are improved with respect to more efficient excitation and radiation of localized surface plasmons.

The basic characteristics of TERS are related to the fact that the Raman scattering cross sections of the sample molecules just below the enhanced electric field at the metallic tip are selectively enhanced and detected to provide a sufficiently high spatial resolution (Fig. 3). The field confinement is readily achieved if the field polarisation vector of the excitation laser is orientated along the direction of the tip axis.[53] This electric field, localized to a volume of ~ 20 nm^3, is used as nanoscale Raman excitation source. The field enhancement is much weaker if the exciting laser field is not polarized along the tip axis.

So far, TERS has mainly been applied to pure substances. Developments in tip enhanced coherent anti-Stokes Raman scattering (TE-CARS) have allowed measurements from nanometric structures of DNA.[54,55] More recently, Deckert and co-workers[56] reported a study on cells of the Gram positive bacterium *Staphylococcus epidermidis* using a silver coated AFM-tip based TERS. The outside cell surface is composed of a variety of sugar and peptide components. The experiment was performed firstly with the AFM in the tapping mode (tapping amplitude 4 nm). A topographic image of the sample was recorded as shown in Fig. 4A. To excite the surface plasmons at the silver coated non-contact, ultra-sharp AFM tip of ~ 50 nm, a krypton laser (568 nm, 1.5 mW) at the sample was used. TER spectra were readily observed from the cell surface (Fig. 4B–D).

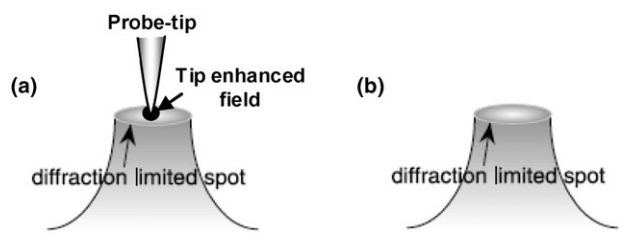

Fig. 3 Tip enhanced field confined in a nanometric space to the tip apex (a) and conventional diffraction limited field (b). (Reprinted with permission from ref. 54.)

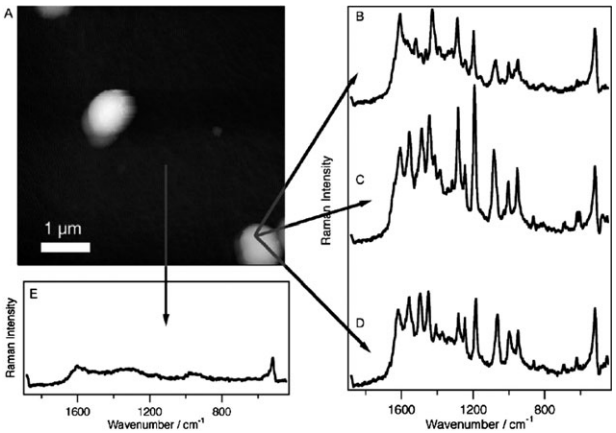

Fig. 4 (A) Topographic image of *S. epidermidis* cells with marked locations of the corresponding TERS measurements. (B–D) denote TERS spectra measured with a silver coated AFM cantilever on top of a bacterium whereas (E) corresponds to a reference TERS experiment on the glass surface. The ever present band around 520 cm^{-1} is attributed to Raman scattering of the silicon tip. (Reprinted with permission from ref. 56.)

While pronounced Raman spectra were obtained on the cell, no signal was recorded from the background. An enhancement factor for the experiment can be estimated from the signal-to-noise ratio (SNR) and the probed sample area and volume to be around 10^4–10^5.

TERS is therefore an exciting concept using modern vibrational spectroscopic methods along with exploiting near-field optical technology, providing single-molecule sensitivity and well beyond the diffraction limited spatial resolution. A major challenge in the application of TERS is the optimization of probes with respect to high enhancement factor, excitation and collection optics as well as of imaging techniques.

3. Variability of the SERS signal

Variability of the SERS signal from a given intracellular region is expected with both nanoparticle and tip-like substrates because of the dynamic nature of analyte(s) near the metal surface. This variability concerns both intensity and spectral pattern. For the same molecule, different adsorption orientations and different types of adsorption sites can result in quite variable spectra. Furthermore, in complex samples such as cells, several analytes could compete for adsorption on the SERS substrate. For metal nanoparticles, one can increment changes in their optical properties[57] and thermally activated diffusion of individual molecules on the metal surface. The equilibrium of the adsorbed/replaced species on the silver surface within a given cell compartment could evolve with time. In addition, inside a living cell, the position of nanoparticulate substrates could also change with time under the laser excitation. In this way, a multitude of qualitatively and quantitatively different SERS spectra should be observed from the same intracellular SERS substrate.[24] In those cases, one can apply multivariate statistical signal processing protocols like PCA (principal components analysis) for discriminating between stable and unstable information. By way of example, PCA with an agglomerative clustering approach has been recently used to distinguish SERS spectra of structurally similar molecules like penicillin analogues.[58]

Conclusions

Recent developments in SERS and TERS mark big steps forward for accessing highly resolved spatial and chemical information from prokaryotic and eukaryotic cells without the need for labelling techniques. Such information is crucial for understanding many biological processes, for example, cell recognition or drug–cell interaction, and opens new possibilities for the design of new strategies in drug development.

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