Nanoprobes for intracellular and single cell surface-enhanced Raman spectroscopy (SERS)

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Surface-enhanced Raman spectroscopy (SERS) is a promising and powerful label free technique for high resolution analysis of single cells. For intracellular analysis, there is a need for SERS-active nanoprobes that are minimally invasive to cells, do not affect cell viability, and provide reproducible signals. This work reviews the state-of-the-art tools currently available for intracellular SERS. Various types of SERS probes are considered, including colloidal gold and silver nanoparticles, metallized optical fibers, and tip-enhanced Raman probes. We also discuss recently developed SERS-active nanopipettes implemented on the basis of pulled glass microcapillaries. Finally, the critical aspects of selecting an optimal SERS nanoprobe for single-cell analysis depending on a particular application are summarized. Copyright © 2012 John Wiley & Sons, Ltd.

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Traditional methods for analyzing intracellular processes are indirect and require millions of cells in order to extract and analyze a specific protein or a cell organelle. It is well known that extraction procedures can result in significant artifacts in the final data. Moreover, the behavior of a single cell varies even in a small population. Therefore, the data averaged over millions of cells might actually obscure the true mechanism of cell metabolism. In view of these problems, there is a need for single-cell techniques, which could enable the analysis of intact cells with the organelles preserved in their native environment. Investigating proteins and other biomolecules in a living cell environment is a major goal of molecular biology, which has been achieved to a very limited extent so far. Single cell analytical techniques can facilitate early detection of diseases, such as cancer, and promote the development of more specific and efficient drugs.

Imaging and spectroscopy of single cells relies mainly on fluorescence-based methods. Confocal laser scanning fluorescence microscopy provides high lateral image resolution, thus enabling the nondestructive analysis of individual cell organelles (e.g. nuclei, mitochondria) in their native state. However, the need for specific fluorescence dyes required for labeling each subcellular unit poses a limitation on using these methods for parallel intracellular analysis. Toxicity of fluorescent dyes remains a significant problem, especially in the case of prolonged time-dependent studies. At the same time, the nonspecific binding of certain fluorescent dyes might be a source of strong background signal and lowered detection sensitivity. Quantum dots coated with a protective polymer layer have significantly lower effects on cells compared with the dyes, but they need surface functionalization, e.g. with antibodies, to make binding specific.

Surface-enhanced Raman spectroscopy (SERS) offers the advantage of detecting molecules without the need for labels. Moreover, unlike fluorescence, SERS provides information about multiple molecular functional groups simultaneously. This is particularly important for analyzing biochemical processes in living cells, which are usually associated with a highly complex expression/suppression of multiple molecules.

Figure 1 shows the number of peer-reviewed papers dealing with various applications of SERS published per year from 1980 to 2010. The data were collected using the Science Citation Index Expanded database. The general interest to SERS has risen significantly after 1997 when the single molecule detection capability of this technique was demonstrated by two research groups. Cellular applications of SERS, however, received a broad attention only in the last several years as shown in Fig. 1. In 2004, there were only seven papers published on this subject, whereas already 81 publications were available in 2010.

The capabilities of SERS for single-cell studies were demonstrated as early as 1991 by Nabiev et al. This work had not received due attention until 2002 when Kneipp et al. demonstrated the application of SERS for the detection of various biochemical components in single cells, thus reviving the interest to this subject. At the same time, the number of publications dealing...
SERS enhancement mechanisms

The Raman scattering intensity \( I(\nu_S) \) of a molecular vibrational mode \( Q_j \) is proportional to the number of molecules in the sampling volume \( N \), the intensity \( I(\nu_0) \) and the wavenumber \( \nu_0 \) of the excitation field, and the Raman scattering cross section \( \sigma_j \) (Eqn 1). \[ I(\nu_S) \propto N(\nu_0 \pm \nu_s)^4 I(\nu_0) \sigma_j \] The latter is proportional to change in molecular polarizability \( \alpha \) during the vibration induced by the incident light (Eqn 2). \[ \sigma_j \propto \frac{\partial \alpha}{\partial Q_j} \]

The typical Raman scattering cross section is on the order of \( 10^{-30} \) cm\(^2\) molecule\(^{-1}\), which results in very low signal intensity. \[31]\] The enhancement of electromagnetic field on a metal nanostructure is considered as the main contributor to the amplification of Raman scattering observed in SERS. \[33\] The amplification provided by this mechanism is twofold. First, because Raman scattering intensity is proportional to the intensity of the excitation electromagnetic field, the increase in magnitude of this field results in the Raman enhancement. Second, it has been noted that the scattered Raman field undergoes the enhancement as well. \[31\] Another mechanism of SERS enhancement is related to the charge transfer between the analyte molecule and metal nanostructure, which causes the change in the analyte's molecular polarizability. \[21,34\] This also creates the resonance conditions for Raman scattering. \[35,36\] The level of SERS enhancement due to charge transfer is significantly lower than that attributed to the electromagnetic mechanism, or 10–100 times versus \( 10^6 \). \[31\] For this reason, SERS-active substrates are engineered depending on the requirements dictated by the electromagnetic mechanism.

Electromagnetic SERS enhancement is related to the effect called plasmon resonance, the frequency resonance between the metal electron cloud and the incident electromagnetic field. Experimentally, plasmon resonance exhibits itself as an absorbance band in the UV–vis spectrum of the metal nanostructure. Silver has plasmon resonance in the close-to-UV wavelength range (approximately 350–550 nm), and gold exhibits the resonance in the visible and near-infrared wavelength range (520 nm and higher). In order to achieve Raman enhancement, the laser excitation wavelength should be within the plasmon resonance band. According to the electrostatic theory, only the dielectric permittivity of the metal and its surrounding medium defines the conditions of the plasmon resonance. \[12\] However, it is well known that the size, shape, and mutual orientation of metal nanoparticles (i.e. the elements of the nanostructure) also affect the plasmon resonance (Fig. 2). \[13,17,40\] By varying these parameters, it is possible to tune the plasmon resonance to the desired wavelength range and also achieve higher SERS enhancement.

Colloidal SERS probes

Gold and silver nanoparticles with close-to-spherical shapes are the most widely used substrates for intracellular SERS. Typical spherical gold nanoparticles are shown in Fig. 3(a). Recently, a variety of nanoparticles with different shapes, including nanorods, \[41\] nanoprisms, \[42,43\] and nanocubes \[44\] have been developed. Figure 3 (b–d) exhibits scanning and transmission electron micrographs of these types of nanoparticles. All of them can potentially be used inside cells and should be taken into consideration when a new SERS probe is designed. Figure 3(e) demonstrates one of the applications...
of colloidal nanoparticles. Gold nanoparticles, functionalized with 4-mercaptobenzoic acid, were introduced into living cells, and the SERS maps of intracellular pH levels were obtained.\[45\] Silver nanoparticles

The first intracellular SERS analysis was conducted using silver colloid by Nabiev et al.\[16\] Incubation of erythroleukemic cells with nanoparticles allowed internationalization of colloidal particles inside cells by the mechanism of endocytosis. This technique has become the most widely used method for introducing metal nanoparticles into cells. It was applied for studying the interaction between several antitumor drugs (doxorubicin,\[16\] amsacrine\[46\]) and cells. The adsorption of pre-aggregated silver nanoparticles on the cell plasma membrane enabled SERS to analyze cell resistance to an antitumor drug mitoxantrone.\[47,48\] Silver colloids are usually prepared by the reduction of silver nitrate AgNO\(_3\) with either sodium citrate\[49\] or sodium borohydride.\[49,50\] Detailed reviews of colloids’ applications for intracellular SERS are provided elsewhere.\[19,28\]

Gold nanoparticles

Colloidal gold nanoparticles have several advantages over silver ones for intracellular measurements because they are (1) chemically inert and (2) unlike silver colloids, allow the use of near-infrared laser excitation wavelengths for SERS.\[17\] The latter is important for reducing the heating effects on cells and minimizing the fluorescence background in Raman spectra. Moreover, near-infrared wavelength range is the most suitable for optical imaging of tissues because both water and hemoglobin, the main light absorbers in biological samples, are transparent in this range.\[51,52\] Synthesis of gold nanoparticles largely relies on the citrate reduction of chloroauric acid HAuCl\(_4\) proposed by Turkevich.\[53\] Sodium citrate also serves as a stabilizer of gold colloid by providing the negative surface charge of the nanoparticles. By varying the amount of sodium citrate, it is possible to obtain nanoparticles of different sizes.\[54\]

Core–shell nanoparticles

Metal nanoshells with dielectric cores are a very promising type of nanoparticles for biological applications introduced by the
research group of Halas.\cite{55,56} By varying the ratio between the core radius and shell thickness, the optical properties of the nanoparticles, i.e. their plasmon resonance wavelength, can be tuned.\cite{57} Most importantly, this tunable plasmon wavelength range is very broad and can be varied from approximately 600 nm to 1 μm while the nanoparticle diameter stays below 200 nm.\cite{56,57} These parameters hold for nanoparticles having gold shells. Silver nanoshells without a dielectric core exhibit plasmon resonance in the UV range.\cite{58} Such variability in optical properties is unattainable with solid silver or gold nanoparticles. Plasmon resonance wavelength of solid nanoparticles is proportional to their size. Large nanoparticles or nanoparticle aggregates must be used in order to achieve plasmon resonance in the near-infrared wavelength range.\cite{38,59,60}

To date, core–shell nanoparticles have been mostly applied as contrast enhancing agents, targets for photothermal tumor ablation, and drug carriers.\cite{61,62} The reports on applications of core–shell nanoparticles for intracellular SERS are very limited. Nanoshells with silica core/gold shell, with an average diameter of 150 nm, have been employed for analysis of fibroblast cells.\cite{63} Functionalization of nanoshells with 4-mercaptobenzoic acid (4-MBA) enables measurements of intracellular pH by using the calibration SERS data from 4-MBA in solution. This approach to intracellular pH sensing has been previously reported on 4-MBA-functionalized silver\cite{64} and gold\cite{45} nanoparticles and optical fibers coated with a silver film.\cite{65} The work of Kneipp et al. demonstrates the spectral mapping of intracellular pH levels calculated using the calibration SERS spectra recorded from 4-MBA-coated nanoparticles in solution.\cite{45} Overall, core–shell nanoparticles hold great promise for spectroscopic applications, and the full range of their capabilities is yet to be explored.

Limitations of using colloidal nanoparticles for intracellular SERS

The main obstacle in using colloidal nanoparticles inside cells is their aggregation, uncontrollable localization,\cite{28,66} and irreversible uptake.\cite{67} Kneipp et al. have demonstrated that although they are instrumental in analyzing individual biochemical components in cells, colloidal nanoparticles tend to aggregate with time.\cite{66} Figure 4(b) shows the transmission electron microscopy images of immortalized rat renal proximal tubule cells incubated with gold colloid. The size of aggregates clearly increases over time. SERS spectra collected at the same location in a cell but at different time points exhibited significant variation as seen in Fig. 4(c). The reason is that the aggregation of gold colloid modifies the configuration of SERS-active ‘hot spots’ formed by the nanoparticles.\cite{68} This results in the change of SERS spectra intensity and appearance of new spectral peaks. Therefore, the changes in SERS data are likely to be attributed not to the change in the molecular composition in the vicinity of gold nanoparticles but rather just to the rearrangement of the SERS-active substrate. This is a major obstacle in using colloids for intracellular SERS. Overall, colloidal nanoparticles are particularly useful in intracellular SERS applications, especially in cases when SERS analysis is performed right after the introduction of nanoparticles inside cells and time-dependent studies are not the main goal. Otherwise, the colloid aggregation, inevitably occurring with time, results in data uncertainty.

Overcoming the limitations of colloidal nanoparticles

Functionalization of SERS-active nanoparticles, which allows selective targeting of specific cellular subunits, is one of the ways to overcome the problems associated with uncontrollable aggregation of nanoparticles.\cite{69} For example, spherical gold nanoparticles\cite{70,71} and gold nanorods\cite{72} were conjugated with nuclear localization peptides, which enable the targeting of nanoparticles to the nucleus. Nanorods functionalized with a peptide through the thiolalkyl-triazole linker were applied as SERS-active probes in cells.\cite{72} However, the localization of the nanoparticles inside a cell was shown to be dependent on the cell type and was different for normal and cancerous cells.\cite{71,72} This presents a critical limitation of this technique. In addition, it is important to remember that the functionalized particles are likely to be a source of a background SERS signal, which may interfere with the cellular signal. For example, the SERS spectrum collected from peptide-conjugated nanorods has multiple similar features with the SERS spectra of the same particles inside the cells.\cite{72} It would be interesting to explore...
the use of metal nanoparticles that are functionalized with a molecule, which plays two different roles simultaneously. The first one is the targeting of the nanoparticle to a particular location inside a cell. The second one is to act as a SERS beacon.\textsuperscript{[73]} To the best of our knowledge, no such particles have been reported for intracellular applications. However, it is important to note the recent work of Qian et al.\textsuperscript{[74]}, which described gold nanoparticles for tumor targeting. Nanoparticles of 60 nm were functionalized with malachite green as a Raman reporter, coated with a protective layer of polyethylene glycol to minimize the toxicity, and conjugated with antibodies to enable selective tumor targeting. Similar silver-based nanoparticles with about 200-nm diameter specially designed for tumor targeting were developed earlier by Kim et al.\textsuperscript{[75]}

Another way to solve the problem of colloid aggregation is to create a nanoprobe with a fixed configuration of SERS-active metal nanostructure. This has been achieved by attaching nanoparticles to glass nanopipettes\textsuperscript{[76]} and carbon nanotubes\textsuperscript{[77]}, coating fiber-optic probes\textsuperscript{[78]} and microelectrodes\textsuperscript{[79]} with silver films, and using sharp metal nanotips.\textsuperscript{[80]} All these approaches are discussed in detail in the next section.

SERS probes with the fixed controlled configuration of metal nanostructure

Fiber-optics-based SERS probes

Fiber-optic tips with SERS functionality, enabled by silver film or immobilized silver colloids, provided the first steps for colloidal-based methods in single cell studies.\textsuperscript{[78,81–83]} Scaffidi et al. demonstrated the application of a silver-coated optical fiber for intracellular pH SERS sensing enabled by functionalization of the probe with 4-mercaptobenzoic acid.\textsuperscript{[65]} Although the reported diameter of the probe tip is on the order of 100 nm, to the large apex angled, a conic tip will likely incur cell damage when left inserted for a prolonged time. The further optimization of the probe shape for cellular studies can be realized by making longer and thinner fiber tips by means of laser pulling.

Tip-enhanced Raman spectroscopy

Tip-enhanced Raman spectroscopy is based on the electromagnetic field enhancement in the vicinity of a sharp metal nanoscale tip.\textsuperscript{[84,85]} While relying on the same principle for Raman signal amplification as SERS, this technique allows superior spatial resolution of 10–30 nm, which is well below the optical diffraction limit.\textsuperscript{[86]} The critical advantage of tip over surface enhancement is that the signal amplification is provided by a single ‘nanoparticle’, i.e. a metal tip. This ensures that the electromagnetic field is consistently distributed, thus eliminating the problem of nonuniform field enhancement associated with size variability of a typical SERS probe (colloidal nanoparticles, island films).\textsuperscript{[84,86]} However, enhancement caused by a single particle also presents the main limitation of TERS. In apertureless TERS, a metal tip in close contact with an analyte molecule is illuminated with a focused laser beam through a high numerical aperture objective, and the scattered light is collected in the far field with the same objective.\textsuperscript{[86]} In this setup, the spatial TERS resolution is limited only by the diameter of the probe tip, which defines the dimensions of the enhanced electromagnetic field.\textsuperscript{[87]} Other TERS schematics involve near-field detection and/or illumination, as discussed in the following paragraphs.

Tip-enhanced Raman spectroscopy not only enables high-resolution Raman spectroscopy but also allows collecting near-field chemical images of surfaces. This technique emerged as a result of near-field Raman scattered imaging.\textsuperscript{[88]} By employing the principle of a near-field scanning optical microscopy (NSOM), the first near-field Raman spectra were collected using a single optical fiber for sample illumination and signal collection. SERS spectra collected using NSOM were demonstrated on cresyl fast violet and p-aminobenzoic acid deposited on a silver film.\textsuperscript{[89]} The low collection efficiency, however, is known to be the major problem associated with NSOM when the signal is collected through an optical fiber. The use of a larger optical fiber increases the amount of collected signal; however, this also results in the decreased spatial resolution.

One way to solve this problem is to illuminate the sample in the near field through a fiber and collect the scattered light with a high numerical aperture objective.\textsuperscript{[90]} This method enabled SERS imaging of dye-labeled DNA with 100-nm spatial resolution.\textsuperscript{[90]} The more efficient approach is to use a free-space illumination and collection geometry. Apertureless near-field SERS was first measured with a silver-coated optical fiber for enhancing the scattered signal.\textsuperscript{[91]} The excitation and signal acquisition were performed using a single objective. However, this was still carried out in conjunction with using a silver island film for Raman signal amplification. Finally, a true tip-enhanced Raman scattering was demonstrated by Stockle et al., where the enhancement was provided only by the metal tip.\textsuperscript{[84]}

Tip-enhanced Raman spectroscopy has been largely implemented using silicon atomic force microscope (AFM) cantilevers coated with gold or silver films, as well as gold\textsuperscript{[87]} and silver\textsuperscript{[92]} tips produced by electrochemical etching, which are similar to typical probes for scanning tunneling microscopy (STM). These TERS probes have been successfully applied for imaging single-walled carbon nanotubes,\textsuperscript{[93]} aminoacids,\textsuperscript{[96]} DNA components,\textsuperscript{[94]} viruses,\textsuperscript{[95]} bacteria,\textsuperscript{[96]} and cells.\textsuperscript{[92]} In some cases, metal island films were still used for enhancing the Raman scattering in conjunction with a metal tip.\textsuperscript{[91,97]} In the original work of Stockle et al., TERS spectra of a thin layer of brilliant cresyl blue were obtained using a silver-coated AFM cantilever.\textsuperscript{[84]} As shown in Fig. 5(a), the TERS signal was collected with the cantilever brought in contact with the sample. In case when the cantilever was retracted, only a low background signal could be observed. Importantly, the TERS spectra still exhibit certain variability with time. Figure 5(b) demonstrates the pseudocolor map of the intensity of tip-enhanced Raman signal collected from one point of the bacterial surface at different times.\textsuperscript{[96]}

Although TERS has been applied for analyzing the outer surface of a single-cell plasma membrane,\textsuperscript{[80,96]} no intracellular measurements using TERS have been reported so far, although AFM cantilever-based probes, similar to the ones normally used in TERS, have been previously applied to penetrate a cell and, for example, to introduce a fluorescent protein into a cell.\textsuperscript{[98]} There are several reasons for the lack of reports on intracellular TERS. First of all, the primary application of TERS for Raman mapping of surfaces is difficult to realize inside cells because currently available TERS probes have large apex angles and cannot be scanned inside the cell without damaging it. Second, the ability of a probe to penetrate a cell does not necessarily imply the applicability of this probe for prolonged intracellular measurements. Typically, cell microinjection or AFM assisted transfection is performed in seconds in a shot-like manner. This is required for reducing the cell damage, although even in this
case a certain number of cells suffer fatal damage. In intracellular measurements often require a prolonged monitoring of cell activity on the order of tens of minutes or even hours. This implies a significantly higher risk of cell damage, thus calling for a minimally invasive intracellular probe. TERS probes, which utilize AFM cantilevers, are not suitable for this application because, although very small at the tip (tens of nanometers), their conical or pyramidal shape results in fatal rupture of a cell membrane in case of deep penetration and extended probe presence in a cell. In addition, an AFM cantilever can be inserted in a cell only at 90° angle, which is not optimal for cell interrogation. Typically, the penetration angle of 30°-45° is used for cell microinjection.

In summary, TERS holds a great potential for high resolution Raman analysis. Its main application is the analysis of cell organelles. Although it is not well suited to do intracellular mapping, TERS can definitely be applied for point-of-interest spectroscopic analysis of cell activity and characterization of cell organelles. The design of intracellular TERS probes requires careful consideration of the size and shape of a probe and the angle of the probe insertion into a cell. At the same time, it should be noted that intracellular mapping with TERS probes could potentially be realized with nanometer-sized tips, which can be scanned with a comparable resolution. Such a setup will also require a laser scanning Raman microscope for tracking the scattered signal from a probe tip.

**Figure 5.** (a) Tip-enhanced Raman spectra of brilliant cresyl blue on a glass support measured with a silver-coated atomic force microscope probe. The top spectrum was measured with the tip in contact with the sample, while the bottom spectrum shows the background Raman signal collected with the tip retracted from the sample. Adapted from Stockle et al. (b) Evolution of the tip-enhanced Raman spectra on one spot of the bacterial surface with time. Adapted from Hering et al. and Neugebauer et al.

**TERS-active glass nanopipettes**

The nanopipette-based SERS probe for in situ intracellular analysis of living cells was recently developed by Vitol et al. The nanopipette design employed a hollow glass capillary with a 150-nm tip, which was coated with gold nanoparticles. The interparticle distance was optimized to ensure the probe sensitivity for intracellular applications. The overall length of the capillary was on the order of 10 cm and the outer diameter was 1 mm. Glass pipettes with such dimensions can be fitted into a standard Eppendorf micromanipulator and fluid injector, which are used for cell microinjection. Importantly, the tip of the nanopipette remains open after the functionalization with gold nanoparticles. This allows the SERS-active nanopipette to be used for concurrent drug injection and monitoring of cell response. This is a critical advantage compared with fiber-optic and TERS probes. Moreover, the glass nanopipettes have a vast number of other applications including scanning ion conductance microscopy for characterization of ion transport in cells which could supplement SERS.

The SERS-active nanopipette was demonstrated to be highly sensitive to its microenvironment in a cell, enabling the detection of characteristic spectral signatures of cell nucleus and cytoplasm. Moreover, this probe enabled the intracellular detection of protein expression in response to a change in cell osmolarity (Fig. 6). This is the first demonstration of using SERS for in situ analysis of living cell function. Figure 6 shows the SERS spectra collected immediately after the insertion of the nanopipette into a cytoplasmic region of a HeLa cell and the time sequence of the spectra recorded from the same point after the addition of aqueous KCl solution into the cell medium. The dynamic modulations of SERS spectra observed in a treated cell signify the changes in the intracellular protein conformations, which were a result of increased environmental osmolarity and cell plasma membrane depolarization. The reason for the appearance of 1319, 1260, 1515, and 1526 cm⁻¹ SERS peaks at different time points can be associated with the induced expression of various types of stress proteins. The change in environmental osmolarity triggers the cellular adaptive mechanism, which leads not only to the induction but also to the suppression of specific proteins.

Figure 7(a–c) compares the tip geometry of a SERS-active nanopipette, fiber-optics-based SERS probe, and a typical TERS probe. The SERS-active nanopipette shown in Fig. 7(a) has an almost cylindrical tip optimal for intracellular probing. The fiber-optic probe, as shown in Fig. 7(b), not only has a very large tip but is also highly conical. With these dimensions, this probe is not suitable for intracellular probing. The TERS probe shown in Fig. 7(c) has a very fine tip, which ensures high spatial resolution. However, compared with the SERS nanopipette, this probe has a large apex angle, which would be an obstacle in using it for intracellular studies. In contrast to AFM-based TERS probes, SERS-active nanopipette is compatible with standard micromanipulators and allows for more maneuvering freedom during cell probing. Figure 7(d–e) illustrates this fundamental difference between SERS nanopipettes and TERS probes.

In addition, it is important to note that there exist commercially available TERS probes that feature a bent fiber-optic tip, which allows to combine AFM with near-field optical microscopy and Raman spectroscopy. Most importantly, the bent geometry of this tip potentially allows to probe cells in a less invasive manner than a standard AFM cantilever.
Figure 6. Schematic of measuring cell response to the change in osmotic pressure with a surface-enhanced Raman spectroscopy (SERS)-active nanopipette before (a) and after (c) treating cells with aqueous solution of KCl. Panel (b) shows the SERS spectrum collected from the nanopipette tip inserted in the HeLa cell cytoplasm. The representative time-resolved spectra showing HeLa cell response to treatment with aqueous solution of KCl measured with the SERS-active nanopipette are depicted in panel (d). Time-dependent variation of the cytoplasmic signal has been observed. The dynamic changes in the SERS spectra represent the cell activity in response to the osmotic changes. The spectra in (d) are offset for clarity. Adapted from Vitol et al.\textsuperscript{[76]}

Figure 7. Comparison of the tip geometry (a–c) and navigation schematics for cell interrogation (d–e) for different surface-enhanced Raman spectroscopy (SERS) probes. Scanning electron micrographs of (a) SERS-active nanopipette (adapted from Vitol et al.\textsuperscript{[76]}), (b) fiber-optic probe coated with silver nanoparticles (adapted from Gessner et al.\textsuperscript{[78]}), and (c) TERS silver probe prepared by electrochemical etching (adapted from Zhang et al.\textsuperscript{[125]}). Panel (d) shows a SERS-active nanopipette interrogating a cell. The nanopipette allows for controlled insertion at different angles, whereas atomic force microscopy-based tip-enhanced Raman spectroscopy probe (e) permits cell penetration only at one angle. The arrows show the possible directions for probe movements. Fiber-optic tips can also be employed for cell probing in the same manner as nanopipettes.
Carbon nanotube-based intracellular cell probes

Nanopipettes, having tip diameters on the order of 200–500 nm, are largely suitable for probing cell nucleus and cytoplasm. Further development of SERS-active nanopipettes are directed towards reducing their tip size. Smaller nanopipettes with the tip diameter below 200 nm have several advantages: (1) they have a minimal effect on cell integrity and (2) they enable more localized intracellular probing and potentially enable the analysis of smaller cell organelles, such as mitochondria.

Carbon nanotube-based cellular probes offer an attractive alternative to the glass-based ones. Their major advantages are superior mechanical properties at high aspect ratios and cylindrical shape, which, in conjunction with small diameter below 200 nm, should minimize the potential cell damage. Attaching a single carbon nanotube to an AFM cantilever tip is one way of creating small, cylindrical cellular probes. One potential difficulty of using such a probe for intracellular SERS is the 90° insertion geometry, which has been discussed in the previous section. A more versatile probe was developed by attaching a single carbon nanotube to a glass micropipette. Nanotubes with 50–200 nm diameters have been functionalized with Au nanoparticles and tubes with ~20 nm Au particles were used for cell probing as SERS-active pipette tips. Another suitable platform for intracellular SERS is carbon nanopipettes. Carbon nanopipettes have been successfully functionalized with gold nanoparticles and also applied for single-cell electrophysiological measurements; however, the actual SERS applications of these probes are yet to be demonstrated. Recently, carbon nanopipettes with 10–30 nm tip diameters were reported.

For SERS applications, the main obstacle in designing very small probes is the metal particle size limitation. According to the theoretical calculations, arrays of nanoparticles having diameters of 20–100 nm yield the best Raman enhancement when the appropriate interparticle distances are selected. Nanoparticles smaller than 10 nm have very small scattering cross sections and do not provide sufficient SERS enhancement. Micron-sized aggregates of such nanoparticles could provide SERS hot spots; however, this is not suitable for intracellular applications because of the size limitations. Possible solutions can be found by using gold polygonal nanoparticles or using nanorods and nanowires with high aspect ratios.

Data analysis for intracellular SERS

Reproducibility of SERS spectra depends on several parameters. As discussed earlier, SERS enhancement, and therefore the SERS signal, is inherently connected to the configuration of the SERS-active metal nanostructure. First, it is critical to use the probes with uniformly distributed nanoparticles in order to obtain reproducible spectra. The second important factor is the orientation of analyte molecules with respect to the SERS substrate, which can change depending on the concentration of the molecules. For example, it was demonstrated that the intensity of the most prominent spectral band of adenine, the ring breathing mode, shifts depending on the adenine bulk concentration in solution. At 9.0 μM, this mode corresponds to 735 cm\(^{-1}\), whereas after decreasing the adenine concentration to 0.75 μM, the ring breathing mode shifts to 746 cm\(^{-1}\). At lower concentration, adenine molecules are oriented more horizontally with respect to the substrate but tend to orient vertically as the concentration increases. Similar phenomenon has been observed on DNA molecules. The third important factor in intracellular SERS measurements and potential source for data variability is cell heterogeneity. Because of cell heterogeneity, a SERS sensor with submicrometer resolution will inevitably produce different SERS spectra from different locations inside a cell. However, the difference between the spectra should still be within the same range if the same cell compartment is being analyzed.

Statistical data analysis is usually implemented for interpreting SERS spectra. Multivariate analytical techniques take into account the integrated variability of the SERS spectra. Principal component analysis (PCA) has been successfully applied for extracting information from SERS spectra. PCA facilitates identification of hidden relationships between data sets by reducing their dimensionality and representing the data in the new coordinate system. The main application of PCA is the differentiation between characteristic spectral signatures of various samples, for example, normal and cancerous cells. At the same time, PCA has been applied for data classification problems. For this application, PCA is the most effective for analyzing N data sets with p data points when N > p. However, in localized intracellular SERS measurements, only a small number of SERS spectra collected from one cell might be available (N = 5–10) because, in some cases, the probe can be inserted in a cell a limited number of times. Because a typical SERS data set contains multiple data points (p ≥ 1000), it is actually preferred to use other data analytical methods, such as partial least squares (PLS), using a tumor classification problem as an example, it has been demonstrated that PLS analysis results in less false positives than PCA.

Guidelines for selecting a SERS-active probe for intracellular applications

To summarize the review of intracellular SERS probes, this section presents a brief summary on the most critical aspects of selecting a SERS nanoprobe for intracellular analysis for a particular application. The decision process is outlined in Fig. 8.

First, it is necessary to consider the ultimate application of the probe and evaluate the potential implications of using a particular probe (Fig. 8(a)). For example, if the main goal is to obtain general cytoplasmic signatures of cancerous versus normal cells, colloidal SERS probes can be selected. The advantages of colloids are their ease of fabrication, small size, and their ability to be introduced inside cells by natural cellular uptake from the medium. However, the aggregation of nanoparticles with time might result in SERS data uncertainty. It is therefore preferred to use this kind of SERS probes when only the fingerprint information is of interest. In case when a more localized analysis is required and/or long-term, time-dependent study will be performed, it is recommended to use a probe with a controllable configuration of nanoparticles. Fiber-optic probes with optimized tip geometries (small apex angle, tip diameter below 200 nm) can be used as cellular probes when no injection in a cell is needed. Nanopipette-based SERS probes, described in Vitol et al., have the advantage of smaller tips (Fig. 7(a)) and they potentially allow fluid injection in a cell. This might be important for studying cell response to drugs and analyzing biologically active molecules. Furthermore, tip-enhanced nanoprobes can also be employed for highly localized cell analysis. Currently available TERS probes implemented on the basis of AFM or STM cantilevers are difficult to use for cell probing. However,
can potentially be used for probing individual cell organelles, such as mitochondria.

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References


Figure 8. Guidelines for selecting a surface-enhanced Raman spectroscopy (SERS) probe for intracellular applications.

TERS probes could be made from nanopipettes or optical fibers, which will facilitate the control over their navigation inside cells and will reduce their effect on cell integrity. Second, the choice of material of the SERS-active metal nanostructure is based on several considerations. This applies both to colloidal and nanopipette-based probes. In terms of SERS enhancement, silver is known to provide stronger signals compared to gold. However, for intracellular applications gold is preferred (see section Colloidal SERS probes). At the same time, the important practical limitation in selecting the metal for a SERS probe is the available laser wavelength. (Fig. 8 (b)) In case only a green Ar+ (λ = 514 nm) or UV lasers are available for SERS, silver is the only choice. Gold-based SERS substrates can be employed when the lasers with λ > 520 nm are accessible. This is dictated by the plasmon resonance of gold nanostructure, which occurs at the wavelengths larger than 520 nm, thus necessitating the use of corresponding lasers for Raman scattering excitation. The most widely used laser wavelengths are 633, 785, and 830 nm.

In addition to the material of the SERS-active part of the probe, it is important to evaluate the material of the metal-carrying substrate, i.e. the nanopipette or optical fiber (Fig. 8(c)). Glass-based and carbon-based nanopipettes as well as carbon nanotube-tipped probes have been developed. The latter might be more advantageous for probing cells with a stiff plasma membrane. In addition, these probes...