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Surface-enhanced Raman spectroscopy at single-molecule scale and its implications in biology

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Single-molecule (SM) spectroscopy has been an exciting area of research offering significant promise and hope in the field of sensor development to detect targets at ultra-low levels down to SM resolution. To the experts and developers in the field of surface-enhanced Raman spectroscopy (SERS), this has often been a challenge and a significant opportunity for exploration. Needless to say, the opportunities and excitement of this multidisciplinary area impacts span the fields of physics, chemistry and engineering, along with a significant thrust in applications constituting areas in medicine, biology, environment and agriculture among others. In this review, we will attempt to provide a quick snapshot of the basics of SM-SERS, nanostructures and devices that can enable SM Raman measurement. We will conclude with a discussion on SERS implications in biomedical sciences.

1. Introduction

Surface-enhanced Raman spectroscopy (SERS) is based on the phenomena in which molecules adsorbed onto noble metal surfaces generate a million-fold or even higher enhanced signal under favourable conditions [1–4]. Since its first observation in the 1970s [5–7], scientists and engineers have been fascinated by its complexity and possibility. In spite of the numerous advances, the technique has remained elusive for the most part. With the surge in micro- and nanofabrication techniques and the tremendous interest in nanoparticle synthesis, significant developments in both fundamental and applied research in SERS have been possible by scientists and entrepreneurs.

Theoretical studies have indicated that the basic enhancement mechanisms for SERS include chemical enhancement (CM) [8–10] and electromagnetic enhancement (EM), with the EM playing a predominant role in enhancement [11–17]. Based on this concept, SERS-active substrates have usually been found to support plasmonic fields and enhance the Raman signal with exquisite sensitivity. The unique and versatile advantages of SERS include the tremendous multiplexing capacity for simultaneous target detection due to the narrow width of the vibrational Raman bands, quantification based on a specific SERS fingerprint of the corresponding labels, conformation and structural studies of the targets, high photostability and optimal contrast possible with the red or near-infrared (NIR) excitation to minimize background owing to autofluorescence from biological samples, such as blood, cells or tissue. More importantly, only a single laser excitation is necessary to excite the Raman reporters for multiplex detection compared with the multiple wavelengths necessary for multiplex fluorescence signal. Owing to the high sensitivity, less interference from the environment and amplified fingerprint of the SERS signals, the application of SERS to obtain an in depth understanding of a variety of systems, particularly those that are biological in nature, has proved to be invaluable.

Leveraging the findings from SERS, single-molecule (SM)-SERS was first observed in 1997 by two independent groups using silver and gold nanoparticles as SERS substrates together with a dye for enhanced Raman signal

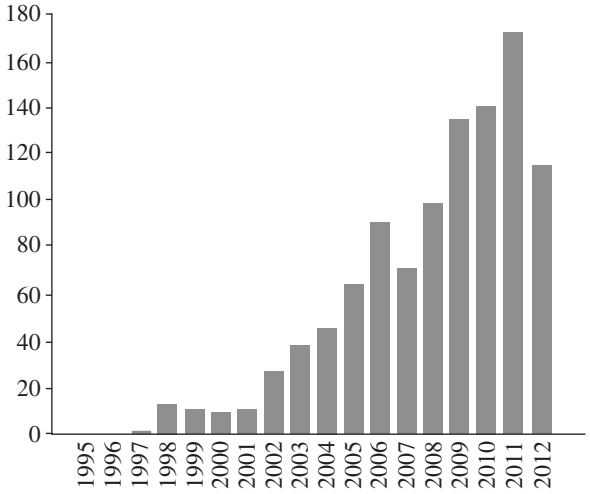

Figure 1. Statistical graph to show publications based on 'SM-SERS' by year.

recording [18,19]. The enhancement factor (EF) for SM-SERS was estimated to be as high as 10^{13}–10^{14}, sufficiently large in principle to induct Raman spectroscopy into the coveted club of SM spectroscopies. It should be noted that recent experimental and theoretical results have claimed the limit of the EM to be less than 10^{11} [20]. Such independent reports on SM-SERS triggered a renewed interest in this technique to expand the technology and applications to pathways traversed by its fluorescence counterpart. The statistics from the ISI database depicted in figure 1 shows continued interest in SM-SERS. A brief survey of the literature will indicate that development spans the entire realm of SM-SERS science, which is especially encouraging. Stimulated and coherent Raman techniques are also in place, predominantly to examine deeper sections and will not be a part of this discussion.

Our intent in this review is to discuss the basics of SM-SERS and summarize its applications in biomedical sciences. Specifically, we will introduce the relevant physical and chemical concepts relevant to the approaches to achieve SM-SERS and the key role of hot spots. Topics such as the basics of SM-SERS (§2), the nanostructure for SM-SERS and their role in enhancing Raman scattering of surface molecules (§3) are discussed. The importance of devices and systems for SM Raman sensing will be discussed in §4. We conclude by highlighting key biological applications in §5.

2. Basics of single-molecule surface-enhanced Raman spectroscopy

Owing to the persistent efforts of various groups, SM-SERS is now becoming a well-established technique and a subfield of SERS as well as a technique in the broader scope of SM laser spectroscopy. Cues to the conditions under which SM-SERS could be observed are being increasingly well understood, and have been discussed and reviewed in several works [21–24]. Herein, only the basic properties and the most important aspects and features will be summarized and highlighted.

As discussed in the initial study in 1997, measurement of low concentration is simple and easily understood and has its origins in SM fluorescence measurements. Monitoring SMs is based on the simple idea of affording the measurement at ultra-low concentration of the analyte [18,19].

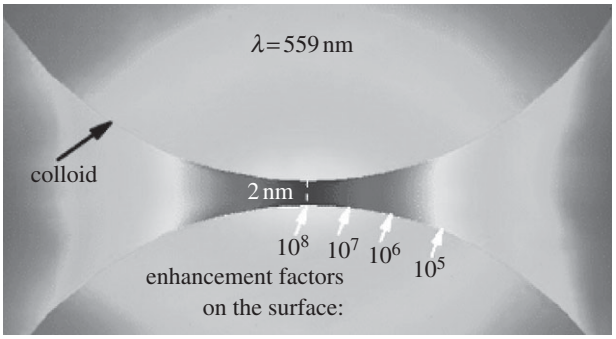

Figure 2. Enhancement factor distribution in the region of the gap (2 nm) between two gold colloids (radii = 30 nm) calculated in the electrostatic approximation with finite-element modelling (adapted from [21]).

However, the problem in developing tools to measure low concentration is the non-uniform distribution of molecules on the metal surface, where molecules need to be adsorbed to create hot spots to produce a signal at the so-called SM resolution. Briefly, SM-SERS is not just a property of the molecule, but is a combination of the response of the molecular microcosm and the localized plasmon-supporting nanostructure, both of which contribute to the success of this field [25]. The bi-analyte approach was then developed to resolve problems that might be encountered in detecting low concentrations by using two distinguishable SERS analytes, reported first by Le Ru *et al.* [26], and normally aided by suitable statistical analysis of the fluctuations [27]. The simultaneous use of two analyte molecules enables a clear confirmation of signals from a single or few molecules and eliminates most of the uncertainties associated with the Raman signal relating to low molecule concentrations in previous experiments. This approach provides a relatively simple and general direction to assess the concentration at which the largest number of SERS can be sampled [26]. By combining the Langmuir–Blodgett approach with the bi-analyte SERS method, an individual fingerprint could be distinguished at SM resolution by SERS [22,28]. A more detailed explanation of the advantages of the bi-analyte approach is provided in the study of Etchegoin & Le Ru [21]. More recently, Dieringer and colleagues presented a convincing frequency domain proof for SM detection using isotopologues of rhodamin 6G, which provides a clear evidence for single-molecule SERS detection owing to frequency rather than intensity correlation [29,30]. This approach has been applied and substantially extended by Etchegoin and co-workers to analyse two or multi-analytes and extract SM events from a large dataset [31–35].

The second very important aspect for SM-SERS is the EF and the distribution of EM. It is well known that SM-SERS was achieved due to the presence of hot spots, which are regions with highly localized plasmon emitting to show a significant electromagnetic coupling effect between particles, typically the gap junctions between metallic nanostructures. For SM detection by SERS, an EF of approximately 10^8 is required or desirable. A more important consideration is the distribution of hot spots (or EF), which should be uniform enough to produce a reproducible SERS signal. Figure 2 shows an example of the spatial EF distribution in the gap region between two colloidal gold nanoparticles by finite-element modelling [21,25]. As indicated, the EF value can vary by an order of magnitude over distances comparable

to a few molecular dimensions, which is a universal property of gap hot spots resulting in a long tail probability distribution that determines most of the basic characteristics of SM-SERS statistics. More recently, Le Ru *et al.* [36] proposed a simple scheme based on selective adsorption of the target analyte at the SERS hot spots for detection of every single target molecule in solution and verified by comparing the average and maximum (SM) SERS EF.

Another very intriguing characteristic of SM-SERS is the temporal fluctuation, observed in the past work [18,19]. While SM-SERS has been found to exhibit drastic temporal fluctuations in both line intensity and frequency [37,38], the vibrational mode emission from SM-SERS has been shown to undergo a characteristic intermittent behaviour that might encode both the dynamics of the molecule and the details of its interaction with the environment [39]. This phenomenon is also called as the SERS blinking effect [40–42]. However, it is still not clear whether it is a defined aspect of SM-SERS since it is an instantaneous process and cannot be 'turned off'. Therefore, the nature and the origin of SM-SERS blinking effect is still a hot topic in this field. One typical explanation for this phenomenon is that the blinking contains both a thermo-activated component and a light-induced component, which means that the blinking is caused from thermally activated diffusion of individual molecules on the particle surface coupled with photo-induced electron transfer and structural relaxation of surface active sites [42]. Therefore, an investigation of blinking effect (spectra fluctuations) will help in addressing some of the fundamental issues, such as the statistical ageing and entanglement of vibrational modes, etc. [43–45]. Detailed knowledge of the fundamental dynamic processes in molecular systems, the charge or electron-transfer mechanism regulating the molecule–metal interaction under light excitation might be helpful in addressing blinking effects [46–49].

3. Nanostructures for single-molecule surface-enhanced Raman spectroscopy

It is well known that SERS actually is a nanostructure-enhanced Raman scattering phenomenon [50]. Therefore, SERS signals (EFs) produced on the nanostructure are strongly related to the size, shape, aggregation state and the type of nanostructures. Typically, silver nanostructures usually generate higher enhanced signal than gold with the same size and shape at the visible laser excitation, while less in the red laser excitation, which depends on the localized surface plasmon resonance [51]. The comparison of EFs based on various size, shape and hot spots has been discussed in a recent review [52–54] and will not be discussed here.

As discussed in §2, an EF of at least approximately 10^8 is required in most situations or desirable for SM-SERS detection. To achieve this EF, nanostructures with hot spots are the prerequisite as demonstrated since the first observation of SM-SERS in 1997 [18,19]. A class of silver and gold nanostructures that has provided large EFs is colloidal aggregates [55], as well as metal evaporated films [56] and, most recently, modified scanning tunnelling microscopy (STM) tips [57]. Although the easiest route to achieve SM-SERS is to use the aggregated metal colloid, which could be obtained by simply changing the solvent and the ionic strength, the aggregation state is hard to control because signals from randomly aggregated nanoparticles often suffer from uncertainty in the size of aggregates and geometry. In contrast, composite nanostructures with well-defined geometry are a good alternative to achieve controlled and reproducible signal. As reported by our group, periodic and dynamic three-dimensional gold nanoparticle–DNA network structures have been fabricated for SERS-based quantification, which could also be grown at the cell surface marker sites and applied for the detection of cancer stem cells [58,59]. Electron-beam lithography technique is an excellent tool for the fabrication of composite nanostructures with desired geometry, and has been used for single pathogen detection [60]. Meanwhile, significant efforts have been spent on the chemical synthesis of composite nanostructures with well-defined geometry, such as dimers, trimers and complex nanoassemblies. Metal nanoparticle dimer is the simplest discrete assembly, which exhibits one hot spot between two particles upon laser illumination. Synthesis of dimers could be achieved either by biomolecular-directed assembly or by using chemical linkers. Loweth *et al.* [61] and Park *et al.* [62] have demonstrated the assembly of gold nanoparticles into dimers, trimers and larger structures based on a DNA-programmed procedure. Chemical linkers, such as the rigid, multivalent thiol-linkers [63], phenylacetylene [64,65], thiol-terminated hydrophobic ligand [66] and so on [67] have been demonstrated for the fabrication of dimers with a relatively high yield. For efficient SERS applications, Xia and co-workers proposed a simple and one-step method to generate dimers without any additional assembly steps, which were obtained by introducing a small amount of sodium chloride into the reaction solution. The formation of dimers was achieved due to the change in the colloidal stability [68]. However, the grand challenge is in the synthesis of dimer metal nanoparticles with high yield because a population of the mixed nanoclusters (monomer, dimer and trimer) is often generated. Most recently, Lim *et al.* [69] developed a high-yield synthetic method for the synthesis of SERS-active gold–silver core–shell nanodumbbells (GSND, or heterodimers) for SM detection. As indicated in figure 3*a*, GSND were synthesized by the hybridization of DNA onto the gold nanoparticle surface first, where the Raman dye resides between the two-particle gap junctions. And then a silver shell layer was allowed to grow around the heterodimer structure to form the GSND particle as shown by high-resolution transmission electron microscopy (HR-TEM; figure 3*b*). The interparticle distance between the two particles and the shell thickness of the silver layer is clearly evident in the image. Figure 3*c* illustrates the SM behaviour using GSND particle surface with the shell thickness of 5 nm. It should be noted that an on–off cycle for Raman signals, indicative of the presence of an SM, was clearly observed (figure 3*c*). Meanwhile, recent studies have emerged on the use of metallic junctions in the so-called nanoparticles on metal film or nanoparticles on mirror system for SM-SERS. For example, the generation of hot spot with silver nanocubes for SM-SERS on gold film was reported by Xia and co-workers [70]. Park and co-workers [71,72] report the SM-SERS study, particularly the charge transfer enhancement in SM on the junction between individual gold nanoparticle–aminobenzenethiol–gold film. Notably, tip-enhanced Raman scattering (TERS) is another typical nanoparticle–metal film system for SM-SERS study since most of the TERS studies were achieved

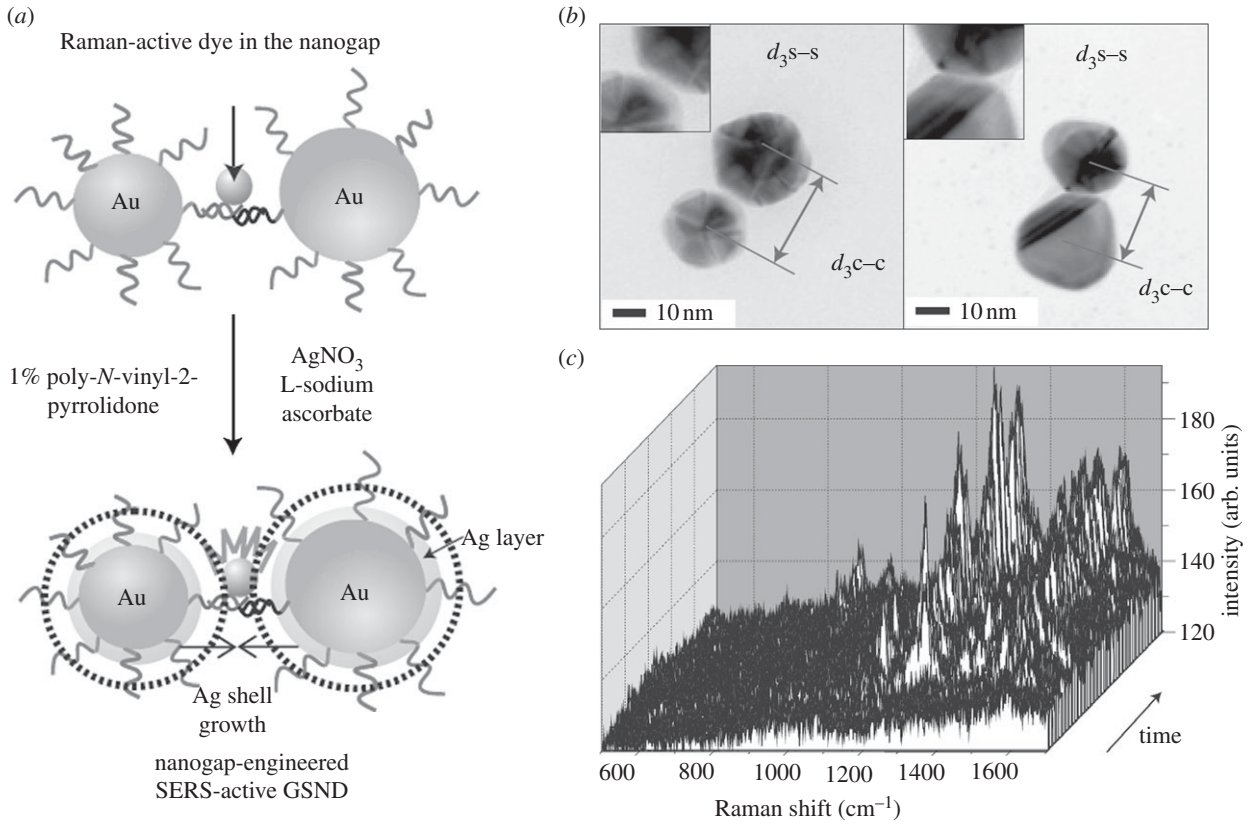

Figure 3. (*a*) Schematic of the fabrication of GSND particles. (*b*) Typical HR-TEM images of the synthesized GSND particles with 5 nm Ag shell thicknesses. Here ds–s and dc–c indicates the distances between two particle surfaces and cores, respectively. (*c*) Single-molecule behaviours from the GSND with a 5 nm Ag shell. Blinking SERS spectra taken from the GSND (accumulation time = 1 s, 100 times) (adapted from [69]).

on the junction between the nanoparticles on the tips and the metal substrate. This will be discussed in detail in §4.

Nanoassemblies with multiple hot spots have also been fabricated according to colloidal chemistry reported by Schlücker's group [73]. The electromagnetic field and the EF around the nanoassemblies were simulated and calculated by the finite-element method, which indicated that the EF value on the nanoassemblies can be as high as 10^{10}, demonstrating its potential to be used as a SERS substrate for SM sensing. With the advancement of nanotechnology, it was believed that more and more SM-SERS substrate could be fabricated with well-defined geometry for reproducible and uniform SERS signal generation. A review of SERS based on molecularly-mediated assembly of plasmonic particles can be found in a recent report [74].

4. Devices/systems for single-molecule surface-enhanced Raman spectroscopy

To achieve SM-SERS, several systems were devised and employed. Examples include microfluidic (figure 4*a*) or opto-electrofluidic approaches with the advantages of fast and dynamic on-demand generation of SERS-active sites for sensitive signal, and a magnetic pull-down system (figure 4*b*) with the advantages of concentrated samples for sensitive detection. As indicated in figure 4*a*, by implementing a flow-based system with higher throughput, the detected event can be accomplished in a very short analysis time [75]. Due to the advantages of the microfluidic system in SERS, immunoassays for protein detection have been developed based on microfluidics by SERS [76,77]. By using a magnetic pull-down system, proteins and DNA from viruses were detected effectively and such a system was very beneficial for SM or single-particle SERS study [78,79].

Most recently, with the development of optical tools and electron microscopy, super optical resolution imaging and STEM imaging have also contributed to SM-SERS development. As reported by Stranahan *et al.* by using super-resolution imaging as a powerful new tool the centroid position of the SERS field could be mapped to within 10 nm resolution, revealing a spatial relationship between the SM-SERS centroid position and the highest SERS intensity [80]. Meanwhile, by employing electron-energy-loss spectroscopy (EELS) in a scanning transmission electron microscope (STEM), Mirsaleh-Kohan *et al.* [81] obtained maps of the localized surface plasmon models of SM-SERS-active nanostructures, in which the SM characteristics were confirmed by the bianalyte approach. Another more promising and most widely used system for SM and single-particle SERS is the combination of Raman microscopy with atomic force microscopy (AFM) as indicated in figure 4*c*, where, an AFM-coupled nano-Raman spectroscopy setup is described for nanoscale detection [69].

By combing the STM/AFM with Raman microcopy, a new subfield of SERS called TERS was developed, which was first reported by the Zenobi group in 2000 for the observation of the Raman spectrum from brilliant cresyl blue upon moving a silverized AFM tip into the focal region and in contact with a thin dye layer [82]. The attractive properties of TERS lie in its ultrasensitivity (down to SM) and the visualized image of the substrate at nanometre resolution. More details on TERS can be found in the earlier studies [83–85]. Furthermore, by using silver tips and the monolayer

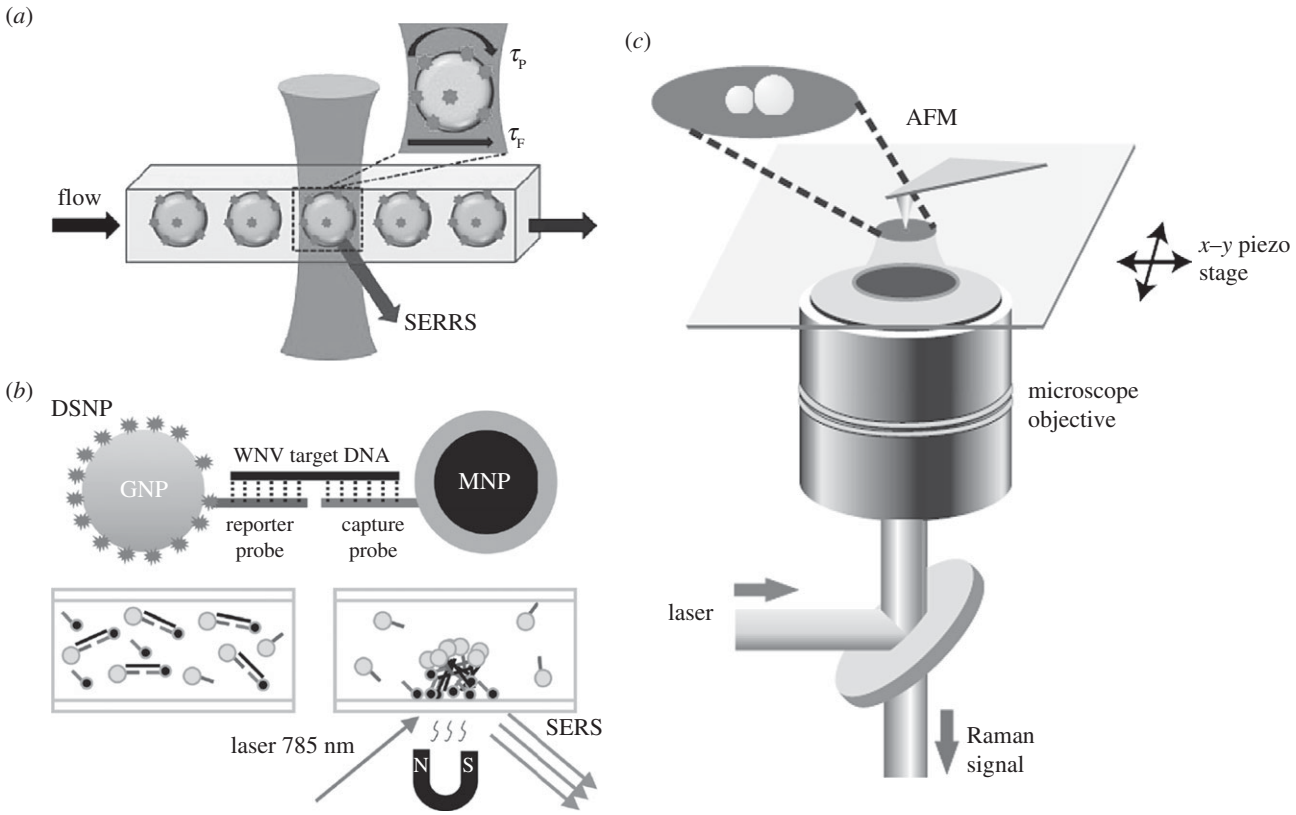

Figure 4. (*a*) Systems for the SM-SERS detection such as microfluidic system, (*b*) magnetic pull-down and (*c*) AFM-correlated nano-Raman spectroscopy (adapted from [69,75,79]). GNP, gold nanoparticle; MNP, model paramagnetic nanoparticle.

of the same dye made by span-coating on Au substrate, Zenobi's group observed the SM-TERS signal from brilliant cresyl blue in 2007 [86]. They claimed that they can attain SM sensitivity because the Raman band from brilliant cresyl blue shows fluctuations in intensity and to a minor extent also in frequency [83,86], which is a possible indicator for SM behaviour as discussed in §2 (the characteristics of SM-SERS). Another popular dye used for SM-TERS study is malachite green isothiocyanate [58,87,88], which has a strong adsorption band centred around the excitation line at 632.8 nm and exhibits strong fluorescence as reported by Domke *et al.* [89]. Therefore, dyes are a specific class of molecules for SM-SERS or SM-TERS investigation because of the resonance contribution. However, TERS has also demonstrated SM sensitivity in detecting small molecules such as biological samples (DNA strands, proteins and membranes) [83,90]. It should be pointed out that the key indicators to achieve SM-TERS are the modification/fabrication of the tips and the controlled distance between the tips and the substrate with molecule adsorption [83]. For example, Zhang *et al.* [86] proved the near-field property of TERS by recording the distance dependence of TERS, which exhibits a strong signal increase when the tip–sample distance is lowered below 30 nm. Owing to the advantages of TERS in SM detection, significant applications in biology could be forthcoming.

5. Surface-enhanced Raman spectroscopy in biology

Owing to the significant advantages of SERS, including the extreme sensitivity, inherent molecular specificity and narrow bandwidth, the technique has made significant inroads in many fields, including catalysis, environmental science, basic sciences and medicine. The sensitivity of SM-SERS has been very beneficial in exploring a range of topics, for instance, electrochemistry, resonant Raman spectra, conduction, heating property and homogeneous Raman broadening. In particular, it is reasonable to state that SERS has enhanced biology. For example, the detection of proteins and DNA, *in vivo* or *in vitro*, to observe targeting has been achieved because of the unique advantage of SERS over fluorescence in biology, including multiplex detection, photostability, and particularly the optimal contrast enabled by the use of red to NIR excitation to minimize autofluorescence from biological species, such as blood, tissue and cells. In this section, we will summarize the application of SERS for multiplex and quantitative *in vitro* and *in vivo* studies in biological sciences, and monitoring of environmental species and ions that are implicated in pollution and contamination.

It should be noted that SERS has been used extensively for label-free fingerprinting and in conjunction with appropriate Raman active labels for detection and quantification. Label-free detection is attractive and has the advantages of simplicity and direct observation, as demonstrated by several applications in biology. Of these, one of the most promising applications of SM-SERS is in the rapid detection and identification of individual DNA bases with Raman structural specificity and characterization of individual base pairs in DNA fragments without the use of fluorescent or radioactive labels, as reported by Kneipp *et al.* in 1998 [91], where adenine was detected by using silver colloid (label-free). Meanwhile, single HIV-1 DNA and single base extension reaction has been detected for DNA methylation analysis

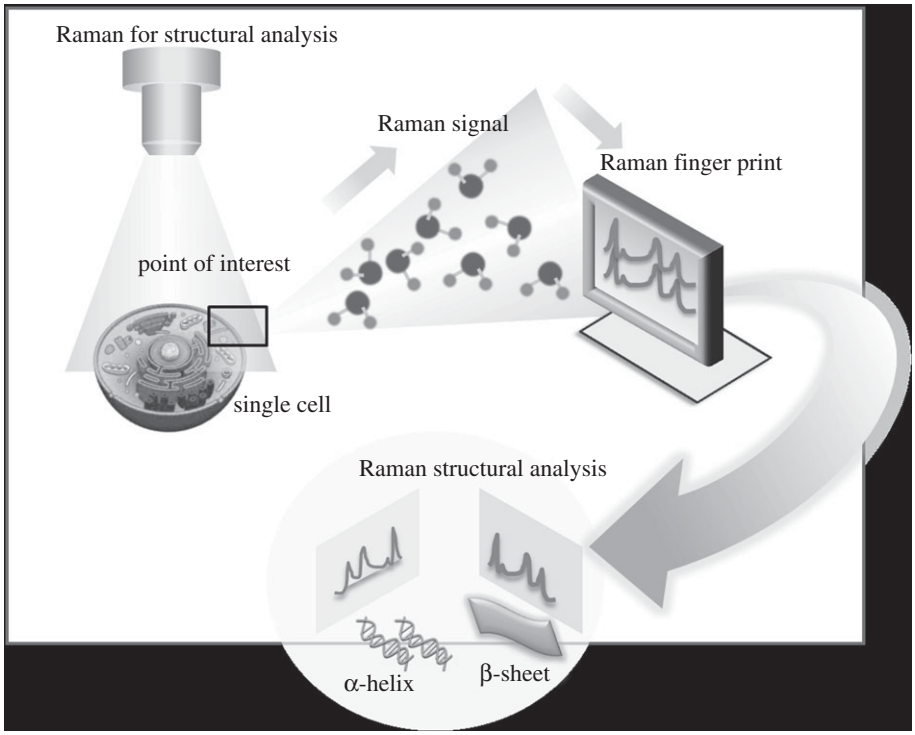

Figure 5. SM-SERS for structural and conformational studies.

[92,93]. Ultrasensitive detection of proteins such as haemoglobin was also reported by using the aggregated silver colloid [94]. Organisms such as pathogens were detected successfully by a label-free approach with high sensitivity using the highly resonant plasmonic nanostructures that can give rise to the formation of multiple hot spots. For instance, pathogens were detected with silver nanospheres as SERS particles, which exhibit multiple hot spots within the structure [95]. Figure 5 demonstrates the possibility of using SM-SERS to examine the intracellular conformation and structure of biological components, a field that has not been significantly explored up to now. Intracellular growth of gold nanoislands can serve as an excellent SERS agent for label-free detection and monitoring of intracellular molecular components, with potential benefit in direct and rapid detection [96]. Such systems may eventually have significant impact in cancer diagnostics through rapid genetic screening or label-free detection of dilute low molecular weight biomarkers.

SERS labels have been more beneficial in ultrasensitive detection because of the high Raman cross section of Raman reporters (dyes) and the selectivity achieved by target elements, such as antibodies, aptamers and DNA. Several groups have reported on the detection of protein, DNA and other components *in vitro* based on SERS labels [97–101]. For example, our group has reported a SERS aptasensor approach from the nanorod–nanopaticle junction for protein detection by labelling the junction with a Raman dye [102]. Quantitative SERS for gene expression estimation has also been accomplished [103] using Raman multiplexers developed for alternative gene splicing [104]. Screening based on SERS labels shows excellent promise in rapid multiplex detection and targeting as shown in figure 6. In this concept schematic, we envision a highly sensitive and high-speed Raman system for simultaneous sorting of cells based on the exquisite cellular signatures paralleling the speed of fluorescence sorting (by flow cytometry) for simultaneous analysis of proteins, DNA or other cellular targets at SM sensitivity in single cells for quantification. Several reports on the application of SERS for multiplex and quantitative monitoring or detection of whole cells and organisms using this highly sensitive and high-speed Raman system have been reported. In 2008, Nie's group illustrated an *in vivo* multiplex targeting approach using SERS probes with NIR laser excitation [105], which is free from autofluorescence. Meanwhile, Zavaleta *et al.* [106] have demonstrated the multiplexed imaging in living mice with encoded SERS nanoparticles with multiplexing ability for molecular imaging. The ability of Raman spectroscopy to separate the spectral fingerprints of up to 10 different types of SERS particles in a living mouse has been demonstrated [106]. Dynamic monitoring of the biodistribution of SERS particles in zebrafish embryos for *in vivo* and multiplex imaging [107] has also been attempted. In the future, these might move to the SM realm with the incorporation of powerful software tools as in fluorescence super-resolution microscopies.

SERS has also been demonstrated as a powerful tool for environmental monitoring, to detect contaminants in the ppb–ppm range to monitor the interaction of chemistry with the biology or the ecology of various systems. For example, the intracellular bioreduction of chromate-decorated gold nanoparticles and intracellular grown gold nanoislands by *Shewanella oneidensis* MR-1 have been monitored by Raman and SERS imaging at very high sensitivity [108,109]. Lead ion detection has been achieved by fabricating the electromagnetic coupling between the gold nanoparticles and the surface plasmon polarization substrate (gold surface) based on DNAzyme. The detection limit for lead ion was demonstrated at 20 pM sensitivity [110]. SERS could play a very significant role in environment monitoring because very few systems could afford the sensitivity offered by chemical fingerprinting. Onsite SM-SERS devices, with reusable enhancer templates,

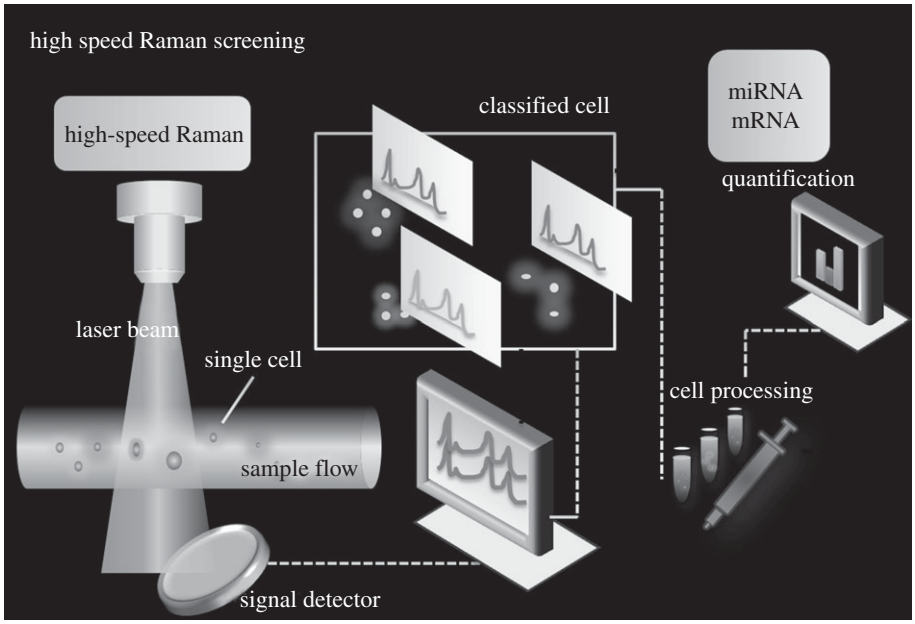

Figure 6. SERS-based high-speed Raman for simultaneous sorting of cells based on cellular fingerprint and SM quantification of targets.

could be constructed for monitoring toxic substances that might be present in trace levels.

6. Conclusions and perspectives

In summary, the basic properties of SM-SERS and the nanostructures, devices and systems used for detection have been discussed and summarized. SM-SERS is now accepted as a well-established subfield of SERS and could emerge as a separate field because of the significant understanding of the plasmon coupling effect and hot spot formation. The implications of SERS in biology, including advances in *in vivo* and *in vitro*, have been highlighted based on the properties of Raman signal generation and enhancement. Although significant improvements have been possible in the synthesis of complex and highly efficient plasmonic SERS nanoparticles with controllable sizes and shapes, challenges in the preparation of stable, well-defined nanoassemblies with consistent hot spots exist, implying an opportunity for further research. Despite the explosion in the fabrication methods of nanostructures, both chemically synthesized and patterned substrates that are tailored to specific applications might be necessary for routine use, as SM sensors. A tremendous opportunity exists in quantitative SERS and for the detection of analytes at ultra-low concentrations to advance healthcare. Attempts in hyperspectral and high-speed Raman imaging to provide chemical data rivalling the speed and sensitivity possible by fluorescence methods could be envisioned. The entrepreneurial efforts of scientists and engineers involved in Raman research will be critical for translation. Funding as expected will drive the research and a more significant contribution from around the globe can be foreseen.

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