Received: 25 December 2021 | Revised: 5 March 2022 | Accepted: 27 March 2022

DOI: 10.1002/wnan.1802

ADVANCED REVIEW

WIREs NANOMEDICINE AND NANOBIOTECHNOLOGY WILEY

Surface-enhanced Raman scattering: An emerging tool for sensing cellular function

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Funding information
National Institute of Biomedical Imaging and Bioengineering, Grant/Award Number: 2-P41-EB015871- 31; National Institute of General Medical Sciences, Grant/Award Number: DP2GM128198; National Institutes of Health, Grant/Award Number: R01 EB030376

Edited by: Dipanjan Pan, Associate Editor and Nils G. Walter, Co-Editor-in-Chief

Abstract

Continuous long-term intracellular imaging and multiplexed monitoring of biomolecular changes associated with key cellular processes remains a challenge for the scientific community. Recently, surface-enhanced Raman scattering (SERS) has been demonstrated as a powerful spectroscopic tool in the field of biology owing to its significant advantages. Some of these include the ability to provide molecule-specific information with exquisite sensitivity, working with small volumes of precious samples, real-time monitoring, and optimal optical contrast. More importantly, the availability of a large number of novel Raman reporters with narrower full width at half maximum (FWHM) of spectral peaks/vibrational modes than conventional fluorophores has created a versatile palette of SERS-based probes that allow targeted multiplex sensing surpassing the detection sensitivity of even fluorescent probes. Due to its non-destructive nature, its applicability has been recognized for biological sensing, molecular imaging, and dynamic monitoring of complex intracellular processes. We critically discuss recent developments in this area with a focus on different applications where SERS has been used for obtaining information that remains elusive for conventional imaging methods. Current reports indicate that SERS has made significant inroads in the field of biology and has the potential to be used for *in vivo* human applications.

This article is categorized under:

Diagnostic Tools > *In Vitro* Nanoparticle-Based Sensing
Nanotechnology Approaches to Biology > Nanoscale Systems in Biology
Diagnostic Tools > Biosensing
Diagnostic Tools > *In Vivo* Nanodiagnostics and Imaging

KEYWORDS

Cellular imaging and sensing, SERS, Plasmonic nanoparticles

1 | INTRODUCTION

Cellular processes are the result of synchronized changes in the concentration, distribution, and interaction of several biomolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins, metabolites, and biochemical pathways inside the cell and in its immediate vicinity (Qiu et al., 2014; Zong et al., 2018). The ability of real-time monitoring of these dynamically evolving events would serve as an important tool for a comprehensive understanding of complex biological processes (Zong et al., 2018). This may further aid in decoding intracellular and cell-to-cell interactions, determining the onset of various diseases, developing sensitive diagnostic methods, and more efficient drugs (Zong et al., 2018). Investigating subcellular organelles, proteins, and other biomolecules inside a living cell in their native state are some of the major aims of molecular biology still requiring novel strategies that can further help advance the current understanding of biological systems.

Continuous single-cell analysis forms the cornerstone of our knowledge in the field of cell biology or drug discovery. So far, fluorescence-based optical techniques have been the technique of choice for cellular monitoring due to their high sensitivity, spatial resolution, fast acquisition speeds, and ability to provide high contrast crisp images (Lee et al., 2014). However, their applicability is limited by serious drawbacks such as autofluorescence from cellular constituents, photobleaching, and crosstalk between fluorophores that hinders multiplex measurements (Loftus & Seliger, 1975). In lieu of these problems, the pursuit of developing more sensitive and specific imaging modes for real-time visualization of molecular and cellular processes has been an ongoing effort.

In recent years, surface-enhanced Raman scattering (SERS) has emerged as a promising and powerful alternative tool for monitoring live cellular processes. Raman spectroscopy, named after the Indian physicist C.V. Raman, detects inelastically scattered photons from their vibrational energy levels, revealing molecule-specific information that helps in the quick identification of analytes from complex biological mixtures (Raman & Krishnan, 1928). However, as 1 out of 1 million photons scatters inelastically, the intensity of Raman signals of most molecules is inherently weak, limiting its wide application, especially in biological systems (Lee et al., 2019). SERS, the plasmonic counterpart of spontaneous Raman spectroscopy, requires placing of the analytes nearby metal nanostructures, whereby a huge enhancement in Raman signals is observed owing to plasmon-assisted scattering of molecules (Haes et al., 2005; Jeanmaire & Van Duyne, 1977; Kaur et al., 2021; King et al., 1978; Lee et al., 2019; Liang et al., 2021; Moskovits, 1978; Schatz & Van Duyne, 1980; Willets & Van Duyne, 2007). This enhancement factor can reach up to 14 orders of magnitude, enough for the identification and quantification of analytes at ultra-trace levels (Kneipp et al., 2005; Schlücker, 2014; Tanwar et al., 2017). SERS-based detection and imaging methods have now gained enormous attention because of their ability to generate and distinguish spectra for similar molecules offering single-molecule sensitivity with minimal sample preparation. (Pandey et al., 2017; Tanwar et al., 2017; Tanwar, Kaur, et al., 2021; Tanwar, Paidi, et al., 2021). With its rich molecule-specific information encoded in the Raman spectra and high sensitivity contributed by its plasmonic counterpart, SERS makes it an ideal technique for multiplexed optical imaging, targeted imaging, and studying complex biological systems (Li et al., 2016; Zong et al., 2018). Recently, nanoparticle-based delivery systems have emerged as a new addition to the field of biomedicine, especially for drug delivery and photodynamic therapy (Arvizo et al., 2010; Rennick et al., 2021). Yet, very little is known about the mechanism of their cellular internalization and further movement inside cells (Liu et al., 2017). Understanding these mechanisms, their effect on live-cell functions, and gaining control over such effects have now become critical challenges in the field of nanobiotechnology and nanomedicine. The advantages of SERS make this an ideal technique for real-time monitoring of drug uptake in pathological tissues including cancer.

The first SERS-based intracellular studies were performed by Nabiev et al. (1991) where they probed modes of interaction of doxorubicin with the nucleus and cytoplasm in living cancer cells. Their method received more widespread attention within the cellular context after Kneipp et al. in 2002 demonstrated SERS-based mapping of the distribution of the native chemical constituents phenylalanine and DNA in a single cell (Kneipp et al., 2002). Since then, the interest in the cellular application of SERS has increased substantially with many emerging new developments including SERS-based monitoring of metabolites, imaging intrinsic biomolecules, studying molecular dynamics, and probing cell surface receptor–ligand binding interactions (Ando et al., 2011; Koike et al., 2020; Zhang, Lin, et al., 2020; Zheng, Zong, et al., 2019). Efforts are being carried out to further improve the sensitivity and reproducibility of SERS by designing novel SERS-detectable nanostructures that provide comprehensive insights into biological systems (Kang et al., 2015; Zong et al., 2018).

In this review article, we analyze recent SERS developments and applications of recently engineered methods for intracellular monitoring. Summarized perspectives on SERS-based biodetection are presented to provide a comprehensive understanding of current challenges and future directions in the adoption of SERS technology.

2 | SERS USING INTRACELLULAR PROBES

2.1 | Sensing intracellular pH

Intracellular pH (ipH) has a key role in maintaining normal cellular function including transporting substances across the cell membrane, metabolism, growth, division, apoptosis, and so on (Lagadic-Gossmann et al., 2004). To perform their functions, cell organelles stringently maintain a distinct pH value and regulate it tightly, as it acts as an important determinant of their function (Casey et al., 2010). Unusual changes in pH are associated with cellular dysfunction and the onset and progression of disease (Fang et al., 2010; Schwartz et al., 2020; Swietach, 2019; Yin et al., 2015). The usual approach for SERS-based pH measurements is to coat plasmonic nanoparticles with pH-sensitive Raman reporter molecules and allow them to be internalized inside the cell (Shen et al., 2018; Zhang, Bando, et al., 2019; Zhang, Jimenez de Aberasturi, et al., 2020). The reporters have functional groups such as carboxyl, amino, and pyridine rings whose changes in vibrational modes upon protonation or deprotonation are reflected in the Raman spectrum and form the basis of SERS-based pH sensing. Because of its sensitivity toward pH, the probe can deliver information from its immediate environment (Scarpitti et al., 2020). In one of the earliest attempts, Talley et al. were able to detect pH values inside live Chinese hamster ovary cells within 1 unit on the pH scale (Talley et al., 2004). They used MBA-coated nanoparticles, where peaks at 1423 and 1700 cm^{-1} corresponding to symmetric COO^- and C=O stretching vibrations were used for pH determination. Subsequently, by changing the probe design, they were able to reduce the sensitivity down to 0.5 pH units (Schwartzberg et al., 2006). Kneipp et al. have shown that by using an MBA-coated SERS nanosensor it is possible to determine pH values in subcellular structures of single live NIH/3T3 cells within a range from pH = 5.4–6.8 (Kneipp et al., 2007). By two-photon excitation, they were able to increase the response range of the pH sensor between 2 and 8, suitable for probing different subcellular organelles. Furthermore, the approach was extended to studying dynamics of local pH changes in the endosome of a single live NIH/3T3 cell (Kneipp et al., 2010). At present, 4-mercaptobenzoic acid (MBA), para-aminothiophenol, and 4-mercaptopyridine are probably the most commonly used molecules for developing pH-sensitive SERS-based probes because of their ability to be easily conjugated to the surface of metal nanoparticles and their structural high stability. Many studies claim that these molecules are highly sensitive for their use as SERS-based pH probes; however, they have vibrational modes in the fingerprint region 500–1800 cm^{-1} which overlaps with that of endogenous biomolecules. This makes it difficult to distinguish whether the detected peak is derived from the probe or from the inherent cellular background. One approach for addressing this problem could be the use of molecules that have vibrational modes in the cell silent region (1800–2600 cm^{-1}) where the possible presence of interfering cellular signals is automatically excluded. For example, bisarylbutadiyne derivatives that contain C≡C bonds have been reported by Wilson et al. as ipH sensors for Raman microscopy (Wilson et al., 2020). Although, compared with other Raman tags such as C–D, the peak intensity of alkynes is higher, the signal is relatively weak for imaging live systems that may leave many targets undetected (Wei et al., 2014, 2017). SERS can offer ultrahigh sensitivity; therefore, inserting additional functional groups such as sulfhydryl can enable easy conjugation of the developed molecules with metal nanoparticles boosting their ipH detection efficiency.

When performing *in vitro* cell studies using nanoparticles, it is imperative that the nanoparticles are highly stable and do not agglomerate in cell culture media before being endocytosed as aggregation can significantly impact their *in vitro* behavior and fate. Furthermore, the ability to distinguish the Raman signal of internalized nanoparticles from particles adhered to the cell surface is paramount for the precise analysis of the intracellular environment. Methods such as excessive washing or treatment with trypsin are recommended to avoid this problem, but these methods can lead to detachment of cells from the surface or destroy them. Several strategies are being adopted to get accurate intracellular information. For instance, Bai et al. designed an etchable SERS sensor whose signal can be turned off using an etchant when present on the surface of the cell, allowing selective measurement of ipH values (Bai et al., 2019). In another approach, Zheng et al. used cell-penetrating peptides to increase particle stability and facilitate nanoparticle entry inside the cell with an even distribution (Zheng, Zong, et al., 2019). The strategy was further advanced to measure the pH of mitochondria and nucleus using Au nanorods modified with mitochondria and nucleus targeting peptides, respectively (Shen et al., 2018).

Changes in ipH values can be associated with a myriad of cellular factors including intracellular stress and cell division, as well as administration of exogenous drugs and therapeutics. Now attempts are being made to correlate SERS-based pH sensing with cellular responses and functions. For example, using SERS, Guo et al. found that HeLa cells have better regulatory control over their ipH values compared with normal cells and adapt quickly to a weak, acidic environment (Guo et al., 2020). Bando et al. observed a decline in ipH when endosomes fused with lysosomes during

endocytosis (Bando et al., 2020). Other reports include monitoring dynamic ipH changes as a function of hypoxia-induced excessive glycolytic activity (Ma et al., 2016), extracellular pH changes (Jaworska et al., 2015), photothermal therapy (Luo et al., 2016), at different stages of the whole cell cycle (Figure 1a; Zheng, Zong, et al., 2019), and during the process of autophagy and apoptosis (Li et al., 2019). Recently, efforts have been made for SERS-based detailed three-dimensional (3D) profiling of ipH values. As an example, Zhang et al. achieved 3D visualization of ipH acidification during nanoparticle diffusion from endosomes to lysosomal compartments (Figure 1b,c; Zhang, Jimenez de Aberasturi, et al., 2020).

2.2 | Sensing redox potential

Cellular redox potential is highly regulated by striking a balance between the generation and elimination of several species such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and their scavengers (D'Autréaux & Toledano, 2007; Menon & Goswami, 2007; Paulsen & Carroll, 2010). Mounting evidence has demonstrated that a slight dysregulation can result in the onset of pathologies such as diabetes, cancer, and neurodegenerative disorders (Andersen, 2004; Klaunig & Kamendulis, 2004; Lowell & Shulman, 2005). Therefore, the design of novel methods for measuring intracellular redox potential with suborganelle resolution is highly warranted. Auchinevole, Thomson, and their coworkers introduced a series of nanosensors containing quinone moieties that can undergo reversible $2e^-$, $2H^+$ redox reactions which are reflected in their SERS spectra (Auchinvole et al., 2012; Thomson et al., 2015). Voltage-dependent changes in vibrational bands of Au nanoparticle-coated 1,8-diaza-4,5-dithia-1,8-di(2-chloro-[1,4]-naphthoquinone-3-yl)octane (NQ-NS) and 2-mercaptobenzene-1,4-diol (HQ-NS) were recorded and correlated with density-functional theory (DFT) calculations. The nanosensors were used for cytoplasmic redox potential measurements in single live cells and were found to be effective under oxidizing conditions including hypoxia. Unlike other known optical techniques that are only able to qualitatively assess large changes in redox potential, the reversible nature of SERS-based nanosensors being developed makes them suitable for monitoring the

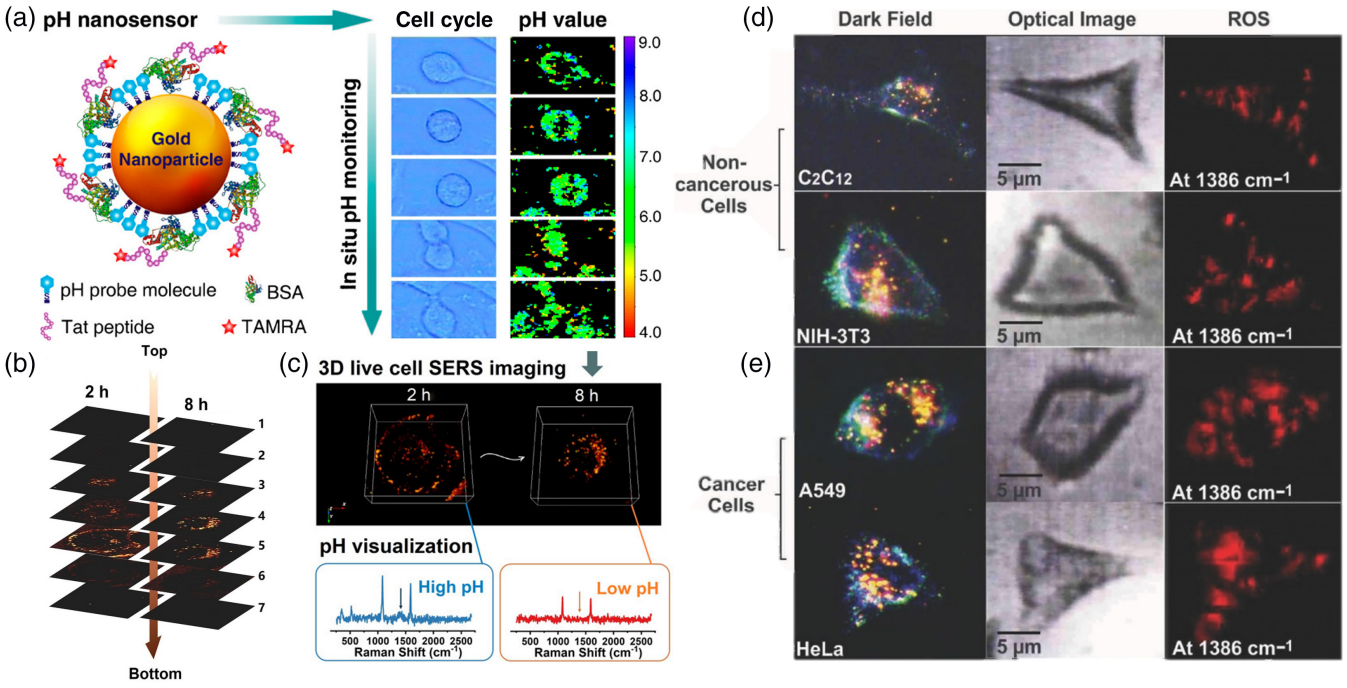

FIGURE 1 SERS-based ipH and redox potential sensing. (a) Schematic illustration of the structure of a pH-sensitive Raman reporter and real-time pH monitoring during the cell cycle of a single living CaSki cell. Reprinted with permission from Zheng, Zong, et al. (2019). Copyright © 2019. American Chemical Society. (b,c) Stacked slides of 2D SERS imaging at 2 and 8 h after exposure to AuNS-4MBA-PA for the same cell and their reconstruction into 3D SERS images. Reprinted with permission from Zhang, Jimenez de Aberasturi, et al. (2020). Copyright © 2020. American Chemical Society. (d,e) SERS-based ROS imaging in live cancerous and noncancerous cells. Shown are (from left to right) dark-field, bright-field, and Raman mapping images (corresponding to the band at 1386 cm^{-1}). Reprinted with permission from Kumar et al. (2017). Copyright © 2017. WILEY-VCH Verlag

redox state of a cell serially at different time points. Continued improvement in the nanosensor design has been reported for simultaneous measurement of redox potential and pH values in live cells, useful for gaining multiplexed information from intracellular environment (Jamieson et al., 2015). With this advancement, using the same nanosensors, the investigators were able to employ SERS for understanding intracellular redox potential changes in a hypoxic environment. They established a correlation between computed and redox potential-measured free energy and identified miRNAs in MCF7 cells whose expression level changes in the process of a cell's adaptation to hypoxia (Johnston et al., 2019).

ROS, hydrogen peroxide (H_2O_2), oxygen superoxide (O^{2-}), and hydroxyl radicals ($^{\cdot}OH$) generated inside cells do not show any characteristic peaks in the Raman spectra, and therefore, their sensing is facilitated by selective reaction with certain small chemical molecules or proteins. For instance, Qu et al. demonstrated H_2O_2 sensing in living HeLa and HL-7702 cells using 4-carboxyphenylboronic acid-coated Au nanoparticles with a limit of detection of 80 nM (Qu et al., 2016). The working principle of this nanosensor is based on the chemical transformation of arylboronate to phenol in the presence of H_2O_2, which leads to the appearance of two new peaks at 1365 and 1600 cm^{-1} in the Raman spectra, accompanied by decrease in the peak at 1562 cm^{-1}. Similarly, Peng et al. and Chen et al. successfully employed 4-mercaptophenylboronic ester-coated Au nanoparticles for intracellular live cell sensing of H_2O_2 and peroxynitrite ($ONOO^-$), respectively (Chen et al., 2018; Peng et al., 2016). Since boronate ester-based probes are reactive toward both H_2O_2 and $ONOO^-$, utmost care should be taken during the interpretation of results. For example, if one is monitoring H_2O_2, $ONOO^-$ may induce changes in the Raman spectrum. Therefore, when possible, it is recommended to use more than one reporter molecule for accurate identification of a specific ROS. Cui et al. have demonstrated the applicability of a reaction-based SERS nanoprobe for simultaneous detection of five ROS species including OH, H_2O_2, O^{2-}, $ROO^{\bullet}$, and $^1O_2$ (Cui et al., 2018). The nanoprobe constitutes Au nanoparticles coated with para-aminothiophenol (PATP) and hemin protein, where hemin serves as a Fenton catalyst and converts H_2O_2 into $^{\bullet}OH$ which in turn leads to dimerization of PATP into 4,4′-dimercaptoazobenzene (DMAB), giving rise to new Raman active vibrational modes. Protein-based probes are another class of probes commonly used for redox monitoring because of their selectivity and amenability to subcellular targeting. Kumar et al. used Myoglobin (Mb)-coated strong electromagnetic field generating core–satellite type structures, where Mb protein acted as a ROS-responsive Raman reporter (Figure 1d,e; Kumar et al., 2017). Transformation of six coordinated Fe(III)-OH_2 (resting state) to Fe(IV)-O (Ferryl state) upon reaction with H_2O_2 was the basis of their detection.

Sensing mechanisms involving a series of reactions are limited by two factors, that is, the rate of the reaction and the fleeting presence of chemicals involved in intermediate steps. Ideally, the reaction rate should be fast enough to rule out potential contributions from new ROS species being generated dynamically inside cells. On the other hand, analysis of reactions proceeding at very high rates is often limited by low selectivity, especially in cases where several different species with similar reactivity are present. Therefore, the development of optimal fast reactions that are selective toward one ROS species is an active area of investigation.

2.3 | Sensing the cell surface and intracellular biomolecules

Every cell in an isogenic population may show significantly heterogeneous responses to common stimuli (Llamosi et al., 2016). The differential behavior is due to variations in the expression of proteins, metabolites, and genes among individual cells (Wang & Bodovitz, 2010). Therefore, when we look at single cells, we see a distribution of individual features rather than an averaged single phenotype, making our understanding of the functioning of cellular systems more robust. The disconnect between single and bulk cell measurements has been demonstrated by Toriello et al., where they found two distinct populations of cells with 50%–100% silencing upon lockdown of the GAPDH gene in Jurkat cells. This stochastic variation would have been masked by averaged cell measurements (Toriello et al., 2008). Therefore, new techniques capable of isolating a single cell and studying its proteomic and genomic profile would be highly beneficial. Our laboratory reported a shell-based single-cell analysis platform, termed as mechanical trap surface-enhanced Raman spectroscopy (MTSERS), which allows capturing a single live cell from a complex sample and enables the simultaneous 3D molecular analysis of its plasma membrane (Figure 2a–d; Jin et al., 2017). The presence of Au nanostars on the mechanical trap led to substantial enhancement in the intrinsic Raman signals of the cell surface. Thermoresponsive poly(*N*-isopropylacrylamide) coated ultrathin 2D graphene sheets further facilitated close contact between the encapsulated cell and the SERS substrate, enabling 3D SERS mapping of MDA-MB-231 breast cancer cells (Figure 2e–j; Xu et al., 2019). Subtle changes in the exterior cell surface composition and receptor expression are clinically relevant cell surface markers and are promising targets for the detection of early onset of diseases.

Using the MTSERS substrate, we were able to perform label-free SERS-based imaging of proteins and lipids in the cell membrane of single live cells. The entrapped cells were found to be alive for up to 2 days, showing the efficiency of the SERS substrate for probing long-time dynamic cellular processes. We envisage that the newly developed MTSERS platform can be utilized for nonperturbative cell-surface spectroscopic analysis and disease detection. We recently reported the development of a nano-pillar-based Raman optical detection (nano-PROD) platform for selectively interrogating the molecular composition of live cell membrane (Zhang et al., 2021). We were able to differentiate between phenotypically similar prostate cancer cells that differ only in prostate-specific membrane antigen (PSMA) expression. Studies over the last decade have demonstrated that SERS enables the identification of cancerous cells based on cell-surface-specific molecular signatures. For instance, Au nanorods conjugated to anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies were used for imaging of HaCat normal and HSC cancer cells, demonstrating significantly different Raman signatures that were obtained from cancerous cells as compared with normal (Huang et al., 2007). In other work, Au nanoparticles functionalized with epidermal growth factor (EGF) peptide were employed for identifying circulating tumor cells in the peripheral blood of patients with squamous cell carcinoma (Wang, Qian, et al., 2011). Au nanorods and Au nanospheres, suitable for dark field and SERS dual microscopy, were applied for targeting and imaging of HER2-overexpressing MCF7 breast cancer cells (Park et al., 2009). Yan et al. employed SERS active nanoparticles for characterizing the chemical composition of the cell surface of MCF7 breast cancer cells and monitoring changes in the pericellular matrix upon enzymatic treatment (Yan et al., 2012). In another exciting study, de Albuquerque et al. presented super-resolution SERS imaging of αvβ3 integrin receptors in colon cancer cells using arginine–glycine–aspartic acid–phenylalanine–cysteine coated Au nanoparticles (de Albuquerque & Schultz, 2020). Polyethylene glycol-coated Au nanoparticles were applied for multiplex detection of CD45, CD19, and CD20 cell surface proteins on malignant B cells (MacLaughlin et al., 2013). Similar reporter applications include imaging prostate-specific antigen (PSA), folate, and β2-adrenergic receptor (Figure 3a–d), known to be upregulated in several types of cancer (Kennedy et al., 2009; Wang, Zong, et al., 2011; Zhang, Hong, et al., 2011).

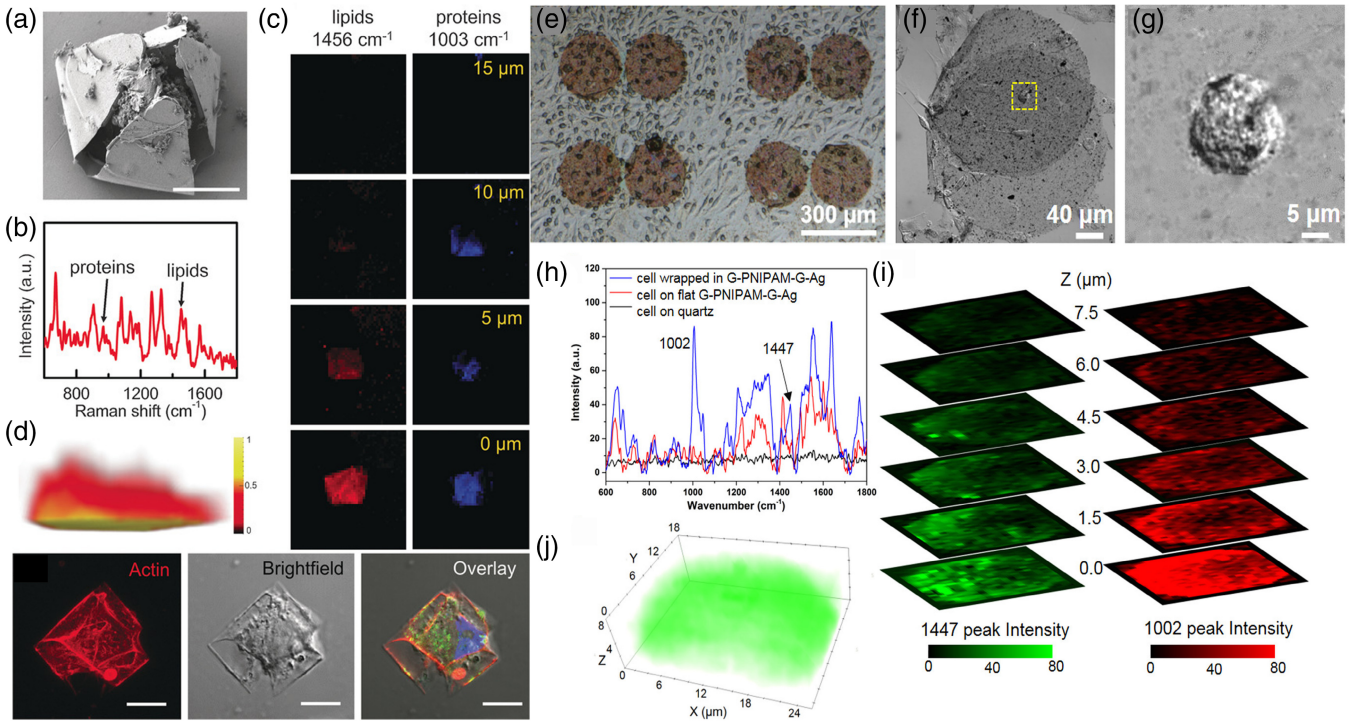

FIGURE 2 MTSERS-based single live cell surface molecular profiling and chemical imaging. (a) SEM image of a single live cell entrapped in a mechanical trap (MT; scale bar = 10 μm). (b) Representative SERS spectrum showing Raman signatures of lipids and proteins. (c) Molecular profiling of trapped single live cells acquired at different confocal planes. (d) 3D SERS image of a single cell. Reprinted with permission from Jin et al. (2017). Copyright © 2017. Wiley-VCH Verlag. Wrapping of single live breast cancer cells inside thermoresponsive graphene skin and 3D SERS spatial mapping. Bright-field images of (e) breast cancer cells cultured on thermoresponsive graphene and (f and g) a single cell wrapped in the folded graphene skin. (h) Raman spectra of breast cancer cells. (i) Raman spatial map of a wrapped cell, with the peaks at 1447 and 1002 cm−1 corresponding to lipid and protein, respectively. (j) Reconstructed 3D map. Reprinted with permission from Xu et al. (2019). Copyright © 2018. American Chemical Society

In the past few years, SERS has also paved the way toward intracellular molecular imaging as it delivers spatial resolution difficult to achieve using other imaging modalities (Zhang, Hong, et al., 2011). Considerable attention has been focused on developing SERS-based multiplexed sensing platforms where multiple biological events can be probed simultaneously. For example, using SERS-based multiplexed targeting, Woo et al. were able to detect three different proteins, CD34, Sca-1, and SP-C co-expressed in bronchioalveolar stem cells (Woo et al., 2009). The ability of SERS-based multiplexed monitoring further enabled simultaneous detection of carcinoembryonic antigen (CEA) and α-fetoprotein (AFP) lung cancer biomarkers and identification of a $CD44^{+}/CD24^{-}$ subpopulation of breast cancer stem cells in MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cell lines (Figure 3e; Chon et al., 2011; Lee et al., 2011). Huefner et al. employed nucleus targeting peptide-capped Au nanoparticles for selective targeting and investigating the nuclear targeting of undifferentiated and fully differentiated SH-SY5Y cells of human neuroblastoma (Huefner et al., 2013). In another recent example, Jiang et al. designed Au nanostar-3.3′-diethylthiatricarbocyanine iodide-silver sulfide nanoparticles, SERS, and fluorescence dual imaging probes for tracking stem cells in hypodermic and myocardial infarction models, crucial for monitoring cell stem therapy (Jiang et al., 2017). Although Raman spectroscopic signatures of every molecule are unique, spectral overlapping from endogeneous biologicals makes analysis cumbersome. To circumvent this, several chemical groups such as alkyne, azide, nitrile, and deuterium having Raman signals in the cellular Raman-silent region (1800–2800 cm^{-1}) have now gained attention. For example, Koike et al. demonstrated the applicability of SERS for real-time quantitative monitoring of drug uptake in live cells, illustrating its potential for

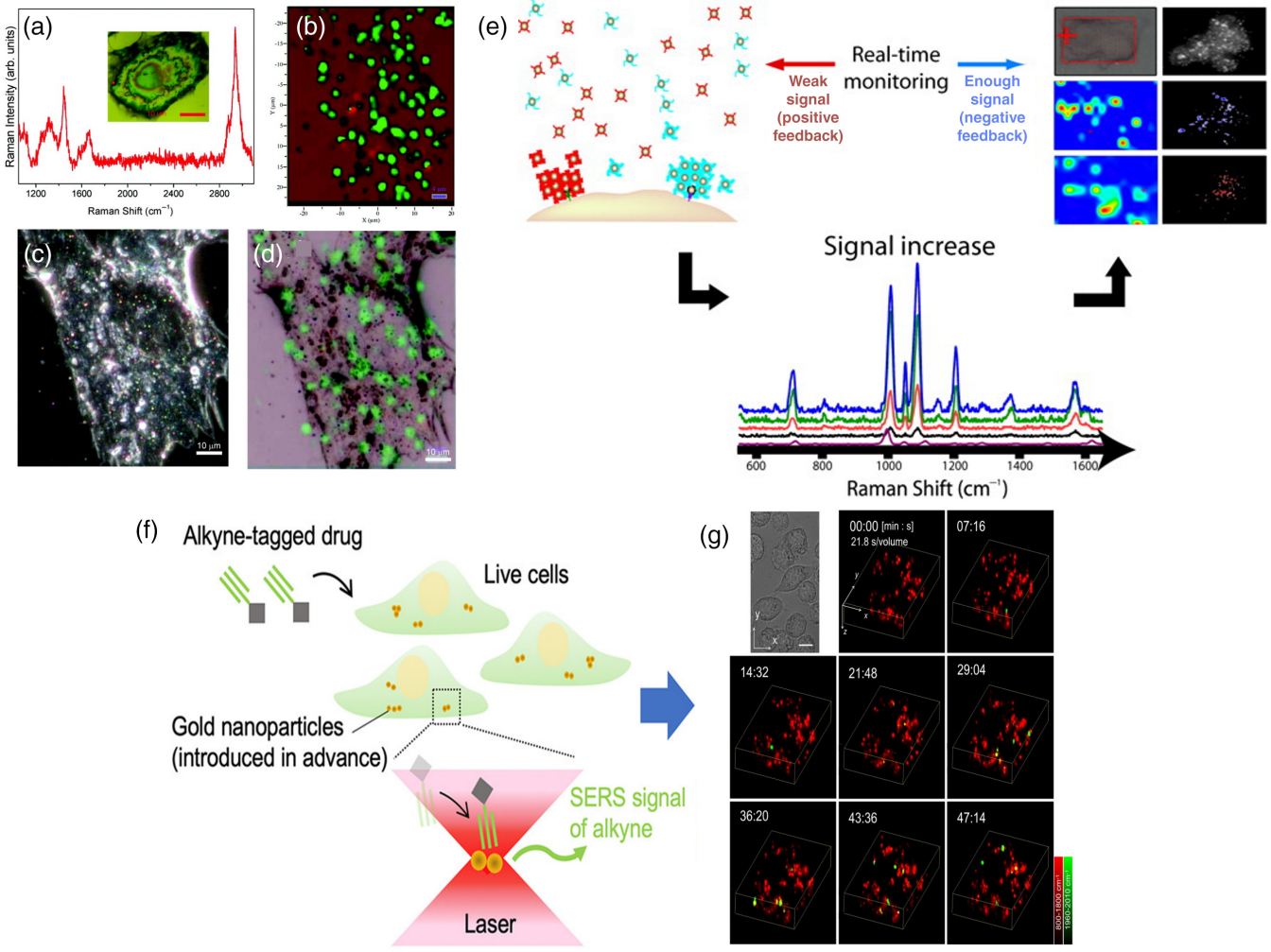

FIGURE 3 SERS-based imaging of cellular β2-adrenergic receptors. (a) SERS spectrum of Raman probe taken from the nuclear region of H9c2 cells. (b) Deconvolution of 4-(mercaptomethyl) benzonitrile SERS spectra using multivariate analysis. (c) Rayleigh's light scattering image of the H9c2 cell is shown in Figure 3b. (d) Overlay of Figure 3b,c. Reprinted with permission from Kennedy et al. (2009). Copyright © 2009. American Chemical Society. (e) Schematic of DNA–Au nanoparticle conjugates-based cell marker detection strategy for multiplex SERS mapping of CD44/CD24 marker profile from three different breast cancer cell lines. Reprinted with permission from Lee et al. (2011). Copyright © 2011. American Chemical Society. (f) Schematic illustration of SERS-based real-time monitoring of alkyne tagged drug uptake in live cells. (g) Time-lapse 3D SERS imaging of living macrophages. Reprinted with permission from Koike et al. (2020). Copyright © 2020. American Chemical Society

pharmaceutical research (Figure 3f,g; Koike et al., 2020). They used alkyne tags exhibiting Raman signals in the cell silent region to avoid overlapping signals from cellular molecules. Similarly, Zhu et al. developed a click SERS strategy for intracellular monitoring of caspase-3 enzyme in live and apoptotic cells (Zhu et al., 2021).

2.4 | Sensing endocytic pathways

Endocytic pathways play a critical role in the processes by which cells uptake nutrients or defend against pathogens from the extracellular environment. Ando et al. demonstrated dynamic SERS imaging of Au nanoparticles inside living cells at 65 nm spatial and 50 ms temporal resolution, enabling the generation of molecular maps of nanoparticle transportation and lysosomal accumulation (Ando et al., 2011). They combined laser-beam scanning Raman and dark-field microscope using a feedback system to simultaneously detect nanoparticle movement and SERS signals in J774A.1 macrophage cells (Figure 4a). In their later reports, with the use of the same strategy, they found that the Raman peak at 1062 cm^{-1}, is always observed during the internalization of nanoparticles inside cells and hypothesized it to be associated with kinesin and dynein proteins used for cellular transportation (Fujita et al., 2013; Huang et al., 2014). The approach adopted is important because it will help in mapping cellular pathways along with molecular information which is otherwise difficult to achieve using other known techniques (Kusumi et al., 1993; Ruan et al., 2007). Additionally, Yilmaz et al. employed SERS for distinguishing three different processes, namely clathrin, caveolae, and micropinocytosis-dependent endosomal trafficking of Au nanoparticles (Yılmaz & Culha, 2021). Distinct spectral signatures were identified for each specific endocytosis pathway in a label-free manner. Kneipp et al. demonstrated the role of adenosine triphosphates and monophosphates in the first 3 h of Au nanoparticle uptake by cells (Kneipp et al., 2006). Huefner et al. used SERS in combination with chemometric methods for distinguishing the molecular content of two different vesicles formed during the process of endocytosis, namely endosomes and lysosomes (Huefner et al., 2016).

2.5 | Sensing apoptotic pathways

Selective induction of apoptosis in cancerous cells is a leading chemotherapeutic strategy, where crucial information on the extent and speed of onset helps in increasing the effectiveness of the therapeutic interventions (Pui & Evans, 2006). Furthermore, real-time visualization and identification of molecular-level events such as DNA damage, protein denaturation, blebbing, cell shrinking, and apoptotic body formation will help in providing a better understanding of pathways involved in apoptosis (Elmore, 2007; Kang et al., 2014). To meet this end, Kang et al. demonstrated real-time monitoring of molecular changes occurring during H_2O_2-induced apoptosis using a plasmonically enhanced Rayleigh/Raman spectroscopy imaging approach (Kang et al., 2014).

Analysis of vibrational bands of DNA and protein molecules not only revealed the occurrence of molecular events such as amide bond hydrolysis, protein unfolding, DNA fragmentation, and cleaving of the disulfide bond in proteins, but also helped in establishing a time-dependent dynamic profile of H_2O_2-induced apoptosis. Further, by integrating SERS with metabolomics and proteomics studies, they discovered significant perturbations in the concentration of phenylalanine and associated metabolites during plasmonic photothermal therapy (Ali et al., 2016). Shin et al. carried out SERS-based monitoring of molecular changes in mitochondria during mitochondrially mediated apoptosis (Figure 4b; Shin et al., 2019). Triphenylphosphine-coated Au nanoparticles were targeted to human oral squamous carcinoma (HSC-3) cells followed by inducing apoptosis either photothermally or chemically. They were able to identify characteristic Raman signatures for both the ways of inducing apoptosis and argued that their strategy can be used as a future drug screening tool. Zhang et al. carried out real-time monitoring of Abrin-induced apoptosis in HepG2 cells using TAT peptide coated Au nanostars (Zhang, Ma, & Wang, 2019). Panikkanvalappil et al. demonstrated label-free SERS-based real-time visualization of ROS-induced damages in genomic DNA of HaCaT cells. In addition, the ability of Pt nanoparticles to prevent ROS-induced DNA aggregation, backbone damage, and residual nucleobase damage was also assessed (Panikkanvalappil et al., 2013).

2.6 | Sensing intercellular communication

Chemical signaling pathways are key to cell–cell communication and mark the onset of several biological and physiological processes. Lussier et al. developed a SERS-based optophysiology technique for monitoring cell secretion events

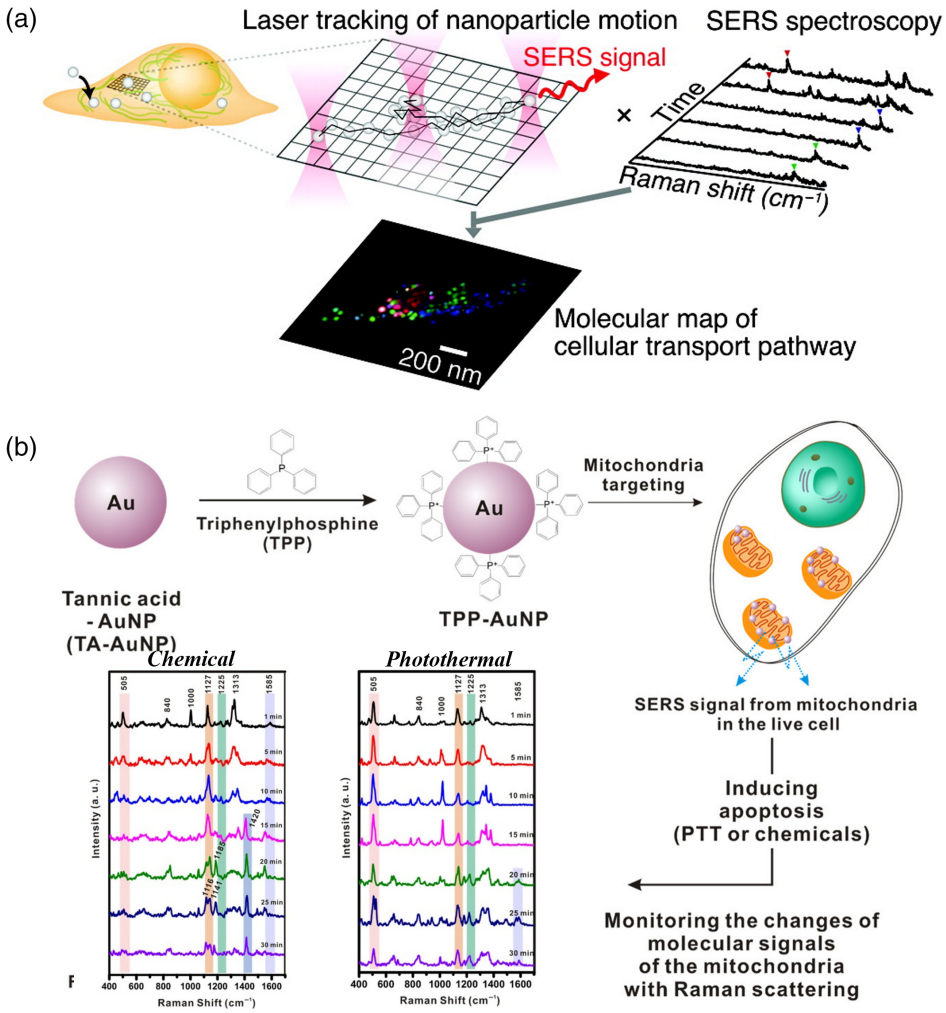

FIGURE 4 SERS-based probing of endocytosis and apoptosis pathways. (a) SERS analysis of the cellular pathway showing the trajectory of endocytosed Au nanoparticle. Reprinted with permission from Ando et al. (2011). Copyright © 2011. American Chemical Society. (b) Schematic depiction of the strategy used for selective targeting of mitochondria and Raman-based spectroscopic analysis for mitochondria-mediated apoptosis induced photothermally and chemically. Reproduced with permission from Shin et al. (2019). Copyright © 2019. American Chemical Society

with a temporal and spatial resolution (Lussier et al., 2016). They performed real-time monitoring of pyruvate, lactate, ATP, and urea in the extracellular media of MDCKII cells. Upon incubation with saponin, a toxin that causes cell perforation, an increased level of pyruvate was observed, indicating significant release by cells. They further extended the approach for neurotransmitter detection under basal conditions and in an activity-dependent manner as a function of membrane depolarization (Lussier et al., 2017). Multiplex analysis of neurotransmitters and neuronal function will pave the way for a better understanding of brain neurochemistry. Recently, Silwal et al. reported a SERS-based approach for probing selective interactions of signaling compounds such as dopamine, amphetamine, methamphetamine, and methylenedioxypyrovalerone with D1 and D2 dopamine receptors in live cells (Silwal & Lu, 2018). It was found that upon interaction with D1, the intracellular levels of cyclic adenosine monophosphate (cAMP) increase. On the contrary, reduced levels were observed upon interaction with D2. Zhang et al. using SERS mapping were able to unravel the roles of two fatty acid receptors, G protein-coupled receptor 120 (GPR120) and a cluster of differentiation 36 (CD36), together as the primary factor in the activation of the fatty acid uptake cycle (Figure 5a; Zhang, Lin, et al., 2020). Other studies include interrogating cellular stress response (Escoriza et al., 2007), monitoring cellular function (Huh et al., 2009), and probing the functional state of the cell nucleus (Xie et al., 2009).

2.7 | Sensing therapeutic drug delivery and uptake

Real-time monitoring of intracellular drug release is important to ensure localization of drugs in diseased tissues, avoid an overdose, and improve therapeutic efficiency (Zheng, Xiong, et al., 2019). Understanding the precise mechanism of drug release from delivery vehicles such as Au nanoparticles is important for gaining further insights into the current knowledge of intracellular events and designing new improved systems. Nabiev et al. demonstrated SERS as a noninvasive technique for monitoring the interaction of doxorubicin (DOX) with cancerous cells as a function of location (Nabiev et al., 1991). Distinct Raman signatures of DOX–DNA and DOX–nanoparticle constructs were utilized for visualizing intracellular drug localization. Kang et al. utilized plasmonic-tunable Raman/fluorescence imaging spectroscopy for monitoring the dynamic release of DOX in live human oral squamous cell carcinoma cells (Kang et al., 2013). DOX was conjugated to the surface of Au nanoparticles with a pH-sensitive hydrazine bond, whose cleavage in acidic lysosomes resulted in reverse Raman and fluorescence signals making detection possible. In recent years, attention has been focused on designing stimuli-responsive nanosensors with integrated real-time SERS-based monitoring and drug-releasing capabilities. Some of the examples include a redox-responsive platform designed by Hong et al. (2014), glutathione (GSH)-controlled intracellular thiopurine anti-cancer drug delivery release studied by Ock et al. (2012), (Figure 5b–d), and pH-cleavable drug delivery systems (Liu et al., 2016). Recently, Koike et al. carried out SERS-based 3D real-time visualization of small drug molecules uptake and dynamics inside macrophages (Koike et al., 2020). To enhance the signal-to-noise ratio, the drug molecule acyloxymethyl ketone-type inhibitor, a cathepsin B inhibitor, was tagged with alkyne groups having a Raman peak around 2120 cm^{-1} in the cell "silent" region, where most of the endogenous molecules do not show any Raman signature.

3 | CURRENT OUTLOOK AND FUTURE PERSPECTIVES

In recent years, SERS has made significant advancements for accessing highly resolved spatial and chemical information from the biological environment. The million-fold enhancement generated in SERS has transformed Raman spectroscopy from an analytical tool to a single molecule detection technique. As demonstrated from recent examples, the ability of SERS to detect, quantify, monitor, and localize molecules at the sub-cellular level has set the stage for unlocking a new treasure of information regarding molecular changes during a range of cellular processes.

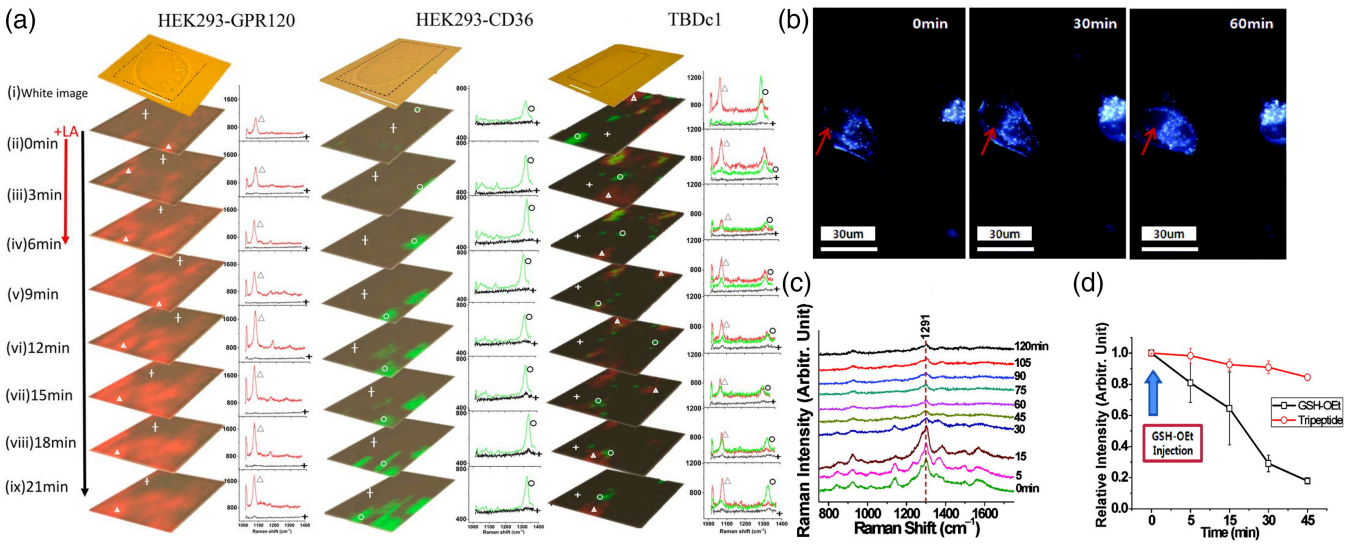

FIGURE 5 SERS-based probing of cell-to-cell communication and drug uptake mechanisms. (a) Real-time SERS mapping of GPR120 and CD36 cell surface receptors upon incubation with linoleic acid. Reprinted with permission from Zhang, Lin, et al. (2020). Copyright © 2020. National Academy of Sciences. (b) Time-dependent dark-field images of Au nanoparticles inside A549 cells after glutathione ethyl ester treatment, indicating the in situ release of thiopurine. (c) Vibrational band of thiopurine at 1258 cm^{-1} at different time points after injecting glutathione ethyl ester. (d) The plot of decrease in the band intensities with time. Reprinted with permission from Ock et al. (2012). Copyright © 2012. American Chemical Society

Furthermore, the absence of signal deterioration due to photobleaching and ultrahigh sensitivity in an aqueous environment allows long-time live-cell monitoring, imperative for understanding cellular dynamics. Advances in nanotechnology, such as coupling nanoparticles with cell-penetrating peptides for efficient cellular delivery, targeting specific subcellular compartments, and applying coatings to avoid pH-induced aggregation, have played a major role in the success of SERS. The field of intracellular SERS studies is still in its infancy; however, it is drawing immense attention from the scientific community and showing growth. Despite its efficiency, a few challenges still need to be overcome before fully implementing SERS for solving complex biological questions. Among all, the biggest concern is nanoparticle-induced toxicity. So far, there have been several investigations probing the toxicity of plasmonic nanoparticles but the results are still controversial. For example, Zhang et al. found 60 nm Au nanoparticles to be not cytotoxic, whereas, Kang et al. reported programmed cell death due to nucleus-targeting nanoparticles (Kang et al., 2010; Zhang, Hitchins, et al., 2011). Some reports claimed that toxicity is associated with the surface ligand rather than the nanoparticles themselves (Cheng et al., 2013). To employ future clinical applications of SERS for diagnosis and therapy, it is important to take necessary steps such as use biocompatible coatings to mitigate associated toxicity, ensuring long-term intracellular stability, and advancing its targeting ability. The second potential concern is thermal damages that may occur due to localized electric fields generated on the surface of plasmonic nanoparticles. This effect is of greater concern in the case of long-term live-cell imaging where heat generation may perturb the biomolecule/biological processes under study. Hence, there is a need to shorten image acquisition times. We believe that this can be achieved by designing Raman nanoprobes that generate a high enhancement factor, where excellent contrasted images can be produced using minimal exposure time and laser power. The size, shape, type, and surface chemistry of nanoparticles are known to influence their intracellular trafficking and fate. For instance, Chitrani et al. probed the effect of size and shape of Au nanoparticles on their rate of cellular internalization. They found that uptake was highest for 50 nm spherical Au nanoparticles out of varying sizes between 14 and 100 nm. The internalization of rod-shaped Au nanoparticles is lower than their spherical counterpart and decreases with an increasing aspect ratio (Chithrani et al., 2006). They further demonstrated that this could be attributed to the fact that small nanoparticles cannot bind to multiple receptors on the cell surface and large nanoparticles, despite their capability of multivalent receptor binding, are not able to be fully engulfed by the plasma membrane required for endocytosis (Jiang et al., 2008). Gratton et al. found that, for a particle size larger than 100 nm, rod-shaped nanoparticles showed the highest uptake followed by spheres, cylinders, and cubes (Gratton et al., 2008). Current findings indicate that nanoparticle–cell interaction is a complex phenomenon dependent on several other factors such as type of ligand coating, noble metal, and cell type (Albanese et al., 2012). Despite all these studies, a comprehensive understanding of these mechanisms is lacking. Probing fundamentals of nanoparticle-biological system interactions would enable researchers designing better smart next-generation intracellular SERS-based imaging and sensing probes with highest possible delivery efficiency and maximum signal strength. Moreover, achieving huge and uniform enhancements of SERS is imperative to perform large-scale measurements with homogeneous and reproducible results. Significant advancements have been made in the synthesis of complex plasmonic nanoparticles and assemblies with controlled sizes, shapes, and stoichiometry, while challenges, such as high stability and uniform hotspots, still exist, implying an opportunity for further improvement.

As discussed in this review, cell-based SERS experiments are usually performed either using a probe or in a label-free manner. In the probe-mediated approach, the SERS active substrate is functionalized with a Raman active label and the detection of biomolecules is performed indirectly. With this approach the specificity toward the target and the analyte is highly enhanced. For example, in SERS-based immunoassays, antibody labeling has enabled specific biomolecule detection in complex biological mixtures. The probe-based approach has been more dominant over the past few years as localization and quantification of molecules of interest becomes easy. However, the plethora of information hidden in other millions of biomolecules present inside the cell remains unrevealed. With significant advancements in computational approaches for complex data analysis, preference is now shifting toward a label-free approach. Building an elaborate molecular database for spectral assignment, developing internal standards for reliable comparison and quantitative analysis, and creating efficient data pre-processing and statistical analysis tools are major goals in this field of study.

We believe that SERS-based cellular sensing studies will continue to be a dynamic research field over the next few decades and address more interesting questions in the field of biological and medical sciences such as identifying new drug targets, decoding complex cellular pathways, and monitoring the metabolism of small biomolecules. In near future, the efforts of scientists and engineers involved in Raman and SERS research will decide when it will be clinically translated.

CONFLICT OF INTEREST

Dr. Jeff W. M. Bulte is an editor of the journal and was excluded from the peer-review process and all editorial decisions related to the publication of this article.

AUTHOR CONTRIBUTIONS

Ishan Barman: Conceptualization (lead); supervision (lead); writing – review and editing (lead). **Swati Tanwar:** Conceptualization (lead); writing – original draft (lead). **Jeong Hee Kim:** Writing – original draft (supporting); writing – review and editing (supporting). **Jeff W. M. Bulte:** Supervision (equal); writing – review and editing (equal).

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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How to cite this article: Tanwar, S., Kim, J. H., Bulte, J. W. M., & Barman, I. (2022). Surface-enhanced Raman scattering: An emerging tool for sensing cellular function. *WIREs Nanomedicine and Nanobiotechnology*, *14*(4), e1802. https://doi.org/10.1002/wnan.1802