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SERS Tags for 3D Bioimaging and Biomarker Detection

Elisa Lenzi,[§] Dorleta Jimenez de Aberasturi,[§]* and Luis M. Liz-Marzán[§⊥]*

[§]CIC biomaGUNE and CIBER-BBN, Paseo de Miramón 182, 20014 Donostia-San Sebastián, Spain.
[⊥]Ikerbasque, Basque Foundation for Science, 48013 Bilbao, Spain

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ABSTRACT: We have recently witnessed a major improvement in the quality of nanoparticles encoded with Raman-active molecules (SERS tags). Such a progress relied mainly on a major improvement of fabrication methods for building-blocks, resulting in widespread application of this powerful tool in various fields, with the potential to replace commonly used techniques, such as those based on fluorescence. We present hereby a brief perspective on SERS tags, regarding their composition, morphology and structure, and describe our own selection from the current state-of-the-art. We then focus on the main bioimaging applications of SERS tags, showing a gradual evolution from two-dimensional studies to three-dimensional analysis. Recent improvements in sensitivity and multiplexing ability have enabled great advancements toward *in vivo* applications, *e.g.* highlighting tumor boundaries to guide surgery. In addition, the high level of biomolecule sensitivity reached by SERS tags, promises an expansion toward biomarker detection in cases for which traditional methods offer limited reliability, as a consequence of the frequently low analyte concentrations.

Major advances on synthetic procedures during the past 20 years have provided a remarkable control over nanoparticle design, thereby allowing us to tailor their properties toward specific applications. As a result of such "a la carte" nanoparticle library, we can realistically devise more advanced bio-applications, bringing up concepts like personalized medicine, diagnostic tests, periodic health check-ups and targeted therapies.[1]

Early diagnosis of any disease is a key factor to provide an effective treatment. Therefore, much effort has been invested in the development and improvement of reliable diagnostic tools. On one hand, quick diagnostic solutions[2] based on point-of-care devices,[3] such as tests for respiratory viral infections,[4] or chronic diseases such as glucose sensors for diabetes,[5] have been significantly improved. Early detection is particularly critical in cancer, for which the efficacy of existing treatments is largely improved when administered at early stages of the disease. On the other hand, slower but extremely precise methods, such as advanced imaging, are also required for precise diagnostics, treatment, long-term controls, or to perform "image-guided intervention", so as to minimize invasive therapeutic procedures.[6]

With the aim of overcoming these hurdles, surface-enhanced Raman scattering (SERS) spectroscopy has revealed itself as a promising analytical tool for ultradetection. Since 1974, when Fleischmann's team observed that the inelastic scattering from pyridine was considerably enhanced when in close contact with a rough silver electrode,[7] SERS has gained ever increasing attention, recently focusing toward biological and medical applications.[8] This high interest arises mainly from a great sensitivity, which can reach detection limits down to attomolar concentrations,[9,10] as well as molecular specificity, provided by the characteristic vibrational fingerprint of each molecule. Instrumentation, *i.e.* spectrometers, have also evolved considerably, with still increasing resolution and sensitivity while their components become more reliable. This development has been mainly oriented toward high-end setups (with highly focused laser beams, efficient filters, highly sensitive detectors, *etc*.) and portable Raman spectrometers.[11]

Although other mechanisms may also play a role, it is currently accepted that the so-called electromagnetic mechanism is the main factor behind the remarkable enhancement of the Raman scattering signal in SERS. This mechanism relies on localized surface plasmon resonances (LSPR), *i.e.* collective oscillations of conduction electrons in nanoparticles in resonance with an external electromagnetic radiation. Apart from large absorption and scattering, the incident light creates high electric fields, confined at the surface of the nanoparticles, which can strongly affect the polarizability of adsorbed molecules, thereby leading to enhancement of their characteristic Raman scattering, by many orders of magnitude. Tailoring the LSPR for the substrate relative to the laser excitation wavelength, so that the measurement is carried out on-resonance, is crucial to achieve a large Raman scattering enhancement. This phenomenon makes it possible to detect arbitrary analytes with high specificity and at very low concentrations. As a result, plenty of investigations have been performed to apply the SERS technique in sensing of low abundant species, or to improve the sensitivity of existing methods, *e.g.* in SERS-based immunoassays which are commonly used to identify the presence of pathogens in clinical samples.[12] SERS additionally features important advantages over commonly used fluorescence-based biological assays, as it is potentially more sensitive and provides stable and reproducible signals with narrow spectral peaks (*ca.* 1-2 nm), which are essential for multiplexed measurements.[13]

On the basis of many reported results and outstanding achievements, it has been predicted that cancer diagnosis should be the most prominent application for *in vivo* Raman spectroscopies, due to its versatility, multiplexing capacity and high sensitivity, related to intense signals and reduced background noise.[14] However, in the context of biological analysis, the extremely low intrinsic Raman cross sections of most biomolecules are commonly compensated by using other

labeling molecules with comparatively higher cross sections, as Raman reporters (RaRs).[15] We can thus distinguish two general methodologies for SERS-based biological and biomedical applications, namely label-free detection and indirect approaches that make use of RaRs attached to a nanoparticle, commonly known as SERS tags.[16] The long-term stability featured by RaR molecules results in highly reproducible SERS signals, which, together with the reduced sample background autofluorescence noise,[17] are key advantages of SERS over other bioimaging techniques.[18,19] SERS imaging has also gained relevance due to its versatility, non-invasive character and negligible photobleaching, which is essential for long-term *in vivo* imaging. It should however be noted that, the inherent complexity of this system makes further development necessary, prior to generalized use.[18] An important limitation is the short penetration depth of the excitation laser light (*ca.* 2-5 mm)[20] which severely hinders application in deep human body tissues/tumors. Therefore, those applications where the laser beam can access the diseased area are the ones with a better chance to succeed. An important advancement in this respect has been the release in 2015, of the first endoscopic probe capable of SERS imaging.[21] It should be noted that SERS is compatible with other imaging techniques,[22,23] so that it can be combined in dual or multimode platforms, for simultaneous imaging and sensing,[24] and/or with therapeutic treatments such as laser ablation (also termed photothermal therapy).[25]

Unfortunately, due to the above mentioned difficulties, most of the analysis and studies performed so far, are related to *in vitro* cell and *ex vivo* biofluid and tissue samples, but still few *in vivo* studies have been reported.[14] Notwithstanding, we can expect that, by relying on the remarkable recent advances in instrumentation and optimized fabrication of plasmonic substrates, SERS will see a crucial leap in the near future, in terms of *in vivo* applications. Apart from endoscopy assisted surgery,[26–28] we propose that a key contribution will be the application of SERS for high resolution imaging of 3D tissue models, which are rapidly gaining popularity due to their ability to reproduce real interactions among different cells, thereby reducing the use of animal testing.[29] Such 3D cell models are dynamic systems that allow cell proliferation and dissemination, which can be monitored *in vivo* using SERS.

In this perspective article, we focus on the promising expectations and possibilities that the use of SERS tags offers for bioapplications. We attempt to describe the most common formulations to fabricate SERS tags and the most recent advances in biomedical applications related to indirect SERS detection.

BASIC ELEMENTS OF SERS TAGS

The SERS tag is the first concept to be described when discussing indirect SERS measurements. A SERS tag, also called SERS-labeled nanoparticle, comprises (**Figure 1A**) a noble metal (typically Au or Ag) nanoparticle (I), which acts as the plasmonic enhancer, covered with a monolayer of Raman reporter (RaR) molecules (II) acting as fingerprint labels,[30] usually surrounded by a protective layer or coating shell (III), which can be in turn selectively functionalized with targeting biomolecules (IV).

(I) *A golden heart:* The core of a SERS tag comprises one or more plasmonic metal nanoparticles, which provide a largely enhanced electric field when an LSPR is excited by the incoming light (selected incident laser light source). The efficiency of this process is an essential element behind the required high signal intensity and reproducibility of Raman imaging experiments. Chemical composition, size and shape are the main factors dictating the optical response of the nanoparticle cores, but the refractive index of the surrounding medium also affects the precise value of the LSPR frequency. Even though silver is the most efficient plasmonic metal,[31] gold is by far the most widely used material in biomedicine; its biocompatibility, low toxicity and excellent control over the synthesis of Au NPs, have resulted in the so-called "new Golden Age".[32] The morphology of Au NPs has a major effect on LSPR, *e.g.* whereas small gold nanospheres display a plasmon resonance centered around 520 nm,[33,34] the LSPRs of anisotropic NPs such as nanorods can be tuned from the visible into the near-IR (NIR) region (up to 1500 nm).[35] Such a wide tunability is particularly relevant for biological applications, because it is precisely in the NIR windows (the first biological transparency window (NIR-I) at 650-950 nm; second window (NIR-II) from 1 to 1.35 μm and third one (NIR-III) from 1.5 to ~1.8 μm),[36,37] where we find optimum light transmission in tissue, with maximum penetration and minimized autofluorescence. Apart from anisotropy, the presence of sharp tips and edges has been found to result in larger electric field enhancements, and therefore star-like morphologies have also acquired a high relevance in this context.[38,39] We do not elaborate further on the synthesis of anisotropic gold nanoparticles for SERS, since excellent reviews are available in the recent literature.[11,40]

Apart from single Au (or Ag) NPs with tailored morphology, much effort has also been spent on the fabrication of multiparticle cores. The rationale behind this choice is the well-known formation of plasmonic hot spots (areas of locally high near-field enhancement) at interparticle gaps, where RaR molecules would be placed to yield high SERS signals.[41–44] Controlled assemblies of nanoparticles such as dimers,[45] larger clusters[42,46] or even switchable core-satellite Au-SiO_2 heteroassemblies,[47] have been reported as suitable candidates to be used as cores for SERS tags. Despite efforts in nanoparticle synthesis, several challenges are still to be overcome to produce highly homogenous NPs and controlled NP assemblies. Recent efforts directed to overcome these issues include significant improvement of morphological monodispersity and reproducibility in Au nanostars,[48] as well as methods to improve encapsulation of nanoparticle clusters, using microfluidic systems equipped with a suitable mixing chamber.[49] The use of automated reactors for nanoparticle synthesis are an attractive development, not only to prepare big batches of nanoparticles but also to avoid human error.[50]

(II) *Raman reporters:* The identification of SERS tags is directly related to the selection of appropriate RaR molecules, which provide a characteristic spectral fingerprint that gets enhanced when in contact with the plasmonic metal core. Under these conditions, a large library of SERS tags with different Raman codes can be fabricated,[15,51] by simply modifying the chemical structure of RaR molecules, thereby offering exciting applications in multiplexed bioimaging. As an additional requisite for RaR molecules, they should feature relatively high Raman cross sections. This restriction still allows a wide variety of molecules to be used, including *e.g.* standard fluorophores (crystal violet, Nile blue, *etc.*). When the dyes display an electronic transition in resonance with the excitation laser wavelength, the occurrence of surface enhanced resonance Raman scattering (SERRS) results in even higher signal intensity.[14,15] On the other hand, thiolated aromatic molecules

can readily adsorb onto the gold surface and provide characteristic Raman spectra, examples including: biphenyl-4-thiol (4-BPT), 2-naphthalenethiol (2-NaT), benzenethiol (BT), 4-Mercaptobenzoic acid (MBA), *etc*. It should be noted that, most of these molecules have an eminently hydrophobic character, which in some cases requires the transfer of Au NPs into an organic solvent, to obtain maximum surface coverage. Ideally, a monolayer of RaR molecules uniformly covering the metal surface would provide intense, stable and reliable signals[13,52] As a rule of thumb, high Raman scattering cross sections are obtained when molecules contain highly polarizable moieties, such as double and triple bonds. For multiplexing purposes the overall number of vibrational Raman bands, as well as the spectral overlap between selected RaRs should be minimized, which is not trivial. Occasionally, the combination of different RaR molecules on the same NP substrate has been reported, so that tags are created which can be detected across a wider range of excitation wavelengths.[53] This approach is also appealing toward anti-counterfeiting applications.[54] Recently, the use of custom made RaR molecules has been introduced as a means to improve the performance of existing RaRs. For example, resonant dyes can be modified to incorporate functional groups that facilitate adsorption onto NPs. Specific synthetic procedures have been developed for novel RaR molecules comprising olefin or alkyne moieties with strong and characteristic vibrational Raman bands.[55] Quantification of the RaR molecules attached to NPs is still a challenge and the use of techniques such as mass spectrometry to determine the ligand shell morphology provide a better understanding of these systems.[56]

(III) *Protective coating:* A third important component of SERS tags is an external layer or coating shell, which helps improving their colloidal stability while providing insulation.[30] Although this protective layer is not always present, it is used in most tags as it provides multiple advantages. Briefly, the main functions of the protecting layer are the following: (i) avoiding leaching of RaR molecules; (ii) preventing potential signal contamination from interfering molecules present in the surrounding medium; (iii) reducing any eventual toxicity of the NPs; and (iv) reducing plasmon coupling that might be induced by NP–NP interactions, leading to undesired intensity variations. The chemical identity of the protecting shell can vary depending on the specific applications, and has typically comprised biomolecules (*e.g.* bovine serum albumin, BSA), amphiphilic or other polymers, such as polyethylene glycol (PEG), and inorganic shells, most often SiO_2.[47,57] Silica encapsulation is indeed one of the most popular coating layers due to its versatility in terms of shell thickness, porosity, reproducibility of the synthesis and biodegradability (**Figure 1B**).[58–61] However, silica often suffers from degradation and agglomeration problems in studies that require long-term exposure to cellular media.[62] Polymers are also suitable candidates for tag coatings; heterofunctional polyethylene glycol (PEG) with a thiol end-group (for binding to Au NP surface) and additional functional groups such as –COOH (which can be conjugated to amine groups of *e.g.* antibodies, through EDC-NHS chemistry) are commonly used as anti-fouling and AuNP stabilizers.[63] However, the binding competition between PEG-SH and the RaR molecules makes it necessary to fine-tune the polymer/RaR ratio to maintain a high SERS signal.[13,64] Alternatively, crosslinked polymers such as poly(N-isopropylacrylamide) (pNIPAM) can be used to entrap RaR molecules.[17] On the basis of hydrophobic interactions between hydrophobic RaR molecules covering NPs surface and hydrophobic sites of amphiphilic polymers (such as dodecylamine/polyisobutylene-alt-maleic anhydride, PMA),[13,65] highly stable and biocompatible coatings can also be achieved (**Figure 1C**).[66] An alternative strategy comprises the coating of NPs with liposomes, which provides high biocompatibility but requires more tedious encapsulation and purification steps.[67] Concerning biocompatibility, high molecular weight proteins are considered to be key biocoatings,[68] which can readily bind to AuNPs through either covalent bonds or physical interactions.[69] Bovine serum albumin (BSA) is a widely used model globular protein that provides stability and protection to the particles.[70] Recently, the deposition of few layer graphene onto AuNPs has been reported to form so-called graphene-isolated–Au nanocrystals (GIANs). Interestingly, graphene serves simultaneously as protective layer and RaR (Figure 1D)

(IV) *Targeting ligands:* The selectivity of SERS tags toward a specific body region or tissue is typically provided by target-specific biorecognition ligands, which in general are specific peptides, antibodies, aptamers or proteins.[71,72] The connection of antibodies and proteins to SERS tags is often based on direct binding to polymeric shells, by means of either 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry, forming stable amide bonds, or click chemistry, whereas silane chemistry is often used for silica-coated tags. Site-specific immobilization of antibodies is important, so as to achieve maximum selectivity while leaving the reactive site of the antibody available for targeting. A usual strategy involves the attachment onto the NPs of a peptide linker, such as Protein A, which presents high affinity toward the Fc fragment of human and rabbit IgG. This method facilitates orderly immobilization. Alternatively, biotin-modified proteins or aptamers can be bound to avidin or streptavidin-modified NPs.[73] Less effective biomolecules can also be linked through direct adsorption or by electrostatic interactions, when NPs and target have opposite charges.[57] Regarding peptides, one of the most common strategies involves covalent coupling of cysteine-terminated peptides to the particle surface *via* S-Au bonds. BSA has also been used as a linker between peptides (*via* the crosslinker 3-maleimido benzoic acid N-hydroxysuccinimide, MBS) and the nanoparticles (*via* electrostatic interactions), providing better solubility and stability to the peptide.[74] The resulting selectively functionalized nanoparticles have been widely used to target and image cancer cells, both *in vitro* and *in vivo*,[75,76] but a variety of applications are still emerging, which are based on the use of SERS tags for immunoassay testing or recognition of tumor cells coupled to endoscopy methods.[21,43]

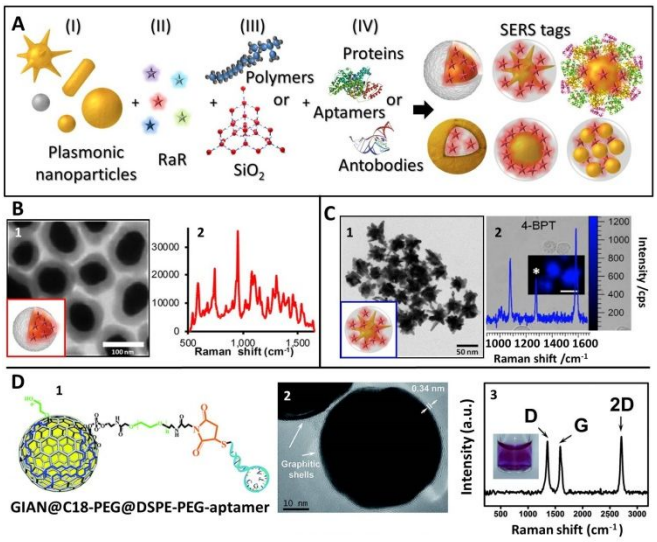

Figure 1. A) Schematic representation of the basic elements of single core and multiple core SERS tags: (I) inorganic cores; (II) Raman reporter molecules; (III) coatings made of different materials (proteins, polymers, silica, etc.); (IV) recognition moieties. B) PEGylated, silica coated SERS-nanotags containing the NIR dye IR780 perchlorate as RaR: B.1) TEM image; B.2) SERS spectrum using 785 nm laser excitation. Reproduced with permission from ref [27]. Copyright © 2019 American Chemical Society. C) BPT-SERS-encoded Au nanostars with an amphiphilic polymer protective layer: C.1) TEM image; C.2) Colored SERS maps of tag-labeled cells. The white scale bar corresponds to 20 µm. Reproduced with permission from ref [13]. Copyright © 2016 American Chemical Society D) Graphene-isolated–Au nanocrystals (GIANs): D.1) Scheme of aptamer-functionalized GIANs; D.2) TEM image; C.3) SERS spectrum of aqueous GIANs suspension highlighting graphene main peaks. Reproduced from ref [77]. Copyright © 2018 The Royal Society of Chemistry.

IMAGING WITH SERS TAGS

The characteristic properties of SERS tags are particularly useful for imaging applications in which long-term experiments are to be performed. A key advantage of SERS tags, as compared to commonly used fluorophore labels, is their resistance to photobleaching.[18,78] Therefore, the localization of biocomponents labeled with SERS tags can be monitored over extended periods of time, with negligible loss of SERS signal. On the other hand, the readily available NIR-responsive SERS tags provide access to deeper light penetration in biological systems, thereby largely expanding the range of potential applications. Finally, the non-invasive character of SERS makes it compatible with *in vivo* experiments, and avoids the need for fixing cells, which is crucial toward understanding cellular behavior under real life conditions. We present below selected examples that clearly illustrate the current interest in using SERS tags, as compared to conventional methods.

From 2D to 3D cell models and *in vivo* SERS imaging

The development of a new material for biomedical applications requires first of all cytotoxicity tests to be carried out,[65] commonly by means of experiments using living cells. In this respect, minimally invasive techniques should be available for toxicity evaluation over extended periods of time. Other studies related to long term monitoring, for example of the effects of anticancer drugs, require the use of methods that do not modify the cellular environment. For such cases, SERS imaging appears as an ideal technique of choice.

The simplest *in vitro* models are single cell cultures, which grow as a monolayer on a flat surface, in the presence of the necessary nutrients provided by a cell culture medium. Over decades, these 2D cell models have been used for all types of bioassays. SERS tags have been demonstrated to be particularly useful in those cases where mixtures of different cell types are to be separately monitored. For example, Jimenez de Aberasturi *et al*.[13] demonstrated that five different cancer cell lines, each labeled with a different SERS tag, can be specifically imaged and followed within a quintuple cell co-culture, over time periods exceeding 24 h, without visible damaging of the cells. In cases where cell discrimination is a requirement, SERS tags with specific cell receptors can be used. Bodelón *et al.* reported a system based on Au@pNIPAM SERRS tags carrying different antibodies, which selectively attach to cellular receptors so that tumor cells can be clearly distinguished from healthy cells.[17] In another interesting study, four different SERS tags comprising silver-coated gold nanorods with a PEG protecting layer, four thiophenol derivatives as RaR molecules, and four different antibodies that are specific to breast cancer membrane receptors and the leukocyte-specific CD45 marker. By integrating multiplex targeting, multicolor coding, and multimodal detection, this approach demonstrated promising improvements in multispectral imaging of individual tumor cells within complex biological environments (**Figure 2A**).[79]

It is well-known that, for medical applications, the microenviroment of cellular models should closely resemble that in the human body. Therefore, preclinical studies are typically carried out using various types of animals. However, ethical considerations require that the use of animal models be reduced as much as possible, which requires the design of realistic *in vitro* models.[80] Unfortunately, commonly used 2D cell models are far from simulating the real situation in humans. Attempts to resolve this issue and achieve a more realistic model included the addition of extracellular matrix (ECM) or mixing different cell types, so that communication and intercellular contacts are improved, but this is still insufficient. When cells are cultured in 3D systems, they tend to form aggregates or spheroids in which cell-cell interactions are closer to a real *in vivo* situation.[81] Such 3D cell cultures are often assisted by scaffolds through which cells can migrate and recreate real dynamic situations[82] As a result of these developments, the use of 3D cell culture models has rapidly increased. However, our ability to characterize the internal structure or events within tissue in 3D is still rather limited. Confocal fluorescence microscopy is still the most commonly used technique, but it presents various drawbacks related to limited penetration depth, autofluorescence from cells, photobleaching and overlapping signals due to broad emission bands. Therefore, SERS imaging is emerging as a promising alternative technique to monitor 3D cell ensembles. The biggest challenge for 3D SERS imaging of bio-samples is related to the spatial and temporal resolution of existing instruments. Several components determine the sensitivity and resolution of the measurements. Light source parameters such as wavelength, spectral linewidth, frequency and power stability, spectral purity, beam quality, output power, *etc*. are highly relevant in any measurement. In biological samples, irradiation wavelengths are restricted to the NIR, so as to increase the penetration depth, even if the Raman-scattering efficiency is weaker than at shorter wavelengths. The 785 nm laser is most

often used, as it presents a good balance between scattering efficiency, fluorescence, detector efficiency and cost-efficiency.[83] Regarding detectors, wavelength specific CCD devices with improved precision are increasingly available.

We should say however that, despite its interesting prospects, the development of 3D-SERS imaging is still in progress.[84] Only few reports have used Raman imaging with high spatial resolution in 3D.[85,86] In a recent example, Strozyk *et al.* used confocal Raman microscopy to image the 3D distribution of SERS-encoded AuNSs within multicompartment microgel particles.[18] Altunbek *et al.* used SERS to analyze biochemical information from 3D culture systems, while maintaining their integrity. In this case, rather than SERS tags, a label free SERS based approach was used for the analysis of intracellular responses at different depth layers of a 3D culture model upon exposure to different drugs (**Figure 2B**).[87] We propose that, combination of both approaches, SERS tags for 3D imaging and label free SERS analysis to monitor selected biomolecules (biomarkers) will become a suitable pathway to obtain a complete characterization of the biological system.

Related techniques are coming up, to overcome the spatial resolution problem. For example, confocal Raman microscopy (CRM) is a spatial filtering technique which uses pinholes to eliminate the out-of-focus signal, enabling resolutions of 0.5-1μm.[88] To push this limit to the nanoscale, it is possible to implement atomic force microscopy (AFM), coupled to a confocal Raman spectrometer. This coupled technique, also called tip-enhanced Raman spectroscopy (TERS), is based on an opto-mechanical coupling that confines the laser on a metal-coated tip, which acts as a nano-source of light and a focal enhancer.[89] Moreover, combining this method with non-linear coherent Raman scattering phenomena, tip-enhanced coherent anti-Stokes Raman scattering (TECARS) is possible, leading to detection of single molecules with high sensitivity and resolution.[90] On the other hand, the combination of SERS with spatially offset Raman spectroscopy (SORS) has been shown to yield enhanced Raman signals at much greater sub-surface levels. By means of this so-called SESORRS technique, tracking of SERRS-tags through porcine tissue at depths up to 25 mm has been achieved.[91] Similar to two-photon fluorescence microscopy, surface enhanced hyper Raman Scattering (SHERS) is capable of reaching high resolution levels by exploiting non-linear excitation and surface enhancement phenomena. The non-linear process permits reducing the illumination area and consequently obtaining more precise data in deep 3D samples.[92] For rapid imaging (30 msec per pixel) of large tissue areas, immuno-SERS microscopy is also being developed, with single-nanoparticle sensitivity.[93] To study molecular motion and molecule-plasmon interactions at a single molecule level, all these techniques can be equipped with ultrafast lasers reaching femtosecond or picosecond timescales.[94,95]

High resolution, fast acquisition time and increased penetration depth are all highly desirable features in bioimaging. Consequently, due to the high amount of produced data, improved data analysis methods are required, particularly for 3D imaging applications. Multivariate analysis is increasingly used to focus on the degree of correlation or covariance among data. For example, principal component analysis (PCA) is used to reduce the data dimensionality without losing information related to the variability of the sample.[96] Other classifiers and regression methods, such as linear regression analysis, are used to discriminate different elements inside the specimen, on the basis of a model predicted from data features.[97]

In vivo imaging

In vivo SERS imaging has been largely used to identify the presence and location of tumors, thereby guiding surgical tumor removal,[28] occasionally in combination with complementary imaging techniques, thereby leading to multimodal imaging.[23] The majority of works aiming to *in vivo* SERS imaging involve the use of SERS tags,[2] which are injected into the animal and either accumulate in a tumor due to the enhanced permeation and retention (EPR) effect[98] or specifically target certain cells or tumors *via* direct injection or biorecognition surface ligands (**Figure 2C**). Again, the main limitation is given by the short penetration depth of the excitation laser beam, therefore much effort is being devoted toward developing new instrumentation for *in vivo* SERS imaging. For example, Zabaleta *et al.*[99] developed a small animal Raman imaging (SARI) system that allows for rapid imaging over a relatively large area (>6 cm^2), with high spatial resolution.[100] The same group developed a fiber optic probe designed to couple with a clinical endoscope, the combination of both systems being a significant advancement for simultaneous *in vivo* imaging and detection of different types of cancer, as explained in more detail below.

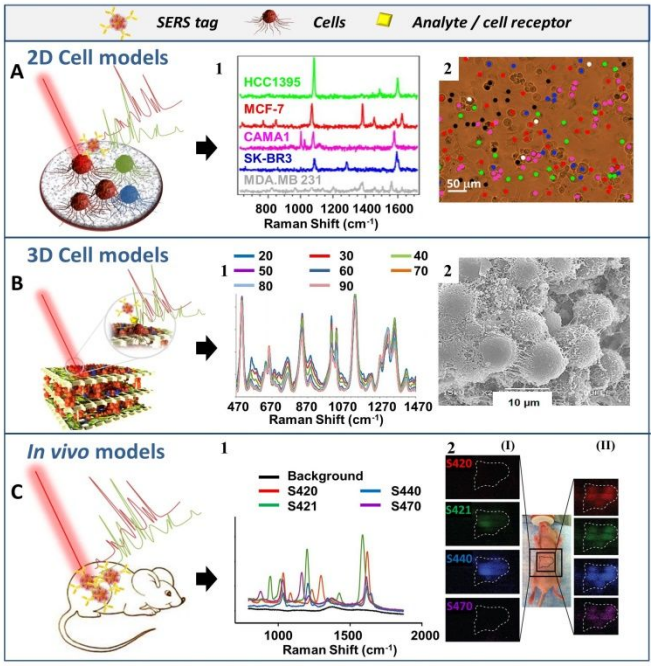

Figure 2. A) SERS imaging in a 2D cell model: A.1) Reference RaR fingerprints recorded from single cultures; A.2) large-scale SERS single-point mapping to quantify the composition of 147 cells within an area of 0.22 mm^2 using references from A.1. Black dots correspond to cells with insufficient signal. Reproduced with permission from ref [13]. Copyright © 2016, American Chemical Society. **B)** Toward 3D cell models: B.1) SERS spectra from different depth layers of Doxorubicin-treated HeLa spheroids; B.2) SEM images from spheroids formed by AuNP-treated HeLa cells. Reproduced with permission from ref [87] © 2018 Elsevier B.V. **C)** *In vivo* SERS imaging: C.1) SERS spectra of four different SERS tags passively accumulated in the liver after injection; C.2) SARI system to image SERS tags: two tags are seen at 1h post-injection (I) whereas all four probes are evident at 2h post-injection (II). Reproduced from ref [100].

Multimodal imaging

The combination of mutually compatible imaging techniques has become one of the preferred solutions, so that we can obtain as much information as possible, toward accurate disease diagnosis. Each technique can provide important and complementary information, so that platforms enabling the use of multiple techniques are highly desirable. For example, the combination of magnetic and plasmonic properties within a single nanosized object should allow us to use magnetic resonance imaging (MRI) through contrast provided by *e.g.* an iron oxide component, while a plasmonic part, such as RaR-labeled gold, can be monitored by SERS imaging, providing high sensitivity and spatial resolution. Reguera *et. al*[23] developed Janus plasmonic–magnetic (Au–Fe_2O_3) NPs and showed their multimodal imaging performance. Photoacoustic (PA) imaging can also be considered as a complementary tool, where the signal would again be assisted by the plasmonic response.[23,101] Kircher *et al.* reported a combination of SERS, PA and MRI to visualize brain tumor margins with high precision. In this case, the system comprised Au nanotags, functionalized with Gd organometallic complexes.[102]

The combination of SERS with other imaging techniques has also been explored, where particles containing fluorescence and Raman labels are probably the most common ones. For example fluorescent polystyrene beads coated with SERS-labeled AuNSs have been demonstrated efficient systems as dual imaging probes.[22] For a recent review on SERS-fluorescence encoded particles, see Guerrini *et al.*[103]

SERS imaging and sensing can not only be combined with other imaging techniques for diagnosis, but also with selective treatment techniques such as photothermal therapy (PTT). Theranostic platforms combining both processes are actively being explored. Examples include the work by Liu *et al.* using plasmonic Au nanostars for *in vivo* tumor imaging and photothermal therapy,[104] as well as the Raman reporter-coupled Ag@Au nanostars reported by Zeng *et al.* for the same purpose.[105]

BIOMARKER DETECTION

Biomarkers play a vital role in disease detection and treatment follow-up; however, their detection is sometimes limited due to technical issues.[106] The development of techniques that enable accurate biomarker detection can be considered as a trending topic. The ultrasensitivity provided by SERS is of great interest in this field, SERS tags being again attractive candidates for several applications such as *in vivo* detection or point-of-care (POC) devices.

In vivo detection

The high sensitivity and specificity of SERS tags are considered as the essential properties behind their use for detection at the single-cell level. The target cell would be recognized *via* binding through a targeting moiety carried by the SERS tag, while the acquired Raman signal provides identification and localization of the target. Despite the high sensitivity, direct clinical application of SERS tags still requires the development of inexpensive and easy handling instrumentation. Therefore, the application of such probes in *vivo* has been largely focused on endoscopic systems for imaging and/or detection. These systems enable real-time *in vivo* identification of tumor receptors,[24] ultimately leading to real-time image-guided resection and/or drug delivery (**Figure 3**).[107]

During endoscopic observation, SERS tags become a powerful biomedical tool that allows improving the set of molecular information toward detecting cancer cells more accurately and studying the molecular changes associated with progression of the disease. Biomarker-targeted nanoparticles are inoculated inside the body and a spectral-imaging endoscope is used to acquire the Raman signal, as schematically shown in **Figure 3A**.[108] For example, using the multiplexing capabilities of silica-coated nanoparticles functionalized with monoclonal antibodies (mAb), in combination with a single wavelength laser, imaging of cell-surface biomarkers of oesophageal cancer has been possible (**Figure 3B**).[109] Additionally, image analysis provided quantitative information on the relative concentration of SERS tags that were present in the luminal surface of hollow organs.[21] Harmsen *et al.*[27] have recently developed a mouse Raman endoscope and a clinically applicable Raman endoscope for larger animal studies that serve to detect premalignant gastrointestinal lesions using SERS-tags.

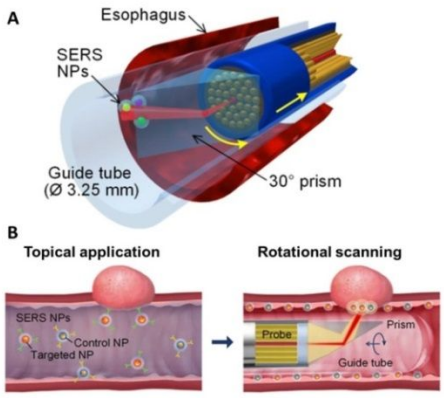

Figure 3. A) Schematic illustration of a SERS-endoscopy system: a fiber-bundle imaging probe within a glass guide tube, inserted in a rat esophagus. Reproduced from ref [108]. Copyright © 2014 Optical Society of America. B) Endoscopic imaging of a rat esophageal tumor model. Left: Topical application of multiplexed SERS tags on the lumenal esophagus surface, for ratiometric biomarker detection; Right: endoscopic imaging of SERS tags *via* rotational pull-back of the fiber-bundle imaging probe. Reproduced from ref [109]. Copyright © 2015 Optical Society of America.

Another improvement has been the detection of cancer biomarkers in blood, which is a particularly difficult environment for optical detection, since blood features strong absorption and scattering of light, as well as autofluorescence, therefore strongly hindering the signal. An approach toward overcoming these problems comprised silica-coated SERS tags coupled with a fiber-optic bio-sensor (FOB), based on an evanescent wave detection mode.[110,111] Another key application is open surgery: SERS image–guided resection requires intravenous injection of the nanotags, which circulate in the blood stream until reaching the tumor mass. After several hours, the area is examined with a handheld fiber-optic device that excites and collects the signal from the SERS tags. Karabeber *et al.*[28] showed that SERS tags can accurately outline the extent of a tumor, thereby allowing real-time scanning and detection

of single tumor cells. These studies validate the use of SERS tags for targeted ultrasensitive *in vivo* detection and imaging.

In vitro diagnostics

Moving away from living clinical samples, recent years have seen extensive application of SERS tags for indirect analyte detection. For example, immunoassays are used to detect a specific target molecule in solution, using antibodies and antigens, such as the ELISA test, which however suffer from drawbacks including long detection times, large sample volumes, and poor limits of detection. SERS based immunoassays using SERS tags with high signal intensity offer improved detection in cases where extremely low amounts of solution lead to failure in standard systems.[112] Such devices have shown detection of different types of biomolecules and microorganisms, including proteins, bacteria and viruses, with limits of detection that are *ca.* two orders of magnitude more sensitive than conventional immunoassays. Additionally, SERS-based immunoassays do not require sample culture or amplification steps and are characterized by reduced sample consumption and short assay times.[113] The procedure is usually divided in two steps: a small amount of analyte is first mixed with functionalized SERS tags for selective binding, resulting in SERS probe-analyte complexes for the subsequent measurement of biomarkers. Depending on the capture method, we can identify various types of immunoassay devices. A numerous group of devices exploits magnetic interactions to collect the SERS probe-analyte complexes, *e.g.* those based on magnetic beads. These systems allow the antibody-antigen interaction to take place in an aqueous environment,[114] but are limited by the required multiple washing steps and by the difficulties in controlling the assay conditions.[115] The work by Choo and colleagues[116] is an example of a magnetic bead-based device: PEGylated Au NPs carrying two different RaRs were employed to detect two different prostate specific antigens (PSAs) and create magnetic sandwich immune-complexes for both biomarkers, thereby leading to a more specific diagnosis.

A different capture method relies on biofunctionalized sandwich SERS probes adsorbed onto a planar substrate. The biomarker expression could be identified at the single cell level,[117] by using a substrate-based biosensor (**Error! Reference source not found.A**) which allows monitoring sub-nanomolar concentrations of the cancer biomarker epithelial cell adhesion molecule (EpCAM), in two different cancer cell lines. The device is characterized by AuNSs substrates functionalized with an EpCAM aptamer receptor, which leads to formation of a sandwich with the SERS tags, characterized by the (mercapto-hexanol) RaR molecule and the EpCAM aptamer molecule. Similarly, a ultrasensitive SERS immunoassay for ultra-small serum volumes (≈2μL) has been developed toward exosome-based diagnosis, classification and metastasis monitoring of pancreatic cancer.[112] Polydopamine (PDA) encapsulated, antibody-reporter-Ag(shell)@Au(core) SERS tags were employed to form a sandwich structure with a porous hydrophilic PDA layer, where specific antibodies were previously encapsulated (**Figure 4B**). Some other immunoassays[118,119] have been reported, which make use of SERS tags in lateral flow assays, for detection of specific biomarkers. For example, silica coated AuNS functionalized with detection antibodies were incorporated in a paper-based lateral flow strip, to detect the neuron specific enolase (NSE) biomarker, directly from blood plasma.[120]

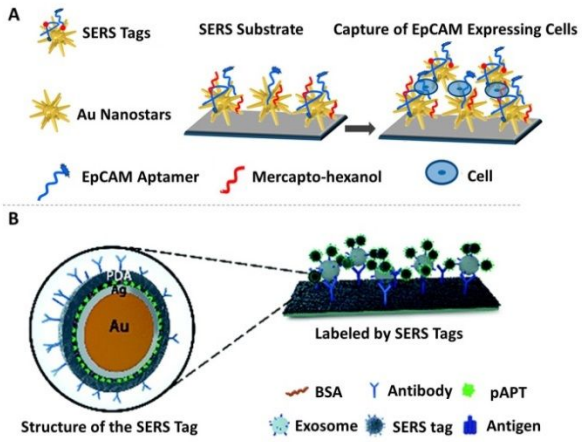

Figure 4: A) Schematic representation of a substrate-based biosensor employing AuNSs functionalized with EpCAM aptamer molecules and mercapto hexanol as RaR molecule, to quantify subnanomolar concentrations of EpCAM protein in solution. Reproduced with permission from ref [117]. Copyright © 2018, American Chemical Society. B) Schematic view of polydopamine-modified immunocapture substrates and ultrathin polydopamine-encapsulated antibody-reporter-Ag(shell)-Au(core) multilayer (PEARL) SERS-tag. Reproduced from ref [112]. Copyright © 2018 The Royal Society of Chemistry.

Notwithstanding, application of SERS in clinical assays which require precise quantification, suffer in many cases from poor reproducibility. Better control of particle aggregation, particle monodispersity in terms of both the inorganic core and RaR distribution, are key factors that must be overcome. An emerging solution is the use of portable point of care (PoC) devices for fast and accurate diagnosis.[3] While nanotechnology has provided the possibility of reducing device size, SERS can offer high analytical sensitivity, reducing detection limits and providing multiplexing capabilities. Therefore, SERS based clinical tests are increasingly reported[121] (**Figure 5**). The integration of SERS within microfluidic chips using portable readers is a promising alternative.[122] This combination allows quantification by SERS of nanoliter volumes in an automatic and reproducible manner, assisted by homogeneous mixing. Moreover, the fabrication of such assays by means of 3D bio-printers, which allow reproducible fabrication of complex substrates and bio-architectures with a precise amount of material, opens up a whole world of possibilities.[123]

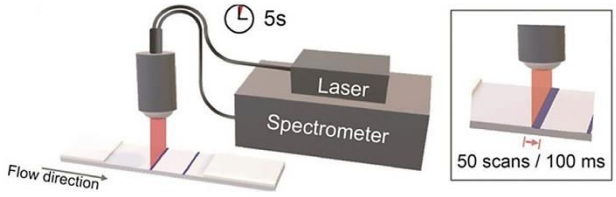

Figure 5: Sketch of a portable Raman/SERS reader design with a customized optical fiber probe with laser line focus. Reproduced from ref [121]. © 2018 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA.

OUTLOOK

SERS tags have become an essential component in SERS-related biomedical applications. This is mainly due to the difficulties often encountered during direct SERS sensing in the biomedical and clinical fields, where the environment is highly heterogeneous and in constant change. Most often, well-defined SERS-labeled elements with specific signals that are not altered over time, can be used as a reference in such complex biological conditions and in particularly for SERS imaging. Still, direct SERS would provide complementary information related to analyte changes. We thus propose that, combination of both approaches should be highly beneficial.

In this context, recent progress in the controlled and reproducible synthesis of anisotropic metal nanoparticles, combined with reliable encoding, protecting and targeting methods, are leading to highly sophisticated systems that can be designed "*a la carte*" for each specific case. The highly reproducible production of nanoparticles is based on better understanding of the growth mechanisms while avoiding spurious errors by the implementation of automated synthesis reactors.

The field of tag-based SERS imaging is currently undergoing a significant input, largely based on advancements related to improving spatial and temporal resolutions. High spatial resolution techniques such as TERS, combined imaging methodologies (multimodal imaging) and the application of ultrafast lasers, are pushing the limits to reach nanometer resolutions and femtosecond timescale. The implementation of well-defined 3D cell and tissue models and multivariate data analysis are essential tools required to reach these aims. Additionally, SERS-based imaging can not only be paired with other imaging procedures for more accurate diagnostics, but also with related therapeutic techniques. We thus expect a burst of new advances in the near future, related to ultrafast and highly accurate SERS imaging.

Another field where SERS tags have much to offer is *in vivo* biomarker detection, which relies on open surgery, where endoscopic SERS probes can complement the analysis and detection of tumor cells, thereby assisting selective removal from the damaged tissue. On the other hand, SERS based point-of-care assays are seeing increased development because of the enhanced sensitivity that can be obtained in those cases where analytes such as cell biomarkers are present at very low concentrations. Although reproducibility is still the main drawback, integration of SERS within microfluidics devices allows obtaining more reproducible measurement conditions, leading to reliable quantitative measurements. We can thus expect increased SERS-based clinical applications along with further development of portable instruments for *in situ* essays, *i.e.* smaller dimensions, more sophisticated elements and automated analysis.

AUTHOR INFORMATION

Corresponding Author

* E-mail: djimenezdeaberasturi@cicbiomagune.es (DJA); llizmarzan@cicbiomagune.es (LML-M)

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

SERS: surface-enhanced Raman scattering; LSPR: localized surface plasmon resonance; RaR: Raman reporter; AuNP: gold nanoparticle; AgNPs: Silver nanoparticle; 4-BPT: biphenyl-4-thiol; 2-NaT: 2-naphthalenethiol; BT: benzenethiol; MBA: 4-Mercaptobenzoic acid; pNIPAM: poly(N-isopropylacrylamide); PMA: dodecylamine modified polyisobutylene-alt-maleic anhydride; BSA: bovine serum albumin; GIANS: graphene-isolated–Au nanocrystals; TEM: transmission electron microscopy; SEM: scanning electron microscopy; SARI: small animal Raman imaging; MRI: magnetic resonance imaging; PA: photoacoustic; PTT: photothermal therapy; mAb: monoclonal antibody; PSA: prostate specific antigen; EpCAM: epithelial cell adhesion molecule; PDA: polydopamine; PEARL polydopamine-encapsulated antibody-reporter-Ag(shell)-Au(core) multilayer; ECM: extracellular matrix; EPR: enhanced permeation and retention; NSE: neuron specific enolase.

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Table of Contents (TOC)