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Multimodal Gold Nanoprobes for SERS Bioimaging

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Abstract

Growing number of studies report on the improved sensitivity of various imaging modalities in detecting abnormalities within tumours. Surface enhanced Raman scattering (SERS) microscopy is a novel optical imaging technique which is advantageous in terms of greater multiplexing capability, minimal or no photobleaching of the Raman reporters, better spatial resolution and low signal-to-noise ratio within complex biological environment. For the enhancement of the Raman vibrational signal in SERS bioimaging, gold nanoparticles (GNP) are the most viable among metal nanoparticles because of comparable ease in controlling its size distribution and biocompatibility, among other parameters. GNP based SERS nanoprobes can be synthesised by tagging Raman reporter and conjugating with target specific biomolecules. Because of GNP's wide-ranging optical properties and narrow and distinct signal from SERS, other labelling methodologies like fluorescence microscopy, magnetic resonance imaging (MRI), etc. can also be implemented along with SERS bioimaging, by tagging fluorophores, magnetic nanoparticles, etc. This review focuses on various structures and shapes of GNP, fabricating GNP based nanoprobes and the multiplexing and multi-modality capability of GNP based SERS nanoprobes.

Keywords: Surface Enhanced Raman scattering (SERS); Bioimaging; Gold nanoparticle; Raman reporter; Fluorophores; Multi-modality imaging

Introduction

In recent years, surface-enhanced Raman scattering (SERS) has emerged as a versatile technique that can enhance the intensity of the vibrational spectra of a molecule by several orders of magnitude when in the close proximity of nano-roughened metal surfaces such as gold/silver or when it is adsorbed on to these metallic nanoparticles. These enhanced vibration spectra are often referred as the 'fingerprint' of the molecular species. Therefore, this technique is widely adopted for the identification of molecular information in complex biological matrices. Moreover, Raman imaging based on SERS tags has attracted growing interest as it has shown many advantages over other optical bioimaging techniques including the multiplexing capability due to the narrow width of Raman bands, better photostability of the Raman active molecules than fluorescent dyes, higher spatial resolution due to the confinement of the intensified electric field around the surface of the nanoparticles, and high signal-to-noise ratio in complex biological system at single wavelength excitation in the near infrared (NIR) region [1,2]. Furthermore, SERS modality could be combined with other imaging techniques such as magnetic resonance imaging and fluorescence microscopy to achieve multi-modal imaging capability by rational design of multi-modal nanoprobes. The central challenge in this field is to develop high performance SERS tags and realize high sensitive cell or molecular imaging. The schematic illustration of multi-modal SERS imaging using gold nanoparticles is presented in Figure 1.

Typically, the SERS tags are constructed by metal nanoparticles such as gold, silver, platinum, etc, which could enhanced electromagnetic (EM) field and amplify the efficiency of Raman scattering up to 12 orders of magnitude due to their localized surface plasmon resonance effect (LSPR). The LSPR not only depends on the type of noble metal, but also the shape and size of metal nanoparticle. Gold and silver colloidal particles are the popular substrates widely used for SERS studies [3,4]. However, despite silver nanoparticles having higher scattering cross-section resulting in large enhancement of Raman signal [5,6], it has shown to be highly toxic towards biological samples [6-8] because of its surface oxidising property. Gold nanoparticles (GNP) has been safely used to treat rheumatoid arthritis for 50 years and has exhibited better biocompatibility due to the chemical inertness with sizes above 5 nm [9,10]. Moreover the maximal light extinction of GNP can be artificially shifted from the visible to NIR region, thereby improving the optical penetration depth and avoiding possible auto fluorescence from biological samples [1,2,10]. In addition, the technique for the preparation and bioconjugation of various gold nanostructures with tunable LSPR has been well-established. Thus in this review, we will focus on SERS tags fabricated using various GNP.

SERS tags are realized by anchoring strong Raman active molecules (also known as reporter molecule, RM) on the surface of GNPs. Therefore, its sensitivity will inherently depend on the signal intensity generated by the Raman reporter molecules. The resonance Raman (RR) spectroscopy where the electronic transition of the molecule coincides with the scattered light could result in large increase in Raman signal by as much as a factor of 106. Thus, the electronic structure of RM and LSPR of GNP should be carefully selected to satisfy the resonance Raman condition. Most of the currently used RMs could only generate RR signal in the visible light region, while RMs in the NIR region is preferred for in vivo SERS imaging. Also,

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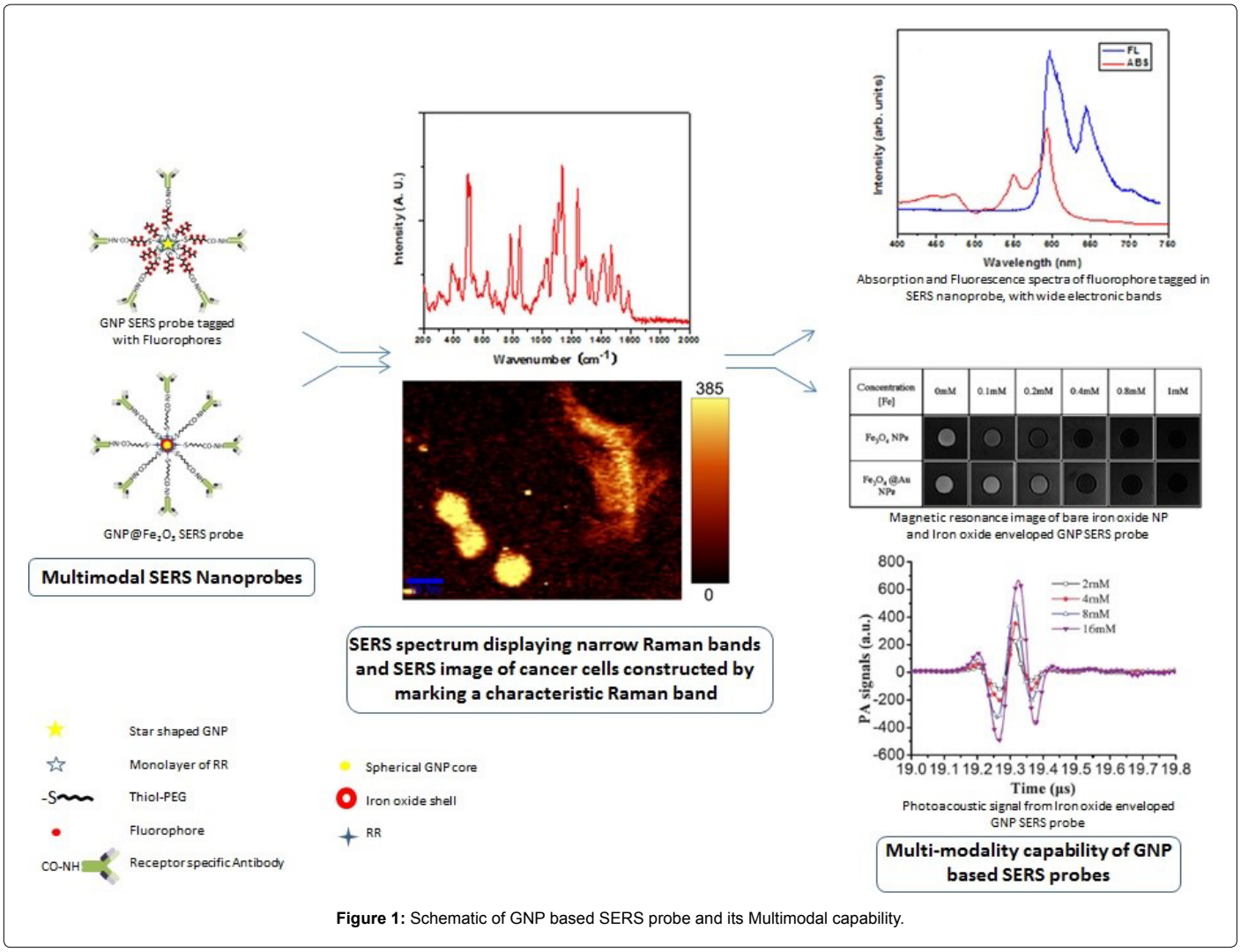

Figure 1: Schematic of GNP based SERS probe and its Multimodal capability.

the active RM satisfying the RR condition should have high chemical affinity towards metallic surface [11]. Few of the commercially available dyes reported to be used as RMs were mercaptobenzoic acid, crystal violet, rhodamine 6G, cyanine dyes Cy 3, Cy 3.5, Cy 5, texas red and diethylthiatricarbocyanine iodide (DTTC). The NIR SERS imaging is largely restricted by the availability of RM that can be excited by light in the NIR region. Olivo's group recently reported triphenylmethine and modified cyanine based Raman reporters that exhibited superior Raman vibrational signatures at NIR region [11-13] that will be further discussed later. Besides the RR selection criterion, the vibrational frequency of RM is also important. The preferential vibrational peak of RM should be around 2000 cm^{-1} to avoid possible peak overlapping from proteins and water. Further studies are necessary to develop novel RMs for future *in vivo* SERS imaging.

In order to improve the biocompatibility and stability of SERS tags and the conjugation of tumor-targeting ligands proper surface coating, termed as surface functionalization, is required. The popular coating agents includes (poly) ethylene glycol (PEG), silica, bovine serum albumin (BSA), etc. [1,2,9]. The SERS tags would then have to be bio functionalised by conjugating with target receptor specific biomolecules such as single-chain variable fragment (ScFv) antibodies. Similar surface functionalization has been widely studied for quantum dots (QDs) fluorescent tags. However, since it is an inevitable step for the practical application of SERS tags, herein we also review the surface functionalization of GNPs to target tumours.

Gold nanoparticles and its role in SERS imaging

Spherical GNP: Spherical GNPs are one of the well-studied SERS substrates although their SERS enhancement is only moderate. Aggregation of spherical nanoparticles produces nanogaps between particles, known as "hot spots" where the LSPR greatly increase resulting in significant enhancement in SERS intensity. Aggregation of particles can be easily achieved but controlling the particle aggregation has been shown to be difficult [1,14]. Few research groups have successfully addressed this issue by enveloping the spherical GNPs with protein-A [15,16] and a polymer polyacrylamide [14], and the Raman enhancement factor (EF) increased with the aggregation size.

By using SERS tags, Lucas et al. reported imaging the distribution of epidermal growth factor receptor (EGFR) in epidermoid cancer cells, which over-expresses EGFR [17]. His work made use of the Raman signal enhancement of malachite green by spherical GNP sized around 30-34 nm in average. The SERS spectra were collected in x- and y- direction with step size of 300 nm with an accumulation time of 1 s using 633 nm excitation line. Two separate SERS images were

constructed, (Figure 2) by marking the intensity of Raman peaks of the RM around 1583 and 1450 cm^{-1}. Another cell line that does not express EGFR was used as a negative control. The SERS image showed good colour contrast between positive pixels (spots that showed good intensity of the concerned Raman peaks) that marked the area around endosome and cell membrane, and negative pixels that marked other regions within and outside the cells. This SERS mapping was found to be consistent with the expected biodistribution of EGFR within epidermoid carcinoma cells.

Thus obtained SERS images had an interesting feature, that the SERS spectra of the various positive pixels displayed different degrees of Raman enhancement. The possible reason, as reported, might be that the EGFR was broken down within endosomes that resulted in change in environment immediately around the GNP and the level of enhancement varied [3] from EGFR-tagged GNP around cell membrane. This implies that the SERS spectra reflected the distribution of aggregated GNPs, which holds true to the fact that spherical GNPs tend to aggregate easily [14].

Gold nanorods: Gold nanorods (GNR) based SERS was reported to have been used for imaging ovarian cancer tissue, with the goal of identifying tumour margin and monitoring the removal of tumour during resection [18]. In this case, SERS spectra were collected using 785 nm laser excitation and the mapping scans were collected for 15 minutes with 500 micron resolution. SERS mapping was constructed using least square analysis by comparing the SERS intensity at

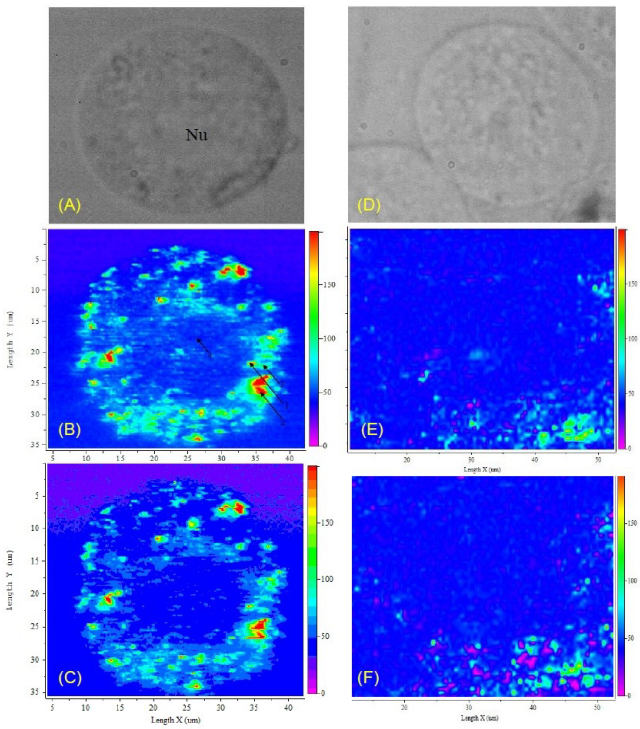

Figure 2: Spherical GNP as SERS tag: (A) White light image of an A431 cancer cell incubated with 30 nm anti-EGFR labelled GNP; (B) SERS image of the cancer cell obtained by an intensity map of the 1583cm-1 peak; (C) SERS image of the cancer cell obtained by an intensity map of the 1450cm-1 peak; (D) White light image of an NHBE normal cell incubated with 30 nm anti-EGFR labelled GNP; (E) SERS image of the normal cell obtained by an intensity map of the 1583cm-1 peak; (F) SERS image of the normal cell obtained by an intensity map of the 1450cm-1 peak [18].

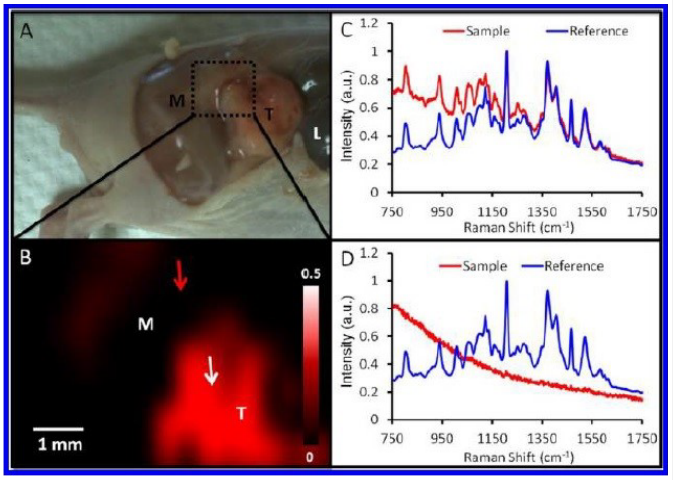

Figure 3: Tumour margin identification with SERS imaging: (A) White light image of the mouse bearing tumour, with epidermis removed. (B) SERS image of the area indicated in (A). SERS image was constructed by comparing the SERS spectra at different spatial locations (red curve in (C) and (D)) with a reference spectra (blue) collected ex-vivo. Close match of the sample and reference spectra yields an intense signal in the SERS image (white arrow) and a poor match with much lighter signal (red arrow) [19].

characteristic 1202 cm^{-1} peak of IR792 dye before and after injection of nanotags into tumour tissue. As shown in Figure 3, the SERS image thus constructed correlated well with the white light image of the tumour, in which the brighter pixels corresponds to the well matched SERS intensity. When the tumour is gradually resected, the SERS signal went down as well, imaged with lesser positive pixels – 65.7% before resection and 6.2% after resection.

GNP in core-shell and multi-branched configuration: To make use of the advantages of both gold and silver nanoparticles, Ag/Au core-shell nanostructures have recently gained prominences which are found to be stable, biocompatible and exhibit excellent LSPR properties. Guo et al. recently reported the Raman enhancing property of Ag/Au core-shell nanostructures in SERS bioimaging [19]. By coating methylene blue as RM on SNP and then covering with a protective layer of gold, such SERS tag could not only be able to detect Raman enhancement of methylene blue by SNP, but also the Raman enhancement of biomolecules in the immediate vicinity of GNP surface [19,20]. With 785 nm excitation, two different SERS images was constructed by mapping the Raman peak of methylene blue at 1602 cm^{-1} and Raman peak of proteins at 1254 cm^{-1}. These SERS images not only can localize the nanostructures within cells, but also enable sensitive and locally confined probing of cellular structures.

One merit of hiding the RM between layers of metallic NPs is that the outer shell prevents desorption and degradation of RM, thereby stabilising the SERS signal. Similar to the aforementioned study, Gandra et al. synthesised plasmonic core-shell nanostructure with dual layer of gold with the RM sandwiched in between. Apart from protecting the RM-1, 4-benzenedithiol (BDT), this nanostructure called Raman-intense gold nanostructures with hidden tags (BRIGHT) also displayed in-built hot spots (with constructive EM interference from 2 layers of gold) [21].

After stabilising the nanostructure with PEG, SERS spectra were collected using the excitation of 785 nm laser with the power of 3 mW

and exposure time of 1 s. Intra-cellular SERS image of BRIGHT was then constructed by mapping BDT's Raman peak at 1078 cm^{-1} and the image revealed uniform distribution of BRIGHT around the cell surface, as shown in Figure 4. Individual SERS spectrum of BRIGHT from different spots within cells showed remarkable uniformity, due to its in-built hot-spots.

Gold nanoshells have been experimentally shown to yield higher SERS intensities compared to similar sized nanospheres. This shows the shape dependence of LSPR, which tends to red-shift and increase in intensity with complex geometries. Also the extinction cross-section of nanoparticles in general increase with increasing size, with the contribution of scattering dominating over absorption. This shape and size dependence of LSPR, and thus the SERS capability, has been experimentally shown on various nanostructures including nanospheres, nanoshells and nanorods [1].

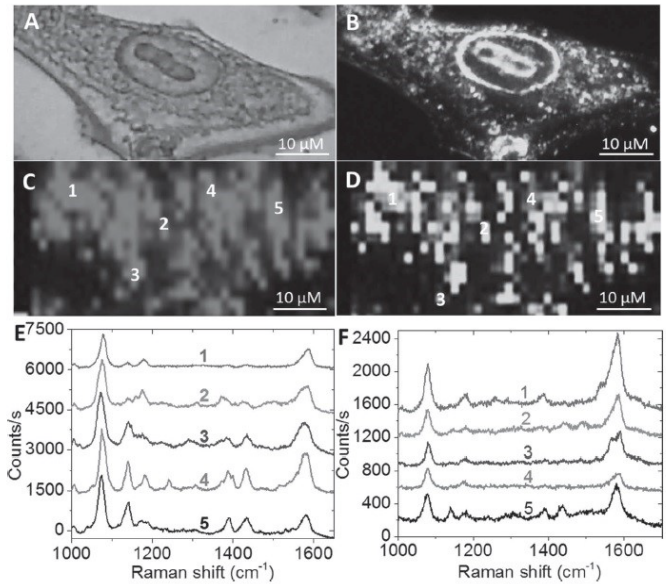

Figure 4: Plasmonic core-shell GNP as SERS tag: Bright-field (A) and Dark-field (B) images showing intense Rayleigh scattering from BRIGHTs. SERS images mapped with 785 nm excitation (C) and 633 nm excitation(D). (E and F) Representative SERS spectra of the spots marked in (C) and (D) respectively, showing higher SERS intensity with 785 nm (E) excitation than with 633 nm (F) [22].

Multi-branched star shaped GNPs have also been synthesised, the use of which as plasmonic nanostructures has two important advantages. Firstly, their LSPR falls in the longer wavelength region of the spectrum thereby avoiding majority of autofluorescence signal. Secondly, the multiple sharp branches act as hot spots for enhanced EM due to lightning rod effect without the need for aggregation. Such plasmonically active gold nanostars synthesised by Schutz, et al. (Figure 5) was successfully used as SERS tag after conjugating with the derivatives of DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) [22]. The colloidal nanostars were stabilised by self-assembled monolayer (SAM) of ethylene glycol conjugated DTNB derivatives. SERS spectra were obtained using 633 nm laser excitation; the resulting Raman scattering were found to be in resonance with the broad surface plasmonic band of the nanostars which is around 670 nm. SERS tags were then bio-functionalised with anti-p63 antibody for targeting the tumour suppressing p63 antibody in prostate tissue. SERS image was constructed by marking the RM's Raman band at 1340 cm^{-1} and the mapping was performed with accumulation time of 0.1 s and step size of a micron. As shown in Figure 5, SERS pseudo-colour image displayed positive pixels (signal from anti-p63 tagged SERS label) in the basal epithelium of the benign prostate tissue, thereby locating the abundance of p63 antibody; individual SERS spectra from different regions confirmed the same. Yuan et al. and Luo, et al. also reported separate SERS studies using multibranched GNPs using different synthetic strategies [23,24].

The nanoparticle LSPR's dependence on shape and size is reiterated over a comparative study by Jain et al., where the Raman enhancement by gold nanoparticles of three different shapes and sizes was analysed.

Raman reporters

As mentioned earlier, ideal Raman reporter that can elicit stable signal in/near the NIR region and high affinity towards metallic nanoparticles are urgently needed. Raman active dyes like crystal violet (CV) and malachite green isothiocyanate (MGITC), which has a triphenylmethine (TM) core, found wide usage as RM during early periods of SERS as a sensitive detection technique. Their absorption maxima in visible region around 600 nm, limited NIR Raman enhancement. At the same time, their relative high toxicity [13,25]

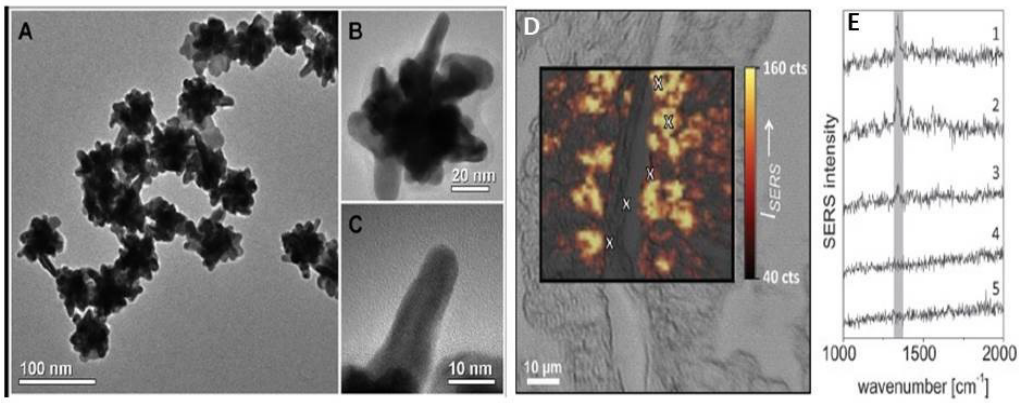

Figure 5: Multibranched GNP as SERS tag: TEM images of 60 nm gold nanostars (A), a single gold nanostar (B) and one of its tips (C) at larger magnification, (D) White light image of a prostate tissue specimen with an overlaid SERS false colour image based on the intensity of the 1340 cm-1Raman marker band of the SERS label. (E) Five representative SERS spectra from different locations in (D), indicated by white crosses in the SERS false colour image (from top to bottom). [Reprinted with permission from RSC Publications [23].

lead researchers to develop new and safe Raman active compounds in NIR region with better Raman characteristics. Cho et al. reported a combinatorial approach in synthesising TM based library of compounds and screening of those compounds revealed thirteen of them displayed stronger SERS signal than CV [13]. Maiti et al. took one such TM-based compound (B2) and linked it to lipoic acid (LA) for better chemisorption onto 60 nm GNPs (B2LA nanotag) [26]. LA linked B2 (B2LA) exhibited better stability of SERS signal over a period of time than MGITC. After bio-functionalising with EGFR and HER2 antibodies, animal studies substantiated the sensitivity and stability of B2LA nanotag. As an extension of this work, Maiti et al. also compared B2LA with another LA linked Raman active cyanine based dye, Cy3LA [13]. This study showed the multiplexing capability of both the Raman active dyes, which showed the Raman compatibility under a single laser excitation at 633 nm.

To further utilise the near-infrared window where light has maximum depth of penetration in biological tissues, RMs in this range were investigated. When the RM has electronic transition around the excitation wavelength of the laser (RR condition), it further improves the Raman enhancement and is called surface enhanced resonance Raman (SERRS). IR-792 is one such NIR RM that was used successfully for SERRS by Jokerst et al. [18], with 785 nm excitation. But its weak non-covalent adsorption on to GNP leads to its elimination during the ligand exchange reaction using PEG (or any biocompatible stabiliser) for stabilising the nanoparticle [27].

DTTC (3,3'-diethylthiatricarbocyanine iodide) is another efficient NIR RM [28] that is stable enough after bio-functionlisation and elicit good enhancement of Raman signal even after incubation within cells [4]. By a combinatorial approach, Samanta et al. screened 80-member tricarbocyanine library of compounds that revealed six aromatic amine containing derivatives (marked as A5, A6, C6, C8, E9 and G9 in Figure 6) had higher SERS intensity than DTTC [29]. Further analysis of the aforementioned six derivatives identified that a tricarbocyanine derivative – lipoic acid linked amine acetylated tricarbocyanine-381 (CyNAMLA-381) – had 12-fold higher sensitivity than DTTC. CyNAMLA-381 had absorption maximum around 800 nm, close to that of the excitation wavelength of 785 nm. CyNAMLA-381 was tagged onto 60 nm GNP and was stabilised with bovine serum albumin (BSA). Analysis of the stability of SERS intensities across the CyNAMLA library revealed that CyNAMLA-381 showed better intensity and consistent stability of SERS signal over a period of a month. Using gluteraldehyde, Her-2 antibody fragment (scFv) was then conjugated with BSA encapsulated CyNAMLA-381 – GNP (CyNAMLA-381) nanoprobe and the entire nanoconstruct was then incubated with Her-2 positive breast cancer cells. Strong SERS signals were observed, whereas negligible SERS signal observed when the nanoconstruct was incubated with Her-2 negative cells. Equally good Raman enhancement of CyNAMLA-381 was observed when CyNAMLA-381 nanoprobe incubated cells were xenografted subcutaneously into nude mice [29].

Multiplexing and multimodality capability of GNP

Unlike molecular fluorophores, SERS nanoprobes suffer negligible loss in signal over time and do not suffer from saturation conditions. The linewidth of the vibrational Raman bands are much narrower than electronic absorption bands of fluorophores, thus making it possible to simultaneously detect spectral fingerprints of estimated 10-30 SERS reporters [1]. Neng et al. reported the detection of surface envelope and capsid of two different viruses based on multiplexed SERS spectroscopy [30]. The detection was based on antibody recognition using nanoprobes - RM coated onto GNP and paramagnetic particle that are conjugated with two antibodies specific for each antigen target. When excited at 785 nm, Raman enhancement of Nile blue (NB) and Infrared-792 (IR-792) were detected and their spectral signature was preserved when both the nanoprobes were mixed. Multiplex assay reaction was performed by incubating the sample containing both NB and IR-792 based nanoprobes and magnetically concentrating the nanoprobes for laser excitation. Detection specificity was based on the characteristic peak of NB at 591 cm^{-1} and that of IR-792 at 556 and 524 cm^{-1}. Though the characteristic peak of NB and IR-792 falls within a small range, the narrow vibrational Raman bands enabled the combined spectra to have three distinct peaks with no spectral overlap.

Quantitative multiplexing of four NIR RM was also achieved using bovine serum albumin (BSA) stabilised multibranched gold nanoparticles (Nanostars) as the core. In multiplex SERS detection, early methods like simple peak comparison and spectral fitting will not be suitable for NIR SERS spectra because of the fluorescence background

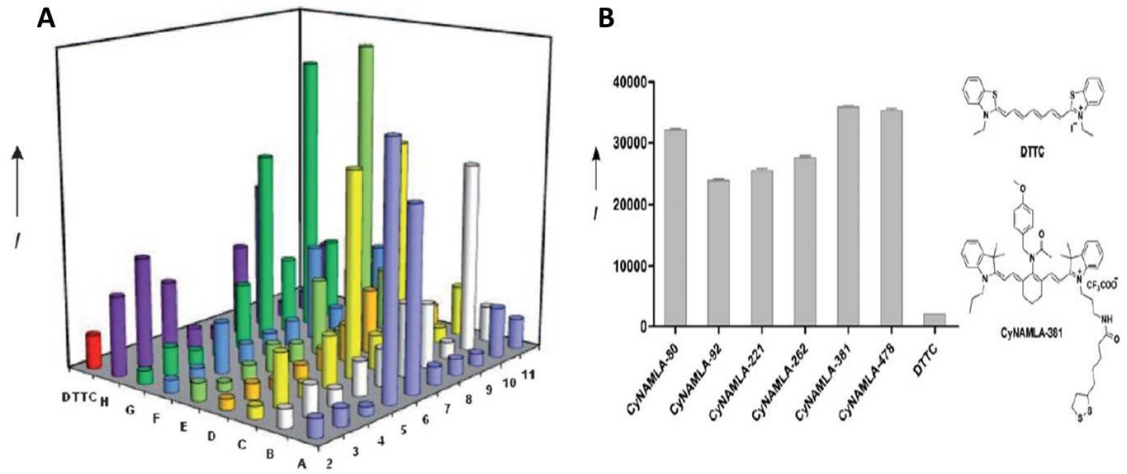

Figure 6: Tricarbocyanine derivatives as Raman reporters: (A) Comparative SERS intensities of the entire CyNAMLA library, with intensity of DTTC, as reference, plotted in red. All the SERS spectra were acquired with excitation at 785 nm using a 60 mW laser. (B) SERS intensities of the six selected aromatic amine containing BSA-encapsulated CyNAMLA coated GNPs, and organic structures of DTTC and CyNAMLA-381 [30].

and complex peak overlaps of the RMs. To quantify the signal fractions of individual RM, Yuan et al. used spectral decomposition method based on least-squares regression and free-fitting polynomial. The measured signal fractions thus obtained, as shown in Figure 7, were in good agreement with the pre-determined probe ratios [23].

By conjugating GNP with fluorophores or magnetic particles, SERS can be used simultaneously with other modalities like fluorescence microscopy, magnetic resonance imaging, computed tomography, etc. Ciu et al. synthesised such a dual-modal nanoprobe containing spherical GNP as core and coated MGITC as RM and stabilised with silica shell that is conjugated with a fluorophore, FITC (fluorescein isothiocyanate). Raman signal decreased in intensity with increasing shell thickness which may be because of the reduced light transmission; fluorescence signal increased with increasing thickness, which the researchers owed to the increased amount of FITC molecules. But the fluorescence quenching capability of the metallic NPs was not considered and that might be an important reason in that increased shell thickness resulted in increased spacing between fluorophore and GNP surface thereby retaining its fluorescence. The confocal laser scanning microscope (CLSM) images showed the internalisation of the nanoprobes in cytoplasm of the HeLa cells (cervical cancer), even though the nanoprobes were not targeted towards any specific receptor indicating passive uptake by the cells. SERS spectroscopy then revealed stable Raman signal of MGITC, from spots that showed intense fluorescence signal, with no obvious interference from FITC [31].

Since GNP have higher X-ray attenuation coefficient due to high electron density on its surface, the contrast between soft tissues it provides enhances the sensitivity in cancer diagnosis. Thus GNP can be used as a contrast agent for computed tomography (CT) based whole body imaging, along with Raman enhancing core for SERS detection. One such proof-of-principle study demonstrated the potential of GNP based nanoprobe for dual-modality CT and SERS imaging of tumours [32]. Different sizes of quasi-spherical GNP were tested and GNP with diameter of 40-60 nm displayed better X-ray attenuation than Iohexal, a standard iodine-based clinical contrast agent. Dual modal nanoprobes were synthesised by coating DTTC onto GNP and further stabilisation using SH-PEG-COOH, with thiol group enabling steric adsorption on GNP's surface and carboxylic group enabling further conjugation with biomolecules that preferentially conjugates with cancer-specific biomarkers. In-vivo dual modal imaging was demonstrated by injecting nanoprobes with 65 nm GNP core subcutaneously into nude mice, as shown in Figure 8. With whole body CT scan at 24 hour post-injection, spleen was clearly visible with GNP based nanoprobes as contrast agent, and its internalisation and preferential accumulation within endosomes was confirmed by TEM images of the spleen tissue slices. At 785 nm excitation, SERS spectra were recorded in those positive areas and the characteristic Raman bands of DTTC was retained. This dual-modal capability will enable the physicians to localise the tumour using whole body CT imaging and analyse the tumour at molecular level using SERS imaging by profiling cancer-specific biomarkers.

Iron oxide nanoparticles (Fe_3O_4) have been used as contrast agent for MRI and in microwave induced thermoacoustics due to their strong absorption of microwaves. Along with GNP's biocompatibility and plasmon properties, when Fe_3O_4 nanoparticles are conjugated with GNP the complementary strengths of different modalities can be taken advantage of for various biomedical applications. Zhou et al. synthesised such hybrid nanoprobe, Fe_3O_4 core with GNP shell (Fe_3O_4@Au NP) that had stable structure and controlled size distribution [33]. When excited with 515 nm laser, Raman signal of 4,4'-dipyridyl was enhanced by GNP in Fe_3O_4@Au NP, though the SERS intensity was less compared to 4,4'-dipyridyl coated onto bare GNP. The T2-weighted MR images of Fe_3O_4@Au NP were comparatively lesser in intensity and the relaxivity value was weaker compared to Fe_3O_4 NP, due to the weaker magnetic property because of the presence of gold nanoshell. But the Fe_3O_4@Au NP had comparable contrasting property as other Fe_3O_4@Au nanoconstructs have longer circulation time, and thus longer imaging period than clinically used gadolinium complexes. The capability of Fe_3O_4@Au NP as an effective contrast agent for microwave induced thermoacoustic tomography has also been demonstrated. The thermoacoustic signal had a linear dependence on iron concentration and the intensity of the signal reached 180% of that of water at iron concentration of 20 mM. Considering that high contrast thermoacoustic image can be constructed when the signal is 1.5 times that of water, Fe_3O_4@Au NP evidently has potential as contrast agent for microwave induced thermoacoustic tomography. FITC tagged integrin $\alpha_v\beta_3$ antibody was then conjugated with Fe_3O_4@Au NP for the purpose of studying the internalisation of the nanoprobes within integrin $\alpha_v\beta_3$ overexpressing human glioblastoma cells (U87-MG). Confocal

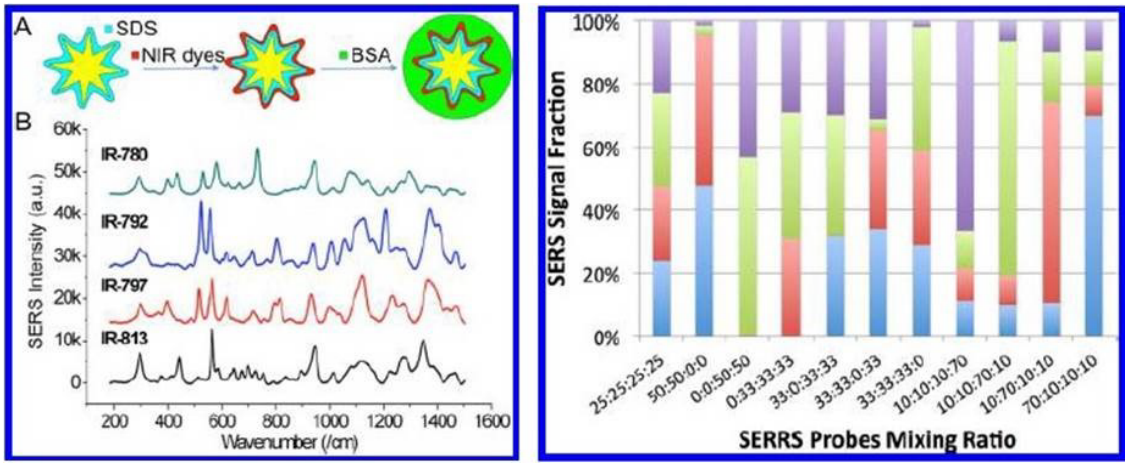

Figure 7: Multiplex SERS spectra of four RM: (A) Schema of star-shaped GNP, coated with NIR dyes and stabilised with BSA. (B) SERS spectra of four different NIR dyes used in this multiplex assay. (C) Quantification of SERS intensities of four nanoprobes used in multiplex SERS assay; blue: IR-780, red: IR-792, green: IR-797, purple, IR-813[24].

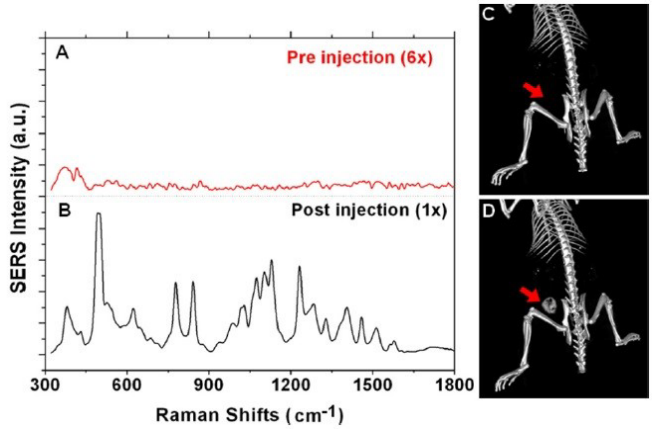

Figure 8: *In vivo* dual modal SERS imaging with CT: (A) and (B) are recorded SERS spectra pre- and post-injection of 60 nm sized GNP –based nanotags. (C) and (D) 3D CT images of pre- and post-injection respectively. The arrows in (C) and (D) point to the site of injection and CT contrast generated in spleen [33].

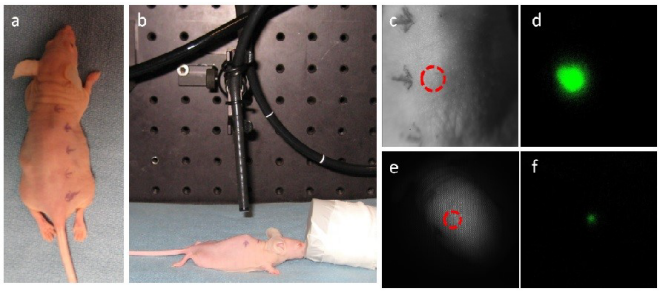

Figure 9: Endoscopic Raman imaging: (a) Arrows indicate injection sites of serially diluted nanotags. (b) mouse positioned for endoscopic SERS imaging. (c) and (d) white light and SERS wide-field images using epi-imaging mode respectively. (e) and (F) white light and SERS wide-field images using endoscopic mode respectively [19].

fluorescence microscopy images showed high fluorescence signal from U87-MG cells but no evident fluorescence in MCF-7 cells that express very few integrin $\alpha_\gamma\beta_3$ antibody [33]. Though it can be seen that the SERS capability of GNP and the magnetic property of Fe_3O_4 NP was hampered to a small extent by the GNP nanoshell enveloping the Fe_3O_4 core, the structural stability, circulation time in tumour neovasculature and enough detectable signals of Fe_3O_4@Au NP has shown promise as nanoprobe for multi-modality bioimaging.

Afore mentioned multi-modality studies proved that SERS can be an effective adjuvant technique for optical biopsy, although Raman analysis is restricted to single spot focus. Background fluorescence can be reduced by taking advantage of the narrow vibrational Raman bands, the enhanced intensity by SERS and the possibility of target specific imaging by conjugating with antibodies and peptides, and thus making it possible the conception of wide-field Raman imaging [34]. McVeigh et al. modified the optics with NIR corrected objective lens for epi-imaging mode that can be exchanged for a fibre bronchoscope for endoscopic mode, as shown in Figure 9. By exciting with a 785 nm laser, in-vivo imaging detected picomolar concentration of nanoprobes against broad inherent autofluorescence. This study showed that by coupling a narrow bandpass filter to the clinical bronchoscope, wide-field SERS images can be acquired at sub-video rates.

Surface functionalization

As discussed earlier, aggregation control of isotropic nanostructures is important for their plasmonic properties, as 'hot spots' form only as result of plasmon coupling between adjacent nanoparticles. Stabilisation of NP's surface is thus of prime importance, even for aninsotropic nanostructures, which are also prone to solvent derived aggregation and may precipitate. Encapsulation also prevents the desorption of the RR and adsorption of undesired biomolecules in the close vicinity of GNP's surface [1]. Various biopolymers like protein [15,16], DNA [35], organic polymers like polyacrylamide [14], PEG [21,27,32,36], BSA [23,30,37], silica [1,38], were reported to have been used to encapsulate GNP, thereby stabilising the structure. But to enhance biocompatibility, PEG and BSA have been exploited. Thiolated PEG (SH-PEG) covering has been shown to stabilise GNP and shield RR due to the specific adherence of thiol compounds on GNP's surface that spontaneously forms a monolayer on gold [21,36]. But BSA was found to be more advantageous as the -thiol binding is much stronger that maintained the non-aggregated isolated state of the nanostructures even after multiple washes [23,29]. Nanoparticle tracking analyses (NTA) confirmed the same and also showed that PEG stabilised nanoconstructs tend to form small clusters after multiple washes [23]. Along with the favourable plasmonic properties of GNP core, NIR absorption and narrower vibrational Raman bands of RR and steric stabilisation of the nanoconstruct with biopolymer, bioconjugation of the SERS nanotags to tumour specific biomarkers also plays an important role in enhancing the sensitivity of SERS bioimaging. EGFR is often over-expressed in most of the epithelial cancers and is the widely targeted biomarker along with Her-2 receptor (another receptor in the same epidermal growth factor (EGF) family), a well-known breast cancer biomarker. These biomarker specific proteins (usually monoclonal antibodies) can be conjugated to the stabiliser or, in the absence of encapsulation, conjugated directly with the RM. EDC/NHS chemistry is the widely used method for bio conjugating antibodies with biopolymers. The carboxyl terminal of PEG/BSA was first activated by adding N-ethylcarbodiimide (EDC) and N-hydroxysuccinamide (NHS) and equal concentration of antibody will then be added; thus bio functionalised stabiliser (PEG/BSA) will then be added to GNP coated with RM [21,22]. Recent studies that reported GNP based SERS bioimaging of tumours mostly used antibodies for aforementioned receptors.

Summary

Synthesis of GNP SERS nanotags for bioimaging should thus consists of following steps: 1) synthesis of GNP core (preferably anisotropic) for Raman enhancement, 2) coating of Raman reporter with characteristic vibrational Raman bands that absorb light in NIR region, 3) surface stabilisation (and RM shielding) using thiol-PEG or BSA, and 4) conjugation of target specific biomarker. Future direction on SERS based bioimaging should primarily concentrate on optimising the aforementioned steps in SERS nanotags fabrication, as there is no consensus yet on a gold standard process. An area of research that would take SERS bioimaging closer to clinical utility would be testing for very minimal or no toxicity of all components of the SERS nanotags.

Multi-modal approach for therapy and diagnosis (theranostics) is considered the way forward. As the possibility of integrating SERS nanoprobes with other modalities has been demonstrated as proof-of-concept studies (as explained in Section 4), research has to be focussed on comparative analysis of various SERS nanotag constructs that elicit optimised signal when multiple modalities were tested simultaneously. For example, comparative analysis of SERS enhancement from

spherical GNP aggregation and hot spots from individual anisotropic GNP, keeping in mind the optimal multi-modal signal generation (fluorescence or magnetic property) from both the nanoconstructs.

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