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Review Article

In vivo and *ex vivo* applications of gold nanoparticles for biomedical SERS imaging

Mehmet V Yigit, Zdravka Medarova

Molecular Imaging Laboratory, Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129

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Abstract: Surface enhanced Raman scattering (SERS) is a signal-increasing phenomenon that occurs whenever Raman scattering on a metal surface is enhanced many orders of magnitude. Recently SERS has received considerable attention due to its ultrasensitive multiplex imaging capability with strong photostability. It provides rich molecular information on any Raman molecule adsorbed to rough metal surfaces. The signal enhancement is so remarkable that identification of a single molecule is possible. SERS has become a genuine molecular imaging technique. Gold nanoparticles, encoded with Raman reporters, provide a SERS signal and have been used as imaging probes, often referred to as SERS nanoparticles. They have been used for molecular imaging *in vivo*, *ex vivo* and *in vitro*. Detection of picomolar concentrations of target molecules has been achieved by functionalizing the nanoparticles with target recognition ligands. This review focuses on recent achievements in utilizing SERS nanoparticles for *in vivo* molecular imaging. In the near future, SERS technology may allow detection of disease markers at the single cell level.

Keywords: SERS, gold nanoparticle, Raman, molecular imaging, cancer, SERS nanoparticle

Introduction

Applications of nanotechnology in different parts of our life are expanding significantly -- especially in biomedicine. Nanotechnology offers great promise in both biomedical imaging and drug delivery. Metallic nanoparticles have different physical properties from those of their bulk state [1-3]. These physical properties have been examined thoroughly by physicists and chemists and the underlying differences have been identified. Engineers, biologists and medical scientists have been quick to exploit the advantages of nanotechnology as evidenced by the increasing number of publications, patents and commercial products in the last decade [4]. For instance, iron oxide nanoparticles have been used for cellular separation [5-9] as biosensors [10, 11], drug delivery vectors [12-14], contrast agents [15, 16], and therapeutic components [17, 18]. Quantum dots have been used for single molecule imaging of individual proteins [19], biological sensing [20] and *in vivo* biomedical optical imaging [21-24]. Other metals form suitable nanoparticles including gadolinium [25], silver [26] and gold [27]. Among all the metallic nanoparticles used so far, gold nanoparticles hold a particularly important place. Their facile synthesis and bioconjugation procedures, along with gold's unique surface plasmon properties, made gold nanoparticles practicable in labs without expensive or sophisticated equipment.

Gold nanoparticles have unique physical properties; they shift their surface plasmon peak between the dispersed and aggregated state, which can be observed by the naked eye [28, 29]. This feature is largely used for designing colorimetric sensors with gold nanoparticles [28-30]. By putting a recognition element on the surface of gold nanoparticles (with thiol and gold chemistry), these particles become especially useful in bio-sensing applications. Besides the improvements garnered in sensing applications there is also a significant effort in using gold for *in vivo* biomedical imaging and delivery purposes [31].

Gold nanoparticles have another unique property; they enhance the Raman signals of adsorbed dye molecules on their surface [32]. This

signal enhancement, which is referred to as Surface Enhanced Raman Scattering (SERS), has been reported to be as high as 10^{14} to 10^{15} fold [32-34]. The Raman reporters adsorbed on the gold surface are protected by a polymeric or silica coating. This ensures the prolonged adsorption of the Raman tags on the gold surface and the stability of the nanoparticle [35, 36]. With this enhancement not only can picomolar amounts of target analyte be detected, but also a single target molecule can be identified [37]. Due to such a capacity for enhancement, it has been shown that these nanoparticles are significantly brighter than quantum dots in near-infrared spectral region [35]. Since SERS offers picomolar sensitivity and has multiplexing capability, it will attain an important role in the molecular imaging field [38].

SERS provides detailed spectroscopic information, which can be translated into imaging signal and adapted to an *in vivo* imaging system [35]. Although the SERS nanotechnology is still in its infancy, due to its extreme sensitivity and the spectroscopic information it provides; it has captured many researchers' attention [39, 40]. It has a deep tissue light penetration challenge, but the extraordinary signal enhancement generated by SERS gold nanoparticles makes it still very attractive. Gold nanoparticles are generally considered safe and have been safely administered to humans [41] and used in clinical trials of cancer therapy (http://www.cytimmune.com). Considering that high dosages of gold nanoparticles have been widely and safely used as contrast agents for Computed Tomography (CT) [42, 43], the amount of gold nanoparticles required for SERS will not be a serious concern. Moreover, the fact that nanoparticle systems have been widely used for both imaging and therapy [44] this type of enhanced signal from a SERS nanoparticle platform holds remarkable potential for image-guided therapy [45, 46].

In this review we will focus on applications of gold nanoparticles for *in vivo* or *ex vivo* biomedical SERS imaging. Efforts in transforming nanoparticles into targeted multiplexed contrast agents are expanding and we believe this trend will continue.

In vivo imaging applications

SERS nanoparticles for in vivo multiplexed imaging

One element of design, when using SERS nanoparticles for molecular imaging, is the selection of Raman reporters. Different Raman reporters adsorbed on the rough gold surface provide different Raman spectra. This enables us to design SERS nanoparticles with more easily interpretable spectroscopic information. By simply changing the adsorbed Raman tags on the gold surface, different SERS nanoparticles [36] with a multiplexed imaging property can be created. In one study Gambhir and coworkers designed ten different SERS nanoparticles. Each one was composed of a gold core, a different Raman label and silica coating [36]. Each SERS nanoparticle produced a distinct Raman spectrum in solution. The authors sought to test the bioavailability and the signal generating capability of these nanoprobes *in vivo*. After injecting the SERS nanoparticles subcutaneously (s.c) in nude mice, they obtained ten different optical signals consistent with those obtained in solution. The signals could be separated using spectroscopic information (**Figure 1**).

After identifying the brightest SERS nanoparticles, the authors tested their ability to read signals in deep tissue. Five of the brightest nanoparticles were administered intravenously (i.v.) in order to observe their spectral separation in the liver where they naturally accumulate. Each of these five SERS nanoparticles was both identifiable and resolvable in the liver using an optimized *in vivo* Raman system. The authors were also able to correlate the signal intensity with the injected dosage. Due to the signal enhancement achieved, nanoparticle accumulation in deep tissues could be measured semi-quantitatively. The authors concluded that simultaneous noninvasive imaging of multiple diseases would be possible by combining the ultrasensitivity of Raman spectroscopy with the multiplexing properties of SERS. This work is important for molecular imaging as it delivers information about multiple different anatomical or physiologic phenomena by using the same nanoparticle template.

In vivo molecular targeting of cancer markers

In vivo administration of nanoparticles for tumor imaging or therapy utilizes either of two targeting methods, active or passive. In active targeting, nanoparticles are functionalized with a targeting moiety for receptor mediated uptake by over-expressed surface antigens on cancer cells

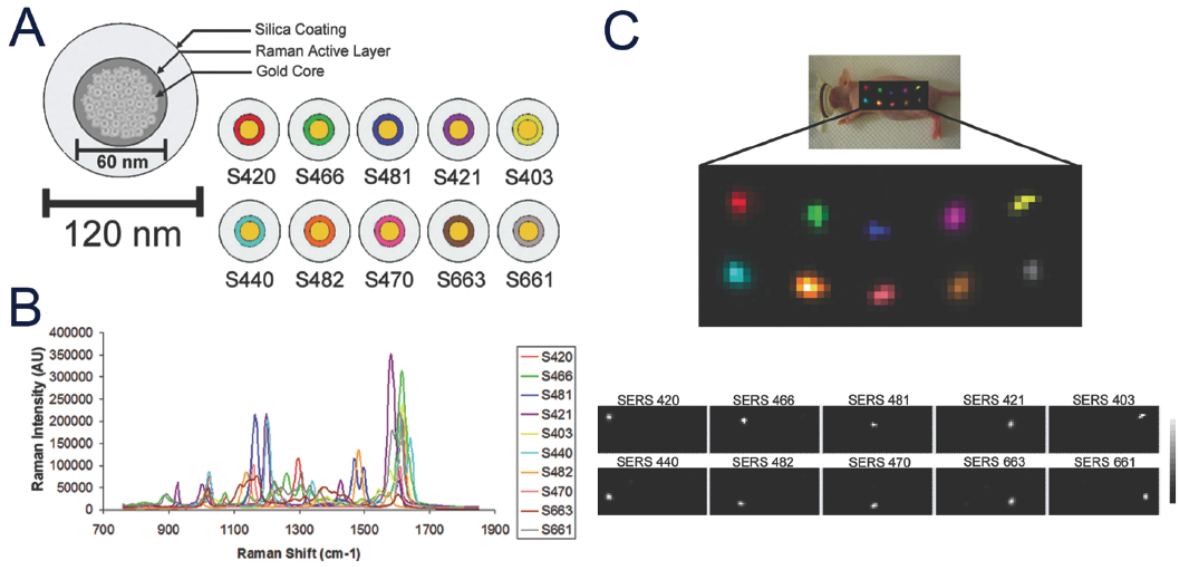

Figure 1. Schematic representation of a SERS Raman nanoparticle and graph depicting unique Raman spectra associated with each of the 10 SERS nanoparticles. (A) Schematic of a SERS Raman nanoparticle consisting of a 60-nm gold core with a unique Raman active layer coated with glass. The trade name of each SERS nanoparticle is depicted to the right, where a color has been assigned to the Raman active layer of each SERS nanoparticle. (B) Graph depicting Raman spectra of all 10 SERS nanoparticles; each spectrum has been assigned a color corresponding to its unique Raman active layer. (C) Evaluation of multiplexing 10 different SERS nanoparticles in vivo. Raman map of 10 different SERS particles injected subcutaneously (s.c.) in a nude mouse. Arbitrary colors have been assigned to each unique SERS nanoparticle batch injected. Panels below depict separate channels associated with each of the injected SERS nanoparticles (S420, S466, S481, S421, S403, S440, S482, S470, S663, and S661, respectively). (Reprinted with permission from Ref 36 by Proc Natl Acad Sci, USA).

[47]. In passive targeting, uptake is achieved by nanoparticle escape through leaky vasculature -- an enhanced permeability and retention effect (EPR) [48-50]. Both of these methods have been widely used. However some researchers report that active targeting results in better therapeutic effect due to receptor-mediated uptake [51, 52]. Others prefer passive targeting since active targeting risks loss of the probe to the reticuloendothelial system (RES) whenever incorporated ligands on the nanoparticle surface bind to blood proteins nonspecifically [53, 54].

In one study authors used active targeting to specifically direct gold nanoparticles into human tumor xenografts, subcutaneously implanted in nude mice. The authors reported that active targeting achieved more nanoparticle accumulation in the tumor they examined [35]. In this study, gold nanoparticles functionalized with Raman labels were used for SERS imaging in order to validate the nanoparticle accumulation in the tumor. The nanoparticles were synthesized with gold ion salts. After nanoparticles with an optimum size for SERS were obtained, they were engineered as SERS probes. The nanoparticles were mixed with Raman reporter molecules, which were then covered with polyethylene glycol PEG molecules for: (a) protecting the nanoparticles from aggregating; (b) sealing the Raman tags onto the gold surface; (c) providing a terminal functional group for further functionalization with a targeting moiety; and (d) increasing the circulation time in the blood stream.

As a last step, nanoparticles were functionalized with ScFv B10, an antibody fragment specific for human EGFR. The nanoparticles were first incubated with EGFR-positive cancer cells (Tu686) and EGFR-negative cancer cells (human non-small cell lung carcinoma NCI-H520). The EGFR-positive cells showed internalization of nanoparticles, which was validated by SERS. However, EGFR-negative cells did not show any detectable SERS signal.

After validating the detection of SERS signal from the nanoparticles taken up by cancer cells,

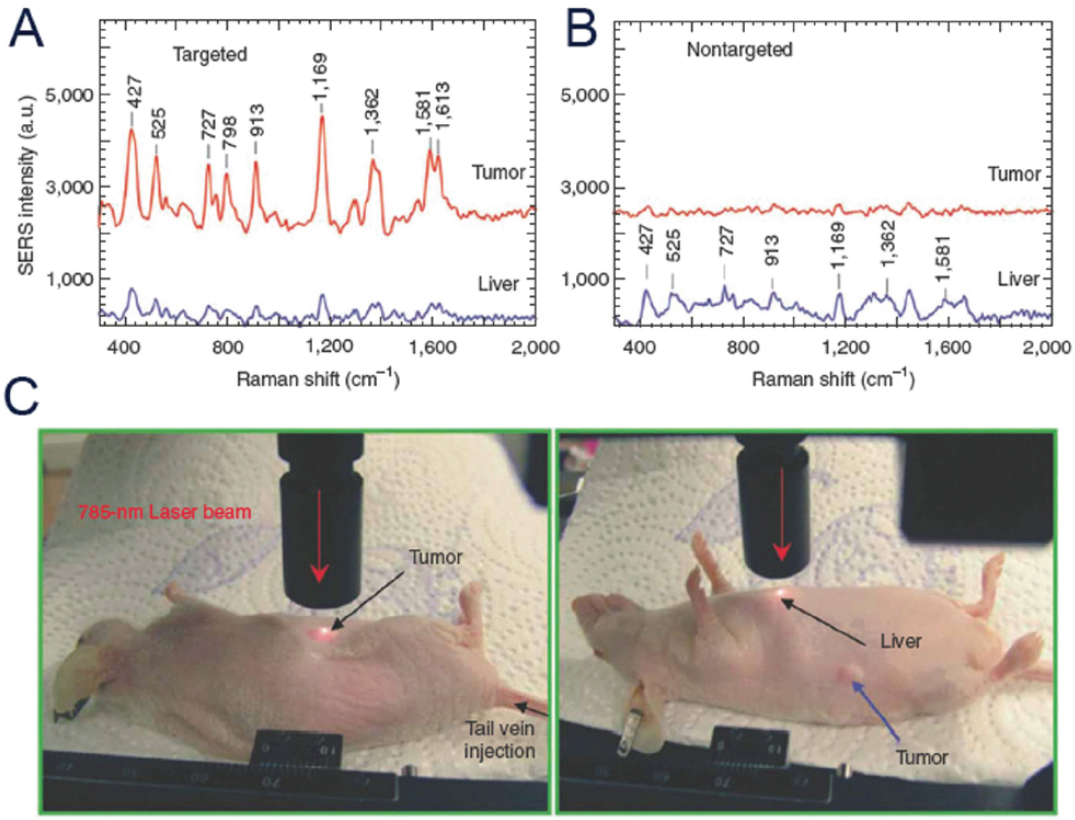

Figure 2. In vivo cancer targeting and surface enhanced Raman detection by using ScFv antibody conjugated gold nanoparticles that recognize the tumor biomarker EGFR. (A, B) SERS spectra obtained from the tumor and the liver locations by using targeted (A) and nontargeted (B) nanoparticles. Two nude mice bearing human head-and-neck squamous cell carcinoma (Tu686) xenograft tumor received ScFv EGFR-conjugated SERS tags or pegylated SERS tags via tail vein injection. (C) Photographs showing a laser beam focusing on the tumor site or on the anatomical location of liver. In vivo SERS spectra were obtained from the tumor site (red) and the liver site (blue) with 2-s signal integration and at 785 nm excitation. The Raman reporter molecule is malachite green, with distinct spectral signatures as labeled in A and B. Laser power, 20 mW. (Reprinted with permission from Ref 35 by Nature Publishing Group).

the authors moved to a mouse model. Nude mice were implanted with Tu686 tumor cells, injected subcutaneously into the flank. Nanoparticles with or without targeting ligands were injected systemically into the mice. After the nanoparticle injection, tumors were monitored with an *in vivo* SERS imaging system. The tumors of the mice with targeted-nanoparticle administration showed strong SERS signals, which suggested a successful delivery of probe to the tumor, **Figure 2**. However tumors in the mice with nontargeted-nanoparticle injections did not show any readable SERS signal. The authors presented the first and only *in vivo* molecular imaging study of tumor biomarkers using SERS. This report has been a cornerstone in the translation of SERS into a noninvasive molecular imaging modality for detection of different carcinomas.

SERS/MRI nanoprobes for in vivo multimodal imaging

Designing contrast agents for multimodal imaging is an emerging and important field. Any given imaging modality could be powerful in certain respects and weak in others. Therefore combining two or more modalities may allow the offsetting of one modality's weakness with the strength of another. For instance MRI is a very powerful biomedical imaging modality in terms of image resolution. However MRI suffers from poor sensitivity and has a low detection limit. High dosages of contrast agents are required to

obtain useful image contrast. SERS, on the other hand, has poor resolution but very high sensitivity, which can identify single molecules. Hence a combination of MRI and SERS should constitute an imaging modality with high resolution and multiplexed sensitivity.

After administration of a low dose of SERS/MRI active contrast agent (AuMN-DTTC), the localization of the probe in tissue can be identified with very high precision. For this purpose, we engineered a contrast agent, which has distinct SERS signals and T2 magnetic relaxivity [55]. The nanomaterial consisted of dextran coated iron oxide nanoparticles, complexed and surrounded with gold nanoparticles. The gold nanoparticles were tagged with a Raman reporter molecule (DTTC) and capped with PEG chains. The iron core served as a T2-weighted MRI contrast agent and the gold nanoparticles with Raman tags provided the SERS signal. The nanoparticles in aqueous solution generated strong SERS signals, captured with a hand-held portable Raman imaging system. T2-weighted MR imaging of probes showed loss of signal (T2 shortening).

The nanoparticles were then intramuscularly (i.m.) injected into the flanks of nude mice and imaged both by the portable Raman system and a 9.4 T MRI, **Figure 3**. The nanoparticles, tagged with the Raman reporter, showed a sharp and strong SERS signal. MRI revealed T2-shortening at the site of injection consistent with localization of the agent in tissue. By functionalization with molecular targeting elements, the nanoparticle could be used as a bimodal molecular imaging contrast agent. This is the first work that reports a bimodal probe with a SERS and MRI imaging capability for *in vivo* imaging application. However the engineered probe would be more suitable for *in vivo* imaging applications after molecular targeting is achieved.

Preclinical endoscopy imaging

Even though enhanced Raman spectroscopy is a powerful and promising method for preclinical imaging, the challenge of limited light penetration in deep tissues cannot be overlooked. Therefore in one recent study the authors proposed to use Raman spectroscopy for gathering *in vivo* or *ex vivo* information by coupling Raman spectroscopy with endoscopy. This approach potentially addresses the challenge of limited light penetration in Raman imaging. In this study, the nanoparticles were functionalized with DOTA and radiolabeled with 64Cu for imaging their localization, utilizing a well-established clinical imaging modality, micropositron emission tomography (microPET) [56]. The SERS nanoparticles were injected either intravenously (i.v.) or intrarectally (i.r.) to discover their natural accumulation in mouse tissues by each administration route.

The investigators also addressed the potential toxicity associated with each administration route. The intravenously injected nanoparticles mostly accumulated in the liver, lung, kidney and spleen of administered mice, as evidenced by PET. The mice receiving a topical intrarectal injection had nanoparticle localization largely observed in the intestines and cecum. Other tissues revealed minimal uptake of probe after intrarectal administration. After acquiring biodistribution data across time using microPET, the animals were sacrificed; the tissues were excised and imaged by SERS. The biodistribution data gathered by *ex vivo* SERS imaging correlated with that obtained from PET.

The authors conclude that their data indicate that topical intrarectal injection produces minimal unintended uptake of the probe and is the safer route of administration. The data also suggest that combining endoscopy with SERS provides a safe method for imaging suspected diseases of the colon in humans. Validating the *in vivo* PET results with *ex vivo* SERS using the same imaging-capable nanoparticles provides stronger evidence for data interpretation. This was established in this work.

Imaging zebrafish embryos with SERS nanoparticles

Cell labeling is a useful tool for following embryonic development and differentiation. In one recent study, researchers engineered a gold SERS nanoprobe for this purpose. The authors showed that detection and imaging of zebrafish embryos at the one-cell stage, with microinjected nanoprobes, is possible [57]. In this study gold nanoparticles 40 nm in diameter were used as the SERS template. This size produced the greatest signal enhancement capability of all sizes tested. The nanoparticles were conjugated with either MPA or MPY as the Raman reporter label. The microinjected embryos

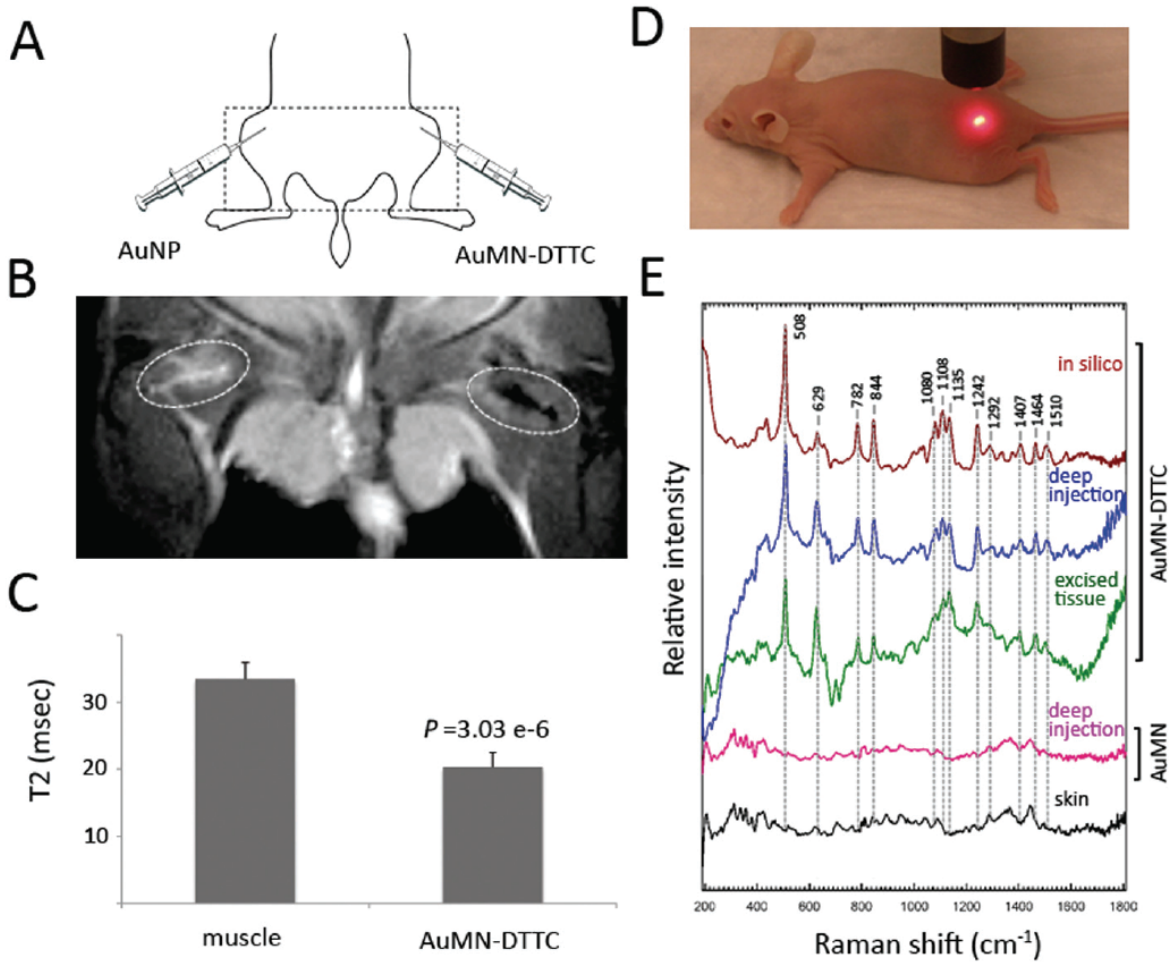

Figure 3. MRI and Raman spectroscopy in vivo. (A) A schematic of the probe injection setup. The experimental AuMN-DTTC probe was injected in the deep right gluteal muscle. A control probe was injected in the contralateral muscle. (B) In vivo T2-weighted MR image of a mouse injected intramuscularly (i.m.) with AuMN-DTTC and the control probe, AuNP. (C) Calculated T2 values based on multiecho T2-weighted MRI. The T2 relaxation time of AuMN-DTTC was significantly lower than both noninjected muscle and muscle injected with AuNP (n = 3; Student's t test; $p < 0.05$). (D) A photograph demonstrating the Raman spectroscopy experimental setup. E) In vivo Raman spectra of a mouse injected i.m. with AuMN-DTTC and the control probe, AuMN. The in vivo Raman spectrum of muscle injected with AuMN-DTTC has a clear SERS signature. (Reprinted with permission from Ref 55 by American Chemical Society).

produced a distinct SERS signal with their Raman imaging system.

The authors constructed Raman maps that mirrored the development of the embryos into different cell types, tissues and organs. The location of nanoparticles in the tissues of developing zebrafish was determined by SERS. Visual examination of the morphology of the microinjected embryos, their development, gene expression, and organ function revealed no toxicity or biocompatibility problems associated with the probe. In this study multiple targets were simultaneously detected, with single molecule sensitivity, using SERS. This study is an important step towards translating SERS into a multiplexed imaging modality for studying embryonic development.

Ex vivo imaging applications

Molecular imaging of EGFR by SERS nanoparticles

EGFR is over-expressed in most human colon cancers. In one recent study researchers syn-

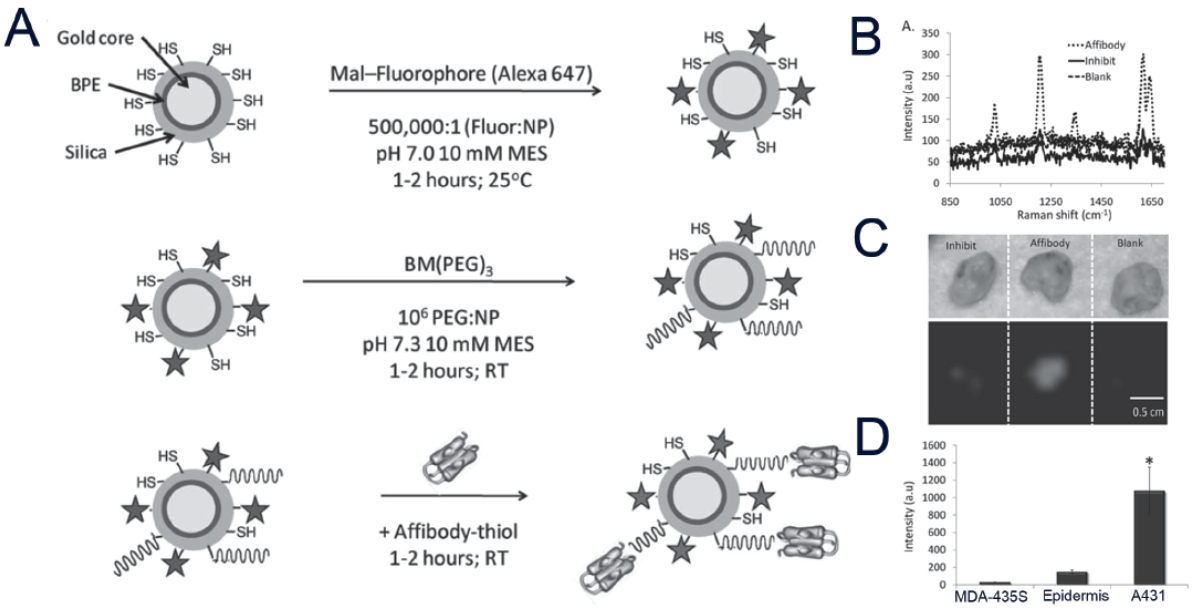

Figure 4. (A) Preparation of NPs: a gold core with Raman-active molecules is coated with a silica shell for stability and SERS intensity. The NPs are first coated with a fluorophore (star) and remaining thiols react with a bis-maleimido short (n =3) PEG (coil) cross-linker. Affibodies specific to EGFR or Her2 are bound to the NP surface. RT denotes room temperature reactions. (B) Validation of SERS NPs with explanted xenograft tumors: Representative spectra for A431 tumors labeled ex vivo with three different NP types: targeted (dots; via anti-EGFR affibody), targeted with competitive inhibition (solid), and untargeted (dashes; blank). Specific signals arise from the targeted NPs. (C) Collections of spectra create Raman maps from whole tumors and signal quantified in (D). Error bars represent the standard error and * indicates significance at $p < 0.01$. (Reprinted with permission from Ref 58 by John Wiley and Sons).

thesized multimodal SERS nanoparticles for monitoring colon tumors with different EGFR expression levels [58]. The nanoparticles were targeted to EGFR using affibodies, which have nanomolar binding affinity for the antigen. For imaging purposes, the nanoparticles were conjugated to NIR fluorescent dye (Alexa 647) on their silica coating and Raman reporter; trans-1,2-bis(4-pyridyl)ethylene (BPE); sealed between gold surface and silica shell. The resulting nanoparticle provided both NIR fluorescence and SERS signals. The author incubated the purified nanoparticles with two different tumors *ex vivo*. They showed that EGFR-positive A431 tumors produced a SERS signal, which was 35 times higher than that of EGFR-negative MDA-435S tumors. The authors were also able to show that adjacent tissues with low-level EGFR expression had seven-fold lower signal than EGFR-positive tumors.

In order to show that the signal was due to molecular targeting of EGFR, the authors also incubated the EGFR-positive tumor with a competitive probe. It did not generate any SERS signal. They observed that competitive inhibition resulted in six-fold lower SERS signal than the tumors incubated with the engineered SERS probe, **Figure 4**. The authors used fluorescence imaging in order to correlate the SERS signal intensity with fluorescence intensity. A good correlation was observed. The authors concluded that imaging cancer markers in excised human tumors is not only possible but also quantitative with SERS. With this work, the potential of SERS for molecular imaging is clearly demonstrated.

Imaging tissue biopsies with immuno-SERS microscopy

In one recent study researchers engineered 60 nm sized gold/silver nanostars encoded with Raman active reporters. These nanoparticles were used for molecular imaging of a tumor suppressor marker, utilizing a new modality dubbed immuno-SERS microscopy [59]. The star-shaped nanoparticles were chosen be-

cause (a) their plasmon bands are observed in the red region of the spectrum with minimal interference from tissue autofluorescence; and (b) the star shape has sharp tips and edges, which enhance the Raman signals remarkably. The nanostars were labeled with an anti-p63 antibody to target the p63 tumor suppressor marker in prostate tissue biopsies. The authors were able to locate the marker on the basal epithelium of prostate tissues. Immunostaining tissues with SERS nanoparticles thus offers a new method for ultra-sensitive molecular imaging in tissue biopsies.

Conclusion

Surface enhanced Raman scattering is a very sensitive imaging method with a single molecule detection limit. It has multiplexing capability such that a Raman signal is enhanced up to 10^{14} to 10^{15} fold. It provides spectroscopic information with strong photostability and low background. Even though it is still in its infancy, it is receiving careful attention due to these extraordinary properties. Translating SERS into a molecular imaging technique or combining it with a biomedical imaging modality should benefit patients dramatically, affording them disease detection at a very early stage. Therefore a substantial effort needs to be made to transform SERS into a more clinically relevant modality. There are several challenges that need to be addressed.

Deep tissue penetration of light is a major obstacle lying between SERS and preclinical applications. Another important challenge is to discover biocompatible and biodegradable SERS probes with minimum cytotoxicity. Even though several groups have shown minimal toxicity with gold nanoparticle based SERS contrast agents, more thorough studies need to be performed. Finally increasing the length of time nanoparticles stay in circulation will increase their interaction with the tissue of interest and decrease the clearance rate by the RES.

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Address correspondence to: Dr. Mehmet Yigit, Research Fellow in Radiology, Molecular Imaging Laboratory, MGH/MIT/HMS Athinoula A. Martinos Center for Biomedical Imaging, Massachusetts General Hospital/Harvard Medical School, Bldg. 75, 13th St., Charlestown, MA 02129 Tel: 617-643-4889; Fax: 617-643-4865; E-mail: myigit@nmr.mgh.harvard.edu. Dr. Zdravka Medarova, Assistant Professor of Radiology, Molecular Imaging Laboratory, MGH/MIT/HMS Athinoula A. Martinos Center for Biomedical Imaging, Massachusetts General Hospital/Harvard Medical School, Bldg. 75, 13th St., Charlestown, MA 02129 Tel: 617-643-4889; Fax: 617-643-4865; E-mail: zmedarova@partners.org

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