Raman spectroscopy is an optical technique that offers unsurpassed sensitivity and multiplexing capabilities to the field of molecular imaging. In the past, Raman spectroscopy had predominantly been used as an analytic tool for routine chemical analysis, but more recently, researchers have been able to harness its unique properties for imaging and spectral analysis of molecular interactions in cell populations and preclinical animal models. Additionally, researchers have already begun to translate this optical technique into a novel clinical diagnostic tool using various endoscopic strategies.

Key Words: Raman’s effect; spectroscopy; molecular imaging

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Raman’s “Effect” on Molecular Imaging

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In his inaugural address delivered to the South Indian Science Association, Chandrasekhara Venkata Raman gave a presentation on “a new radiation” that he had observed on February 28, 1928 (1). Using nothing more than a beam of sunlight converged by a telescope objective, a flask of benzene, a few filters, and a direct-vision pocket spectroscope, Raman discovered a new light-scattering phenomenon that would win him the Nobel Prize in 1930 and, several decades later, influence an entirely new diagnostic strategy. This light-scattering phenomenon has been affectionately coined the Raman effect. When light interacts with matter, most of the light is elastically scattered, meaning that the scattered light maintains the same energy, frequency, and wavelength of the incident light. However, a small fraction of light is inelastically scattered, meaning that the scattered light has a lower optical frequency or wavelength than the incident light (Fig. 1A). The process leading to this inelastic scatter is termed the Raman effect, and objects made of various molecular compositions will inelastically scatter light differently. Therefore, every molecule has a different Raman signature, allowing Raman spectroscopy to be used as an analytic tool to identify various substances. This process is entirely different from optical fluorescence, which requires light absorption and is not based on light scatter per se.

One drawback, though, is that the Raman effect is weak, producing only 1 inelastically scattered photon for every 10 million elastically scattered photons. However, major technological advances, such as the invention of the laser in the 1960s and the discovery of resonance-based enhancements in the late 1970s, have generated a resurgence of interest in using the unique properties associated with Raman spectroscopy. Lasers offer a high-intensity monochromatic light source that is necessary because of the low fraction of inelastically scattered photons and has become the only real practical excitation source for Raman spectroscopy. Another important advancement in the field was the discovery of the surface-enhanced Raman scattering (SERS) effect. SERS is a plasmon resonance effect in which small molecules adsorbed onto a nano-roughened noble metal surface experience a dramatic increase in the incident electromagnetic field resulting in a Raman effect several orders of magnitude higher. This effect has been used to create Raman active gold or silver SERS nanoparticles that have the potential to be used as molecular imaging contrast agents in conjunction with Raman spectroscopy (Fig. 1B). In addition, several “flavors” of nanoparticles can be produced by modifying the Raman active layer (composed of small molecules) that is adsorbed onto the metal surface, resulting in several unique spectral signatures (Fig. 1B). This allows detection of multiple flavors of SERS nanoparticles simultaneously (multiplexing).

Over the years, Raman’s great discovery has been used for all kinds of interesting applications, including the detection of narcotics and explosives at airports, the characterization of archaeological artifacts, the analysis of bodily fluids during forensic investigations, and even as a tool to search for life on Mars.

In more recent years, biomedical researchers have been using this inelastic scattering process as a diagnostic tool. Two main strategies have predominantly been used thus far for diagnostic applications and will be discussed further in this review of the literature. The first involves assessing intrinsic Raman spectral signatures between diseased and normal tissues in order to interrogate specific chemical changes due to the presence of an invading disease (Fig. 1C). The other
strategy involves using Raman active nanoparticles as molecular imaging agents (Fig. 1B) or disease-targeting beacons that can assess various targeting ligands and their ability to effectively bind to biomolecular targets on disease.

IN VITRO RAMAN IMAGING IN CELL STUDIES

Intrinsic Approach

Raman imaging has been used on a microscopic scale to interrogate detailed intrinsic information about various cell populations using a relatively rapid and nondestructive technique. Spectral maps acquired with micrometer resolution can provide an intrinsic molecular fingerprint of individual cells revealing lipid content (2,800–3,000 cm\(^{-1}\)), phosphate content (1,100–1,250 cm\(^{-1}\)), and protein content (1,500–1,700 cm\(^{-1}\)) (Fig. 1C) (2). Researchers have been able to successfully differentiate between various cell types solely based on their intrinsic characteristic Raman peaks, which are due mostly to differences in the composition of DNA, protein, and lipids within the cells. This differentiation includes discerning certain cancer cells from normal cells (3) and even the differences between human embryonic and adult mesenchymal stem cells (4). In addition, researchers have been able to use Raman microscopy imaging to localize the intracellular distribution of metabolites and metabolic changes induced by certain therapeutic drugs. For example, Raman chemical mapping revealed that the nucleus was the site of action for certain HIV protease inhibitors in human papillomavirus–expressing cancer cells (5).

Molecular Imaging Agent Approach

Raman microscopy imaging has also been used to assess molecular targeting to various cellular proteins expressed on cancer cells using conjugated Raman nanoparticles that act as tumor-targeting contrast agents. Various Raman nanoparticle constructs have been developed to assess multiple cancer biomarkers in live cells. SERS-based gold and silver core-shell Raman nanoparticles were conjugated with PLC\(\gamma\)1 antibodies and showed highly sensitive biologic imaging of HEK293 cells, which overexpress the cancer
marker PLCy1 (6). Other researchers have used multimodal approaches involving the use of fluorescence and Raman spectroscopy with fluorescent surface-enhanced Raman spectroscopic nanoparticle dots as molecular imaging agents. This group successfully detected 3 cellular proteins that are simultaneously expressed on bronchioalveolar stem cells using a multiplexed array of these dots (3 different flavors), each containing a different Raman active layer for multiplexed imaging (7). Another group has successfully demonstrated multiplexed Raman imaging using 5 different $^{13}$C and $^{12}$C isotope compositions of single-walled carbon nanotubes (8). These are unique in that they express their own intrinsic Raman signature based on their carbon composition and do not require the use of metallic surface enhancer. Each of the 5 sets of nanotubes was conjugated with various antibodies that were specific to various cancer cell lines overexpressing different surface receptors (epidermal growth factor receptor [EGFR]/Her1, integrin αvβ6, carcinoembryonic antigen, CD20, and Her2). Raman microscopy imaging revealed that each of the 5 sets of conjugated single-walled carbon nanotubes successfully targeted its respective cellular protein overexpressed on its respective cancer cell line.

IN VIVO RAMAN IMAGING IN PRECLINICAL ANIMAL MODELS

Intrinsic Approach

More recently, investigators have been using the intrinsic chemical features of intact tissues to create Raman images of living mice. Kirsch et al. have been able to successfully localize cortical and subcortical tumor cell aggregates using this intrinsic approach in a mouse model of induced brain metastasis (9). This was achieved by collecting Raman spectra while raster scanning an entire region of the brain and focusing on differences in spectral contributions from proteins, lipids, blood, water, bone, and melanin. The pigment melanin displayed additional spectral contributions that were not seen in normal tissue and thus could be used as an inherent marker of these brain metastases. Further development of this intrinsic Raman technique could prove to be invaluable in helping surgeons to delineate infiltrating tumor margins during tumor resection.

Although not the focus of this review, it is worth mentioning that alternative nonlinear Raman imaging techniques such as stimulated Raman scattering and coherent anti-Stokes Raman scattering have also been used to assess the intrinsic chemical features in tissues and offer much stronger signal enhancements than that of spontaneous Raman scattering. Saar et al. have recently shown the ability to perform video-rate molecular imaging of living mice with stimulated Raman scattering, providing microscopic maps of lipid, protein, and water content in the skin (10). For more information on the use of coherent anti-Stokes Raman scattering, please refer to a key review by the Huser group (11).

Molecular Imaging Agent Approach

Using Raman nanoparticles as molecular imaging agents in conjunction with Raman spectroscopy has proven to be a novel molecular imaging technique offering ultrahigh sensitivity and multiplexing capabilities in preclinical animal models. Eighty years after the discovery of the Raman effect, our group showed the first noninvasive Raman image of a living mouse using a tail-vein injection of SERS Raman nanoparticles (12). The image revealed a bright liver, the primary organ responsible for the natural uptake (because of the reticuloendothelial cells) and excretion of these 120-nm spherical Raman nanoparticles after tail-vein administration (Fig. 2). This in vivo approach showed ultrasensitive picomolar detection of SERS nanoparticles, and depths of 5.5 mm were achieved with low nanomolar concentrations (12). It was also shown that SERS nanoparticles could be used for multiplexed imaging. Several Raman nanoparticle batches have been evaluated, each containing a unique Raman active dye that exhibits a distinct spectral fingerprint. Thus far, researchers have successfully multiplexed 10 unique SERS nanoparticles in a living mouse (13).

Various Raman nanoparticle constructs have recently been developed as molecular imaging agents for several kinds of preclinical applications. One important application...
being tumor targeting, researchers have developed different conjugation strategies with their SERS Raman nanoparticles to evaluate tumor targeting to the EGFR, which is overexpressed in several types of malignant tumors. One group conjugated its SERS nanoparticles with single-chain fragment antibodies that recognize EGFR (14), whereas another group conjugated its SERS nanoparticles with an EGFR-binding Affibody (Affibody AB) (15). Each group successfully demonstrated effective tumor targeting of its respective Raman nanoparticles in tumor-bearing mice, thus showing the potential of SERS Raman nanoparticles as diagnostic agents for cancer detection.

Another application involves the use of Raman nanoparticles for sentinel lymph node mapping. Gold nanorods have recently been developed for both Raman and fluorescence imaging. This bimodal approach successfully demonstrated the ability to localize the sentinel lymph node after an intradermal injection of these fluorescence-SERS gold nanorods into the forepaw of a mouse (16). Another multimodal approach has used a unique gold-based Raman nanoparticle modified with gadolinium, which can be used in conjunction with MRI, photoacoustic imaging, and Raman imaging. This unique triple-modality nanoparticle has shown the ability to accurately target and delineate the margins of brain tumors in living mice (17).

Theranostic nanoparticles have recently been evaluated preclinically incorporating Raman spectroscopy for diagnostic imaging and photothermal heating for therapy (18). Three batches of SERS-coded gold nanorods have been developed, and multiplexing was successfully demonstrated in a living mouse. This multifunctional diagnostic and therapeutic approach holds great potential for preclinical screening of various tumor-targeting strategies simultaneously.

CLINICAL UTILITY OF RAMAN SPECTROSCOPY

In the last 5–8 y, there has been an explosion in the number of studies reporting on the clinical use of intrinsic Raman spectroscopy. Undoubtedly, being able to differentiate malignant from benign tissues and to potentially classify tumor grades with a noninvasive, nondestructive, real-time method that does not require administration of any molecular imaging agent (label-free) would pave new avenues for clinical cancer diagnosis and staging. Although issues due to the weakness of the intrinsic Raman signal persist, particularly in a confounding in vivo scenario, label-free Raman spectroscopy has shown remarkable progress due to advances in instrumentation and spectral postprocessing. With Raman spectroscopy being an inherently surface-based method, skin cancer has been an early target for the evaluation of its usefulness. For example, confocal Raman mapping enabled accurate identification of basal cell cancer tumor margins (100% sensitivity, 93% specificity) with a sample size of 15 specimens (19). Confocal Raman spectroscopy has also been used to discriminate basal cell carcinoma and squamous cell carcinoma from normal skin and melanoma samples. In vivo studies featured Raman spectroscopy as a tool for real-time diagnosis and guidance in nonmelanoma skin cancers (95% overall classification accuracy in 21 suspected nonmelanoma skin cancers) (20).

In the neurologic–oncologic field, Raman spectroscopy has been used to differentiate glioblastomas from tissue necrosis and holds promise in defining resection margins intraoperatively. In the head and neck region, Raman spectroscopy was able to discriminate between normal, dysplastic, and malignant changes in squamous epithelial cells of the larynx (94%, 91%, and 90% specificity, respectively, and 83%, 76%, and 92% sensitivity, respectively) (21) and has shown promise in differentiating benign from malignant thyroid lesions, as well as adenomas from hyperplasia of the parathyroid (22).

Breast cancer would be a natural target for clinical Raman spectroscopy because of the limited depth of the cancer from the skin surface and the importance of staging axillary lymph nodes, which are also located superficially. Of particular interest is the potential of Raman spectroscopy to distinguish between the different chemical makeups of malignant versus benign breast calcifications. Spatially offset Raman spectroscopy (SORS) enables discrimination between different types of calcifications at depths of up to approximately 9 mm. A variation of Raman spectroscopy in which the emitted photons are recorded from the opposite side of the incident laser light (termed transmission Raman spectroscopy) has enabled identification of calcifications at even greater and clinically viable depths of up to 2.7 cm (23). A Raman spectroscopy study to diagnose malignant and benign lesions in human breast tissue was able to differentiate normal tissue, fibrocytic changes, fibroadenoma, and infiltrating carcinoma (94% sensitivity, 96% specificity, and 86% overall accuracy for distinguishing cancerous tissue from benign or normal tissue). Raman spectroscopy has also shown successful differentiation between different nuclear grades (low, intermediate, and high) of ductal carcinoma in situ and invasive ductal carcinoma of the breast (24). In these studies, spectral changes in the intensity of phospholipids, cholesterol, fatty acid chains, proteins, and nucleic acids with increasing nuclear grade were observed. The investigators concluded that low-grade carcinoma tissue is rich in protein, whereas high-grade carcinoma is richer in acylglyceride. A classification model with 59 lymph node sections from breast cancer patients demonstrated high accuracy (91% sensitivity, 93% specificity) for the correct classification of cancerous versus benign nodes. Breast cancer lymph node metastases had increased DNA and tyrosine content and reduced collagen content, whereas benign lymph nodes appeared to have relatively higher carotenoid contents (25).

The gastrointestinal tract is another organ system in which Raman spectroscopy might have great potential as a rapid and high-accuracy diagnostic method, given the natural accessibility via endoscopes. Changes in the relative distribution of glycogen, lipids, proteins, and DNA enable the diagnosis of malignancy in the esophagus and stomach.
(95% sensitivity and 91% specificity for discrimination between normal and dysplastic gastric tissue). The first in vivo study using a fiber-optic probe via the accessory channel of a colonoscope resulted in an impressive accuracy in diagnosing hyperplastic \( n = 9 \) and adenomatous \( n = 10 \) polyps (100% sensitivity, 89% specificity, 95% overall accuracy) (26).

In the urologic field, so far mainly bladder and prostate cancer have been evaluated with Raman spectroscopy. Diagnostic algorithms for bladder tissues differentiated benign samples (normal, cystitis) from malignant samples (transitional cell carcinoma) with an overall accuracy of 84%. Prostate tissue is heterogeneous and complex, and finding and classifying abnormal tissue areas represent a particular challenge for histopathologists. A Raman spectroscopy classification model achieved an overall accuracy of 86% in differentiating malignant from benign prostate tissue (27).

For more detailed information on clinical applications using intrinsic Raman spectroscopy, please refer to the excellent review by Kendall et al. (28).

**FUTURE DEVELOPMENTS**

Another approach toward using Raman spectroscopy for clinical applications that is currently being developed in our laboratory includes the use of an accessory Raman endoscope in conjunction with topically applied tumor-targeting Raman nanoparticles (Fig. 3) (29). The idea would be to use SERS Raman nanoparticles as molecular imaging agents by conjugating them with specific tumor-targeting ligands (e.g., peptides) and then topically applying them to the tissue of interest to increase targeting efficiency while decreasing systemic toxicity. The unbound Raman nanoparticles would then be rinsed away and a Raman endoscope could be used to localize the diseased areas in situ, offering a sort of real-time histopathologic evaluation of the tissue in question. This strategy has great potential for early cancer detection and for helping surgeons to delineate tumor margins while performing intraoperative debulking procedures. Mohs et al. have developed a handheld spectroscopic device that has been shown to have great potential in localizing both fluorescent and SERS contrast agents in preclinical animal models to aid in the surgical resection of tumors (30).

Cell trafficking in living subjects is yet another application for which Raman imaging could be used. There is much to be understood about how certain cells are recruited to various sites in the body and how they interact with other cells in these environments, particularly in tumor settings, as tumor heterogeneity remains a huge obstacle in the treatment of cancer. Researchers have recently monitored the uptake and redistribution of SERS nanoparticles within cells and have revealed that SERS signals remain until the fourth generation of cells (31). Therefore, encapsulating harvested cells with SERS reporters offers a unique approach toward tracking their natural distribution and localization after being readministered into the body. In addition, researchers could use the multiplexing capabilities of SERS nanoparticles to track a variety of cell types simultaneously within a living system.

Researchers in the United Kingdom are performing critical research using a new technique called spatially offset Raman spectroscopy that has the ability to recover Raman signals from SERS nanoparticles at depths of 45–50 mm in tissue noninvasively (32). This achievement is significant because most optical techniques are limited by their depth of penetration, which often keeps them from clinical translation. This new technique offers a completely noninvasive approach toward using Raman spectroscopy as a clinical diagnostic tool without the need for minimally invasive endoscopic instrumentation. It is not hard to imagine that with SERS signals acquired at these depths, a noninvasive tomographic imaging approach in small-animal models could be developed. In fact, Raman spectroscopic diffuse tomographic imaging has already been demonstrated in a piece of excised bone harvested from a limb of a canine (33). This was the very first demonstration of Raman tomography, but it used suboptimal conditions for in vivo applications, including acquiring weak intrinsic Raman signals of bone mineral at a high laser power and long integration times. Finally, we have been conducting detailed toxicity studies of gold and silica SERS nanoparticles in small-animal models using both an intravenous and an intratumor injection. These studies have shown that SERS nanoparticles can be used in a variety of applications, including tracking cell trafficking in living subjects, monitoring cell uptake and redistribution, and noninvasive imaging of tumor tissue.
intrarectal administration route (JZ). These studies show encouraging results for clinical translation of these types of particles, and further studies are under way.

CONCLUSION

It is clear that Raman’s great discovery has had quite an “effect” on the field of molecular imaging. It has been shown to be a powerful preclinical imaging tool for both in vitro and in vivo studies. The ability for elemental, chemical, molecular, and morphological analyses of single cells. Anal Bioanal Chem. 2010;397:2051–2065. Pijanka JK, Kumar D, Dale T, et al. Vibrational spectroscopy differentiates between multipotent and pluripotent stem cells. Analyst. 2010;135:3126–3132.


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