Chapter 3

APPLICATIONS OF SERS IN CANCER DIAGNOSIS
AND THERAPY MONITORING

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ABSTRACT

Cancer is a heterogeneous group of diseases characterized by abnormal cell growth with concomitant acquisition of mutations. Poor therapeutic response and subsequent low patient survival rates are hallmarks of cancer-related morbidity. Cancer imaging is a powerful tool that can help to improve overall patient survival rate by allowing for early cancer detection, facilitating a way to monitor therapeutic response to treatment, and providing assistance with surgical interventions. Recent discoveries in the field of cancer biology include the identification of novel biomarkers which have both diagnostic and prognostic value, highlighting the need for technologies that can provide reliable and sensitive real-time measurement of these biomarkers. Current developments in image-guided surgery are focused on differentiating tumors from normal tissue, and improving surgical outcomes for cancer patients. Surface-enhanced Raman spectroscopy (SERS) offers an excellent platform for development of in vivo diagnostic and optical imaging tools. Some of the advantageous attributes of SERS include non-invasiveness, high sensitivity, possibility of using a single excitation source for multiple probes, minimal photobleaching, and low background. Based on these characteristics, SERS-based imaging can be a useful alternative or adjuvant to traditional cancer imaging techniques like fluorescence, MRI, positron emission tomography (PET), and X-ray computed tomography (CT). This chapter describes the recent advances made in the application of

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preclinical SERS-based technologies to the development of *in vivo* diagnostics, imaging, and theranostic platforms in clinical care settings.

**Keywords**: SERS, image-guided therapy, cancer theranostics, clinical translation

## INTRODUCTION

Cancer is a complex multifactorial disease with distinct characteristics, such as aberrant cellular alterations along with gene mutations and dysregulation of signaling pathways. These microscopic processes play a significant role in cancer development and progression. Elucidating these complex and intricate relationships that lead to cellular modifications and changes in cellular behavior, proliferation, and survival still remains a challenge. The overarching goal of early cancer screening is to reduce cancer morbidity by diagnosing the disease before it reaches its advanced stages. However, effective early screening is often difficult because of vague clinical symptoms, or even lack of symptoms [1]. To overcome this barrier, early cancer screening that focuses on detection of microscopic changes and disruption of biochemical pathways can play a pivotal role in cancer prevention and timely treatment. The discovery of novel non-invasive diagnostic and prognostic biomarkers could thus provide potential tools for screening early stage cancer, especially in asymptomatic individuals. In general, the task of developing clinically validated biomarkers for cancer has been difficult to accomplish, despite other advances in the field of cancer molecular biology. The presence of these biomarkers in extremely low amounts *in vivo* (in the order of femtograms) makes their measurement challenging, and accentuates the need for techniques that can sensitively and specifically detect markers in biological samples. Table 1 consolidates a list of FDA-approved clinically employed biomarkers, and the conventional methods employed to detect and measure them [2-4]. Multiplexed biomarker protein detection (i.e., detecting multiple biomarkers using the same detection technique) holds great promise for clinical cancer diagnostics in order to increase measurement sensitivity using available devices and meticulously validated protein panels [5].

Cancer screening based on traditional biopsy-based methods has been shown to decrease mortality and still remains the main screening method for clinically occult malignancies. Biopsies, however, suffer from well-recognized limitations such as invasiveness, a frequent need for repeat procedures, and the inability to biopsy solid tumors that have not yet grown enough to become detectable. Many traditional imaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound have been routinely employed as adjuvant cancer screening tools (particularly in high-risk patients) and to monitor therapeutic effects of cancer intervention. These imaging techniques play a key role in clinical oncology and the clinical management of cancer...
patients, but they are limited to anatomical information about tumors. Within the medical imaging field, molecular imaging has emerged as a promising tool that helps in the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems [6]. By using targeted imaging agents, molecular imaging enables clinicians to localize tumors, as well as to visualize the expression and activity of specific molecules such as proteases and protein kinases, and obtain information on biological processes such as apoptosis which influence tumor behavior and/or therapeutic response. The rich and complex biological information provided by molecular imaging techniques can thus aid in cancer detection, customized treatments, and therapy and drug development [7]. Molecular imaging modalities include molecular MRI, magnetic resonance spectroscopy, optical bioluminescence imaging, optical fluorescence imaging, targeted ultrasound, single-photon emission computed tomography (SPECT), and positron emission tomography (PET). These non-invasive imaging techniques allow for early diagnosis and better prognosis, opening avenues for personalized medicine;[6] but on the other hand, they often lack sufficient sensitivity and specificity in target detection. This highlights the need for a sensitive non-invasive imaging technique that can detect the target analyte (tumor-associated metabolite or tumor cell itself), as well as provide information regarding changes in the biochemical profile of tumors.

Table 1. Examples of FDA-approved cancer biomarkers [2-4]

<table>
<thead>
<tr>
<th>Biomarker name</th>
<th>Specimen</th>
<th>Clinical use</th>
<th>Cancer type</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcino-embryonic antigen (CEA)</td>
<td>Serum, plasma</td>
<td>Monitoring therapeutic response</td>
<td>Colorectal cancer, breast cancer metastasis</td>
<td>Immuno-assay</td>
</tr>
<tr>
<td>Total prostate-specific antigen (PSA)</td>
<td>Serum</td>
<td>Diagnosis, Monitoring therapeutic response</td>
<td>Prostate cancer</td>
<td>Immuno-assay</td>
</tr>
<tr>
<td>CA.125</td>
<td>Serum, plasma</td>
<td>Monitoring disease progression, Monitoring therapeutic response</td>
<td>Non-small lung cancer, ovarian cancer</td>
<td>Immuno-assay</td>
</tr>
<tr>
<td>CA19.9</td>
<td>Serum, plasma</td>
<td>Monitoring disease progression, Monitoring therapeutic response</td>
<td>Pancreatic cancer</td>
<td>Immuno-assay</td>
</tr>
<tr>
<td>CA15.3</td>
<td>Serum, plasma</td>
<td>Monitoring disease progression, Monitoring therapeutic response</td>
<td>Breast cancer</td>
<td>Immuno-assay</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>Formalin-fixed paraffin-embedded (FFPE) tissue</td>
<td>Monitoring therapeutic response</td>
<td>Breast cancer (stage IV)</td>
<td>Immuno-histochemistry</td>
</tr>
</tbody>
</table>
Raman Spectroscopy (RS) is a vibrational spectroscopy technique which provides details of chemical composition, molecular structure, and molecular interactions in cells and tissues. Disease states often result in changes in molecular composition of the affected tissues, with accompanying changes in the associated Raman spectra. These spectral changes specific to disease state, in principle, could be utilized as a precise phenotypic marker of the disease. Current research substantiates the difference observed in ex vivo Raman spectra of healthy and diseased tissues [8]. Raman spectroscopy can hence be used as a label-free imaging modality that provides information about basic chemical bonds (e.g., in hydrocarbons or nucleic acids) through the analysis of nominally weak inelastic scattering of photons by such molecular motifs in vitro or in living tissues [9]. The detection of this weakly inelastic scattering of photons in tissues typically requires long integration times (minutes) and physical contact between the optical probe and the target tissue. Surface-enhanced Raman scattering (SERS) using nanoparticles (NPs) of about 120 nm in diameter enables enhancement of the weak inelastic scattering of specific Raman-active molecules by several orders of magnitude. The amplification mechanism is through a plasmonic enhancement effect, thereby allowing SERS NPs to serve as a source of significant Raman signals [10, 11]. There have been several reports of SERS tags exhibiting femtomolar detection sensitivities [12, 13]. The sensitivity achieved by SERS is unmatched by medical imaging techniques such as MRI (micromolar) [14] or CT (picomolar) [15]. Although PET imaging has high sensitivity (femtomolar) [16], it also has limited resolutions of a few millimeters [17]. Raman imaging, at the diffraction limit, offers resolutions of a few hundred nanometers, so that it can provide highly sensitive imaging without compromising image resolution. SERS, by delivering an enhanced Raman signal, can thereby serve as a good candidate for in vivo imaging as a diagnostic tool or when used in conjunction with surgery. An example is image-guided surgery, where SERS can be used to delineate tumor margins during resection procedures. This book chapter describes the recent advances made in the application of SERS technology from the preclinical stages towards the development of in vivo diagnostics, multimodal imaging, therapeutic monitoring, and theranostic platforms in clinical care settings.

**SERS AND LIQUID BIOPSY**

Tissue biopsy is a widely-used technique in clinical detection of a variety of cancers, and it is recognized as the best method to customize patient treatment [18]. Tissue-based biopsy, however, suffers from serious limitations such as invasiveness, cost, and inaccessibility or inability to use the technique in certain cancer types. Furthermore, tumor tissues are often heterogeneous so that tissue biopsies may provide misleading diagnostic information, thereby affecting patient treatment. These limitations of tissue biopsy thus prompted the development of liquid biopsy [18].
Liquid biopsy is a novel method of detecting cancer biomarkers in body fluids (such as blood and urine) that can assist in cancer screening, diagnosis, and therapy. Apart from being non-invasive and relatively inexpensive, this technique provides the additional versatility of allowing for practical repeated assessment of disease stage and treatment response in real-time. Further, liquid biopsy offers an unbiased assessment of tumor biology because biological fluids possess information from all the tumor cells, unlike a small proportion of tumor extracted through tissue biopsy. Additionally, liquid biopsy provides genetic profiling in addition to tissue biopsy [19].

The biomarker analytes used in liquid biopsy can be broadly classified into four classes: circulating tumor cells (CTCs), circulating tumor vesicles (CTVs), which are comprised of exosomes and microvesicles), circulating nucleic acids, and proteins. The diagnostic combination of these markers gives the best assessment of the various aspects of cancer, but the detection of cancer biomarkers is an arduous task as they are found in very low proportion in complex biological fluids [20]. The traditional methods used to detect circulating cancer biomarkers are Western blot, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), flow cytometry, mass spectrometry, and immunofluorescence. The methods used for nucleic acid detection include Polymerase Chain Reaction (PCR) and reverse transcription Polymerase Chain Reaction (RT-PCR).

Although great advancements have been made in the measurement of circulating biomarkers, the techniques described above often lack sensitivity to detect relevant cancer markers at an early stage, and they require laborious processes involving expensive instrumentation and skilled professionals. For example, typical concentrations of biomarkers in the early stages of cancer lie between $10^{-16}$ M to $10^{-12}$ M [21], whereas the limit of detection (LOD) of traditional detection methods is only at the picomolar ($10^{-12}$M) level, thus rendering them ineffective for early detection [22]. As a result, there is a clear need for the development of ultrasensitive techniques for the detection of cancer biomarkers.

Nanotechnology serves as a unique platform which has opened several avenues for ultrasensitive detection of cancer biomarkers. One distinctive feature of nanomaterials is their high surface-to-volume ratio for highly efficient interactions with a target, and this capability can be exploited to outperform traditional methods in cancer biomarker detection in complex biological fluids [23]. Among the various nanomaterials, plasmonic nanoparticles such as gold and silver nanoparticles are being widely studied due to their ease of synthesis, facile surface chemistry, excellent biocompatibility and exceptional optical properties [24]. One of the most remarkable features of plasmonic nanoparticles is the existence of localized surface plasmon resonance (LSPR). LSPR is the collective oscillation of conductive electrons on the nanoparticle surface induced by the electric field of the incident light. Due to LSPR, plasmonic nanoparticles exhibit strong absorption and scattering coefficients [25]. LSPR also plays a predominant role in enhancing the electric field of incident and scattered light, thereby directly augmenting the Raman signals of
absorbed molecules [26]. These unique attributes of plasmonic nanoparticles are well suited for the development of ultrasensitive SERS-based detection platforms for circulating cancer biomarkers (circulating nucleic acids, proteins, vesicles and tumor cells). Table 2 details the advantages and limitations of surgical biopsy, liquid biopsy and SERS-based liquid biopsy methods [27].

**Table 2. Summary of advantages and limitations of surgical biopsy, liquid biopsy, and SERS-based liquid biopsy methods [27]**

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Limitations</th>
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<tr>
<td>Surgical biopsy methods</td>
<td></td>
</tr>
<tr>
<td>• The data obtained can be stratified according to tumor type, receptor status and margins</td>
<td></td>
</tr>
<tr>
<td>• The entire tumor (or part of it) is removed</td>
<td>• Local anesthetic administration is required</td>
</tr>
<tr>
<td>• Surgical biopsy methods</td>
<td>• Image guidance is required</td>
</tr>
<tr>
<td>• Less invasive</td>
<td>• Invasive and uncomfortable for patients</td>
</tr>
<tr>
<td>• Enable real-time monitoring</td>
<td>• No real-time monitoring</td>
</tr>
<tr>
<td>• Do not require the expertise of a pathologist</td>
<td></td>
</tr>
<tr>
<td>Liquid biopsy methods (in general):</td>
<td>Collecting rare biomarkers is challenging</td>
</tr>
<tr>
<td>• Do not require appreciable symptoms for</td>
<td>Tumor tissue is not removed</td>
</tr>
<tr>
<td>diagnosis</td>
<td>There are very few FDA-approved technologies</td>
</tr>
<tr>
<td>• Less invasive</td>
<td>No information on tumor size and tumor margin location</td>
</tr>
<tr>
<td>• Enable real-time monitoring</td>
<td></td>
</tr>
<tr>
<td>• Do not require the expertise of a pathologist</td>
<td></td>
</tr>
<tr>
<td>SERS-based liquid biopsy methods:</td>
<td>In vivo clearance of SERS substrates has not yet been validated</td>
</tr>
<tr>
<td>• Allow for the measurement of rare biomarkers</td>
<td>Signal reproducibility is still a challenge</td>
</tr>
<tr>
<td>• Enable multiplexed biopsy-based biomarkers</td>
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</table>

**SERS for Detection of Circulating Nucleic Acids**

Moribund tumor cells release small fragments of their DNA into the bloodstream, which are known as ctDNA. These fragments contain genetic and epigenetic alterations present in tumor cells that are different from those obtained from normal cells. ctDNA are emerging as a novel biomarker for cancer detection, and are becoming extensively used for tumor genotyping, early cancer detection, patient prognosis, therapy evaluation, and evaluation of disease recurrence. However, the analysis of ctDNA is challenging because it forms a very small proportion (< 1%) of wild-type cell-free DNA. Another known class of circulating nucleic acids are circulating RNAs (ctRNA). Circulating RNAs are comprised of a myriad class of RNAs (such as mRNA, microRNA and other non-coding RNA) which are repeatedly released into the plasma of patients with different cancer types. Based on serum samples from cancer patients, circulating RNAs have been identified as another novel biomarker for cancer staging, diagnosis, prognosis and monitoring.
therapeutic response. Compared to other types of RNA, microRNA levels are found to be more reproducible, stable and consistent in the serum and plasma of cancer patients. Still, the presence of low amounts of circulating RNAs in plasma means that extensive isolation and enrichment techniques are required. After extraction and concentration, the RNA is quantified using PCR, fluorescence or spectrophotometry-based approaches. PCR-based methods often require expensive and complex procedures and provide false positive signals.

Alternatively, nanomaterials can simplify the measurement of ctDNA and ctRNA by being more cost-efficient and specific. Wee et al. reported a SERS-based detection method in conjunction with PCR to specifically detect 10 mutation alleles amongst a background of 10,000 wild-type cell-free DNA sequences (0.1%) [28]. Three primers specific to clinically important point mutations were encoded with respective Raman reporters. The presence of ctDNA was specifically detected using SERS encoded nanotags. This technique was able to detect ctDNA from as low as 5 ng of genomic DNA, and also successfully detected ctDNA from serum samples of melanoma patients. This multiplexed method (which incorporates several antibodies for detection onto a single SERS nanoparticle platform) had assay times comparable to conventional PCR, and sensitivity comparable to digital droplet PCR (ddPCR). Multiplexed detection techniques for ctDNA measurement are desirable because they can simultaneously detect several DNA sequences and can also be useful in detecting several cancers. Wang and coworkers designed a sensor wherein AgNPs were specifically immobilized onto glass slides previously tagged with a Raman reporter and molecular beacon. Upon interaction between the molecular beacon and miRNA, the molecular beacon will unfold, thus emitting out fluorescence. Thus, this technique helped in detecting microRNA using an inverse application of both SERS and fluorescence [29]. In another study, Driskell et al. performed label-free SERS detection of microRNA using a silver nanorod (AgNR) based substrate. AgNR was able to specifically detect and distinguish between microRNAs that differed in ≤ 4 nucleotides, and a partial least squares technique was successfully employed to specifically detect microRNA from a mixture of microRNA [30].

**SERS for Detection of Circulating Proteins**

Proteins circulating in blood have been widely investigated in the search for potential cancer biomarkers, because proteins released by cancer cells provide a “snapshot” of their pathophysiological condition. Elevated levels of certain proteins in the serum obtained from cancer patients are known to be well correlated with metastasis, prognosis and therapeutic response [31, 32]. Some proteins that are considered useful in cancer detection include prostate specific antigen (PSA) for prostate cancer, cancer antigen 125 (CA125) for ovarian cancer, alpha-fetoprotein (AFP) for liver cancer, and CA19.9 for gastric/
pancreatic cancer [33]. As a defense mechanism against innate immunity, cancer patients also exhibit higher levels of serum IgG and IgE autoantibodies, which can also serve as potential protein biomarkers for the respective cancer. Conventional detection methods for these circulating proteins include western blot, ELISA, RIA, and mass spectrometry [34]. Unfortunately, these methods are technically complicated and labor-intensive, and they are unsuitable for early detection of cancer biomarkers because their limits of detection (LOD) are well above the biomarker levels present in early stages of cancer [21, 22].

High sensitivity, good photostability, large working range, and excellent multiplexity make SERS an ideal choice for early detection of serum cancer protein markers [35, 36]. SERS detection methods employ a sandwich between an antibody-coated SERS substrate and an antibody-coated surface (glass), or antibody-coated magnetic binding as shown in Figure 1 [37-41].

Non-specific binding of incorrect targets could be resolved by using subsequent washing steps or magnetic separation. Chon et al. showed that spherical SERS gold nanoparticles, also known as gold nanospheres (AuNSs), and hollow gold nanospheres had a LOD of 1pg/ml, which is comparable to available immunoassays [41]. Thus, anisotropic AuNPs (known as nanostars or NSTs) can be used to improve detection sensitivity due to their higher SERS enhancement in high curvature tips and edges. Employing NSTs and Au triangle nanoarray substrates can further enhance the plasmon coupling and subsequent SERS electromagnetic enhancement, thereby significantly reducing the LOD of the assay. The LOD using NSTs was hence significantly reduced to 7 fg/ml (≈ 0.3 fM) for human IgG proteins in buffer in comparison to AuNSs [42]. Further, this assay was used to estimate the level of vascular endothelial growth factor (VEGF) in blood obtained from breast cancer patients to assess for angiogenesis [43]. These SERS substrates, when arranged in a series of arrays, exhibited a potential for multiplexed detection. For example, Li et al. constructed a 3X7 array of Au-coated glass slides with parafilm-defined wells, which allowed for simultaneous detection of tumor-associated antigens cancer antigen 15.3 (CA15.3), cancer antigen 27.29 (CA27.29), and carcinoembryonic antigen (CEA) at different concentrations. These antigens are regularly measured in metastatic breast cancer patients to provide important clinical information and monitor response to therapy. This assay showed a much lower LOD than conventional immunoassays. The results obtained from this novel SERS-based immunoassay showed that levels of CA15.3, CA27.29 and CEA in clinical serum samples from breast cancer patients were significantly higher than those obtained from healthy subjects [42].
Employing labels, as done by the studies described in the previous paragraph, can sometimes provide erroneous results, which led Lin and coworkers to design a label-free SERS sensor intended to probe cancer specific biomolecular changes rather than direct quantification [44]. This method involved direct interaction of patient serum samples with gold nanoparticles to detect serum levels of proteins, saccharides, nucleic acids and lipids. The results obtained from this assay showed that colorectal cancer patients had higher levels of nucleic acids and lower levels of saccharides and proteins in their serum compared to healthy subjects. Combining this technique with principal component analysis (PCA) and linear discriminant analysis (LDA) further improved the sensitivity to 97% and specificity to 100% for colorectal cancer. This method can also distinguish between different stages of nasopharyngeal cancer, with 83.5% detection accuracy for stage T1 (tumor confined to the nasopharynx, or with no parapharyngeal extension) and 93.3% for stages T2-T4 (more extensive tumors) [44]. Given that Ag displays higher SERS enhancement than gold, other researchers have utilized Ag for similar purposes. Feng et al. demonstrated the use of AgNPs for specific detection of biochemical SERS signatures found in serum obtained from nasopharyngeal, gastric and colorectal cancer patients [45-47]. Pazos et al. also used AgNPs for rapid, highly specific and sensitive detection of oncoprotein c-myc in blood samples [48]. The c-myc gene is a regulator gene that plays a role in cell cycle progression, cell metabolism, and universally increases transcription of all active promoters. This gene is found to be deregulated in 70% of cancers, and evaluating its level in blood is important for cancer diagnosis. The Pazos et al. sensing scheme relied on silica microparticles coated with Ag nanoparticles. The metallic surface was bio-derivatized using a peptide against c-myc modified with 4-mercaptopbenzoyl antenna (MB-H1) that transduced specific interaction with c-myc in blood through spectral changes in MB-H1. The spectral changes were correlated with the amount of c-myc in complex biological solutions, such as blood. These results hence demonstrate that SERS can serve as a novel tool with the ability for label-free non-invasive detection of circulating proteins in a variety of cancers.

**SERS for Detection of Circulating Vesicles**

Extracellular vesicles are membrane-bound vesicles that are frequently released into the extracellular environment by a wide spectrum of cell types. Extracellular vesicles include exosomes (EXOs) and microvesicles (MVs). EXOs and MVs are respectively derived from multivesicular bodies and the plasma membrane compartment of cells [49, 50]. These vesicles are known to carry molecular constituents of their originating cells [51, 52], and they also aid in cell-cell communication through horizontal transfer of proteins and nucleic acids in between cells. Extracellular vesicles also play an important role in cancer initiation and progression, thus having prognostic value in cancer diagnosis. They
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are widely found in body fluids such as blood, urine, saliva and cerebrospinal fluid, and both EXOs and MVs are found to be significantly higher in patients with advanced cancers than in healthy subjects. Thus, extracellular vesicles serve as novel cancer biomarkers used for clinical diagnosis and monitoring therapy. EXOs and MVs in plasma require extensive purification before detection and characterization. Density gradient centrifugation, filtration, and immunomagnetic isolation are some common methods used to capture and concentrate vesicles. The detection and molecular characterization of circulating vesicles is challenging due to their small size (40-100 nm for EXOs and 100-1000 nm for MVs), because neither conventional optical imaging techniques nor flow techniques can be effectively used for vesicle sizes less than 300 nm. Also, measurement techniques such as western blot and ELISA require very large volumes of concentrated EXOs (e.g., 3 ml from plasma and 300 ml of cell culture media), making their detection impractical in clinical settings.

AuNP-based detection platforms exploit the LSPR shift, which is a direct assessment of the refractive index of the surrounding media. Using this LSPR based detection strategy, Im and coworkers designed a novel sensor called the nanoplasmonic exosome (nPLEX) assay [53]. The sensor was made by stacking several Au nanohole arrays on a glass substrate with a hole of diameter of 200 nm and periodicity of 450 nm. Using exosome surface marker CD63, the assay achieved a LOD of 670 aM (attomolar), which is 104 times more sensitive than Western blot and 102 more sensitive than ELISA. By combining this nanohole array with a multichannel chip, one can achieve high throughput quantification of a panel of surface biomarkers. In another study, Stremersch and coworkers exploited single EXO detection using SERS technology [54]. Briefly, 10-nm gold nanoparticles capped with cationic 4-dimethyl amino-pyridine (DMAP) were allowed to interact with exosomes derived from B16F10 melanoma cells through electrostatic interaction. The dense packing of gold nanoparticles led to specific detection of molecules within the exosomes such as lipids, proteins, carbohydrates and nucleic acids, which were significantly different from the spectral features of DMAP. These fingerprints of the molecules from tumor-derived exosomes were different from those obtained from red blood cells (RBCs). Thus, this SERS based technique could specifically distinguish between tumor-derived exosomes and the ones derived from RBCs using chemical analysis. Furthermore, Lee and coworkers showed that tumor-derived exosomes incubated with micrometer plasmonic nanobowls were able to distinguish exosomes based on their membrane composition, providing new insights into trafficking of these vesicles [55].

Tirinato and coworkers also showed that tumor-derived exosomes and those derived from healthy subjects exhibited marked differences in exosome composition. The researchers concentrated and conveyed the exosomes onto SERS-active areas of super hydrophobic surfaces. The results indicated that exosomes derived from healthy subjects exhibited higher peaks corresponding to lipid vibrations, whereas tumor-derived exosomes were rich in nuclei acids. They were also able to detect exosome concentrations as low as
0.2 ng/ml [56]. Zong et al. described a SERS-based method of exosome detection using SERS nanoprobe and magnetic nanobeads [57]. The SERS nanoprobe consisted of a gold core–silver shell as the SERS active probe, with its surface decorated with exosome-specific antibodies. Magnetic nanobeads are comprised of Fe3O4 core-silica shell with the shell coated with specific antibodies. Both SERS nanoprobe and magnetic nanobeads were used for selective detection of exosomes by forming a sandwich-type immunocomplex. The immunocomplex is not formed in the absence of exosomes. Thus, their method was used for quick (within 2 h) qualitative and quantitative detection of tumor-derived exosomes in complex fluids, such as cell media, in contrast to traditional time-consuming detection methods. Kerr et al. also utilized SERS as a tool to distinguish exosomes derived from tumor cells grown in different oxygen environments, i.e., normoxia and hypoxia conditions [58]. This study has clinical relevance in utilizing exosomes as biomarkers to identify tumor composition and provide insight into tumor environments, which can help improve patient treatment through personalized medicine. Exosomes derived from tumors grown in hypoxic conditions exhibited high levels of phenylalanine, guanine, cytosine, tryptophan and tyrosine. Exosomes derived from tumors grown in normoxic conditions showed higher levels of thymine, collagen and desmosine/isodesmosine. These results thus show that there are distinct biomolecular features of exosomes derived from normoxic tumor cells compared to hypoxic tumor cells, which could potentially be associated with their ability to communicate with surrounding cells.

**SERS for Detection of Circulating Tumor Cells**

Circulating tumor cells (CTCs) are instrumental in propagating a primary tumor from its site of origin to distant secondary organs. CTCs were first identified by Ashworth in the blood of metastatic breast cancer patients in 1869 [59]. Since then, several research groups have reported the identification and characterization of CTCs in peripheral blood samples derived from cancer patients. Circulating tumor cells are very rare cells which are almost non-existent in healthy patients and patients with nonmalignant disease, but are present in variable frequencies in patients suffering from metastatic carcinomas. CTCs are generally heterogeneous in both phenotype and genotype. 2.5% of CTCs develop into micrometastases, and only 0.01% of the population of CTCs develops into macrometastases [60–62]. Viable tumor-derived cells (CTCs) identified in peripheral blood of cancer patients are considered to be the origin of intractable metastatic disease. Previous studies have shown a strong correlation between the levels of CTCs in blood and poor patient survival [58], and the prognostic importance of CTCs has been implicated in several cancers such as breast, colon, and lung cancer [63–65]. The identification of CTC levels in the blood of cancer patients can also help stratify those patients with metastasis who would potentially benefit from systemic therapy [66]. Although they are extremely rare, these
cells provide a viable alternative to invasive biopsy of tumor tissue for cancer detection, characterization and monitoring. The potential identification, isolation and characterization of this subpopulation of cells can thus provide new insights for the discovery of cancer stem cell markers and expand the understanding of the biology of metastases. However, accurate detection of CTCs with ultrasensitivity and specificity has been a major challenge for researchers due to various reasons. During the process of development of metastatic lesions, some CTCs undergo an epithelial-to-mesenchymal transition (EMT), which could allow them to escape from epithelial-based marker detection [67]. Furthermore, CTCs are present at a very low density amongst billions of blood cells per ml of blood [68], which makes them difficult to detect. Currently available strategies for isolating CTCs are limited to complex analytical approaches that generate very low yield and purity [69].

Despite these challenges, progress has been made in developing practical CTC detection methods. An example of such progress is the Cell Search system, which has been FDA-cleared for clinical utilization. This technique employs 12-200 nm Fe nanoparticles which magnetically separate CTCs from blood plasma, and detects tumor cells by immunofluorescence based on cytokeratin expression. CTCs are isolated based on the presence of cell surface epithelial cell adhesion molecule (EpCAM), which is an epithelial marker. Because epithelial cells are generally absent in blood, EpCAM can be used to detect CTCs. The Cell Search system has been used to detect CTCs in blood samples from breast, prostate and colon cancer patients [70]. However, it can detect CTCs only in 50% of breast cancer patients diagnosed with metastatic cancer [71, 72], and it still presents the challenge of epithelial-based detection.

AuNPs have been used to isolate and capture CTCs using two-dimensional substrates such as periodic nanostructured arrays or AuNP deposited surfaces [73]. AuNPs are coated with anti-EpCAM antibodies and aptamers to selectively capture CTCs, and the non-specific cells are washed away from the surface using buffer. The high surface-to-volume ratio of AuNPs allows for capture of almost 90% of CTCs through nanostructured substrates, in comparison to 50% capture through solid substrate [74]. SERS can provide ultrasensitive detection based on AuNPs, with up to single molecule sensitivity due to electromagnetic and chemical based enhancements [75]. SERS provides a sharp molecular fingerprint and gives 10-100 times sharper bands than fluorescence. SERS AuNPs are created by conjugating Raman reporters (generally organic dyes with delocalized electrons) on the surface of AuNPs. These functionalized nanoparticles are further stabilized using heterobifunctional PEG for interaction with AuNP surface and ligand conjugation. The potential of SERS in CTC detection was first demonstrated by Sha et al. [76]. In their study, commercially available 50-nm stabilized SERS AuNPs were conjugated with anti-human epidermal growth factor receptor-2 to capture SKBR3-mimicking breast cancer CTCs. Immunomagnetic enrichment of CTCs with anti-EpCAM labelled magnetic beads was used to isolate CTCs using flow conditions, and both techniques were used together to ensure ultrasensitive detection up to 10 CTCs. EpCAM
antibodies specifically bind to CTCs in the serum, thereby helping in enriching them. Flow conditions can be further mimicked using microfluidics, and polymer membranes labeled with anti-EpCAM antibodies have been used to enrich CTCs before SERS-interrogation [77, 78].

One of the major challenges associated with detection of CTCs is the large background from white blood cells (WBCs), due to their similarity in density and size. The use of antibodies in detection of CTCs often gives false positives due to the non-specific binding of antibodies to white blood cells. To address this problem, Wang and co-authors employed peptide epidermal growth factor labeled SERS nanoparticles to specifically capture EGFR (+) head and neck cancer cells in the blood along with WBCs [79]. They demonstrated for the first time that they could specifically detect EGFR (+) head and neck cancer cells in 17 out of 19 patients, with a limit of detection of 5-50 cancer cells per ml of blood and avoiding the detection of WBCs. The method was developed using iron oxide-gold core shell nanoparticles which had the feature of magnetic separation and enrichment of CTCs, and the SERS signal retrieval was based on the gold shell of the nanoparticles. To ensure high SERS activity, other authors have employed anisotropic gold nanoparticles for high SERS enhancement. In this method, using dual targeting with anti-EpCAM and HER-2 antibodies can capture 90% of SKBR3 cancer cells without interference with WBCs with a LOD of 1-2 cells/ ml of blood [80, 81]. The technique itself was simple and specifically detected CTCs in blood in the presence of WBCs, but on the other hand it involved laborious separation of CTCs through density gradient centrifugation. Nima et al. also exploited the multiplexing capability of SERS for detection of CTCs by using Ag-coated gold nanorods (AuNRs) against breast cancer cells in blood in the presence of WBCs, using the fact that breast cancer cells expressed insulin growth factor-1, EpCAM, anti-cytokeratin and anti-CD44 [82]. Thus, SERS imaging can provide protein topography of multiple markers on single cells, aiding in further understanding their biology and biochemical profile. Additionally, this technique includes not only epithelial markers but also mesenchymal markers such as CD44, making it possible to detect cancer cells after EMT events.

Still, the major limitation of the above-mentioned SERS-based techniques is the employment of EpCAM-based strategies which also bind to macrophages, hematopoietic precursor cells, and plasma cells. These limitations can be overcome with nucleic-acid based detection strategies employing epigenetic changes that are specific to tumor type.

**SERS FOR MONITORING METASTASIS**

The primary reason for cancer-related morbidity is the dissemination of primary tumor tissues to distant organs with the development of distant metastases. Hence, improving the ability to detect and treat metastases is of paramount importance in preventing cancer deaths, and inhibition of the growth of metastases in secondary sites also offers a promising
approach for cancer therapy [83]. Blood flow and other factors influence delivery of cancer cells to specific organs. Table 3 summarizes the different SERS-based approaches that can be employed for monitoring metastasis [11, 84-90].

<table>
<thead>
<tr>
<th>Target analyzed</th>
<th>Approach</th>
<th>Type of cancer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-βand FOXC1</td>
<td>EMT (epithelial to mesenchymal transition) regulators in mesenchymal CTCs (circulating tumor cells)</td>
<td>Breast</td>
<td>84</td>
</tr>
<tr>
<td>Cellular components</td>
<td>EMT</td>
<td>Lung</td>
<td>85</td>
</tr>
<tr>
<td>Integrins</td>
<td>Targets of angiogenesis</td>
<td>Breast</td>
<td>86</td>
</tr>
<tr>
<td>Vascular endothelial growth factor (VEGF)</td>
<td>Targets of angiogenesis</td>
<td>Breast</td>
<td>87, 88</td>
</tr>
<tr>
<td>Epidermal growth factor receptor (EGFR)</td>
<td>Breast</td>
<td>Colorectal cancer</td>
<td>89, 90</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>Feature of angiogenesis</td>
<td>Breast</td>
<td>11</td>
</tr>
</tbody>
</table>

The epithelial-to-mesenchymal transition (EMT) of adherent epithelial cells to a migratory mesenchymal state has been implicated in tumor metastasis in preclinical models. In support of this rationale, EMT has been characterized in circulating tumor cells (CTCs) from breast cancer patients. Primary tumor cells rarely show simultaneous expression of both mesenchymal and epithelial markers on their cell surface, but CTCs have much higher proportions of mesenchymal cells. The mesenchymal CTCs in breast cancer patients were also found to be associated with disease progression. Mesenchymal CTCs often express known EMT regulators, including transforming growth factor (TGF)–β pathway components and the FOXC1 transcription factor. These data thus support the role for EMT in the blood-borne dissemination of highly invasive and metastatic cancers such as breast cancer [84]. EMT is also a definitive characteristic of several lung disorders including pulmonary fibrosis. Conventional spectroscopy offers limited options in discriminating cells of closely associated phenotypes in a non-invasive and label-free manner. In spite of extensive studies, clearly defined methods in distinguishing differentiated epithelial cells are limited. Raman spectroscopy techniques can provide vibrational fingerprints that enable identification of chemicals and biologicals. SERS enhances the Raman signal of analytes present in close proximity to Au or Ag NP surfaces, thereby allowing for sensitive detection of analytes at the single molecule level. SERS is highly resistant to extraneous factors such as autofluorescence, photobleaching and interference from water, making it appropriate for detecting analytes in aqueous systems. AuNPs provide additional favorable attributes such as high biocompatibility and excellent optical properties, making them good candidates for biomedical detection applications.
Cao and coworkers demonstrated the application of SERS in characterizing EMT processes in alveolar epithelial cells (ATII) [85]. Briefly, ATII cells were incubated with bleomycin to cause differentiation into fibroblasts. TAT- functionalized AuNSs were used to characterize the changes of cellular components during the EMT process. These intracellular SERS probes demonstrated low toxicity and high stability in vitro. The internalized SERS probes exhibited SERS signals from ATII cells as early as 24 hours. The differences in spectral features of the cells at different stages of EMT demonstrated that different cellular biochemical components are modulated in the EMT process. Principal component analysis (PCA) allowed researchers to clearly distinguish differentiated from undifferentiated ATII cells based on the SERS spectra obtained, and biological assays confirmed the results obtained through SERS spectral data. Thus, these results demonstrate that SERS can facilitate non-invasive monitoring of EMT processes.

Angiogenesis, i.e., growth of blood vessels, is a normal physiological process that also plays a crucial role in tumor progression [91]. Once the tumor reaches a definite size of 1-2 mm³, diffusion of oxygen and nutrients remain insufficient for its growth, thereby inducing the tumor to generate its own blood vessels. Tumor-induced angiogenesis has the potential to convert a dormant tumor into an active metastatic tumor with the help of a multistep cascade mediated by growth factors, cellular receptors, proteases and adhesion molecules [92, 93]. The typical nutrient-deprived tumor environment triggers the activation of certain transcription factors that upregulate several proangiogenic factors, such as vascular endothelial growth factor-A [94]. In turn, this activates endothelial cells to secrete matrix metalloproteases which degrade extracellular matrix (ECM), and to express adhesion molecules to promote cell migration [95, 96], resulting in new blood vessel formation and angiogenic remodeling. Tumor angiogenesis can be probed using several non-invasive techniques such as dynamic contrast enhanced computer tomography (CT), magnetic resonance imaging (MRI), ultrasound, positron emission tomography (PET) and optical imaging [97, 98]. Traditional functional imaging involves hemodynamic characterization of immature neovessels through measurement of microvascular features such as tissue blood flow, blood volume, perfusion, capillary wall permeability, and vessel density; whereas novel targeted molecular imaging based techniques employ specific biomarkers present on endothelial cells [99]. The targets used in molecular imaging of angiogenesis are often the targets for anti-angiogenesis therapy. Thus, direct assessment of these targets can provide us with valuable information about therapeutic response to anti-angiogenesis therapy.

Common molecular targets for imaging belong to specific families of proteins such as integrins, vascular endothelial growth factor (VEGF-A/VEGF-R2) and matrix metalloproteases (MMPs). Integrins are heterodimeric transmembrane cell adhesion molecules that play a critical role in cell-cell and cell-matrix interactions. They are composed of one α subunit and one β subunit [100]. Several members of the integrin family are implicated to function in tumor angiogenesis regulation, but αvβ3 is the most widely
studied molecule. When αvβ3 is present on activated endothelial cell surfaces, it mediates cell migration and survival during angiogenesis. When present on the surface of tumor cells, αvβ3 facilitates blood vessel invasion during metastasis. Therefore, integrin-targeted probes can help monitor angiogenesis and metastatic activities. αvβ3 binds to the ECM via an RGD peptide sequence, so that RGD peptides can serve as angiogenesis inhibitors by blocking the activity of αvβ3 [101]. In vivo integrin targeting and imaging using single-walled carbon nanotubes (SWCNTs) was first reported by Liu et al. Briefly, SWCNTs were non-covalently conjugated with PEGylated lipid and labeled by Cu radioisotopes for microPET imaging [86]. The PEGylated SWCNTs were further conjugated with an RGD peptide sequence to specifically target integrins. The in vitro targeting potential of RGD-peptide-conjugated SWCNTs showed a strong binding affinity to integrins in comparison to their untargeted counterparts. Furthermore, the RGD-peptide-conjugated SWCNTs showed higher tumor uptakes of ≈ 13% of injected dose per gram of tissue, in comparison to an uptake of 4-5% of injected dose per gram of tissue for untargeted SWCNTs. Ex vivo tumor vascular staining suggested that the targeted SWCNTs specifically localized within tumor vasculature with limited extravasation, thus demonstrating their potential application in angiogenesis imaging [86]. The Keren-Gambhir group further imaged RGD-conjugated SWCNTs in tumor-bearing mice using a Raman microscope, wherein the targeted SWCNTs preferentially sequestered into tumor sites in comparison to the untargeted (without RGD peptide) SWCNTs [11]. This work opened up new avenues for in vivo angiogenesis imaging using SWCNTs.

VEGF/VEGFR is another extensively studied pathway in tumor angiogenesis. VEGF-A is a key player in angiogenesis, and gets activated by environmental stress such as hypoxia [94]. The overexpression of VEGF/VEGFR is a prognostic indicator for several cancers [102]. Angiogenesis mediated by this pathway includes specific interaction of VEGF with VEGFR or activation of the kinase activity of VEGFR2. Thus, molecular imaging of angiogenesis through this pathway includes imaging of VEGF-VEGFR [103]. Ko et al. described a novel SERS- based sandwich immunoassay employing DNA-aptamer conjugated hollow gold nanospheres and gold-patterned microarrays for sensitive detection of VEGF angiogenesis protein markers [87]. The hybrid microarray was comprised of hydrophilic gold wells and a hydrophobic SERS substrate. DNA aptamers that fold into G-quadruplexes were used as the target recognition unit. When compared with conventional ELISA, the sensitivity of detection was 2 to 3 orders of magnitude less, and the dynamic range of detection was 3 to 4 orders of magnitude greater. The total reaction time was less than 3 hours including all washing, incubation, and detection steps. Zhao et al. also demonstrated ultrasensitive detection of VEGF based on self-assembled Ag ornamented Au-pyramid superstructures [88]. Under optimized conditions, the SERS signal from the superstructure was negatively related to VEGF concentration over the range 0.01–1.0 fM, and the limit of detection (LOD) was found to be as low as 22.6 aM.
The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase belonging to the human epidermal growth factor family of receptors. The activation of this biomarker is strongly correlated with tumor progression, angiogenesis, and metastasis [104]. Cetuximab is a chimeric human murine immunoglobulin 1 which binds to the extracellular domain of EGFR with a high affinity (Kd= 0.1 to 0.2 nM) [105], and specifically downregulates EGFR by blocking its tyrosine kinase activity. Thus, Cetuximab-EGFR binding can help in monitoring receptor status and therapeutic response. Chung et al. synthesized a SERS-based Si-AuNP imaging system to quantify EGFR receptor expression before and after Cetuximab administration [89]. The distribution of EGFR and hence the amount of EGFR inhibited by Cetuximab treatment can be rapidly and accurately determined using their assay. Xiao et al. also demonstrated that an ultrasensitive SERS-based technique employing gold nanorods enabled detection of EGFR localization and internalization in living cells, up to the single cell detection level [90]. Furthermore, Sinha and coworkers demonstrated that dual-probe surface-enhanced Raman scattering nanoparticles (SERS NPs) can quantify the binding potential of cell surface biomarkers in fresh tissues, enabling demarcation of tumor margins intraoperatively [106]. Fluorescence endomicroscopy is accessible to molecular targets, and Raman spectroscopy allows for multiplexed detection of numerous molecular targets. Thus, combining both these features in a fluorescence-Raman endoscopic system can be utilized for cancer diagnosis, as was shown in an orthotopic colorectal cancer mouse model reported by Kim et al. [107]. This group used a combination of fluorescence and surface-enhanced Raman scattering dots conjugated with anti-EGFR and VEGF. The combined fluorescence-Raman approach, F-SERS, demonstrated fast and multiplexed signal detection allowing for sub centimeter-sized recognition of colorectal cancer (CRC). Thus, these F-SERS dots could simultaneously probe the molecular characteristics of tumor cells (EGFR) and the tumor microenvironment (VEGF) while performing colonoscopy.

**SERS for Monitoring the Tumor Microenvironment**

Cancer is a heterogeneous mass of cells comprised of malignantly transformed tumor cells and non-malignant cells. The non-malignant cells present in the tumor microenvironment are often recruited and corrupted by the tumor cells. The large proportion of non-malignant cells within the tumor microenvironment often possesses a dynamic role in promoting tumor growth at several stages of carcinogenesis [108]. Intercellular communication between tumor cells and the cellular compartment of the tumor microenvironment (TME) is mediated through a complex network of cytokines, chemokines, growth factors, and inflammatory and matrix remodeling enzymes. The TME contains cells of the immune system, the tumor vasculature, and lymphatics; as well as fibroblasts, pericytes, and sometimes adipocytes. Kneipp and coworkers were the first ones
to report early probing of intracellular compartments of macrophages and endothelial cells using a reporter-free SERS sensor. Differences in SERS spectra obtained over time helped identify physiologically relevant molecules, demonstrating the feasibility of reporter free SERS-based approaches for characterization of changing cellular environments [109]. Figure 2 illustrates how SERS can be utilized to probe the TME [41].

![Roles of SERS in monitoring TME](image)


Early detection is highly effective in improving cancer prognosis, and simultaneous detection of multiple biomarkers improves detection accuracy and treatment response monitoring. Multiplexed detection employing fluorescence-based methods often fail due to broad emission spectra which lead to spectrum overlap and strong background autofluorescence, whereas SERS methods can avoid these pitfalls. Dinish et al. demonstrated actively targeted multiplexed in vitro and in vivo detection of three intrinsic breast cancer biomarkers, EGFR, CD44 and TGFβRII [110]. The technique used intra-tumorally injected antibody-tagged SERS nanotags targeted towards these three biomarkers. The nanotags were maximally detected 6 h post injection, with no detectable signal at 72 hours. Nanotags without antibodies were not found to be detectable 6 hours post injection. This study established the prospective application of SERS-based technologies for ultrasensitive multiplexed-based tumor detection to study tumor
progression and early diagnosis. The biomarkers that were selected for this detection technique have important roles in the tumor microenvironment (EGFR, CD44 and TGFβRII). It has been previously demonstrated that EGFR signaling targets tumor growth by mediating autocrine and paracrine signaling of activating cytokines in the tumor microenvironment [111]. It has also been reported that CD44 is a functional molecule and surface biomarker in cancer-associated fibroblasts (CAFs), which support tumor growth in vivo and help maintain the cancer stem cell population in the tumor microenvironment [112]. In particular, CAFs strongly express CD44 in avascular and hypoxic regions of tumors, and CD44+ CAFs are often upregulated upon treatment with angiogenesis inhibitors. Finally, transforming growth factor β (TGFβ) plays a crucial role in promoting tumor growth and modulating the tumor microenvironment through contribution to the epithelial- to-mesenchymal transition, suppression of immune cell function, contribution to the conversion of fibroblasts to myofibroblasts, and promotion of overexpression of extracellular matrix [113]. Simultaneous detection of these biomarkers provides a multifaceted view of the tumor microenvironment.

Recent data show that inflammation is also a critical component of tumor progression and the tumor microenvironment, although the exact mechanism by virtue of which inflammation plays a key role in promoting tumor growth is broadly elusive [114]. Inflammation increases the risk of cancer development by generating bioactive molecules including cytokines, growth factors, chemokines that maintain a sustained proliferative rate, cell survival signals to avoid apoptosis, proangiogenic factors, and extracellular matrix-modifying enzymes. In particular, these ECM-modifying enzymes such as metalloproteinases promote epithelial-mesenchymal transition (EMT) and facilitate other carcinogenesis programs, such as genome instability, reprogramming of energy metabolism, and immune evasion [115]. Macrophages form the major cell population in the immune filtrate of tumors, and are linked to both inflammation and cancer. Pissuwan and coworkers have developed a SERS-based sensor which can detect lipopolysaccharide (LPS)-induced ICAM-1 expression in macrophages as early as 1 hour, in contrast with fluorescent labeling or ELISA which cannot detect any viable changes between induced and uninduced macrophages until 5 h after exposure. The SERS probe was comprised of GNRs conjugated with 4-mercaptophenzoic acid (4-MBA) and anti-ICAM-1 antibody. These results indicate that SERS probes provide greater sensitivity in the detection of macrophage-mediated inflammation in comparison to fluorescence microscopy and ELISA, and hence may be used as a tool for managing disease risk [116].

The endothelium is a specialized unicellular layer lining the blood vessels and lymphatics. One of the main activities of endothelial cells is to trigger innate and acquired immune responses upon activation by pro-inflammatory cytokines. Activated endothelial cells undergo rapid changes in their cellular composition and further mediate tumor growth through expression of pro-angiogenic factors. Thus, understanding the changes in cellular composition that are triggered by pro-inflammatory cytokines can help in the design of
Applications of SERS in Cancer Diagnosis and Therapy Monitoring

novel therapeutic approaches to cancer. The mechanism of triggering endothelial cells involves alterations in lipid compounds, which are characterized by the presence of long non-polar hydrocarbon chains in their structure. Given the large Raman cross-section of lipid molecules, Raman spectroscopy can be employed to study changes in intracellular lipid composition. Furthermore, 3D Raman images of cells can help track new entities formed inside the cell and study their distribution. Czamara et al. used Raman microscopy to demonstrate the presence of significantly high amounts of unsaturated lipids in inflamed cells in comparison to uninduced cells, a major hallmark of inflammation in endothelial cells [117]. This approach is better compared to conventional confocal microscopy, wherein the number of simultaneously monitored molecules is limited. The Raman signatures of intracellular lipid bodies can further be enhanced by employing plasmonic nanoparticles such as gold and silver. SERS has also been utilized to detect inflammation in vivo with gold nanoparticle clusters that are simultaneously conjugated with a Raman reporter and an antibody against intercellular adhesion molecules (ICAM-1). This SERS technique allows for non-invasive assessment of the ICAM-1 biomarker in vivo, with twice the sensitivity of conventional two photon fluorescence, as well as significant improvement in spectral definition, depth resolution, and significant signal-to-noise ratio gains in vitro, ex vivo and in vivo [118].

The generation and distribution of energy by tumor cells and the surrounding tumor microenvironment is highly complex. Characteristic nutrient, pH, and oxygen gradients are predominant pathophysiological features of the tumor microenvironment. The poorly structured and leaky vasculature surrounding a rapidly growing tumor mass often provides a scarcer supply of nutrients and oxygen, thereby creating and supporting the hypoxic tumor microenvironment [119]. Conventional 2D cell culture ignores the effects posed by the tumor microenvironment, whereas 3D cell culture techniques such as the formation of multicellular spheroids creates a radial structure with a quiescent or necrotic core and an outer layer comprising of proliferating cells. This 3D structures have gradients in nutrients, pH and oxygen that emulate tumor gradients present in vivo. Currently, limited knowledge is available about the quantitative nature of redox potential and pH gradients in cancer models. Redox potential is a measure of the oxidizing/ reducing environment of cells, and is defined by several factors such as oxygen concentration and metabolic pathways. Glycolysis provides more reducing equivalents of NADPH than oxidative phosphorylation, and the use of glycolysis is a universal property of most cancers that confers them with a growth advantage. pH is a function of hydrogen concentration, and a result of metabolism, because lactate production is the result of glycolysis. An analytical technique allowing quantitative assessment of redox and pH gradients could be a valuable tool for screening and monitoring response to therapies. Jamieson and coworkers created a novel technique to measure redox potential and pH, with spatial and temporal resolution that allowed them to probe the phenotype of spheroids and their response to drug and radiation therapy [120]. The SERS nanosensor was comprised of gold nanoshells coated with redox- or pH-
responsive molecules which were allowed to interact with the spheroid using a fiber optic Raman probe. The Raman probe was used to measure different SERS spectra under different conditions to provide a quantitative assessment of multicellular spheroids bioenergetics. The response to therapy measured using this technique was correlated with cell viability in the multicellular spheroids. The radial distribution was assessed using targeted SERS nanosensors, providing great potential for automation and scale-up.

There is accumulating evidence suggesting a clear association between hypoxia and intracellular acidification, which results from excessive glycolysis under hypoxic conditions. Several growth factors and chemokines such as tumor necrosis factor-α (TNF-α) in the tumor microenvironment also play an important role in intracellular endoplasmatic acidosis, and failure of intracellular pH regulation can lead to malignancy. Currently available techniques for measuring intracellular pH include pH-sensitive microelectrodes, nuclear magnetic resonance and fluorescence. Fluorescence is the most widely employed technique to measure intracellular pH but applications are limited by low sensitivity, signal from background, and photobleaching of fluorophores. SERS techniques can be used to overcome these limitations. Ma et al. reported a novel activatable nanoprobe that can measure changes in pH. The SERS probe used for this purpose was created with 4-nitrothiophenol (4-NTP) functionalized gold nanorods. Under hypoxic conditions, 4-nitrothiophenol was metabolized to 4-aminothiophenol (a known Raman reporter). This sensor aided in studying intracellular acidification in live cells and tissues. Dynamic pH analysis showed a change in pH from 7.1 to 6.5 as a function of different degrees of hypoxia (ranging from 15% to 1%), due to excessive glycolytic activity triggered by hypoxia [121]. Jaworska et al. reported that targeted gold nanoparticles functionalized with 4-mercaptopbenzoic acid (MBA-AuNPs) allowed detection of intracellular pH changes induced by short-term alkalinization or acidification of the extracellular environment of endothelium [122]. Using SERS-based intracellular pH mapping, the researchers demonstrated that a TNF-α-induced response in the endothelium was associated with intracellular acidosis. Uptake of MBA-AuNPs occurs through endocytosis, making SERS a valuable tool to study different stages of the process. The observed differences in uptake of MBA-AuNPs by healthy and inflamed cells suggested TNF-α-induced activation of uptake. The spectral signature of MBA-AuNPs can measure intracellular pH in the range of 5 to 9, which enables probing of different intracellular environments and different extracellular stimuli.

Tumor cells have a unique ability to evade immune surveillance by creating the appropriate conditions in the associated tumor microenvironment. A tumor microenvironment which is conducive to tumor cell growth and proliferation is driven by secretion of associated cytokines that play a major role in cell-cell communication between tumor cells and the surrounding immune cells, rendering the immune cells ineffective [123]. Therefore, there is a need for probing tumor-secreted molecular intermediates in cell-cell communication. Animal models can serve in understanding the complex
interaction between tumor and immune cells [124], but probing this cell-cell communication in vivo is complicated due to high cost and labor intensiveness. Due to the complexity of cell-cell interaction assessment in vivo, research attention has shifted towards development of in vitro drug screening platforms for simple analysis of intercellular communications. Traditional assessment of tumor-immune cell interaction can be achieved with cell culture dishes and multi-well plates [125]. Practical applications of these techniques are limited by their lack of reliability in mimicking the same interaction in vivo. Moreover, these techniques are not suitable for measurement of in situ cell-cell communication. Cell secretion profiling on traditional platforms involves the measurement of proteins secreted by cancer cells in vitro through ELISA and Western blot. These traditional techniques are often labor-intensive, and are unable to mimic the real extracellular microenvironment and in vivo signaling pathways that participate in the interplay between cancer and immune system. These models have also not been utilized for pharmacological profiling of drug candidates. Owing to the above concerns, there is still an unmet need for an integrated in vitro platform that can enable in situ monitoring of cancer-immune cell communication and allow for monitoring response to therapies.

The complexity of the extracellular environment and the low abundance of secreted proteins calls for an ultrasensitive and specific cancer secretion profiling system to precisely monitor secretion dynamics after drug treatment. Microfluidic chips have several positive attributes such as faster response time, lower sample consumption, precise liquid control, and high reproducibility thanks to the precise design of channels, chamber and valves, thereby facilitating studies on intercellular communications [126]. Wu and coworkers developed a fully integrated SERS-microfluidic platform that simultaneously aids in in situ cancer secretion profiling, analysis of cancer-immune system interaction, and drug screening on a single device [127]. The microvalve networks control the transport and exchange of fluids. Human cervix carcinoma cells serve as a cancer cell model, and their immunosuppressive effect on T lymphocytes is assessed using this integrated SERS-microfluidic platform. The SERS-microfluidic system is based on two modes: cancer cell secretion profiling (Mode 1) and analysis of secretion mediated cancer-immune system communication (Mode 2). Mode 1 employs a lab-on-chip SERS barcode based immunoassay for detecting several proteins secreted by HeLa cells such as TGF-β1, prostaglandin E2, and interleukin 10. Mode 2, on the other hand, measures the immunosuppressive effect induced by HeLa secretions by characterizing immune cell proliferation through fluorescence imaging. Furthermore, the technique allows for pharmacological profiling of drug candidates, carried out by monitoring dynamic changes in cancer cell secretion concentrations and immune response after drug therapy. The sensitivity of this integrated SERS-microfluidic platform was 1 ng/ml. This intercellular on-chip mode of communication preserves the bioactivity and quantity of the secreted proteins, thereby simulating the in vivo process of cancer–immune system interactions. The multiplexing capabilities of this SERS barcode immunoassay can be used for precise and
sensitive detection of multiple proteins secreted into the extracellular environment by cancer cells, minimizing operation errors and providing minimal complexity of handling. Using an ultrasensitive analytical technique with multiplexing capabilities that can probe the tumor microenvironment can provide important information regarding cancer prognosis and management, given the role of the tumor microenvironment in tumor growth.

SERS for Monitoring Therapy

Once cancer has been diagnosed and a plan for treatment has been established, one of the most important cornerstones of patient management is to determine therapeutic response to the treatment of choice. Potential treatment alternatives may include chemotherapy, radiotherapy, hyperthermia, photothermal therapy, photodynamic therapy, immunotherapy, hormone therapy, stem cell therapy, surgery, and combinations of these approaches. Monitoring the effects of therapy provides clinicians with crucial information about effectiveness of a specific treatment approach, and allows them to make informed clinical decisions regarding adjustments of dosages or timing of therapeutic agents for optimal tumor control. The ability to monitor therapeutic response can also assist in deciding whether a treatment option is the best for a specific patient, or other options should be pursued.

When a patient is receiving chemotherapy or other forms of therapy that involve intravenous administration of active agents, the intent of monitoring could be multifold. First, it is important to achieve therapeutic drug concentrations in plasma, so that the dose is enough to be effective, whereas toxicity and unwanted side effects are minimized; although, as further explained below, there are several barriers to routine implementation of this type of monitoring in oncology [128, 129]. Second, injected agents must reach their targets, so monitoring the amount of injected dose that reaches the tumor can provide important information to predict therapy effects. Finally, the actual therapeutic effect of reducing or arresting tumor growth or progression can be monitored to assess the effectiveness of therapy.

Traditionally, monitoring therapeutic drug concentrations in plasma has been limited to specific drugs with narrow therapeutic indices, which need to stay within a very constricted plasma concentration in order to avoid severe toxicity. Cost, analytical limitations, and lack of portable instrumentation have restricted the widespread availability of therapeutic drug monitoring at the point of care [130]. Typically, monitoring is performed with immunoassays, or with separation techniques (such as liquid chromatography) combined with mass spectrometry. Although immunoassays can be quick, simple, and portable, they often lack sufficient specificity and measurements are confounded by other circulating molecules. Separation techniques, on the other hand, are
usually laboratory-based and involve significant cost and time for analyte processing [130]. Recently, SERS techniques have shown promise for use in therapeutic drug monitoring. Sun et al. modified a SERS substrate by supplementing the substrate with two layers, which were specifically designed to improve the ability to detect analytes in plasma [131]. The first layer was a self-assembled monolayer of thiols designed to attract analytes for detection and amplify their signals. The second layer was a zwitterionic brush layer of poly (carboxybetaine acrylamide), with the purpose of avoiding protein aggregation on the surface of the detector. This phenomenon, known as protein fouling, is a common issue encountered when analyzing plasma samples and can impact measurement sensitivity. Using this modified substrate with a microfluidic system, the authors were able to successfully measure undiluted samples of human plasma that had been spiked with different concentrations of doxorubicin (DOX). The system was not only able to quantify DOX concentrations in real time from the samples flowing over the sensor over a period of 3 hours, but also was able to differentiate DOX from its secondary alcohol metabolite doxorubicinol (DOXol). DOX concentrations were between 0.5 and 20 µM. When determining DOX concentrations from these samples, one must consider that only DOX that is not bound to proteins will be measured by the system. The authors determined that the fraction of DOX that remains unbound is between 23 and 33%, and confirmed their observation using liquid chromatography-mass spectrometry. Finally, the authors also discussed different modifications to the first layer, so that its composition can be customized to better attract or probe analytes with different chemical characteristics such as degree of hydrophilicity.

When treatment agents such as chemotherapy drugs start to reach their target, a set of potential methods to monitor therapy at the microscopic level involves the use of SERS substrates in the design of therapeutic agents, so that SERS can be used to quantify intracellular uptake. For example, Chen et al. utilized SERS-fluorescence dual mode imaging to evaluate the uptake of nanocomposites composed of graphene oxide and AuNP core polyaniline shells (GO-Au@PANI) that were loaded with DOX [132]. These nanocomposites can be used for chemo-photothermal therapy, fluorescent imaging, and SERS probing. Polyaniline is a near-infrared probe that can be used for photothermal therapy. DOX release from these nanocomposites can be triggered by near-infrared irradiation and pH changes. Intracellular delivery of the nanocomposites into 4T1 mammary carcinoma cells was monitored by a simultaneous combination of SERS signals from the nanocomposite structures (1100-1200 cm⁻¹, λex: 785 nm) as well as the fluorescent signal from loaded DOX (590-640 nm, λex: 514 nm). The authors also evaluated the potential of the nanocomposites for in vivo therapy in tumor-bearing Balb/c mice, reporting that the combination treatment with the nanocomposites and laser irradiation at 808 nm had a greater therapeutic efficacy compared to DOX alone, or to photothermal therapy alone. However, SERS probing was not utilized in vivo in this study.
Assessing the actual effectiveness of tumor therapies in arresting tumor growth may involve different approaches including macroscopic and microscopic measurements [133]. Reduction in tumor size is a common measure of therapeutic effectiveness for solid tumors, and can be accomplished with traditional imaging techniques such as X-ray, magnetic resonance imaging (MRI), ultrasound, and computerized axial tomography (CAT) [134]. However, it is often desirable to obtain information about the effectiveness of therapy at earlier stages of treatment, when the effect is occurring at the microscopic level and has not yet translated into actual macroscopic changes. Thus, approaches such as the measurement of circulating tumor markers (discussed in section 1), or probing changes that are occurring at the cellular level as tumors are exposed to treatment, are at the forefront of current research and development in this area, and could be important adjuvants to the metabolic information provided by other widespread techniques such as positron emission tomography (PET), which lacks the ability for multiplexing and can lack sufficient resolution [134, 135].

As introduced in our discussion of the tumor microenvironment in section 3, the ability to monitor changes in redox potential and pH of tumor cells can provide important information regarding tumor cell viability, and could potentially guide therapeutic decisions by evaluating the effects of therapy at the microscopic level. Most reports of intracellular SERS probes for redox and/or pH changes involve cell monolayer setups [122, 136-138], which are not necessarily reflective of a 3-D tumor environment and of how those measurements may change during in vivo therapy. However, in 2016, Camus et al. reported that they were able to measure loss of cell viability based on biochemical measurements in a 3-D prostate cancer spheroid model after radiotherapy administration using SERS probes [139]. In this study, the authors used redox- and pH-sensitive probes attached to gold nanoshells (NS) in order to investigate the characteristics of the intracellular environment and determine cell viability. The redox-sensitive probe was 2-chloro-3-[methyl(2-sulfanyl)ethyl]amino]-1,4-dihydronaphthalene-1,3-dione (MeNQ), and the pH-sensitive probe was 4-mercaptobenzoic acid (pMBA). Under varying redox potentials and pH values, these probes show characteristic changes in their SERS fingerprint. MeNQ can measure redox potentials from −100 to −450 mV, and pMBA can measure pH in the range from 6 to 8. Prostate cancer spheroids exposed to a total radiation dose of 12 Gy, in the form of two treatments of 6 Gy each, showed a significant increase in pH (with alkalosis linked to cell death) and more reducing redox potential, as well as loss of morphology of the spheroid. Similarly, Jamieson et al. utilized gold nanoshells coated with pH and redox-responsive probes to investigate pH and redox potential changes in 3-D breast cancer and prostate cancer spheroid models after exposure to radiation, the chemotherapy drug cisplatin, and the apoptosis-inducing drug staurosporine [120]. They were similarly able to measure changes in gradients related to cell viability, as well as to observe the effect of drug resistance on the response to treatment.
At this time, these viability monitoring techniques have only been used for in vitro studies in 3-D tumor models, in order to guide choice of therapy dosages and provide a better characterization of the response to therapy in these models. More work needs to be done in order to translate these methods into real-time clinical monitoring approaches.

**SERS for Multimodal Imaging and Image-Guided Surgery**

Recognizing that cancer is a complex, multifactorial, and multifaceted disease means that no single method can provide all the diagnostic information that is often required to optimally manage care. Combinations of imaging techniques are often used, in an effort to better understand the functional, anatomic, and molecular characteristics of cancer at both the macroscopic and the microscopic levels. The use of multimodal imaging in cancer detection and monitoring can provide simultaneous and complementary information that can assist clinical decision-making. Furthermore, the ability to use imaging to guide surgical treatment approaches also has the potential to enhance surgical outcomes by providing surgeons with real-time information about tumor boundaries and optimal resection borders. Developing techniques that can guide surgical tumor resection is of special importance, due to the difficulty in determining exact tumor boundaries [140]. Although an obvious choice is to excise beyond the visible borders of a tumor in order to maximize the chances of successful resection, this leads to the potential destruction of healthy tissue which may be critical for function (as is often the example in brain surgery), but it still provides no guarantee that all malignant tissue has been removed. Additionally, the color and texture of healthy and tumorous tissue may be similar, creating a challenge for demarcation. Several techniques can be used to enhance the surgeon’s ability to differentiate tumor tissue. Most commonplace is the use of traditional imaging techniques such as MRI, CT, or ultrasound; however, these have disadvantages including practicality of using the equipment within the surgical suite, and issues with penetration and resolution. Thus, alternative imaging techniques such as fluorescence and Raman microscopy have started to attract attention for use during surgical procedures.

SERS-capable nanoparticles have only been used for in vivo imaging studies in animals, due to the lack of approval for systemic administration in humans. However, SERS imaging has shown great promise for clinical translation in the last decade, with the first patent for Raman imaging filed in 2007, and a lot of subsequent work being done in this area to employ either passive accumulation or active targeting for in vivo Raman imaging [135]. Multimodal imaging has also been explored, with several reports of combination of SERS with other imaging techniques, such as fluorescence. We have also achieved a better understanding of the potential for Raman signals to guide surgical resection, since they can provide boundary information after accumulation of nanoparticles within the tumor limits, and delineate healthy tissue from diseased tissue based on their
different Raman signals, as has been demonstrated for many different tissues including brain, breast, skin, tongue, and the oral cavity, among others [141-146]. The use of coherent Raman scattering microscopy, which amplifies Raman signals by a factor of 10,000, can provide a means to obtain high differentiation of tissue borders, and has shown to have very good agreement with histological analysis [147].

In an example of multimodal imaging, Kim et al. used an endoscopic system that combined SERS and fluorescence to specifically detect tumor markers such as VEGF and EGFR in an orthotopic colon cancer mouse model [107], and which could be combined with a regular colonoscopy procedure. In this technology, fluorescence enables localization of tumor lesions (limit of detection 0.5 pM), whereas Raman signals provide information about the tumor microenvironment via targeted interactions with specific receptors (limit of detection 1 pm). Fluorescence-SERS dots (F-SERS dots) were created by using silver-embedded silica nanoparticles bound to Raman-labeling RITC or FITC, which were then encapsulated into a fluorescent dye conjugated shell. The same group also demonstrated the use of fluorescence-SERS technology to target EGFR and HER-2 in an orthotopic breast cancer model [148].

Another multimodal system created by Shi et al. combined SERS with photoacoustic imaging by using folate-targeted SERS nanoparticles S440 and S421, along with magnetic fluorescent folate-targeted silica particles [149]. The system was tested in vitro and in vivo, with HeLa cells injected subcutaneously in the ear region of rats serving as the in vivo model for circulating tumor cells. This method was capable of magnetically trapping tumor cells and providing simultaneous photoacoustic microscopy images and SERS signals. Hence, this approach is capable of structural vascular imaging as well as molecular imaging, and could provide single-cell detection levels if at least 200 SERS nanoparticles are internalized per cell.

Since MRI is a widely used imaging technique, there have also been efforts to combine the structural information from MRI with the rich microenvironment information that Raman signals can offer. For instance, Ju et al. reported the design of dual-mode nanoprobes based on synthetic melanin encapsulated inside hollow gold nanoparticles, which could be used for T1-weighted MRI and SERS imaging [150]. These particles were surface-modified with PEG for increased stability, and complexed with Fe3+ ion to enhance contrast ability. Their uptake and imaging capabilities were successfully tested in vitro using MDA-MB 231 breast carcinoma cells, demonstrating the ability to obtain T1-weighted MRI images as well as a strong Raman signal.

In an effort to move towards clinical translation of SERS application for guided surgery, several groups have developed novel systems that incorporate Raman signal detection in a manner that could potentially be used in a surgical environment, both by itself as well as combined with other modalities. Karabeber et al. reported the use of a combination system which included a static Raman imaging device and a hand-held Raman scanner [151]. The authors simulated an intraoperative scenario in a glioblastoma mouse
model and found that gold-silica SERS nanoparticles allowed for very accurate delineation of the tumors, with better tumor resection when Raman imaging was used to guide the surgery compared to surgery without guidance. In particular, the hand-held scanner not only enabled real time scanning, but also detected some microscopic tumor areas that were missed with the static system. An obvious limitation of this study was that tumor resection occurred ex vivo, after the brains had been extracted from the animal, thus reducing the applicability to a realistic surgical setting. In another study, Jokerst et al. used passive targeting of gold nanorods to achieve combined photoacoustic and Raman in vivo imaging of ovarian cancer xenograft mice models [152]. SERS imaging was used to delineate tumor boundaries, and image-guided resection was successfully performed 24 h after nanorod injection. The authors propose that photoacoustic imaging could be used to complement other techniques such as ultrasound in order to determine tumor size, stage and morphology, whereas SERS would be useful to guide resection limits. An important limitation was that SERS scans required 20 minutes at 500 micrometer resolution, highlighting the need for wide-field imaging systems that can better translate this technology into the operating room.

Mohs et al. developed a novel system they called the “Spectropen,” which connects a sampling head to a spectrometer that can measure Raman and fluorescence signals (Figure 3) [153]. The device has detection limits in the order of $10^{-11}$ M for fluorescent dyes such as indocyanine green (ICG) and in the order of $10^{-13}$ M for pegylated colloidal gold as the SERS agent. This group performed in vivo studies in tumor mice (4T1 breast tumors) and were able to detect tumor boundaries in a precise manner both preoperatively and intraoperatively. The authors also point out that their portable system allows to further explore the surgical cavity in real-time after resection, to ensure detection of any tumor tissue that may have been left behind, and really bring image-guided surgery to its full potential.

In another demonstration of the possible applications of multimodal imaging for surgical applications, Kircher et al. were able to synthesize gold-silica based SERS nanoparticles coated with Gd$^{3+}$ ions, which allowed for triple modality imaging: photoacoustic imaging, magnetic resonance imaging, and SERS detection [154]. In vivo experiments with orthotopic mouse glioblastoma models showed passive accumulation into tumor tissue after i.v. injection, due to the enhanced permeability and retention effect. The particles were detectable in live mice with at least picomolar sensitivity for at least 1 week after injection, using MRI, photoacoustics, and Raman. Raman imaging in this study showed the ability to guide intraoperative tumor resection in mice, as confirmed by histological analysis. This triple combination is particularly interesting for surgical delineation because it provides the macroscopic information from MRI, high 3D spatial resolution from photoacoustic imaging, and the microscopic level information provided by the Raman signal, which offers high sensitivity, specificity, and multiplexing ability [154, 155].
SERS for Theranostic Applications

The combination of diagnostic and therapeutic applications into a single agent has become a promising strategy in cancer treatment, with the vision of providing highly personalized care in a cost-effective and patient-centered manner. Multifunctional approaches may allow for early detection, customized therapy, and therapeutic monitoring which would have the potential to enhance patient outcomes. Current research shows that SERS can be combined with different multimodal imaging as well as therapeutic approaches, as mapped in Figure 4. Additionally, Table 4 provides specific examples of theranostic approaches to cancer management that include the use of SERS.
Table 4. Examples of the use of SERS in theranostic designs for cancer management

<table>
<thead>
<tr>
<th>Agents combined in the design</th>
<th>Tumor model</th>
<th>Theranostic modes</th>
<th>Reference</th>
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<td>EGFR-functionalized PEGylated gold nanoparticles, rhodamine 6G</td>
<td>Lung cancer cells</td>
<td>Photothermal therapy, SERS imaging</td>
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<tr>
<td>Silver-coated gold nanostars functionalized with para-mercaptobenzoic acid (p-MBA), porphyrin IX</td>
<td>Folate receptor positive (FR+) cancer cells</td>
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<tr>
<td>Folate-functionalized silver nanoparticles, doxorubicin</td>
<td>Folate receptor positive (FR+) cancer cells</td>
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<tr>
<td>Folate-functionalized Pd-pyrolipid nanoparticles (PdPL-NPs)</td>
<td>Folate receptor positive (FR+) cancer cells</td>
<td>Photodynamic therapy, SERS imaging</td>
<td>159</td>
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<tr>
<td>Penetrating peptide functionalized gold nanoparticles, doxorubicin</td>
<td>Breast cancer cells</td>
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<tr>
<td>PEGylated gold nanoparticles, 3,3'-Diethylthiatricarbocyanine iodide (DTTC), antibody-drug conjugate Cetuximab</td>
<td>Mouse xenograft tumor model – colorectal adenocarcinoma</td>
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<td>Gold nanostars, p-MBA reporter</td>
<td>Mouse xenograft tumor model - sarcoma</td>
<td>Photothermal therapy, multimodal imaging (SERS, CT, and two-photon luminescence)</td>
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</tr>
</tbody>
</table>

Among the myriad of recently developed systems which attempt to combine diagnostic and therapeutic capabilities, there are several methods that use Raman signals combined with treatment agents. In 2016, Chen et al. used reduced graphene oxide nanosheets as a platform for a porous silica shell. AuNPs were then trapped within the pores of the shell, and the Raman reporter rhodamine 6G was adsorbed on the AuNPs. Further, this design was PEGylated and conjugated to anti-EGFR antibody [156]. During in vitro tests with A549 lung cancer cells, which overexpress EGFR, this design was capable of photothermal therapy after irradiation with a laser at 808 nm. Additionally, it was able to detect cells using a confocal Raman microscope. The authors concluded that these anti-EGFR SERS probes can be used to detect cancer cells as well as effectively kill cancer cells after NIR irradiation, without damage to neighboring tissue thanks to active targeting. In a similar study, Fales et al. [157] reported the use of gold nanostars functionalized with para-mercaptobenzoic acid (p-MBA), coated with silver, and covered with a silica shell where a photosensitizer (porphyrin IX) was loaded. Finally, they functionalized the surface of the shell with folate. This system was then tested in vitro using two folate receptor positive (FR+) and two folate receptor negative (FR-) cell lines, demonstrating that the construct was able to selectively detect FR+ cells. Additionally, photodynamic therapy was
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successfully administered using this design by irradiating with filtered red light (640/30 nm) from a mercury arc lamp for 15 seconds, at a power of 11.5 mW. After this exposure, FR+ cells (where the constructs had accumulated) showed over 80% reduction in cell viability. Srinivasan et al. created a multifunctional silver-nanoparticle-based prodrug system which was capable of folic-acid targeted photodynamic therapy as well as pH-triggered DOX release [158]. In vitro studies showed that uptake into FR+ cells was enhanced, and upon entrance into the cell, pH-triggered release of the chemotherapy drug was achieved. This therapeutic effect was combined with the cytotoxic effect of photodynamic therapy upon light irradiation. This theranostic system also allowed for cell probing using SERS and fluorescence spectroscopy.

Figure 4. Combinatorial approaches of SERS with multimodal imaging (solid green) and therapeutic strategies (dotted orange).

The ability of SERS to be used for monitoring of treatment effects was illustrated by Farhadi et al. [159], who demonstrated that by coupling plasmonic nanoparticles with palladium photosensitizers. They were able to use SERS to measure the damage imparted to cells by photodynamic therapy. Briefly, this group synthesized Pd-pyrolipid nanoparticles (PdPL-NPs) that when excited by 638-nm light become photodynamically active and produce a bright SERS signal. By decorating these NPs with folate, the authors were able to establish the combined therapeutic and monitoring effect in folate-positive KB cells (a subline of HeLa cells). The fact that the same laser wavelength can be used to trigger photodynamic therapy and measure SERS signal facilitates the application of this
approach. In situ-monitoring was also explored by Hossain et al. [160], who used antibody-targeted, DOX-loaded biohybrid AuNPs functionalized with cell penetrating peptide Tat. By measuring the intracellular SERS signals from DOX, the authors were able to monitor chemotherapy release in real time. This system establishes a foundation for potential monitoring of the release of different types of chemotherapy agents inside cells.

In an example that illustrates in vivo capabilities, Conde et al. developed SERS gold nanoantennas by capping 90-nm AuNPs with the Raman reporter DTTC, PEGylated them, and functionalized the PEGylated surface with the antibody-drug conjugate Cetuximab [161]. This functionalization allows the theranostic system to exert its targeted therapeutic effect by interacting with EGF receptors and blocking signals for uncontrolled cell division. Simultaneously, the Raman reporter-capped gold nanoparticles allow for in vitro and in vivo SERS signal detection, which can not only inform the location of the tumor, but also provide insight into the growth inhibition effect brought upon by the therapy. In this study, a xenograft mouse tumor model demonstrated the success of the combined theranostic approach, allowing for diagnostic imaging, tumor treatment, and therapeutic monitoring. Additionally, animals were also followed for up to one month after injection and demonstrated no toxicity effects.

Liu et al. also demonstrated the multifaceted potential of theranostic applications involving SERS [162]. In their work, they created gold nanostars with a p-MBA reporter, which were capable of multimodal imaging (SERS, CT, and two-photon luminescence – TPL) as well as photothermal therapy in a mouse xenograft tumor model. Irradiation for photothermal therapy was performed with a 980-nm laser at 0.7 W/cm² for 10 minutes in two different sites. The researchers were able to detect a strong in vivo SERS signal in sarcomas through the intact skin for both 30-nm and 60-nm gold nanostars, which had SERS enhancement in the order of 100 times stronger than gold nanospheres; and the nanostars were also detectable using TPL and CT, allowing for a broad spectrum of diagnostic interrogation.

LIMITATIONS AND FUTURE PERSPECTIVES

SERS is a powerful tool that is capable of providing rich spectroscopic information at ultrahigh sensitivity and specificity for label-free and multiplexed tagging. But although SERS-based in vivo technologies show great promise, there are still many opportunities for improvement. High cost, a somewhat complex output signal, reproducibility of enhancement, and other issues can create barriers for implementation. In this section, we briefly discuss future directions, challenges, and opportunities.

The main goal in the production of SERS substrates has been to achieve maximum electromagnetic enhancement in order to accomplish single-molecule sensitivity. These regions of enhanced electromagnetic enhancement are very small in comparison to the rest
of the surface, and hundreds to thousands of reporter molecules are adsorbed on every particle. Uniform enhancement over the surface is more desirable than ultrahigh enhancement factors in a few small surface locations, because a widespread interaction area provides more meaningful information about the prevalence of the target analyte than just confirming that it is present, and would enable more exact quantification. This would require the development of uniform SERS-based nanostructures that are devoid of sharp edges or protrusions; while maintaining a large enough inter-particle distance to prevent plasmonic coupling between particles. Methods focused towards the development of substrates with high substrate uniformity as well as strong SERS enhancement demonstrate both uniform signal intensities and high sensitivity [163, 164]. Shen and coworkers also showed how the use of nanostructures with noninterfering internal standards could help enhance the quantitative abilities of SERS substrates [165]. In this approach, the reporter molecule is sandwiched in between the metal core and shell, serving as an internal standard whose signal is unperturbed by the external chemical environment or adsorption of analytes upon the outside layer of the nanoparticle surface. This internal standard can also help correct the variability in signal enhancement owing to variable aggregation. Thus, this method can be used in conjunction with uniform SERS substrates to provide a reliable quantitative assessment of trace analytes.

Another main factor affecting SERS quantification is particle brightness. The development of SERS tags with uniform brightness is a critical challenge to be overcome, because Raman signal enhancement is the result of complex interactions between a multitude of factors such as particle size distributions, local morphology (faceting and/or defects in structure), and the adsorption of multiple Raman reporter molecules per particle. One possible method to overcome this challenge is through the use of monodispersed spherical colloids with less faceting and fewer crystal defects than the conventional citrate-stabilized nanoparticles. Then, Raman reporters should be delivered slowly to allow uniform diffusion of reporters onto the nanoparticle surface [166]. This method, however, suffers from serious limitations, such as the reduction in SERS enhancement when using monodispersed nanoparticles in comparison to citrate-stabilized nanoparticles. The reducing agents and/or surfactants used to synthesize monodispersed nanoparticles are also bulkier than citrate [167], thereby requiring thiol-modified reporters to provide higher affinity towards the nanoparticle surface [168]. Furthermore, the signals obtained from SERS nanoparticles are highly complex and require multivariate analysis software for data assessment [169].

The greatest obstacle in the advancement of SERS for in vivo human applications is the resistance of inorganic nanoparticles to potential biodegradation, raising concerns about their long-term toxicity in vivo. There have been several conflicting reports on the toxicity of silver and gold nanoparticles, even though gold in particular is considered, at least theoretically, to be biochemically inert and biocompatible [170-172]. Contradictory reports on the toxicity of gold nanoparticles could be attributable to the different ligands
adsorbed onto their surface. The weak electrostatic interaction of citrate onto the nanoparticle surface renders them more liable to aggregation in biological fluids, with high ionic strength leading to toxic effects. Additionally, the ligands themselves may be more cytotoxic than the metal nanoparticle core, as in the case of CTAB-stabilized gold nanorods [173]. The cytotoxicity of silver nanoparticles is mainly due to generation of reactive oxygen species (ROS) and potential DNA damage [172]. Smaller and anisotropic nanoparticles exhibit higher surface-to-volume ratios, which can lead to higher toxicity due to increased potential for degradation and silver ion release [174]. Although gold nanoparticles are more stable than silver nanoparticles, their long-term toxicity is still questionable, as long-term exposure to gold nanoparticles does not result in their complete elimination from body, and it can still lead to generation of ROS which are key mediators of nanoparticle-based toxicity. The inherent toxicity of Raman reporters can also be a subject of concern, but this issue can be overcome using more biodegradable Raman reporters, such as ICG.

The toxicity of SERS tags was examined by Thakor and Gambhir’s group, who incubated liver cells with ~ 120 nm silica-coated gold nanoparticles [175]. The choice of liver cells was based on the fact that these cells exhibit high accumulation of nanoparticles upon intravenous administration. Their results showed that possible hepatocellular toxicity was dependent on both nanoparticle number and exposure time. They demonstrated that toxicity was reduced with exposure to a lower number of nanoparticles, because of lower ROS generation compared to when cells were exposed to higher numbers of nanoparticles. Another important finding was that the cells were not able to generate enough antioxidants to counteract the levels of ROS generated through long-time exposure to nanoparticles in vitro. Upon investigation of the in vivo behavior of these nanoparticles, the group demonstrated that gold nanoparticles reached the liver within the first few minutes after administration but started getting cleared through the hepatobiliary system only after 2 weeks. In the spleen, particles continued to accumulate for a 24-h period before gold concentrations started to decline. Potential hepatocellular death due to the interaction of hepatic cells with nanoparticles was induced through generation of ROS. Therefore, although the gold nanoparticles were demonstrated to be eliminated from the system without affecting the target organs in a significant manner, their elimination process was rather slow and could potentially be problematic [176].

Recent reports have suggested that colloidal gold has little or no long-term toxicity or serious effects on vital organs in vivo. However, the long-term effects of these nanoparticles have not been tested in human patients. Phillips et al. conducted the first human clinical trial of ultra-small inorganic hybrid silica nanoparticles in metastatic melanoma patients [177]. These silica nanoparticles were labeled with 124I for PET-based monitoring. Metabolic profiles and laboratory tests of blood and urine specimens were monitored over a period of 2 weeks, and demonstrated that their route of elimination was renal clearance. No toxic or adverse effects were observed with these nanoparticles, thus
suggesting that they would be safe for use in human diagnostics. Choyke et al. demonstrated that particles having a defined hydrodynamic diameter adding up to 10 nm (including thickness of the core and coating) can be efficiently cleared through renal clearance [178]. However, SERS substrates require that the particle size should be greater than 20 nm for efficient SERS-based detection, which means that these nanoparticles may not be efficiently cleared through the renal system. Some SERS tags of about 120-nm diameter have been shown to sequester in tumors through the enhanced permeability and retention effect, and they are cleared through the hepatobiliary system. However, clearance through the liver is very slow (in the order of several months), and this long-term exposure of liver cells to nanoparticles is anticipated to cause significant hepatocellular toxicity through substantial generation of ROS [179]. To circumvent this problem, SERS nanoparticle design is currently focusing on creating particles that are greater than 20 nm, allowing them to preferentially sequester in tumors, but which are also later broken down to smaller particles by cells, so that they can be efficiently cleared through the renal system. Briefly, Chou et al. demonstrated that small SERS satellite nanoparticles (10 nm) could be assembled into large structures through programmed DNA assembly [180]. One of the challenges, however, was that particles which did not have sufficient PEGylation could be taken up by the reticuloendothelial system, where the clearance rates were still prolonged, whereas particles with greater PEGylation had trouble getting cleared through the renal system. Thus, a balance was sought to be made for efficient clearance of these nanoparticles through the renal system. The optimal SERS nanoparticles that can produce effective SERS enhancement and yet, be cleared through renal clearance is a subject of further investigation.

Another issue in SERS-based molecular imaging is achieving desirable levels of tissue penetration. Although SERS-based imaging techniques exhibit high sensitivity and resolution, they are often limited in their clinical applications due to small depth of penetration. The depth of penetration of SERS signals in tissue is dependent on the light source, wavelength, and power. For example, Haka and coworkers showed that a Raman spectroscope employing an 830-nm diode laser with a power of 82-125 W per second could be used to achieve a depth of penetration of 1-5 mm [181]. Recent advancements in optical instrumentation have enabled an increase in penetration depth by employing a novel technique called spatially offset Raman spectroscopy (SORS). This technique involves the collection of incoming backscattered light from the illumination area, thereby collecting the photons from excitation regions which are distanced far from the excitation region. The collection regions that are far from the excitation region are more sensitive to light, as photons diffuse out to larger areas with distance [182]. It has been demonstrated that penetration depths up to 2 cm can be achieved using surface-enhanced SORS [183]. Even though this is still a small level of penetration, it could be useful for surgical planning.

Imaging large areas can also present challenges for practical applications. Most SERS-based imaging involves a focused laser spot, which creates a 2D scan of the sample. Thus,
imaging large areas using a traditional SERS-based imaging technique might involve long acquisition times, and whole animal imaging can be practically impossible in a reasonable time frame. A recent advancement in the field of Raman imaging is wide-field Raman detection. Wide-field devices have illumination spot sizes of several centimeters, which makes it feasible to scan small animals [184]. However, these apparatuses do not match the sensitivities obtained by direct illumination, and their limits of detection are mostly in the picomolar range [153]. Thus, wide-field approaches may be suited for SERS-based anatomical imaging, whereas direct illumination should be used for high-sensitivity, high spatial resolution detection.

**CONCLUSION**

SERS nanoparticles currently offer several advantages over traditional imaging techniques, including their ultrahigh detection sensitivity, ability for spectroscopic multiplexing and label-free detection, multivalent targeting, favorable pharmacokinetics, and the potential for multifunctionality and multimodality. There are several new approaches using SERS that have been explored in the field of cancer detection and image-guided surgery, and which hold great promise for clinical translation. Looking into the future, there is a need for the development of label-free ultrasensitive detection and quantification of cancer biomarkers, whole-body animal imaging with higher sensitivities, and design of SERS substrates with effective signal enhancement and superior *in vivo* clearance properties to reduce the potential for long-term toxicity.

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