Bioanalytical Measurements Enabled by Surface-Enhanced Raman Scattering (SERS) Probes

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Abstract

Since its discovery in 1974, SERS has gained momentum as an important tool in analytical chemistry. SERS has been used widely for analysis of biological samples ranging from *in vitro* cell culture models, to *ex vivo* tissue and blood samples, to direct *in vivo* application. New insights into biochemistry with an emphasis on biomolecule detection, from small molecules such as glucose and amino acids, to larger biomolecules such as DNA, proteins and lipids, has been gained. These measurements have increased our understanding of biological systems and, significantly, increased diagnostic capabilities. SERS probes have displayed unique advantages in their detection sensitivity and multiplexing capability. We will highlight key considerations required when performing bioanalytical SERS measurements, including sample preparation, probe selection, instrumental configuration, and data analysis. Some of the key bioanalytical measurements enabled by SERS probes with application to *in vitro*, *ex vivo,* and *in vivo* biological environments will be discussed.

1. Introduction

1.1 SERS and its application to biological analysis

Raman scattering is an inelastic process that exploits the fact that individual bonds give rise to unique vibrations, resulting in molecularly specific spectra.(1) Due to the fingerprint nature of Raman spectra, this allows individual components within a sample mixture to be identified. However, Raman scattering is an inherently weak process, with only approximately 1 in 10^6 photons being inelastically scattered. Research carried out by Fleischmann et al.(2) in the 1970s, and developed by Jeanmaire and Van Duyne(3), led to the discovery that by adsorbing the molecule of interest onto a roughened metal surface, the Raman signal could be

significantly enhanced(4), establishing the method that is now known as surface enhanced Raman scattering (SERS). Enhancement factors of 10^4-10^8 compared to conventional Raman have been reported.(5-7) The most common materials used to provide the roughened metal surface for SERS are colloidal suspensions of gold and silver nanoparticles due to their surface plasmons existing in the visible region of the electromagnetic spectrum. This is extremely beneficial when using laser excitations of 633 and 785 nm, commonly used for Raman analysis.

SERS has been widely applied for detection of biological molecules and systems such as proteins(8), DNA(9, 10) and cells.(11) One of the many advantages of using Raman for the analysis of biological samples is the amount of information that is obtained about molecular properties. The recent developments in chemometrics and multivariate analysis have made it even more accessible for Raman to be used to analyse complex biological samples.(12)

1.2 Key considerations when making bioanalytical SERS measurements

SERS is an important tool for the analysis of biological samples and this section will highlight the key considerations required when performing bioanalytical measurements with said probes before highlighting some of the seminal advances in the use of SERS probes in in vitro, ex vivo, and in vivo environments. In order to perform optimal measurements for particular application, careful consideration must be made with respect to the selection of preparation methods for the biological samples to be analyzed, appropriate SERS probes, a suitable instrument configuration, and appropriate data processing and analysis methods. These considerations are summarized in Figure 1.

1.2.1 Biological sample preparation

Consideration must be given to how a biological sample will be prepared for SERS measurement. In the case of in vitro samples, cells can be fixed or live. Often measurements will only be physiologically relevant if obtained using live cells for example using SERS probes for pH sensing.(13) Fixed cells are far easier to work with, specifically when SERS probes are utilized, as cell toxicity may be mitigated. However, fixing cells chemically modifies the cells and could introduce artifacts as a consequence of the fixing process. For ex vivo blood samples, the blood can be analyzed as whole blood, or by collecting the red blood cell, plasma or serum components.(14, 15) Ex vivo tissue samples tend to be processed by fixation (either chemical or freezing) followed by sectioning. Chemical fixation is the most common and tends to be followed by paraffin embedding and sectioning, including a number of dehydration and rehydration steps. Again, these steps can introduce artifacts that may interfere with SERS measurements. Excised tissue can be analyzed directly but this is less common. In vivo measurements ultimately require the patient to be alive, and therefore, there are no sample preparation steps; rather, the SERS probes have to be designed to integrate into this living environment. In all cases, consideration must be taken of other biomolecules that might interfere with SERS measurements, such as bovine serum albumin (BSA) in cell culture media and additional blood proteins in blood samples.(16) Background fluorescence, particularly for in vivo samples, can also create measurement interference. However, careful choice of laser wavelength can diminish these effects, as will be discussed in section 1.2.3.

1.2.2 SERS probe selection

When selecting the ideal SERS probe for a particular bioanalytical measurement, consideration must be made to a number of factors. Schlücker(17) concisely summarized the required components of a SERS probe as a metal nanoparticle, SERS substrate, functionalized with a Raman reporter label and a biomolecule. (Figure 2) In some cases, none of these components are added where direct SERS measurement are recorded from biomolecules that are in close proximity to unfunctionalized metal nanoparticles.(18) However, each of these components will be briefly discussed along with their application-dependent presence or absence, and potential variations of each.

Typically, gold and silver are the metals of choice as SERS substrates due to their optimal optical properties as they have plasmon resonances that lie in the visible and near-infrared (NIR) range. The plasmon resonance of nanoparticles can be tuned by careful selection of nanoparticle size, and in the case of nanoshells, core-to-shell ratio.(19) Optimal laser wavelength for particular application can be used by selecting a nanoparticle with the desired plasmon resonance.

Gold(20) and functionalized silver(21) nanoparticles also exhibit low toxicity with application to biological systems. In particular, gold nanoparticles are inert, and have been approved for use in live humans for particular applications already.(22) For this reason, in vitro and in vivo applications will often use gold nanoparticles as the substrate of choice. Silver, however, tends to exhibit superior scattering properties and larger enhancement of Raman signals.(23) Therefore, in ex vivo application where nanoparticles are not being applied directly to living systems, silver may be preferable. While bare silver nanoparticles have displayed cytotoxic effects, and gold is therefore favored for in vitro and in vivo work, studies have shown that

this toxicity can be mitigated after functionalization. The mechanism behind silver nanoparticle toxicity and its reduction after functionalization are not fully understood but have been attributed to characteristics including surface area, size, shape, charge and the use of different capping agents, which can reduce interference of the nanoparticle with the surrounding biological environment.(24)

Most often, measurements are made indirectly *via* a Raman reporter label, often a dye molecule added to the surface of the nanoparticles. In this case the signal from the dye molecule is measured as an indication of, for example, a binding event between the nanoparticle and desired target molecule. In the case of surface enhanced resonance Raman spectroscopy (SERRS) signals are further enhanced, and sensitivity increased, by selecting a dye that absorbs at a wavelength close to the laser excitation wavelength.(25) Raman labels and biomolecules can be attached electrostatically or covalently to the chosen SERS substrate (Figure 2).(19) Additionally, protective coatings, such as poly-ethylene glycol (PEG) and silica, are often applied to nanoparticles for multiple purposes including to avoid dissociation of functionalized molecules, reduce toxicity and aid further functionalization, as discussed extensively by Wang *et al.(19)*. Finally, in order to target a specific molecule, biorecognition molecules can be functionalized onto the surface of the nanoparticles. These include oligonucleotides and antibodies to target specific DNA/RNA sequences and proteins respectively.(26) Both dyes and biomolecules have been attached to nanoparticles using a number of different approaches which include electrostatic attraction between the nanoparticle surface and dye/biomolecule(25), or covalent attachment, via, for example, EDC/NHS coupling(27). Where both a dye and a biomolecule have been used, these can both

be attached directly to the nanoparticle surface(28), or can be attached directly as one species where the biomolecule has the dye pre-attached(29) (Figure 2).

1.2.3 Instrumental considerations

Raman instrumentation is another important consideration when utilizing SERS probes for bioanalysis. The “ideal instrument” for a given assay can vary significantly based on the SERS probes, the sample format and the amount and type of information that is required. Once a probe is selected, the first decision to make is often laser wavelength. One of the many benefits of Raman spectroscopy is the ability to use one or many laser excitation wavelengths to perform a measurement. However, the use of SERS probes may complicate this choice, as the laser excitation wavelength that will result in optimal performance may vary based on the core particle plasmon resonance and resonant contributions from Raman reporter dyes. Further complicating this matter is the presence of auto-fluorescence from biological components and poor tissue penetration depth of visible excitation wavelengths. Accordingly, many SERS measurements are now being performed with near-IR (NIR) wavelength excitation(30-32), which may help to mitigate fluorescence and increase tissue penetration in future applications.

In terms of measurement format, point and shoot Raman spectroscopy can be applied broadly to in vitro, ex vivo, and in vivo measurements. Traditional, backscattering Raman spectroscopy is common in measurements aimed at understanding spectral variations as a result of biological processes, quantification, and measuring “on/off” signal variations.

Applications where one seeks to gain spatial information about biomarker or tag distribution or location, common in in vitro and ex vivo studies, often rely on Raman microscopy, with confocal mapping in two or three dimensions.(33) Recent advances have also demonstrated mapping in vivo.(34) Further, a given measurement may call for a dedicated piece of equipment. Specialized instruments include handheld or endoscopic probes,(35-38) small animal(39) and widefield imaging devices,(40) and instruments for spatially offset Raman spectroscopy (SORS),(41) capable of detecting SERS probes from depth in tissue(42, 43) and bone.(44) Some applications of these instruments will be discussed further in the following sections.

1.2.4 Interpreting the data

As with all analytical chemistry techniques, consideration must be taken into how the resulting data will be analysed. Similar to fluorescence, SERS probes can be used to give an "on"/"off" type response to a binding interaction. This can be quantitative if the intensity response of a signal is calibrated against known or independently measured values. This is an example of univariate analysis. Often more sophisticated chemometric methods are employed when analysing SERS data, involving multivariate analysis. Where SERS probes are used for direct measurement of biochemical signals from native molecules, the resulting spectra will contain information about a number of biological species that were in close proximity to the nanoparticle surface. In this case, principle component analysis (PCA) is often employed to reduce the dimensionality of the data by creating principle components that explain the maximum data set variation.(45) Another technique, partial least squares regression (PLSR) analysis, is a supervised multivariate analysis technique, where spectral response to known incremental experimental changes are modeled.(46) This has been used

to improve multiplex SERS probe capability, by allowing individual probe contributions to multiplex spectra to be better distinguished and quantified.(47) Direct classical least squares (DCLS) analysis is a type of multivariate analysis technique that is also commonly employed to distinguish contributions from different SERS probes in a multiplex assay.(48, 49) The nature of SERS spectra, with multiple sharp distinguishing peaks, providing multivariate data, allows increased multiplexing capability in comparison to univariate fluorescence analysis.

2. In vitro measurements

There is a significant need for the development of non-invasive and non-destructive methods of disease detection and subsequent treatment. The main reasons driving the detection of biological targets in vitro using SERS are the ability to do so rapidly, sensitively whilst detecting multiple targets simultaneously. A strong motivation for performing in vitro studies is to lay the foundations for potential success of future in vivo studies although it should be noted that not all in vitro studies are designed to move in vivo.

2.1 Label free nanoparticles for cellular analysis

Metallic nanoparticles have been utilized extensively as sensors for cellular studies involving SERS. By combining the plasmonic properties offered by gold nanoparticles and the sensitivity of the SERS method, information on the cellular composition and its physical properties can be readily obtained.(18, 50, 51) In 2006, Kneipp et al.(52) used unfunctionalized gold nanoparticles as SERS sensors to measure the variances in endocytotic uptake in two different cell lines, mouse macrophage cells (J774) and immortalized rat renal proximal tubule cells (IRPT). The conclusion was that the rate of nanoparticle uptake through endocytosis can vary notably depending on the cell line used.

Other physiological processes, such as apoptosis, have been successfully monitored in a label free manner as demonstrated by Zhou et al.(53). The concept of their method was based on the deposition of silver nanoparticles directly onto the cell membrane. When apoptosis was induced by drug application, changes in the intrinsic Raman signal were readily monitored, with a view to transferring this method to allow the monitoring of other physiological processes. Measuring cellular drug response in this manner could also prove to be a vital tool for high throughput drug screening detection methods.(54)

2.2 Labeled nanoparticles for cellular analysis

There is no doubt that there are great rewards to be reaped by using gold nanoparticles as sensors for SERS analysis of cells. However, it has been shown that by functionalizing these gold nanoparticles with specific molecules, even more information on the cellular environment can be obtained. Nanoparticles functionalized with specific biomolecules can be designed to target specific cellular components, such as antibody functionalized nanoparticles for detection of specific antigens, resulting in high resolution spatial resolution imaging of specific cellular biomolecules. Lee et al.(55), using gold or silver nanoparticles functionalized with specific monoclonal antibodies and Raman reporter dye to produce highly sensitive Raman images of live HEK293 cells expressing a specific biomarker, phospholipase Cγ1 (PLCγ1), known to be overexpressed in hyperproliferative human tissue, including many cancers. Antibody tagged SERS probes have been widely applied to in vitro cell culture studies, with application to cancer diagnostics, where a biomarker specific to or upregulated in cancerous cells is often imaged using antibody conjugated nanoparticles. Park et al.(56) used gold nanorods functionalized with the Raman dye reporter mercaptopyridine and secondary

antibodies (anti-rabbit IgGs) to detect the presence of HER2 biomarker (upregulated in breast cancer) in MCF7 breast cancer cells targeted with HER2 specific antibodies. In 2013, Lee et al.(57) reported a SERS based method, using silica encapsulated hollow gold nanospheres, functionalized with specific Raman reporter dyes and antibodies to target breast cancer biomarkers epidermal growth factor (EGF), ErbB2 and insulin-like growth factor-1 (IGF-1). They applied these SERS probes for detection of biomarkers in MDA-MB-468, KPL4 and SK-BR-3 human breast cancer cell lines, allowing breast cancer phenotyping. Not only do these studies provide a readily available platform for development of detection assays, which can ultimately be performed for direct in vivo early cancer detection, but they also provide important information in their own right, increasing insight into the biochemical processed behind cancer progression. These platforms could also be used in drug screening studies, where drug candidates could be tested for their effect on cancer biomarker expression. In 2013, Stevenson et al.(58) demonstrated a new and important detection capability of a SERS probe in an in vitro environment where they were able to detect intracellular enzyme activity. Activity of intracellular β-galactosidase enzymes was measured by conversion of applied 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) to 5,5'dibromo-4,4'-dichloro-indigo, detected by SERS via intracellularly applied gold nanoparticles. This study further highlights the vast capabilities of SERS for in vitro analysis.

In addition to detecting specific biomolecules or enzyme activity, unique SERS probes have been developed to measure specific physiological characteristics of cells, particularly pH and redox potential. Early work by Bishnoi *et al*.(59) demonstrated the successful application of SERS for monitoring intracellular pH changes in the cell using a pH sensitive nanosensor consisting of a gold nanoshell with a self-assembled monolayer of the pH responsive

molecule, para-mercaptobenzoic acid (pMBA). By monitoring the change in the protonation state of the pMBA via the SERS spectrum, information on pH changes within the cellular environment were readily obtained. This was quickly followed by Kneipp et al.(60), who further developed the concept of using pMBA as a pH indicator. A two photon excitation method, surface-enhanced hyper-Raman scattering (SEHRS), was used, allowing a wider pH range to be probed. These studies have paved the way to the work by the Campbell group in recent years. In this work, particular attention was given to investigating cellular redox potential, due to its involvement in the regulation of a number of biochemical processes.(61) A library of SERS redox probes was developed by the group based on functionalizing nanoparticles with small redox active molecules, which report in a ratiometric manner on intracellular redox potential. These probes have been used to measure intracellular redox potential in NIH-3T3 fibroblast cells(62), A549 lung cancer cells before and after hypoxia induction(63) and PC3 prostate cancer cells before and after oxidative stress(64). Significantly, for the first time, simultaneous measurement of intracellular pH and redox potential using these SERS nanosensor probes was reported by Jamieson et al.(13) in 2015. Camus et al.(65) and Jamieson et al.(66) have even recently applied these measurements to a three dimensional (3D) in vitro cell culture model, a significant advancement enabled by SERS probes, as current methods for analysing 3D culture models are incredibly limited. 3D in vitro culture models are becoming increasingly significant by providing a more realistic in vivo environment without the ethical considerations required for the use of in vivo or ex vivo samples.

In vitro cellular pH sensing has also been achieved with sensing molecules other than pMBA. Lawson et al.(67) used the multifunctional molecule, 3, 5-dimercaptobenzoic acid that had

the ability to cross link nanoparticles to form controlled aggregates, enhancing the observed Raman signal, but also acting as a pH sensor, similar to benzoic acid pMBA. The cells used in this particular work were human induced pluripotent stem cells (hiPSCs) and with the above substrate, the pH could be monitored across the entire physiological range. Further to this, Nie et al.(68) designed a multifunctional SERS substrate that allowed for pH sensing and drug delivery. The substrate consisted of a gold nanoparticle, conjugated to doxorubicin and polyethylene glycol, Au-dox-PEG. Doxorubicin was modified with a hydrozone linker that allowed for pH sensing, and ultimately the release of the molecule from the surface, which acted as a therapeutic drug for tumors. Additional to the therapeutic effect of doxorubicin, the molecule gave rise to a distinctive Raman spectrum, therefore allowing the drug release to be monitored by SERS. The results demonstrated successful drug release at acidic pH, all monitored by SERS. This work further provided the confidence in using SERS to detect and track specific drug delivery in tumor cells.

A recent paper published by Chen et al.(69) provided an insight into the potential of SERS as a method for analyzing complex physiological processes within a cell, but did not focus on the use of pH sensing molecules. The study involved both label free and labeled SERS substrates, used for nuclear and membrane targeting in HeLa cells. The label free substrates were able to target specific regions of the cell due to the presence of specific targeting molecules, whereas the labeled substrates contained the targeting molecules along with Raman reporters: crystal violet (CV), cresyl violet acetate (CVa) and mercaptobenzoic acid (MBA), to be specific. From Figure 3, the gold nanoparticles are shown to have localized in the desired regions of the cell, and due to the enhanced optical field surrounding gold nanoparticles, the different Raman signatures of the different cellular components (fatty acids, proteins, DNA)

could be readily observed. The second part of the figure shows the Raman image obtained when using the labeled gold nanoparticles for targeting the cell nucleus and cell membrane in the same cell. From the images, the position of the cell membrane and nucleus could be clearly defined due to the different reporters being used: CV (green) and CVa (red) represent the cell membrane and MBA (blue) represents the cell nucleus. The yellow color in the images represents an overlapping are of the two components. This study begins to reveal the multiplexing capabilities of SERS probes, allowing multiple characteristics to be probed simultaneously. In 2013, McAughtrie et al.(48) combined SERS probes and Raman imaging, to image the intracellular location of three different dye labelled silver nanoparticles. Not only did this study emphasize the multiplexing capabilities of SERS probes, which could ultimately by designed to target and image different cellular biomolecules, but cell imaging was carried out in 3D. Therefore, 3D location of the nanoparticles in the cell was obtained, a significant advancement in the field of SERS probes for in vitro analyses.

SERS probes for in vitro bioanalysis have been widely exploited as demonstrated in this section. Much of this work paves the way for ex vivo and in vivo analysis, using a readily available biological model, which does not suffer from the same ethical restrictions as ex vivo and in vivo samples.

3. Ex vivo measurements

A large proportion of current diagnostic tests rely on accurate detection of disease biomarkers in ex vivo biological samples. Blood samples are routinely analyzed using common

bioanalytical techniques including immunoassays (IA), enzyme-linked immunoassays (ELISA), western blots, fluorescence in situ hybridization (FISH), and polymerase chain reaction (PCR).(70) In addition to biofluid tests for diagnostic purposes, immunohistochemistry is a widely used technique for diagnosing tissue samples from a patient biopsy.(71) These well-established bioanalytical techniques have been used as common practice for a number of years, however, the potential of SERS to be exploited in order to improve sensitivity of these current 'gold standard' techniques is becoming an increasingly prominent area of research. SERS probes have been exploited for detection of small molecules such as glucose(72), as well as larger biomolecules, in particular proteins(73) and DNA(25) as disease markers, in biofluid samples. In addition, relatively recent work has demonstrated the scope for SERS probes to be used for *ex vivo* tissue analysis for the detection of disease markers in both tissue sections(74) and excised tissue samples(75). In addition to the potential for increased detection sensitivity using carefully designed SERS probes, a major advantage of SERS probes is their multiplexing capability, which will be discussed in reference to biofluid and tissue samples in the following sections.

3.1 Ex vivo biofluids

Blood is regularly extracted intravenously from patients to undergo a variety of analytical measurements. Whole blood, isolated red blood cells, and serum are commonly analyzed. There is a constant need to develop techniques that allow detection of biomolecules currently eluding detection and to achieve greater detection sensitivity in established assays. SERS-based detection assays have emerged as promising tools for biomolecule detection in patient blood samples with sensitivities that compete with current gold standard methods. While there are examples of SERS assays based on direct measurement of native biomolecules in a

sample, most assays use indirect mechanisms that detect signals from dye labeled nanoparticles targeted for detection of specific biomolecules, particularly oligonucleotide sequences and proteins.(26)

The majority of SERS probes are based on indirect detection mechanisms, detecting SERS signals from Raman reporter dyes attached to the nanoparticles. For examples of direct SERS probe measurements, where native biomolecule signals are enhanced and detected, reference is made to Bantz et al.*(76).* In this review, the application of SERS probes for the detection of small biomolecules including glutathione, nicotinic acid adenine dinucleotide phosphate (NADDP), glucose and lactate are discussed in full and a more complete review of SERS probes for direct bioanalytical measurements is given. Here, we will focus primarily on the application of SERS probes for measurement of DNA and proteins in indirect assays for biofluid sample analysis in this section. Vo-Dinh pioneered much of the work using SERS for detection of organic materials beginning in 1984, when Vo-Dinh et al.(77) reported the use of SERS substrates for quantitative detection of organic compounds including benzoic acid. Ten years later, in 1994, Vo-Dinh et al.(78) went on to report on the first SERS based DNA gene probe. They report on the use of surface-enhanced Raman gene (SERG) probes for the indirect detection of specific nucleic acid sequences, using cresyl fast violet labeled oligonucleotide sequences designed to target specific complementary sequences, followed by SERS detection on silver-coated alumina substrates. In this initial study, the potential for SERS in DNA detection with both high sensitivity and specificity was highlighted, and the potential for high multiplexing capabilities was alluded to but not demonstrated. Detection of specific DNA sequences is a vital tool in molecular biology for application to DNA sequencing, gene

identification, and diagnostics, often applied to detection of bacterial or viral DNA for disease diagnosis.

In 1998, Narayana *et al*.(79) reported the first use of SERS combined with the polymerase chain reaction (PCR) applied to detection of the human immunodeficiency virus *gag* gene sequence. Here, the SERS probe was used as a primer for PCR, and following probe capture, the presence of the SERS probe was measured following silver deposition for SERS enhancement. In an earlier study in 1997, Graham et al.(25) reported, on the use of surface enhanced resonance Raman scattering (SERRS) for detection of DNA at ultralow concentration, mitigating the need for PCR, by using resonant Raman probes. SERRS allows greater enhancement factors, and consequently lower detection limits, achieved by tuning the laser frequency to the maximum absorbance frequency of the dye used in detection. In contrast to the work from Vo-Dinh, where the captured probes were detected by application to a solid SERS substrate subsequent to capture steps, Graham et al. adsorbed the dye labeled DNA probes directly onto the surface of colloidal silver nanoparticles. They applied spermine to create a positive layer on the negatively charged DNA backbone, which allowed efficient attachment of DNA probes to negatively charged colloidal silver. This highly effective method also allowed further enhancement of SERRS signals as excess spermine caused colloidal aggregation, further increasing electromagnetic enhancement. By using these additional enhancement approaches, the requirement for PCR amplification of target sequences could be eliminated.

Graham et al.(80) went on to demonstrate the ability of their SERRS assay to detect two different DNA targets in a single assay, giving the first experimental example of multiplexed

detection of DNA by SERRS. The measurement capabilities of SERS and SERRS for multiple targets has since then been widely demonstrated in a number of studies. In 2002, Cao et al.(81) reported a microarray chip setup for detection of six different specific DNA or RNA sequences, individually immobilized onto a solid substrate, with high sensitivity and selectivity. Preparing six different probes by functionalizing gold nanoparticles with six different specific dye labeled oligonucleotide sequences, they were able to demonstrate high selectivity for detection of the presence of each complementary oligonucleotide sequence using a Raman scanning method, however this assay was not carried out on a true multiplexed sample in solution. This technique also required a silver enhancement step before Raman measurement to allow sufficient SERS enhancement of dye signals. For high-throughput applications minimizing additional steps is desirable, but this additional enhancement step allowed unoptimized detection limits of 20 fM to be achieved. Faulds *et al.(82)* addressed some important considerations for multiplexed SERRS detection, by demonstrating quantitative linear concentration curves for eight different dye labeled oligonucleotides adsorbed onto silver colloid. In this study, sufficient signal enhancement was achieved from silver colloid, removing the need for additional enhancement steps. As in previous studies by Graham et al.(25), the use of spermine, which caused nanoparticle aggregation, also helps to further enhance SERRS signal. The ability to determine quantitative information regarding oligonucleotide concentration, with detection limits down to 0.5 fM, provided further evidence of the benefit of SERS and SERRS as an alternative to fluorescence. Faulds et al.(83) went on to demonstrate the ability to quantitatively detect the presence of five specific oligonucleotide sequences in a mixture of all species using their dye labeled oligonucleotide nanoparticle probes and two different excitation wavelengths (Figure 4). In 2008, Faulds and Goodacre et al.(47) improved multiplexing capability further to six oligonucleotides in a

mixture by employing multivariate analysis, and in 2014 Gracie et al.(46) provided the first example of quantification within a multiplex assay for application to detection of bacterial meningitis pathogens. Finally, a PCR and SERS based DNA detection approach is now being employed clinically to detect fungal disease using the RenDx Fungiplex assay developed by Renishaw Diagnostics.(84)

In addition to oligonucleotide detection, SERS and SERRS probes have been widely studied for protein detection. Most of these approaches are based on the commonly used immunoassay and ELISA assays. SERS probes are being employed in place of fluorescent markers in an attempt to increase sensitivity and multiplexing capability, as already discussed in application to oligonucleotide detection. Again, it is possible to detect proteins directly, however, it is much more common for an indirect approach using a sandwich type immunoassay, where signal from a dye labeled nanoparticle is measured, to be employed to achieve the necessary sensitivity and specificity. Protein detection methods are incredibly important in immunohistochemistry, where tissue sections are stained to investigate expression of various proteins. Therefore, protein detection by SERS will be extensively covered in Section 3.2 when considering ex vivo tissue bioanalysis by SERS. Rohr et al.(73) were the first to report the use of SERS for detection in an immunoassay in 1989. Since then, the advantages of SERS and SERRS for increased sensitivity and multiplexing capability in immunoassays has been demonstrated. Cui et al.(85) used two methods to perform a SERS based multiplex immunoassay capable of detecting two different antigens (mouse IgG and human IgG), one method based on the same nanoparticles with different dyes and the other based on different nanoparticles with the same dye. Wang et al.(86) reported further multiplexing capability by demonstrating ability to use SERS for quantitative detection of four target antigens (mouse

IgG, human IgG, rabbit IgG and rat IgG). In this study, an alternative binding approach was used to covalently attach antibodies to nanoparticles via the reporter dye in order to increase specificity of binding. In another study, Wang et al.(87) demonstrated the advantage of SERS immunoassays in terms of sensitivity and improved limit of detection. They used a SERS based sandwich assay to detect MUC4 in patient sera as a marker of pancreatic cancer, with a ca. 1000 × lower limit of detection and ca. 10 × lower sample volume requirement than gold standard techniques.

The enzyme linked immunosorbent assay (ELISA) is another common method for protein detection, which uses an enzyme labeled antibody during detection, converting a non-colored substrate into a colored product that can be measured. Dou et al.(88) demonstrated an analogous assay using SERS as the detection technique, where the substrate, peroxidase, is converted to azoaniline, which has a strong SERRS spectrum. In addition to general protein detection, SERS and SERRS have been employed for enzyme detection and measurement of specific enzyme activity. Larmour et al.(89) summarized the utility of SERRS for enzyme measurements. Moore et al.(90) demonstrated the ability of SERRS for detection of hydrolase activity at ultra-low levels, down to that found within single cells.

3.2 Ex vivo tissue

In 2006 Schlücker et al.(74) reported the first use of SERS probes for “immuno-Raman microspectroscopy”. Traditionally, a trained pathologist analyses stained patient biopsies through a microscope and makes an appropriate diagnosis. While this is the ‘gold standard’ technique, is relies on a subjective assessment, which is subject to error. Schlücker et al.(74) have set the scene for the use of SERS as an alternative spectroscopic approach for tissue

diagnostics. SERS is subject to fewer background contribution issues than label free spectroscopic imaging techniques, and allows much faster imaging. Instead of relying on native biochemical signals, SERS works in a similar way to fluorescence staining for specific biomarkers; instead of coupling the specific antibody to a fluorescence marker, it is coupled to a SERS probe. The major advantages that SERS probes exhibit over their analogous fluorescence probes is increased sensitivity and increased multiplexing capability for the detection of multiple markers in a single measurement.

The use of SERS probes as immune markers in tissue diagnostic remains a relatively new area of research. However, since it was first demonstrated in 2006, a number of significant studies have emerged highlighting the multiplexing capabilities of a SERS approach. Lutz et al.(91) reported seminal advances in tissue imaging using SERS, where they employed their composite organic-inorganic nanoparticles (COINs)(92) to target two different antibodies (cytokeratin-18 (CK-18) and prostate specific antigen(PSA)) in formalin fixed paraffin embedded tissue sections (Figure 5). Lutz et al.(91) introduced multivariate data analysis methods to allow multiplex spectral signals to be separated and quantified. They used spectral fitting based on least-squares regression and reference pure COIN spectra, background reference, and freely varying polynomial components to extract contribution of individual COIN from multiplex spectra. They were able to demonstrate the ability of their developed technique to quantify four different contributions from PSA-antibody-COIN probes in a plate-based assay (all specific to PSA antigen but with different Raman reporters – acridin orange (AOH), basic fuschin (BFU), Nile blue A (NBA) and tetramethyl rhodamine isothiocyanate (TMR)). They then went on to demonstrate the ability to distinguish two different antigen expressions (CK-18 and PSA) in a tissue section (Figure 5). Salehi et al.(93)

more recently demonstrated the sensitivity of immuno-SERS, with the ability to detect single nanoparticles. They have also demonstrated multiplexing capability of immune-SERS, investigating colocalization of p63 and PSA in non-neoplastic prostate tissue.(94)

Finally, in addition to fixed and sectioned tissue, SERS has also been used for detection of specific antigens in excised tissue sections. This could allow excised tissue to be analyzed intraoperatively to determine if a tumor has been fully excised by targeting nanoparticles to tumor-specific antigens and measuring response in excised tissue to determine if the antigen remains at the outer surface. This can guide further excising intraoperatively to ensure sufficient removal. Sinha et al.(75) investigated the potential of nanoparticles targeted to the EGFR receptor to be used in intraoperative surgery to assess breast cancer tumor margins. Here the presence of non-specific binding of nanoparticles was highlighted as a problem in this approach, and a dual-probe approach was applied, quantifying 'binding potentials' that take into account these non-specific interactions. Wang et al.(95) highlighted the heterogeneous characteristics of tumor xenograft specimens and human breast cancer tumors and demonstrated the use of a number of nanoparticle probes for multiplexed detection of multiple cancer biomarkers (EGFR and HER2) on an excised tumor quantitatively, in order to better guide intraoperative resection.

4. In vivo measurements

In vivo SERS measurements have been developed using different approaches over recent years, including implantable substrates utilized in glucose detection.(96, 97) However, a large portion of recent reports make use of SERS probes, plasmonic metal nanostructures functionalized with one of a number of Raman reporters, an anti-fouling surface coating (poly-

ethylene glycol or silica, among others) and in the case of targeted SERS probes, a bio-recognition molecule capable of binding to a biomarker of interest. Non-targeted SERS probes have also been utilized which lack a bio-recognition molecule. In a similar fashion to in vitro and ex vivo assays, this strategy allows for multiplexed detection, or the detection of multiple biomarkers simultaneously through the use of multiple Raman reporters and bio-recognition molecules.

Much of the ground-breaking work for the use of SERS probes for in vivo applications was first reported in 2008. First, Qian et. al.(98) showed the functionalization of Au nanoparticles with Raman dyes, poly-ethylene glycol (PEG) and single-chain variable fragment (ScFv) antibodies capable of targeting epidermal growth factor receptors (EGFR), known to be over-expressed in specific cancers. Targeted and non-targeted probes were injected into the tail of xenograft tumor-bearing mouse models. The targeted probes showed a greater affinity both for the tumor mass vs. the liver, and for the tumor mass when compared to their non-targeted counterparts. In the same year, Keren and coworkers showed the simultaneous, multiplexed detection of two non-target probes injected subcutaneously into a mouse model.(99) This strategy was later expanded for the detection of 10 tags at separate injection sites, and five tags simultaneously in the liver after intravenous injection.(100) In a promising development, the SERS signal intensity observed from multiple tags in the liver was found to track linearly with the injected probe concentration. Recent studies with novel nanostructure geometries, such as plasmonic Au/Ag hollow shell assemblies(101) and nanostars(102) have followed similar experimental strategies.

Building upon these advances, among others in probe development(103, 104), in vivo, multiplexed detection of targeted nanoprobes in a mouse model was demonstrated by Maiti et al. in 2012.(105) In this study, near-IR SERS reporters were used to label three species of bovine serum albumin (BSA) and glutaraldehyde encapsulated probes; two species served as positive controls with antibodies targeting the same biomarker (EGFR), and a third as a negative control, targeted to human epidermal growth factor 2 (HER2), which is expressed in lower levels in the tumor type. After tail-vein injection, probes targeted with anti-EGFR were shown to localize selectively in the tumor site via SERS spectroscopy and mapping, while all three tags were shown to be present in the liver as in previous non-targeted studies.(99) Dinish et al. have taken this further by detecting multiple, different biomarkers in a multiplexed measurement.(106) Simultaneous detection of three breast cancer biomarkers– EGFR, CD44, and TGF beta receptor II (TGFβII) – was demonstrated by antibody-targeted probes after inter-tumoral injection. Targeted probes were shown to remain in the model mass up to 48 hours before clearing after 72 hours, whereas non-targeted probes with the same reporters were largely cleared within 6 hours, and completely absent after 24 hours. This work is featured in Figure 6. Notably, non-specific binding by an isotype antibody was not investigated in this study.

Wang and coworkers have shown an important step toward biomarker quantification and monitoring in vivo with their work on ratiometric, multiplexed detection via SERS probes.(49) Topical application of three antibody functionalized probe species—anti-EGFR, anti-HER2, and an isotype control—was performed on two tumor types with varied expression of EGFR and HER2. The first tumor type, A431, is known to overexpress EGFR with modest HER2, whereas type two, SkBr3, overexpresses HER2, but with ordinary EGFR. After the topical

application of probes, the tumors were implanted and SERS measurements taken. The Raman data was treated with direct classical least squares (DCLS) processing, allowing for the determination of useful probe, and thus biomarker, ratios—anti-EGFR/isotype, anti-HER2/isotype, and anti-EGFR/anti-HER2—in the two tumor types and control tissue with very positive results. The inclusion of an isotype probe makes for robust measurement by providing a control for non-specific probe binding.

Apart from the cancer field, there has also been interest in the use of SERS probes for the diagnosis of other diseased states in vivo. McQueenie et al. demonstrated one such example in 2012.(107) Here, intracellular adhesion molecule 1 (ICAM-1) was targeted, as its expression in the vasculature is an early indication of inflammation and atherosclerosis. After inducing inflammation with an injection of lipopolysaccharide (LPS), anti-ICAM functionalized nanoparticles were detected via SERS measurements in the ear pinnae of murine models, with favorable results vs. an isotype probe control.

As mentioned in the introduction, the Raman instrument used in a SERS probe experiment is of critical importance, particularly in vivo, where traditional spectrometers or microscopes may not be optimal. Many advances in instrumentation have been driven by the necessity for purpose-built spectrometers for in vivo detection of SERS probes. Notable examples of the synergistic benefits of non-targeted SERS probes coupled with cutting-edge spectrometers include an endoscopic probe for multiplexed detection(36), a handheld probe for guidance in brain tumor resection (34), and a circumferentially scanning probe for the interrogation of luminal probe-treated surfaces(37). Similar to their previous targeted, ratiometric work, Wang et al. recently showed impressive multiple biomarker detection in esophageal cancer

models using a probe-scanning system.(108) Further, two separate configurations for imaging large areas, one utilizing a line-scanning configuration(39) and another with widefield excitation and a tunable filter for multiplexed detection(40) have also been demonstrated. SESORS (surface enhanced, spatially- offset Raman spectroscopy) combines the sub-surface sampling capabilities of spatially-offset Raman spectroscopy (SORS) with the sensitivity of SERS. This technique was first demonstrated by Stone et. al. with probes buried 25 mm into mammalian tissue(42), with later work showing probe detection up to 50 mm(43). Sharma et. al. have recently shown probe detection through bone(44), expanding the potential for SESORS in future clinical applications. Separately, SESORS has also been demonstrated with implanted SERS substrates, in lieu of SERS probes, for glucose detection(97, 109) in vivo.

Finally, many efforts have been made to produce probes detectable via multiple imaging techniques, so-called multimodal imaging SERS probes, for use in vivo. By creating probes that can be visualized via complementary contrast mechanisms, one might attain valuable information absent when a single mechanism is used, leading to better detection or perhaps more accurate tumor resection. In addition to the properties of traditional SERS probes, these probes are comprised of varied materials, allowing them to be detected via fluorescence (FL)(110-112), photothermal (PT)(113-117), magnetic resonance (MRI)(118-120), Fourier-Transform Infrared (FTIR)(121), photoacoustic (PA)(118, 122), or computed X-ray (CT)(116, 117, 119) imaging. An in-depth investigation into multimodal probes is outside of the scope of this review, but has been discussed previously by others.(123-125)

5. Conclusion

In this review we have provided an overview of some of the seminal examples of SERS probes for use in bioanalytical measurements. This is a field that has vastly expanded since the discovery of SERS in 1974, and its subsequent exploitation for analysis of biological samples is the result of the significant advantages it can provide for rapid, sensitive and multiplexed measurements. As with any analytical technique, there are fundamental considerations that have to be made when developing a SERS probe measurement, particularly if the ultimate goal is to translate these techniques into clinical applications. Major considerations required when developing a SERS probe are biological sample preparation, SERS probe selection, instrumental considerations and data interpretation.

We have demonstrated the use of SERS probes as bioanalytical tools, with particular focus on diagnostics, in application to in vitro cell culture models, ex vivo biofluid and tissue samples and in vivo detection. These studies highlight the unique and beneficial properties of SERS probes for highly sensitive, and often multiplexed, detection of biomolecules of interest. As a result of the intense and sharp characteristics of SERS signals, SERS probes are already pushing detection limits for specific biomolecules, such as DNA and proteins. In the future it is likely that these limits will be pushed further, providing increased momentum for translation of SERS-based assays into the clinic. This is particularly true for detection of biomarkers in biofluid samples, where one assay for fungal detection is already commercially available(84). Multiplexing capability is another prominent advantage of SERS over other techniques, and it is likely that multiplexing capabilities will be expanded in coming years from the currently reported 6-plex assay from Faulds et al.(47). As well as paving the way for in vivo studies, in vitro studies have an important place in their own right, and one particular clinical application that could be pursued would be a high-throughput SERS platform for drug screening. Finally,

perhaps the ultimate goal is to be able to perform robust and accurate in vivo diagnostics, and future work for in vivo application will push current work in animal models into humans. While there are clear toxicity considerations, SERS probes show promise for clinical application for early disease detection whilst being minimally invasive. Therefore, SERS probes provide vast scope for addressing some of the key, present-day problems in bioanalysis and clinical assays. Studies featured in this review have demonstrated the strong research base that has established the applicability of the SERS approach in addressing these problems.

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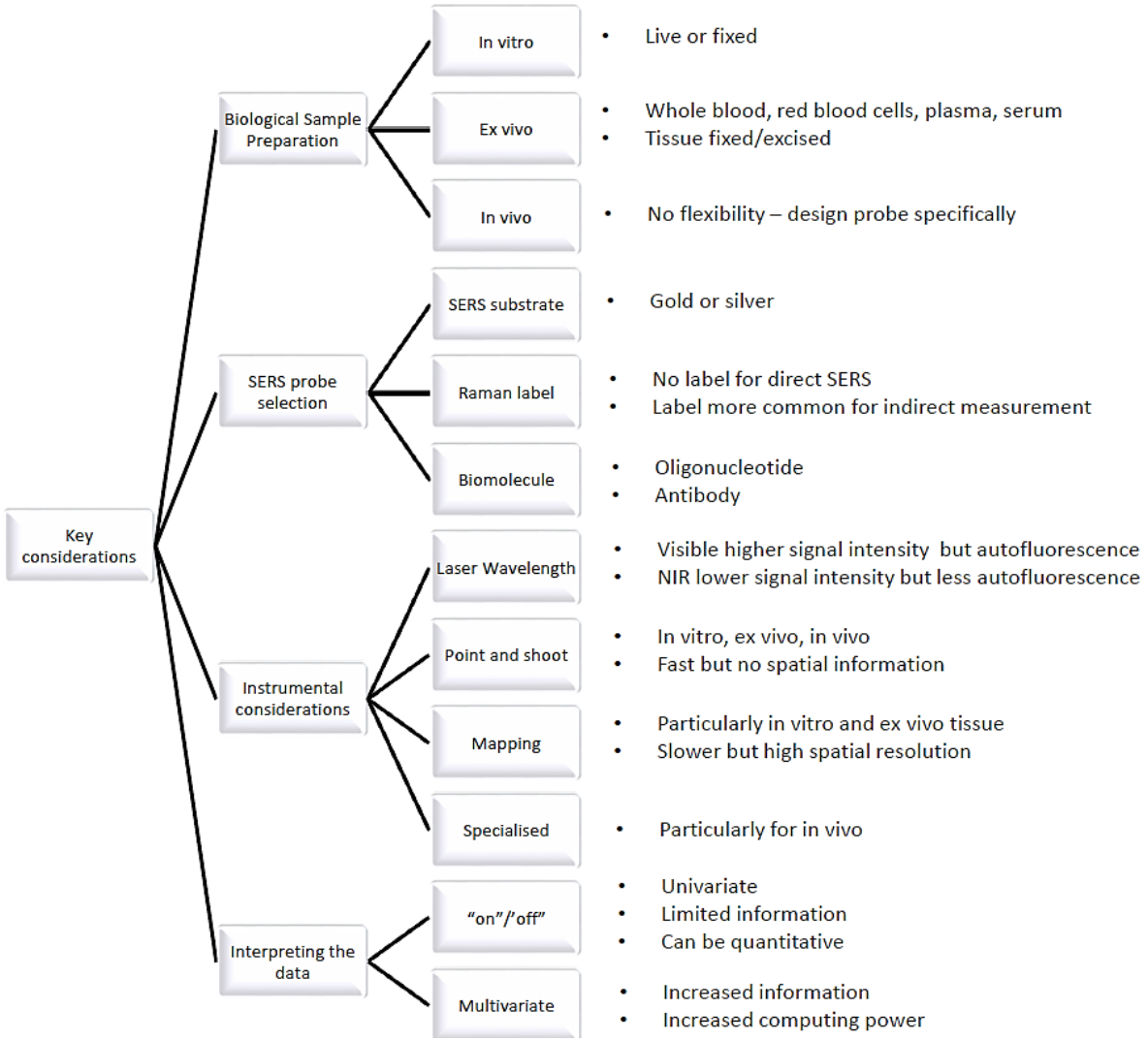

Figure 1 A summary of the key considerations to be made when performing bioanalytical SERS measurements.

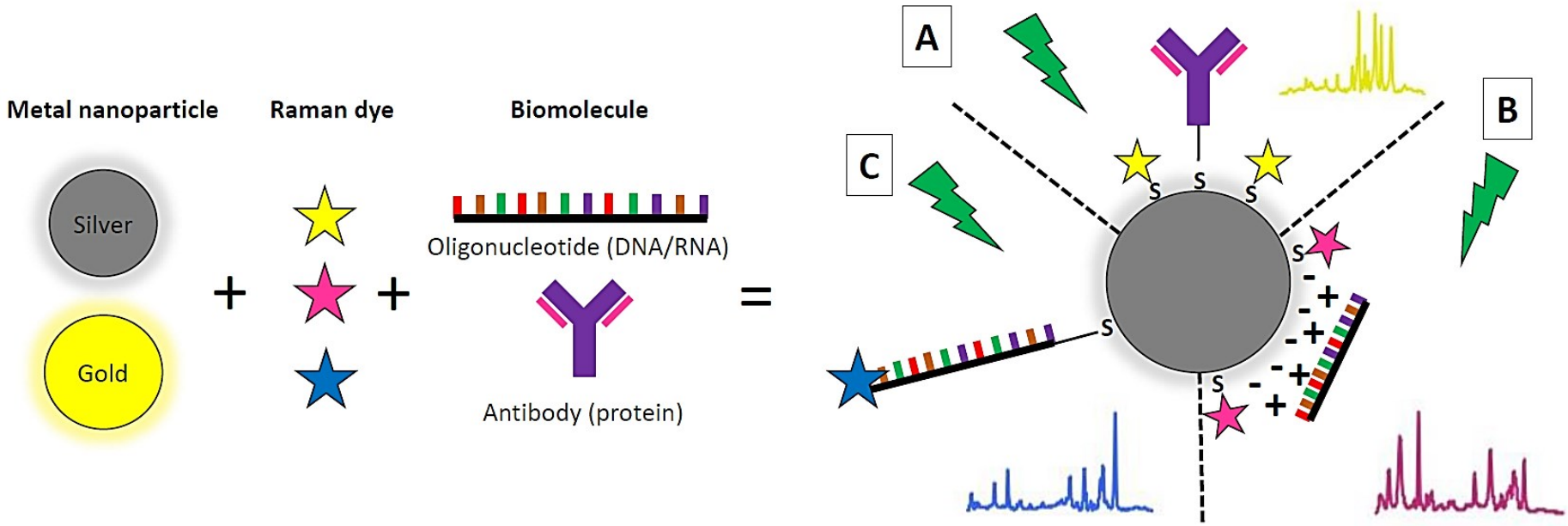

Figure 2 Schematic of the general design of a SERS probe showing the components used: SERS substrate, Raman dye (optional) and biomolecule (optional), and examples of each. Some common SERS probe designs are given showing different attachment mechanisms. (A) Attachment of a specific antibody can be achieved via covalent attachment of a thiol modified polyethylene glycol (PEG) linker. A Raman dye is also covalently attached to the nanoparticle surface via a thiol linker, and gives a characteristic SERS response (yellow spectrum). (B) Attachment of a specific oligonucleotide sequence via electrostatic interaction between negatively charged nanoparticle surface and positively charged modified base sequence. A Raman dye is also covalently attached to the nanoparticle surface via a thiol linker, and gives a characteristic SERS response (pink spectrum). (C) Attachment of a specific Raman dye labelled and thiol modified oligonucleotide sequence via covalent attachment. The Raman dye label gives a characteristic SERS response (blue spectrum).

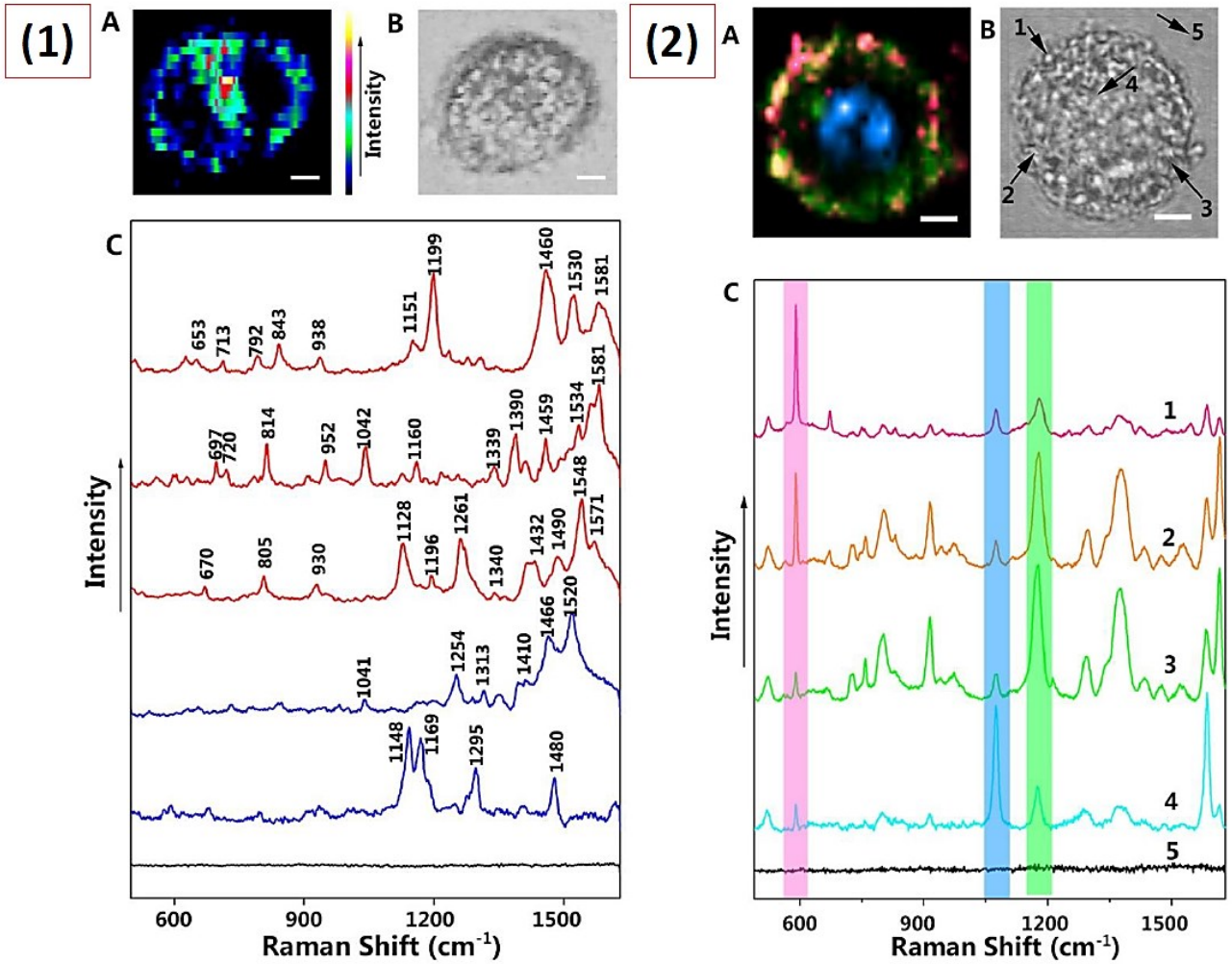

Figure 3 (1) Multi-targeting SERS imaging of a HeLa cell treated with both label free membrane- and nucleus-targeting gold nanoparticles. (A) SERS image and (B) brightfield image of the investigated HeLa cell. (C) SERS spectra obtained from different positions within the cell such as membrane (blue), cell nucleus

(red) and the surrounding environment (black) show significant differences in terms of their intensity and peak positions (scale bar ≡ 4 μm). (2) Multi-targeting SERS imaging of a HeLa cell treated with CVa-coated, CV-coated, and MBA- coated gold nanoparticles. (A) Overlap of SERS images of CVa-coated gold nanoparticles(red), CV-coated gold nanoparticles (green), and MBA- coated gold nanoaprticles (blue). (B) The bright-field image of the investigated HeLa cell. (C) SERS spectra obtained from different positions in the cell (marked in panel B by arrows). The Raman intensities at 595, 1078, and 1175 cm^{-1} revealed the relative amount of CVa-coated, MBA-coated and CV-coated AuNPs at the corresponding positions, respectively (scale bar ≡ 4 μm). Adapted from Chen et al.(69).

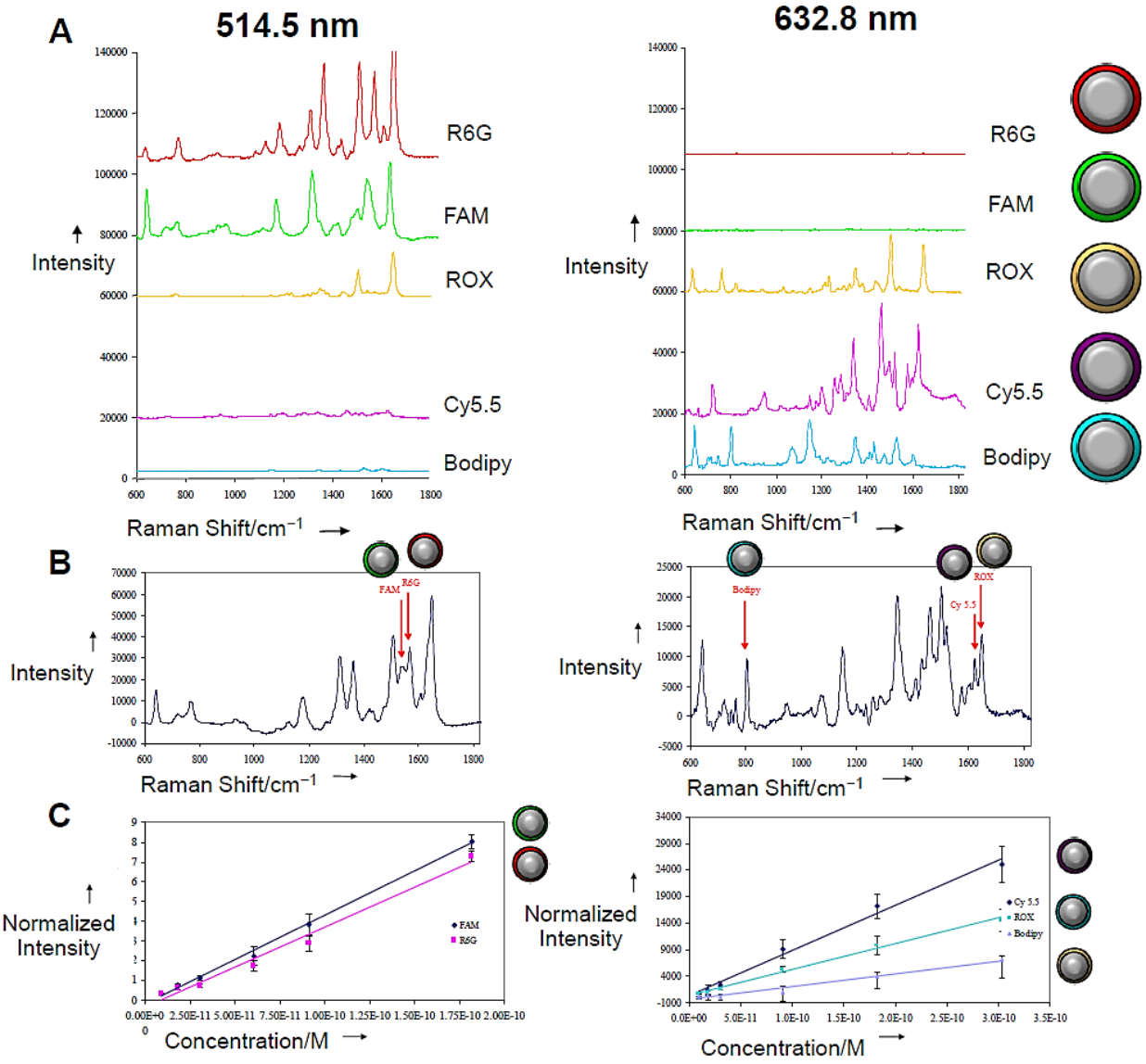

Figure 4 (A) SERRS spectra of silver nanoparticles taken using a 514.5 nm laser (left) and 632.8 nm laser (right) for nanoparticles functionalized with different oligonucleotide sequences each modified with a different Raman dye molecule: rhodamine 6G (R6G) labeled human papillomavirus (HPV) probe (red); FAM labelled universal reverse primer (green); ROX labelled VT2 *E. Coli* 157 gene probe (yellow); Cy5.5 labelled universal reverse primer (purple); and BODIPY TR-X labelled universal reverse primer (cyan). R6G and FAM had the highest SERRS signals at 514.5 nm, while ROX, Cy5.5 and Bodipy had the highest SERRS signals at 632.8 nm. (B) SERRS spectra of a mixture of all five labelled nanoparticle probes at a concentration of 1.82×10^{-9} M taken using a 514.5 nm laser (left) and 632.8 nm laser

(right), indicating SERRS signals from each labelled nanoparticle in each case. (C) Calibration of each labelled nanoparticle in a mixture of all nanoparticle probes using 514.5 nm excitation for R6G and FAM labelled probes (left) and 632.8 nm excitation for ROX, Cy5.5 and Bodipy probes (right). Adapted from Faulds et al.(83).

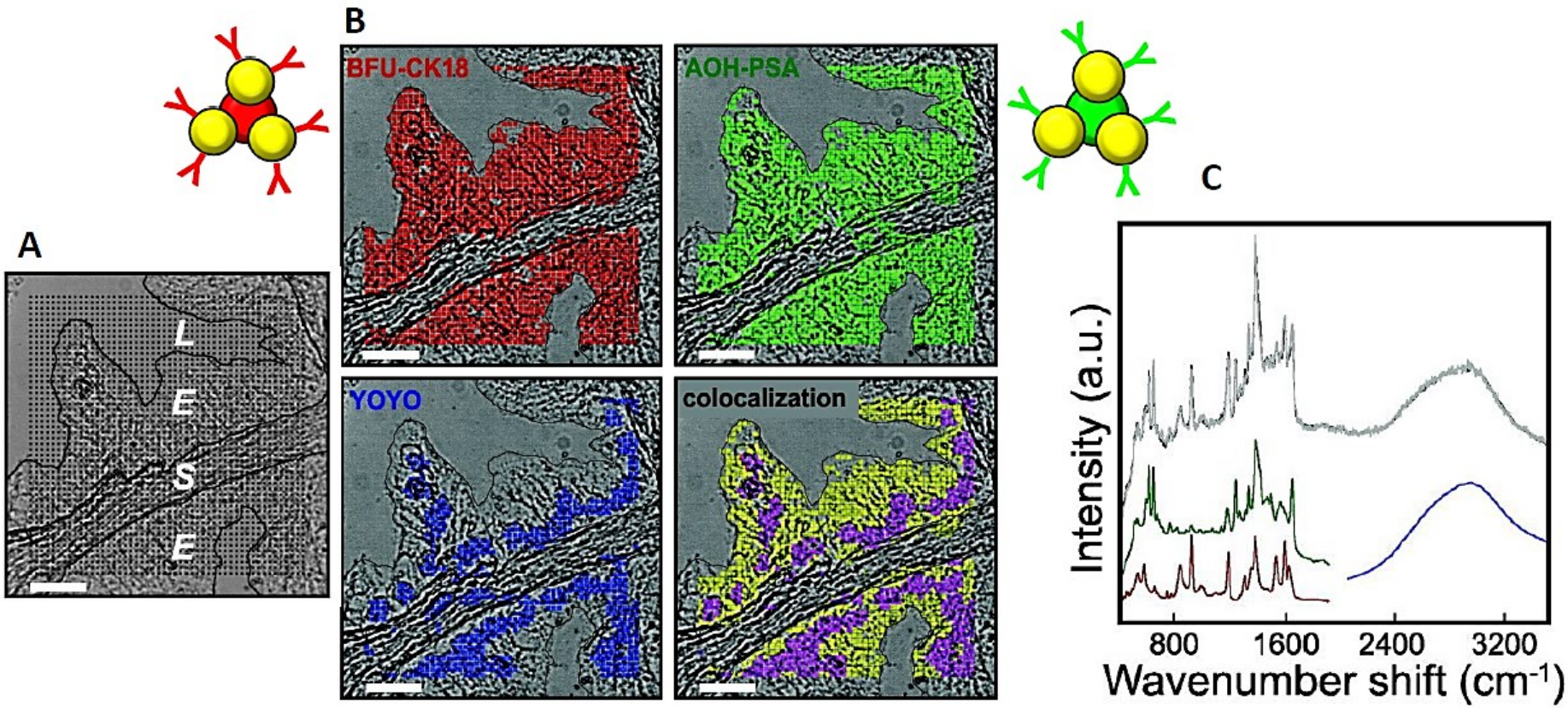

Figure 5 (A) Brightfield image of section of prostate tissue, which was targeted with antibody labelled SERS probes and raster scanned using a 532 nm laser such that a SERS spectrum was taken from each black spot in the 50 × 50 area with 1 µm spacing between points. Brightfield image is labelled to show tissue features – epithelia (E) of two different prostate lands, stromal tissue between the glands (S) and gland lumen (L). Scale bar = 10 µm (B) A SERS probe for detection of cytokeratin-18 (CK-18) by conjugation with anti-CK18 antibody and the Raman dye basic fuschin (BFU) (BFU-CK18, red) was used to locate CK18

antigen on the tissue section (top left). A SERS probe for detection of prostate specific antigen (PSA) by conjugation with anti-PSA antibody and the Raman dye acridin orange (AOH) (AOH-PSA, green) was used to locate PSA antigen on the tissue section (top right). A DNA fluorescent dye (YOYO) was used to detect nuclear regions (bottom left). Colocalization of signals is shown in the bottom right image. Scale bars = 10 µm. (C) Deconvolution of spectrum from a single point in map. The measured spectrum (grey) and best-fit spectrum (black) are shown in the upper spectrum. Extracted spectra for BFU-CK18 (re), AOH-PSA (green) and YOYO (blue) are given below. Adapted with permission from Lutz et al.(91).

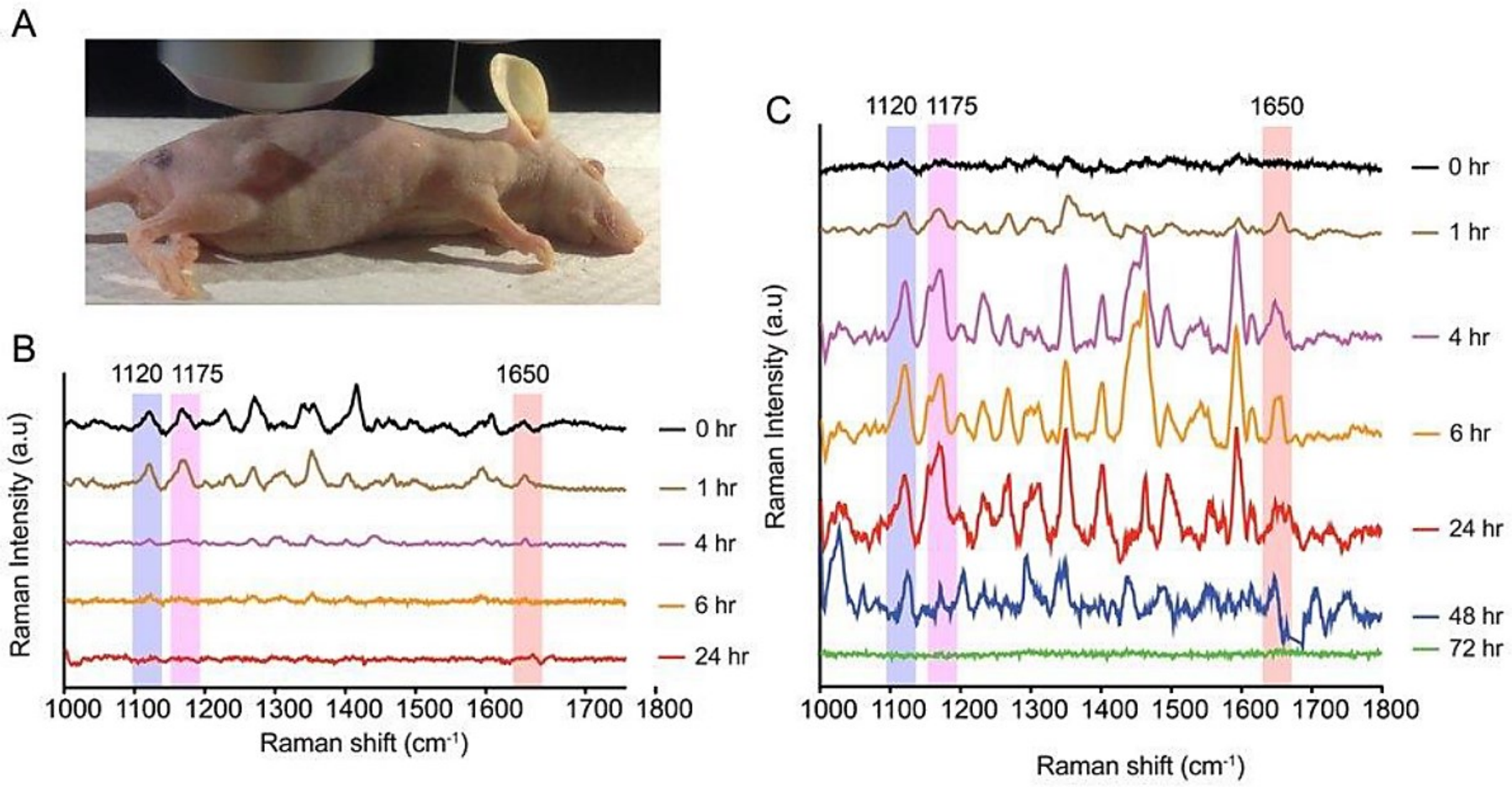

Figure 6 Demonstration of targeted, in vivo, multiplexed detection in a xenograft tumor model. Panel (A) shows an image of a tumor bearing mouse used in the experiments. Panel (B) features SERS spectra from non-targeted nanotags at the tumor site as a function of time after exposure. Peaks at 1120, 1175 and 1650 cm^{-1} correspond to tags with the dyes Cy5, MGITC, and Rh6G, and the disappearance of these peaks

over time suggests clearance of tags from the site after 6 hours. Panel (C) shows SERs spectra from antibody functionalized SERS nanotags as a function of time. The presence of peaks associated with Cy5/TGFbRII, MGITC/CD44, and Rh6G/EGFR targeted tags after 24, and up to 48 hours, after exposure demonstrates the retention effect of targeted nanotags when they are designed to bind to specific tumor biomarkers. Reproduced from Dinish et.al.(106).