

Karen Faulds

1 Overview

Surface enhanced resonance Raman scattering (SERRS) is an analytical technique with several advantages over competitive techniques in terms of improved sensitivity and selectivity. We have made great progress in the development of SERRS as a quantitative analytical method, in particular for the detection of DNA. However, one of the main advantages over fluorescence and other optical detection techniques is the ability to multiplex.

The enhancing surface which is used is crucial and in this case suspensions of metallic nanoparticles will be the focus since they allow quantitative detection to be achieved and are compatible with solution-based diagnostics assays. When using metallic nanoparticles for SE(R)RS, aggregation is required to massively increase the enhancement achieved and the aggregation conditions used are crucial to the success of the analysis. To obtain successful SERRS of DNA, the use of spermine as an aggregating agent is required. The nature of the label which is used, be it fluorescent, positively or negatively charged also effects the SERRS response and these conditions are again explored here. We have clearly demonstrated the ability to identify the components of a mixture of five analytes in solution by using two different excitation wavelengths and also of a 6-plex using data analysis techniques.

This book chapter will explore the ability of SE(R)RS to detect multiple analytes in a mixture, that is, its ability to multiplex. The detection of DNA will be used as the focus for this discussion however the capabilities of SE(R)RS can be easily extended to any biomolecule, or indeed, any mixture of analytes with distinct spectral fingerprints.

K. Faulds

Centre for Molecular Nanometrology, Department of Pure and Applied Chemistry, WestCHEM, University of Strathclyde, 295 Cathedral Street, Glasgow, UK

2 Introduction

Surface enhanced resonance Raman scattering (SERRS) [1–3] is a highly sensitive technique, so sensitive in fact that single molecule detection has previously been reported [4, 5]. It is a very attractive technique for the detection of biomolecules because it produces molecularly specific spectra which make it feasible to easily identify the components of a mixture in a single analysis without extensive separation procedures [6].

SERRS is an advancement over normal Raman scattering which is an inherently weak process with only around one in every million photons being Raman scattered. Used correctly, it can combine many of the advantages of Raman spectroscopy such as molecular specificity and selective identification of a species in situ, with the additional advantages of high sensitivity. SERRS involves adsorbing the analyte of interest onto a roughened metal surface and interrogating the surface using a Raman spectrometer. The most commonly used metals for SERS measurements are gold or silver [3, 7]. The reason for this are that the electronic properties of gold and silver are suitable for SERS as they have surface plasmons which lie in the visible region of the electromagnetic spectrum which coincide with the commonly used Raman excitation wavelengths.

One of the main advantages of SERS, as well as its inherent sensitivity, is in the nature of the spectroscopic data obtained. SERS is a vibrational technique therefore the spectra obtained have sharp, molecularly specific peaks which are unique to the molecule being analyzed. This is in contrast to other highly sensitive optical detection methods such as fluorescence where the spectra produced are not molecularly specific, broad and cannot be used to assign the structure of the molecule being investigated. It is this highly advantageous feature of SERS which allows us to analyze complex samples, for example, to obtain spectra from analytes contained within complex media since if the spectrum of the analyte is known it can be easily distinguished from background signals from the matrix. The other major advantage, which will be focused on within this chapter is the ability to use these unique, fingerprint spectral features to detect, quantify, and identify the components of a mixture when multiple analytes are present, that is, multiplexing. The multiplexing capabilities of SERS will be discussed in the context of biological samples focusing specifically on the example of DNA, however this capability could be easily extended to any analyte or mixture of analytes.

The labeling of biological components to aid in their detection is well established and common practice using a wide range of techniques including micro arrays, ELISA, separation science and real time PCR. The most widely used labels are fluorescent or chemiluminescent in nature. These labels generally offer a high degree of sensitivity, down to single molecule detection levels, therefore they are the most widely used in biological characterization and diagnostics [8]. The same labels can be used to label and detect biomolecules using SERRS as well as using non-fluorescent labels allowing more labeling strategies to be used which are potentially simpler, more extensive and as yet underdeveloped. To achieve maximum surface enhancement the labels must have a chromophore

which is coincident with the excitation frequency and the ability to adsorb onto a suitable metal surface [9]. Many compounds of interest do not possess these properties but addition of a SERRS active label can achieve this and this approach has been used successfully for a number of targets including DNA [10–12]. The use of a metal surface quenches any fluorescence emitted meaning that commonly available fluorescent labels can be used for SERRS. Previously, SERRS detection of oligonucleotides has made use of a covalently attached label followed by a biological event then detection.

2.1 Surface Enhanced Raman Scattering

Surface Enhanced Raman Scattering (SERS) was first reported by Fleischman et al. in 1974 [1]. During experiments using Raman spectroscopy to detect pyridine at a silver electrode, it was noted that the Raman scattering was greatly increased when the surface of the electrode was roughened. Work a few years later, in 1977, by Jeanmaire and Van Duyne [2], and Albreicht and Creighton [13] demonstrated that the Raman scattering from pyridine adsorbed on a roughened silver surface was a factor of 10^6 greater than the same amount of pyridine in solution. This large enhancement in signal stimulated great interest in the technique and it was discovered that the effect was not unique to pyridine. It has subsequently been shown that it is possible to obtain SERS from a large number of molecules as long as they are adsorbed onto a roughened metal surface.

Surface enhanced resonance Raman scattering (SERRS) can be considered to be a combination of two processes, resonance Raman and SERS, and it was first reported by Stacy and Van Duyne [14] in 1983. As a consequence of this, the enhancement in signal that is observed is greater than either of these two processes, with SERRS spectroscopy an enhancement in scattering three to four orders of magnitude greater than SERS is observed. This in turn corresponds to an enhancement of up to 10^{14} in the scattering from some molecules compared to normal Raman scattering.

For an analyte to exhibit SERRS, it must have a chromophore and by tuning the frequency of the laser excitation to the absorption maxima of the analyte, as well as the surface plasmon of the metal substrate, very large enhancements in signal can be observed. This also gives a degree of selectivity as colored compounds with absorption maxima close to the laser excitation will be selectively enhanced over other species that may be present.

2.2 Mechanism of SERS

Since the discovery of the surface enhancement effect, it has been the subject of much debate as to what the origins of the effect are. It is generally understood that there are several mechanisms which are responsible for the observed enhancement. There are two main theories: electromagnetic enhancement and charge-transfer or chemical enhancement which will be mentioned only briefly here.

The collective excitation of the electron cloud of a conductor is known as a plasmon, if the excitation is confined to the surface of the conductor it is known as a surface plasmon. For the excitation of surface plasmons by light, surface roughness or curvature is required. The electromagnetic field of the light at the surface can be greatly increased when the surface plasmon is excited. This results in the amplification of both the incident and scattered and this is the basis of the electromagnetic SERS mechanism [15].

Although electromagnetic enhancement can explain important features of the SERS effect, it does not take into account in any way the chemical structure of the analyte species. Therefore, chemical enhancement models take into account the structural properties of the analyte and it is thought to operate independently from electromagnetic enhancement. Chemical enhancement can result from charge-transfer or bond formation between the metal and the analyte molecule which can result in an increase in the polarizability, α , of the molecule. Therefore, a surface complex between the analyte and the metal must form before chemical enhancement can occur.

It is very difficult to separate the two enhancement effects but this has been attempted by measuring the chemical enhancement from smooth metal surfaces where it was found that the chemical enhancement effect was small [16, 17]. This enhancement process is wholly adsorbate dependent and requires an analyte with a functionality capable of chemi- or physisorption to the metal surface. There is evidence for both these effects and it is widely believed that the enhancement may occur from a combination of these two effects.

2.3 Metal Surface

A wide range of metals have been used to obtain surface enhancement; lithium, palladium, cadmium, and nickel, however, the most commonly used are silver [4, 7, 18], gold [19–22], and copper [23, 24] since they tend to give the largest enhancement in signal and have surface plasmons which lie in the visible region of the electromagnetic spectrum which coincide with the commonly used Raman excitation frequencies.

A wide range of techniques have been used to obtain the roughened metal surface that is required for SERS/SERRS. The original experiments used electrochemical methods to produce roughened metal electrodes [2–4] and this method is still often used. Fiber optics [25], silver coated filter papers [26], nitric acid etching of silver [27], silver coated titanium dioxide [28], and alumina [29], and polymers sol-gels containing silver [30–32] have also been used. Laserna et al. [33] and Rowlen et al. [34] have published papers comparing the effectiveness of some of these SERS substrates.

However, one of the most commonly used substrates for SE(R)RS is colloidal nanoparticle suspensions of the metal, and the most commonly used metals are silver and gold. This is due to the fact that silver tends to give the greatest enhancement in Raman scattering [35, 36], however gold nanoparticles are often

used due to their ease of synthesis and they are often favored for work with laser excitations in the near infrared [21]. Colloidal suspensions of nanoparticles are a particularly attractive substrate for SERS/SERRS as they are relatively simple to make, stable and offer a good degree of reproducibility [3, 37]. The colloid provides a fresh surface for each analysis reducing contamination, which can be a problem due to the high sensitivity of the technique. The colloids are relatively inexpensive and can be made in a batch process that can make enough for several analyses.

The most commonly used suspensions of metal nanoparticles tend to be synthesized by reduction of a metal salt by a reducing agent such as citrate, borohydride, etc., resulting in colloidal suspensions of nanoparticles that tend to be fairly monodisperse and stable over time [3, 7]. Much research has gone into the preparation of silver colloids to give the most reproducible and sensitive colloid. Such citrate reduced silver colloids are thought to be stable for around 6 months due to the colloid being stabilized by a layer of citrate molecules which are bound to the metals surface. This would suggest that citrate reduced silver colloid will have a net negative charge which can make it difficult for negatively charged analytes to get good surface attachment and thus give good SERS/SERRS. Citrate reduced gold colloids are also thought to be stabilized by a citrate layer [38, 39].

This chapter will focus almost exclusively on the use of metal nanoparticles to provide the enhancing surface, however the ability to detect multiple analytes simultaneously can equally be applied to any SERS active metal substrate. Also, as previously stated, the focus here is on the multiplexed detection of DNA since it is a good example of an area where there is a strong need for multiplexing capabilities, however the research reported here could equally apply to another biological system, or indeed, any multianalyte system.

3 Experimental and Instrumental Methodology

Silver Nanoparticles Preparation. A colloidal suspension of citrate reduced silver nanoparticles was prepared using a modified Lee and Meisel [8] procedure.

Sample Preparation. All samples were prepared for SERRS analysis using the following amounts of reagents, 10 μl of dye-labeled oligonucleotide, 10 μl of 0.1 mol dm^{-3} spermine, 250 μl of water, and 250 μl of citrate reduced silver nanoparticles.

Concentration Studies. Concentration studies were carried out using the dye-labeled oligonucleotides. The oligonucleotides were diluted to various concentrations using sterile water. Samples were prepared as above. The samples were analyzed within a minute of the addition of the silver colloid, and each oligonucleotide concentration was analyzed five times. The spectra obtained were the result of a 10 s accumulation time with the spectrometer grating centered at 1,400 cm^{-1} . The spectra obtained were baseline corrected using the GRAMS/32 software and the average peak height of the strongest peak in the spectrum was normalized to the silicon standard peak and plotted against the concentration of labeled oligonucleotide.

Multiplexing. Multiplexing was carried out using six (or five in the case of dual wavelength multiplexing) dye-labeled oligonucleotides. The labels used were TAMRA, ROX, HEX, TET, FAM, and Cy3. The multiplex samples were all prepared using initial stock solutions of labeled oligonucleotides that were prepared to be at a concentration of 10^{-7} mol dm⁻³. The multiplex samples were then prepared by making solutions containing every possible combination of the 6 labeled oligonucleotides resulting in 64 samples. In the multiplex sample mixtures, water was used to replace missing oligonucleotides in the matrix samples, thus allowing the overall concentration of the labeled oligonucleotides in each sample to remain the same. The final concentration of each oligonucleotide in the multiplex sample was 1.92×10^{-9} mol dm⁻³. All samples were prepared for SERRS analysis using the following amounts of reagents, 60 μ l of dye-labeled oligonucleotide, 10 μ l of spermine tetrahydrochloride (0.1 mol dm⁻³, Sigma-Aldrich), 190 μ l of distilled water and 250 μ l of citrate reduced silver nanoparticles. The samples were analyzed within 1 min of the addition of the colloid and spermine and five replicates of each multiplex concentration were prepared and analyzed in a random fashion. The spectra obtained were the result of a 1 s accumulation time with the spectrometer grating centered at $1,400$ cm⁻¹.

Instrumentation. The following Raman instrumentation were used; a Renishaw Model 100 probe system with a 514.5 nm argon ion laser, utilizing a $\times 20$ objective to focus the laser beam into a 1 cm plastic cuvette containing the sample and a Renishaw Microscope System 1000 with a 632.9 nm helium-neon laser utilizing a Vantacon macrosampler to focus the laser beam into a 1 cm plastic cuvette. However, the same dye-labeled oligonucleotide detection limits can be obtained with low cost Raman instrumentation such as the DeltaNu Advantage 532 nm system, with a compromise in the peak resolution which may slightly influence multiplexing capabilities.

4 Key Research Findings

4.1 SERRS of Labeled Oligonucleotides

Detecting target DNA sequences is an extremely important task in various areas of molecular biology, and is a key step to many modern techniques of disease state analysis. Current methodologies require the detection of a specific sequence, or sequences, within a mixture. The most common way to do this is to attach a label to the DNA sequence of interest thus allowing the presence of the label to be indicative of the presence of a specific DNA sequence. The labeled sequence then acts as a probe to the target sequence. In the majority of cases, the polymerase chain reaction (PCR) is used to amplify the DNA sequence of interest during which a hybridization event takes place that allows the labeled probe sequence to be detected and hence infer the presence of the target. The detection is normally then carried out using a spectroscopic method, most commonly fluorescence

spectroscopy, in which case the label attached to the sequence will be fluorescent in nature. However, there are several drawbacks to using fluorescence as a detection technique. The main problem is the nature of the fluorescence emission spectrum which is broad and gives limited characteristic information about the target analyte. This makes the detection of multiple analytes in a mixture difficult due to the large spectral emission overlap that occurs from more than one fluorophore. In practice, using a single excitation light source, only four labels are generally detected at once, three if an internal standard is used unless some sort of physical separation method is employed. Thus, to increase the amount of data obtained per experiment and reduce the number of separate measurements required for DNA analysis, it is desirable to increase the number of DNA sequences that can be detected simultaneously, without separation, in a single experiment.

4.2 Practical Detection of DNA by SERRS

As already stated, to achieve the maximum SERRS enhancement, the analyte molecule must contain a chromophore in the visible region which coincides with the Raman excitation wavelength. DNA does not naturally have this property therefore it has to be modified to allow spectroscopic detection. The chemistry involved in labeling DNA is well characterized since these labeling strategies are routinely used in standard fluorescence detection methodologies.

Custom modifications have previously been developed whereby a non-fluorescent chromophore can be attached to the DNA sequence to provide a strong SE(R)RS signature which is indicative of the DNA sequence present. This has been done previously using DABCYL, phthalocyanines and black hole quenchers (BHQs) as well as specifically designed simple azo dyes which contain moieties to aid in their binding ability to metal surfaces such as the benzotriazole motif which has been shown to be very effective at complexing onto silver nanoparticles [12, 13, 40, 41].

However, the simplest method is to use commercially available fluorophores as the label. This approach is much less synthetically challenging as the labels are commercially available and the procedures for attachment of these labels to sequences of DNA or biomolecules is well characterized and understood. The benefit of this approach is also that there are many fluorophores available which have varying absorption maximum therefore they can be chosen to coincide with the Raman excitation wavelength of your choice. The fact that these molecules are fluorescent does not present the same problem as would be the case in conventional Raman spectroscopy since in surface enhancement the fluorescence emitted by the fluorophore is quenched by the metal surface. The other main advantage of using commercially available fluorophores is that there are a large amount of fluorescent labels available, all with unique spectral fingerprints which make them ideal for multiplexing. For example, the structure and the spectral signature of DNA sequences labeled with FAM, TET, and HEX are given in Fig. 13.1 and it can be seen that although the structure of the three labels are very similar, varying only in

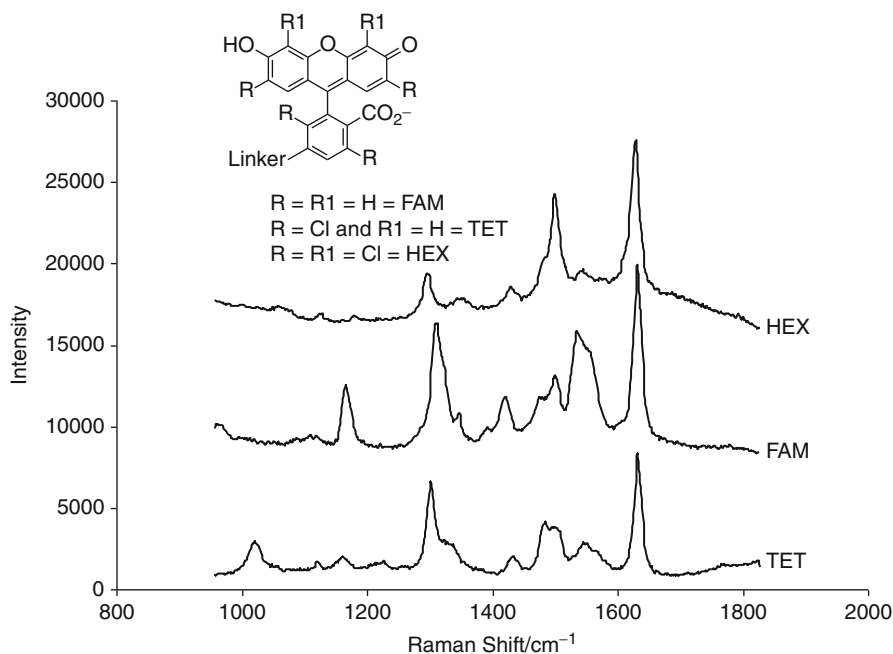


Fig. 13.1 SERRS spectra of FAM, TET, and Hex labeled DNA sequences obtained using silver nanoparticle suspensions and a 514 nm laser excitation

the amount of chlorines on the ring structure, their SERRS spectra are all unique. This opens a vast library of labels which can be used for multiplexing.

The other main requirement to obtain SE(R)RS is that the analyte molecule must come in contact with or be very close to the metal surface used for enhancement. The majority of the work discussed here uses citrate reduced silver nanoparticles which have a net negative charge in aqueous solution due to a layer of citrate that exists on the surface of the silver particles [38]. Since DNA is overall negatively charged, due to the phosphate groups present in the DNA backbone, it is unable to adsorb efficiently onto the surface of the silver colloid. However, the surface charge of the nanoparticles can be altered by addition of a positively charged species which will change the effective surface charge of the nanoparticles due to electrostatic layering. A range of species such as ions, poly-L-lysine, and spermine have been investigated for their ability to allow adsorption of negatively charged DNA onto negatively charged nanoparticles, however the polyamine, spermine has proven to be most effective [42]. Spermine hydrochloride has been shown to interact with the DNA backbone and neutralize the negatively charged phosphate backbone of the DNA allowing it to come down onto the metal surface [43]. The spermine also has the advantage that, when in excess, it will also aggregate the metal nanoparticles to give higher enhancement of the Raman scattering due to the increased electromagnetic enhancement experienced through the coupling of the plasmons when the nanoparticles interact. Therefore, spermine serves a dual purpose in these types of

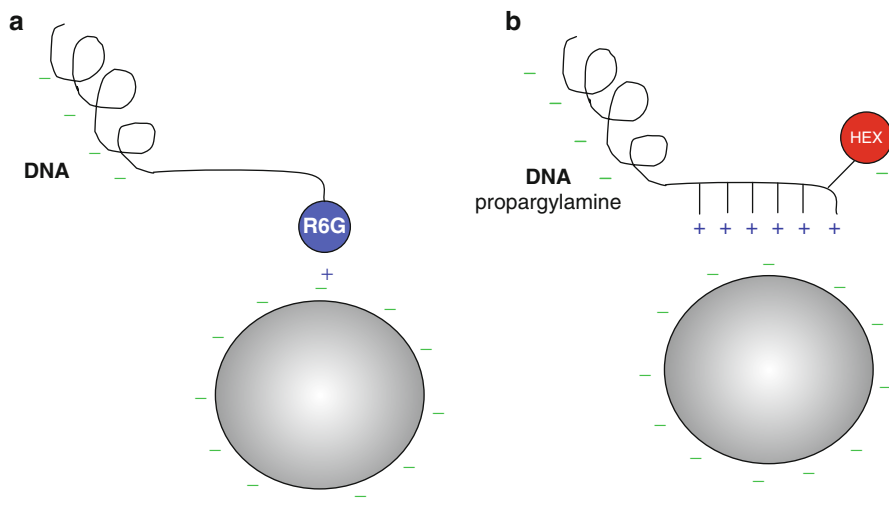


Fig. 13.2 Schematic representation of the process required to achieve detection of labeled DNA by SERRS. Represented is (a) an oligonucleotide labeled with R6G which is already positively charged, therefore no surface modification is required to allow adsorption onto a silver nanoparticle surface and excited with laser light to give a SERRS spectrum and (b) an oligonucleotide modified with a negatively charged dye label, in this case HEX, which requires further modification of the DNA with propargylamine to introduce a region of positive charge to allow adsorption to the metal nanoparticle

studies, namely it promotes surface adsorption of the negatively charged oligonucleotide probe and also aggregates the nanoparticles to provide the higher surface enhancement from the aggregated nanoparticles as opposed to the individual nanoparticles.

The dye labels can also be chosen to aid in the adsorption process to the metal surface, that is, the dye can be specifically designed to have a metal surface complexing group as well as a chromophore such as is the case with azo dyes synthesized to have a benzotriazole group [12, 13] or the charge on the label itself can aid in this adsorption process. Some fluorophores will have a net positive charge in aqueous solution, for example, R6G, ROX, and TAMRA and therefore no further modification is required to allow oligonucleotides modified with these labels to attach to the negatively charged silver surface (Fig. 13.2a). However, some fluorescent labels will have a net negative charge in aqueous solution, for example, Cy3 and FAM therefore further modification of the oligonucleotide is required for effective surface adsorption to occur. Propargylamine modification of DNA sequences utilizing negatively charged dye labels has previously been reported and initially involved the addition of six modified nucleobases at the 5'-terminus close to the dye label (Fig. 13.2b) [44]. When placed in aqueous solution the terminal primary amine groups of the propargylamines will be protonated resulting in a positive charge, allowing the DNA to more effectively adsorb onto the negative silver surface. It has recently been discovered that we do not require six

Table 13.1 SERRS limits of detection for a range of dye-labeled oligonucleotides using citrate reduced silver nanoparticles. No value indicates the experiment was performed but the limit of detection is higher than the base concentration of each dye ($\sim 1 \times 10^{-8}$ M). The fluorescence detection limits were not measured (N.M.) for all of the dyes hence the not measured entries

Dye label	λ_{\max} (nm)	$\lambda_{\text{ex}}: 514.5 \text{ nm}$	$\lambda_{\text{ex}}: 632.8 \text{ nm}$		$\lambda_{\text{ex}}: 785 \text{ nm}$	Fluorescence
		Silver (mol dm^{-3})	Silver (mol dm^{-3})	Gold (mol dm^{-3})	Silver (mol dm^{-3})	LoD (mol dm^{-3})
FAM	492	2.7×10^{-12}	2.0×10^{-9}	–	–	6.5×10^{-8}
R6G	524	1.2×10^{-12}	1.1×10^{-10}	–	–	3.5×10^{-8}
Cy 3	552	2.6×10^{-10}	1.5×10^{-10}	–	–	4.6×10^{-9}
ROX	585	8.1×10^{-11}	3.3×10^{-11}	1.1×10^{-9}	–	2.3×10^{-8}
BODIPY TR-X	588	1.3×10^{-10}	7.9×10^{-12}	4.9×10^{-10}	–	N.M.
PtcCo	625	–	3.2×10^{-11}	N.M.	–	Not fluorescent
PtcAl	640	–	2.8×10^{-11}	N.M.	–	Not fluorescent
PtcZn	680	1.4×10^{-10}	3.2×10^{-11}	N.M.	–	Not fluorescent
Cy5.5	683	–	5.2×10^{-12}	7.3×10^{-11}	–	N.M.

propargylamine-modified nucleobases and that double the SERRS signal can be obtained by adding one modified nucleobase, however at least two are required to get the optimal surface absorption [45]. Therefore, the action of spermine combined with either a positively charged dye or a negatively charged dye and the propargylamine-modified bases allows good absorption of DNA and hence successful SERRS to be obtained.

4.3 Sensitivity of SERRS

When oligonucleotides are designed and analyzed using the conditions described above, it is possible to achieve extremely sensitive detection of labeled oligonucleotides using metal nanoparticles [36, 43]. It is possible to achieve quantitative, linear SERRS responses when the concentration of the labeled oligonucleotide is kept below monolayer coverage of the nanoparticle surface.

A direct comparison between the detection limits using SERRS and fluorescence, when the labels on the DNA are fluorescent, has been carried out, thus allowing a direct comparison of the two techniques. The limits of detection of five different labeled oligonucleotides are shown in Table 13.1 using a range of excitation frequencies and silver nanoparticles [46]. The fluorescence detection limits were calculated using routinely available fluorometers, specifically quantitative PCR instrumentation, thus giving a direct comparison as they are instruments currently found in functioning molecular diagnostic laboratories. The SERRS detection limits were also calculated using a range of different spectrometers,

including Raman microscopes and fiber optic-based systems and the detection limits were approximately the same for each of the different Raman systems, regardless of cost or configuration. This indicated that, although there is a compromise in terms of peak resolution when the specification of the spectrometer is lowered, the absolute sensitivity in terms of limits of detection does not appear to be compromised.

The main point to note from [Table 13.1](#) is that under these conditions SERRS is much more sensitive than fluorescence with SERRS detection limits generally at least three orders of magnitude more sensitive than fluorescence [46]. The other feature of the data presented in the table is that there is a clear correlation between the λ_{max} of the dye and the excitation frequency used. This confirms how important the resonance contribution of the label is to the sensitivity of the detection system and that for maximum sensitivity, a resonance contribution with the dye label as well as a resonance contribution with the surface plasmon of the aggregated silver or gold nanoparticles is necessary for maximum enhancement in signal to be achieved. For example, the lowest detection limits obtained for R6G, which has a λ_{max} of 524 nm, are achieved when an excitation of 514.5 nm is used and this detection limit increases by as much as two orders of magnitude when we move away from resonance and use 632.8 nm excitation. It should also be noted that silver nanoparticles tend to give lower detection limits than gold, even when higher excitation wavelengths are used.

These results highlight that, if the SERRS conditions are carefully and correctly chosen, SERRS is an extremely powerful and sensitive technique. It has improved sensitivity over fluorescence, resulting in detection limits that are generally at least three orders of magnitude lower. However, one of the main advantages of SERRS over fluorescence, or any other optical detection technique for that matter, is that coupled with the sensitivity SERRS produces spectra which are molecularly specific and give a pattern of sharp peaks. Thus, SERRS has a huge advantage over fluorescence when analyzing mixtures of analytes since SERRS spectra contain more spectral features to distinguish the analytes whereas fluorescence spectra of mixtures tend to have broad overlapping features which are more difficult to discern. The ability of SERRS to multiplex, or to detect multiple analytes within the same sample using one analysis will be investigated in the remainder of this chapter using DNA as the example.

4.4 Multiplexing

As well as the inherent sensitivity of SERRS and the ability to obtain quantitative detection of dye-labeled oligonucleotides, one of the main advantages of SERRS is the ability to multiplex. In the context of DNA detection, we use the term multiplexing to mean the ability to detect multiple labels attached to different DNA sequences at the same time, but without using any physical separation procedures. Therefore, the resultant SERRS signal from the label will be indicative of the presence or absence of a particular sequence of DNA. The sharp vibrational fingerprint spectra obtained using SERRS are ideally placed to allow us to separate

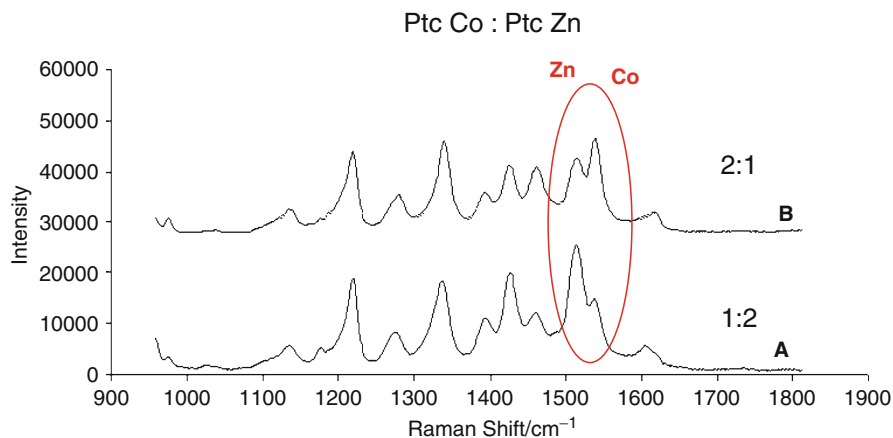


Fig. 13.3 Spectra of mixtures of DNA labeled with phthalocyanine with a cobalt metal center (PtcCo) and zinc metal center (PtcZn) with the following ratios: A – 1:2 and B – 2:1. Spectra obtained using 632.8 nm laser excitation with a 10 s accumulation

the components of a mixture using spectroscopy. This can either be simply done visually when there are, for example, three carefully chosen labels in the mixture, however when the mixture contains more components separation becomes much more complex and impossible by eye and it therefore requires mathematical methods to separate and identify the components of the multiplex. Obviously, if an array-based format is used then the multiplexing capability can be increased infinitely by employing spatial separation, however this chapter will focus on the solution-based multiplexing capability of SERRS and the separation of the spectral features of the components.

We have previously shown how phthalocyanines can be used as unique SERRS labels for DNA and demonstrated how they can be used to successfully discriminate between two different DNA sequences. Phthalocyanines have porphyrin structures with metal centers and by varying the metal center the optical properties of the phthalocyanine can be altered to change their absorbance properties or to become fluorescent or non-fluorescent [41]. Cobalt and zinc phthalocyanines were used to provide different labels attached to oligonucleotides which could then be quantified in terms of their SERRS response and when mixed in a multiplex in different ratios each could be easily identified (Fig. 13.3).

Initial work on the duplex detection of fluorescently labeled oligonucleotides was carried out using HEX and R6G labeled oligonucleotides where it was possible to discriminate the ratio of each oligonucleotide sequence present based on the SERRS signal from the labels [47]. A 3-plex of fluorescently labeled oligonucleotide sequences has also been detected by eye, however in this case a lab-on-a-chip format was used. In this example, microfluidics chips were generated from PDMS and DNA sequences labeled with Cy3, FAM, and TET were introduced into the chip, the SERRS signals were measured at a point further down the channel [48].

A microfluidics approach allowed simultaneous detection of three different DNA sequences corresponding to different strains of the *Escherichia coli* bacterium.

We have also recently carried out a 5-plex of labeled oligonucleotide sequences where we managed to identify five different labeled oligonucleotides in a mixture by careful choice of the label and by using two excitation wavelengths [49]. The sequences used corresponded to a range of different targets. FAM, Cy5.5, and BODIPY TR-X were used to label a universal reverse primer, rhodamine 6G (R6G) was used to label a probe for HPV, and ROX to label a probe to the VT2 gene of *E. coli* 157. The labels were carefully chosen since they have different absorbance maxima (Table 13.1) and because they have unique SERRS spectra.

The spectra obtained from each of the individual labels are shown in Figs. 13.4a and 13.3b, using two different laser excitation frequencies. It can be clearly seen that each label gave a distinctive spectrum, however since the dye labels have different absorbance maxima they will not all be in resonance with the same laser excitation frequency and this property can be exploited to produce a very sensitive and selective method for detecting each of these labels within a mixture of the others using two different laser excitation frequencies. Figure 13.4a shows the spectra of the five labeled oligonucleotides when an excitation wavelength of 514.5 nm was used. Only three of the dye-labeled oligonucleotides (R6G, FAM, and ROX) gave an intense spectrum at this wavelength and this is due to them being in resonance at this excitation wavelength (Table 13.1). Figure 13.4b shows the spectra of the same five dye-labeled oligonucleotides using an excitation wavelength of 632.8 nm, again only three of the labels gave an intense spectrum at this wavelength, however in this case, the labels were Cy5.5, BODIPY TR-X, and again, ROX (ROX could be detected at both excitation wavelengths since it has two absorbance peaks, one at 585 nm and one at ~ 530 nm which will be in resonance with the 514.5 nm laser).

The multiplex spectra obtained using each excitation frequency are also shown in Fig. 13.3. Using 514.5 nm excitation two identifying bands from FAM and rhodamine 6G can be clearly distinguished and that from the multiplexed spectrum of the same mixture at 632.8 nm excitation identifying bands from ROX, Cy5.5, and BODIPY TR-X can be clearly distinguished. The bands chosen allowed simple and fast identification of the oligonucleotides in the mixture by looking for the presence, or absence, of these key marker bands. The bands had sufficiently different Raman shifts to allow this multiplexed identification to be done by eye.

This approach is very powerful since it is possible to manipulate the resonance contribution of the labels to our advantage; however, this is not always possible, particularly when we wish to increase the multiplexing capability or we only have one excitation frequency available. Clearly, multiplexing could be greatly increased by the use of data analysis techniques and it then becomes necessary to use chemometrics methods to separate the components of the multiplex. Figure 13.5a shows the individual spectra of six dye-labeled oligonucleotides and Fig. 13.5b shows the multiplex spectrum of a 6-plex and the difficulty in separating the components of the multiplex by eye becomes apparent due to the lack of visually distinguishable peaks. Therefore, in this case it becomes necessary to adopt a multivariate analysis

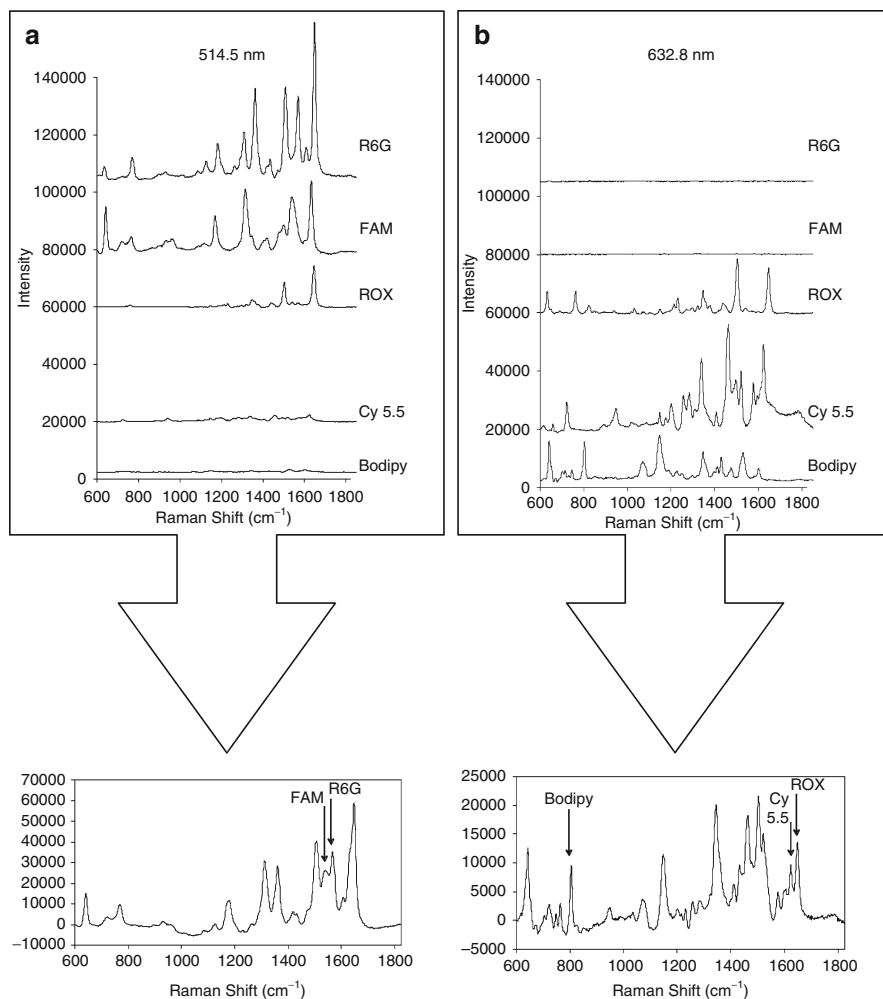


Fig. 13.4 SERRS spectra of each individual dye-labeled oligonucleotide at a concentration of $1.82 \times 10^{-9} \text{ mol dm}^{-3}$ obtained using (a) 514.5 nm and (b) 632.8 nm laser excitation (top spectra). The multiplexed SERRS spectra of the 5-plex using (a) 514.5 nm and (b) 632.8 nm excitation (bottom spectra) are also shown

(MVA) approach rather than looking for specific discriminatory Raman bands [50, 51]. In MVA, the whole of the SERRS spectrum is considered.

Using this approach the first multiplexed simultaneous detection of six different DNA sequences, corresponding to different strains of the *Escherichia coli* bacterium, each labeled with a different commercially available dye label (ROX, HEX, FAM, TET, Cy3, or TAMRA) was reported [52]. In this study, both exploratory discriminant analysis and supervised learning, by partial least squares (PLS) regression, were used and the ability to discriminate whether a particular labeled

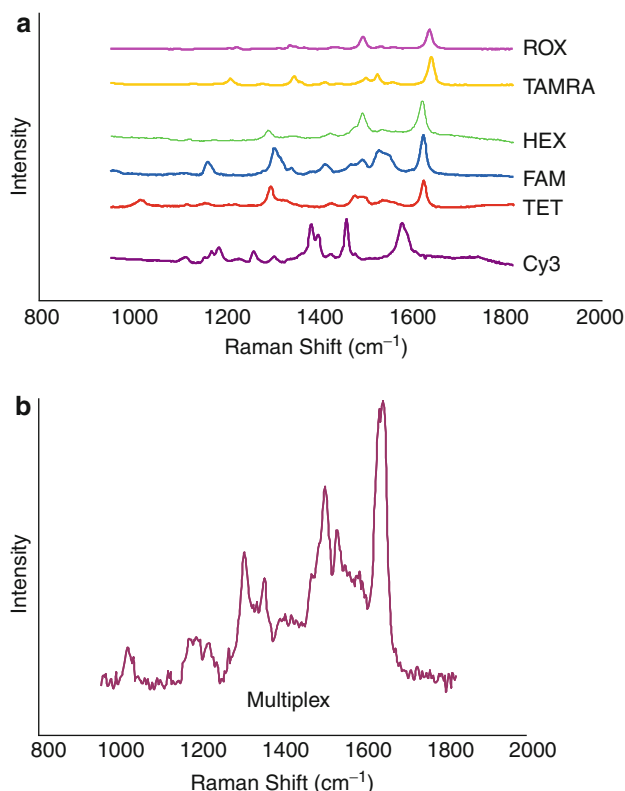


Fig. 13.5 Schematic representation of the use of chemometrics to deconvolute the multiplex spectrum of six dye-labeled oligonucleotides. (a) Shows the individual spectra of six dye-labeled oligonucleotides and (b) shows the spectrum of the mixture of the six labeled oligonucleotides

oligonucleotide was present or absent in a mixture was achieved using PLS with very high sensitivity (0.98–1), specificity (0.98–1), accuracy (range 0.99–1), and precision (0.98–1).

4.5 Multiplexed Assays

All of the work described above involved the direct detection of labeled DNA sequences, however to generate useful detection methodologies, it is required to carry out some molecular biology to create a meaningful assay which will detect a specific, target DNA sequence of interest in a genuine biological sample. For the detection of DNA targets, the most obvious way to do it is to use a PCR-based approach to amplify and detect the presence of a specific sequence. As stated previously, there are studies in the literature relating to array-based formats for specific DNA detection by SERRS, however, this chapter is focusing on

nanoparticle and solution phase-based detection systems and as such the assays reported here relate mainly to this type of format.

An early study for the detection of the mutational status of the cystic fibrosis transmembrane conductance regulator gene used an amplification refractory mutation system (ARMS) approach combined with SERRS [53]. The assay proved to be a good example of the ability to provide a selective 2-plex identification of different gene sequences from genuine patient examples. There are three different possibilities for the genetic status of this particular gene: the wild type, where both alleles are normal, the heterozygote, where one allele is mutated and one is normal, and the fully homozygote mutant where both alleles have the mutated DNA sequence. Specific labeled primers were designed to amplify the region relating to this mutation and used in a multiplexed PCR assay. The PCR amplicons were then identified by SERRS after removal of any unincorporated primers. This allowed the successful determination of the mutational status of these particular samples without the additional separation steps commonly performed in the fluorescence assays [54].

Recent work has developed an assay which has the potential to be used in a closed-tube, homogeneous, and multiplexed assay format [55]. The assay is based on the observation that double stranded DNA has a lower affinity for the surface of silver nanoparticles than single stranded DNA (Fig. 13.6). Therefore, when a single stranded probe sequence labeled with a SERRS active label is added to silver nanoparticles a strong SERRS signal is obtained. However, when the complementary target sequence is present it will hybridize to the labeled probe sequence resulting in a duplex which has a lower affinity for the metal surface resulting in a much reduced SERRS signal as a result of the presence of target DNA. This results in reduced SERRS signal compared to when no target or noncomplementary target is present. The lower affinity of the dsDNA for the metal surface is thought to be due to the increased electrostatic repulsion of the exposed negative phosphate backbone of dsDNA compared to ssDNA.

This assay was used for the detection of three genes which are associated with MRSA and three different SERRS labels namely FAM, HEX, and TAMRA were used to identify these sequences. Using this approach it was possible to detect not only the exact complement DNA but also PCR product. It was also possible to detect every possible combination of the three sequences, present or absent, within a mixture demonstrating the multiplexing potential of SERRS for use in homogeneous molecular diagnostics assay.

Vo-Dinh et al. have previously developed a surface-based assay to detect the BRCA1 gene that codes for breast cancer susceptibility. This approach was used to generate an array of capture sequences immobilized on a 9 nm silver layer on a glass surface using a thiolated DNA capture sequence. The rhodamine labeled BRCA1 target was then captured on the surface by hybridization to the complementary target sequence. This format was only used for the detection of one sequence of DNA however it could be easily extended to give spatial multiplexing of multiple targets with different labels [56, 57]. A more recent approach to DNA detection makes use of *molecular sentinels* which are based upon molecular beacons, Fig. 13.7a [58]. The molecular sentinel is comprised of a region

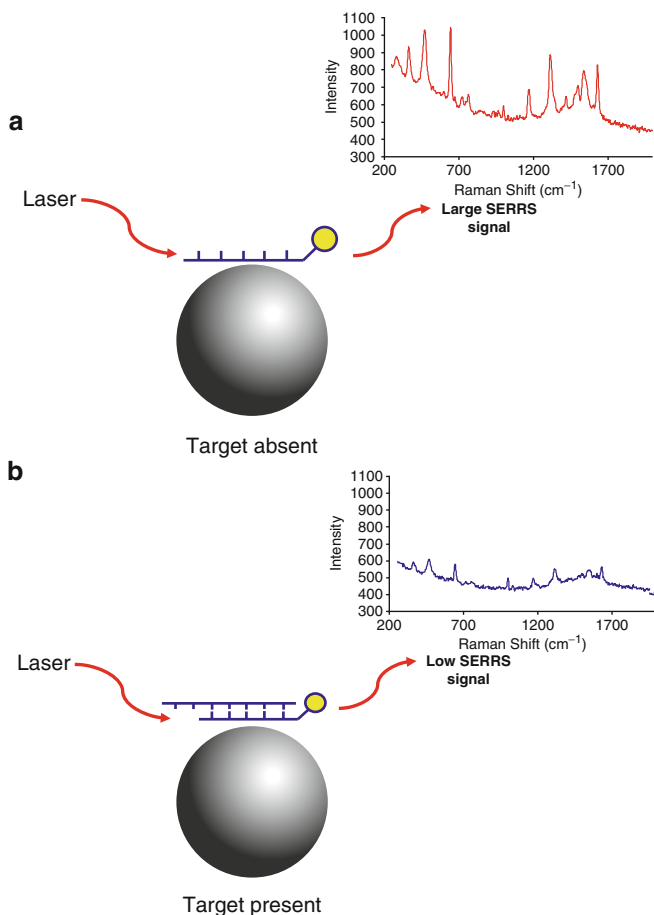
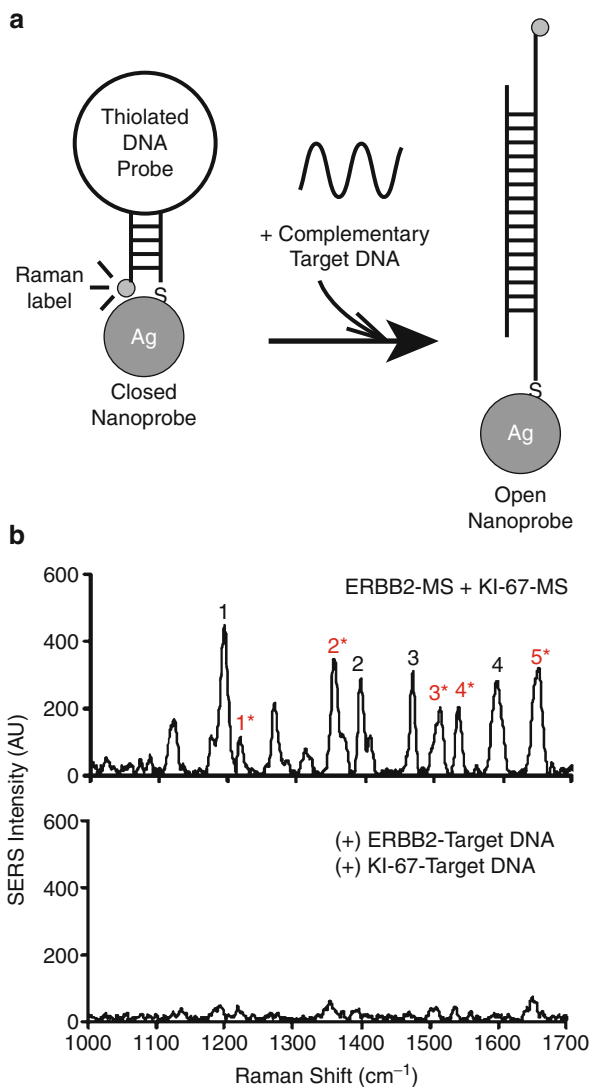


Fig. 13.6 Schematic representation of the SERS detection assay. (a) When target DNA is not present the dye-labeled DNA probe is free to adsorb on the surface of the silver nanoparticles resulting in an intense SERS signal (b) in the presence of target DNA the probe will hybridize to its complement and in this case, the dsDNA is not adsorbed onto the surface of the silver nanoparticles resulting in a reduction in the SERS signal

complementary to the target DNA sequence and a self-complementary stem that holds the sentinel in a closed, hairpin loop conformation. One of the stems of the hairpin is functionalized with a thiol group to allow it to be attached to the surface of a silver nanoparticle and the other end is functionalized with a Raman reporter. In the closed conformation, an intense SERS signal is observed due to the close proximity of the reporter to the metal surface. However, in the presence of the target DNA sequence the sentinel will open resulting in the Raman reporter becoming distal to the surface of the metal nanoparticle. Therefore, this approach results in a reduction in the SERS signal obtained upon binding of target DNA. The molecular sentinels have been used to achieve the multiplexed detection of two

Fig. 13.7 The operating principle of the SERS-based molecular sentinel (MS) nanoprobe. **(a)** The MS nanoprobe is composed of a Raman-labeled DNA hairpin probe and a silver nanoparticle. In the absence of the complementary target DNA, a strong SERS signal is observed due to the hairpin conformation adopted by the MS nanoprobe (*left*: closed state). **(b)** In the presence of the complementary target DNA, the hairpin conformation of the MS nanoprobe is disrupted and the SERS signal is quenched due to the physical separation of the Raman label from the surface of the silver



genes which were biomarkers for breast cancer by creating two sentinels, one labeled with Cy3 and TAMRA (Fig. 13.7b). However, both of these assays have the distinct disadvantage that they are negative assays, that is, the presence of the target DNA sequence results in a decrease rather than an increase in signal.

Recent work by Lowe et al. used the ligase detection reaction (LDR) to detect SNPs in a multiplexed manner using SERS [59]. The so called LDR-SERS approach involved the functionalization of an oligonucleotide primer (which binds downstream of the SNP) with an amine to allow it to attach to the surface of nanoparticles after the ligation. A second oligonucleotide primer sequence

incorporating a Raman active fluorophore and a discriminatory base at the 3' which binds adjacent to the first primer was then used. Since the ligation reaction will only occur when the upstream primer matched perfectly with the template, this means that a Raman signal will only be observed when ligation of the two primers occur since only in this case will the fluorophore be close to the surface of the nanoparticle to give enhancement. Using this approach, a multiplex of two SNP samples were detected, the wild type discriminating primer (labeled with TAMRA) and the mutant (labeled with FAM), which could be detected by eye.

Mirkin et al. have reported the detection of DNA using a microarray-based format. A microarray chip was spotted with capture DNA strands, complementary to half of the target DNA sequence [60]. Gold nanoparticles were functionalized with the other half of the sequence of DNA complementary to the target sequence; however, in this case, a fluorophore, initially Cy3, was attached to the end of the DNA strand in close proximity to the nanoparticles surface. After capture of the target sequence, silver staining was used to further enhance the Raman scattering signal and detection was carried out using a 633 nm laser excitation. Using this approach, it was possible to detect six different target DNA sequences by using different fluorophores for each of the sequences used to functionalize the nanoparticle surface. However, this approach used a spatial multiplex approach to physically separate each of the targets onto different areas of the chip and SERS was used to ensure that there was no nonspecific binding by checking there was only one SERS spectrum per array spot. Using this approach, detection limits of 20 fmol were obtained. During this study, however, the simultaneous detection of SNP targets was carried out. Two RNA targets which bind to the same capture strand of DNA, but have a single base mismatch that lies within the region of the probe sequence, were used to functionalize the nanoparticle; therefore two different sequences labeled with two different fluorophores were used to detect the two target RNA strands. It was possible to use the relative change in the SERS signal of the multiplex of the two reporters to give the relative ratios present in each of the targets.

Irudayaraj et al. have reported the multiplex detection of up to eight different non-fluorescent nanoparticles functionalized with one sequence of DNA [61]. In this approach, a thiolated sequence of DNA was used to functionalize the surface of gold nanoparticles and then non-fluorescent Raman reporters were added to the surface of the nanoparticle to code them with a SERS signal. It was reported that multiplex detection of two, four, and eight differently labeled nanoparticles could be detected in one analysis. However, only one DNA sequence was used in this study to label all the different "flavors" of nanoparticles and the detection of a specific, target DNA sequence by SERS was not reported. However, it was possible to observe the change in surface plasmon by UV-Vis spectroscopy when two batches of nanoparticles functionalized with complementary sequences were hybridized together.

The same group has also reported a multiplexed assay based upon the Mirkin approach but in this case using an array format with non-fluorescent Raman reporters and the reporter molecule was added directly to the surface of the

nanoparticle rather than to the end of the sequence of DNA [62]. Thiolated DNA capture probes were immobilized on the surface of a gold coated glass slide whereupon target DNA was added and allowed to hybridize to the capture probes. The SERRS reporter was then added in the form of gold nanoparticles functionalized with DNA and non-fluorescent Raman reporters, prepared in the same way as described previously [62], the attached DNA sequences were complementary to half of the target sequence, completing the sandwich. A silver enhancement solution was then added to enhance the signal. Using this approach, it was possible to detect four DNA sequences specific to the BRCA1 alternative splice variants by labeling the gold nanoparticles with four different reporters indicative of four different sequences. Detection limits of 1 fmol were also achieved. The same array format was also used to monitor gene expression in cancer cell lines [63]. In this case, a multiplex of two splice junction variants of the BRCA1 breast cancer susceptibility were identified simultaneously by SERS after extraction of mRNA from cancer cells and carrying out DNA/RNA hybridization assay followed by S1 nuclease digestion to remove any single stranded nucleic acids leaving only the target DNA/RNA duplex, before carrying out the SERS array assay as before.

Another approach we have recently developed is also based upon the use of DNA functionalized nanoparticles, however in this case the sequence specific hybridization event is used to “turn on” the SERRS signal through controlled assembly of nanoparticles into aggregates [64]. Assembly of gold nanoparticles by a biological interaction has previously been reported by a number of groups, however in this work silver nanoparticles rather than gold were used due to their high extinction coefficient and the increased level of surface enhancement obtained from silver compared to gold. This has the added benefit that the silver staining step in the previously assays, which was required to increase the enhancement, is no longer required. This study utilizes a solution-based methodology similar in concept to work previously reported by Mirkin et al. with gold in the absence of any SERS [60]. Silver nanoparticles were functionalized with a specifically designed SERRS dye, 3, 5-dimethoxy-4-(6'-azobenzotriazolyl)phenol, which contains a chromophore, a benzotriazole group which complexes strongly to silver metal and a negatively charged phenolic group to prevent nonspecific aggregation of the nanoparticles. The nanoparticles were then further functionalized with 5' thiolated oligonucleotide sequences, complementary to half of the target sequence. Another batch of nanoparticles were also synthesized with the dye and the other half of the probe sequence complementary to the target. Upon addition of target DNA, the hybridization to the two complementary probe sequences occurred which resulted in the nanoparticles being brought close together, that is, the nanoparticles were assembled due to the hybridization event, resulting in a large increase in the SERRS signal due to the increase electromagnetic enhancement experienced by the dye label due to the aggregation event.

This was further extended to allow the multiplex detection of two different target sequences of DNA using two different Raman reporter dyes to code for different probe sequences on the nanoparticles. In this advancement, three batches of

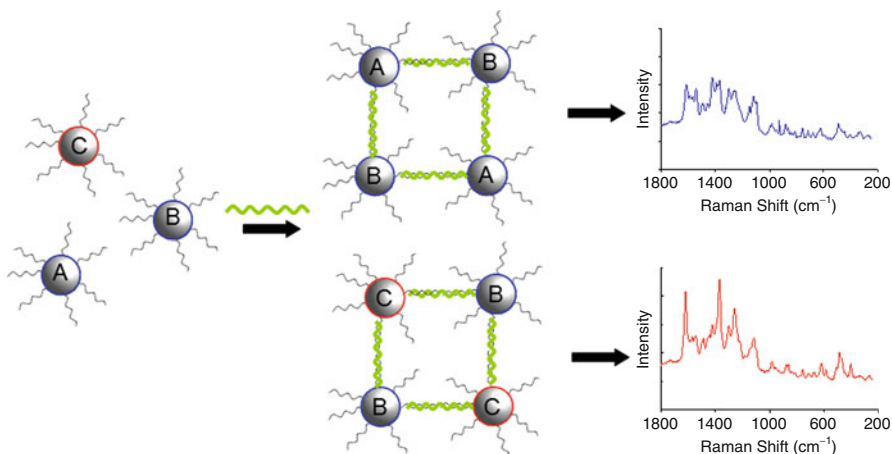


Fig. 13.8 Selective enhancement of specific Raman signals through DNA hybridization. The assay consisted of three different conjugates. Each had a different, noncomplementary oligonucleotide (sequence A, sequence B, and sequence C). Conjugates A and B were functionalized with dye 1, denoted in *blue*, and conjugate C was functionalized with dye 2, denoted by *red*. These three conjugates were mixed together at 30 pM final concentration. When a target complementary to A and B is added, only dye 1 is enhanced (*top right*), and when a target complementary to B and C is added, the spectrum for dye 1 and dye 2 is enhanced (*bottom right*)

nanoparticles were prepared, each functionalized with a different sequence of DNA and coded with two different dye labels, dye 1 and dye 2. Two different target sequences were used to assemble the three sequences such that one hybridization event resulted in SERS signals being obtained from one dye label only, dye 1, whereas the second hybridization event resulting on SERS spectra being obtained from both dye 1 and dye 2, see Fig. 13.8. This is the first time that nanoparticles assemblies have been used to turn on the SERS effect due to a biological interaction as well as demonstrating the potential to use this effect to simultaneously detect multiple target sequences in one analysis.

This chapter has so far solely discussed the multiplexed detection of DNA by SERS as a demonstration of its potential in this area. However, since the multiplexing potential of SERS, as well as its inherent sensitivity, is one of its greatest advantages it has been explored in other areas as well as in DNA analysis.

4.6 Other Multiplexed Formats

Recent work has been published on the use of SERS reporters for *in vivo* analysis. In 2008, Shuming Nie published work on the *in vivo* targeting of tumors in live mice [65]. The SERS particles consisted of 60 nm gold nanoparticles functionalized with a Raman reporter dye molecule and then stabilized with thiolated polyethylene glycols (PEGs). Targeted SERS nanoparticles were prepared by having a mixed monolayer of thiolated PEG and a heterofunctional thiolated PEG with a carboxylic

acid terminal group. The carboxylic acid group allowed the covalent addition of the ScFv antibody, which binds to the EGFR receptor of cancer cells, using EDC coupling. Using these functional nanoparticles, it was possible to target the cancer cell *in vivo* by locating the nanoparticles using SERS. However, this was not carried out to detect multiple targets in a multiplexed manner.

Using a different nanoparticles approach Gambhir et al. used commercially available “nanotags” consisting of gold nanoparticles functionalized with non-resonant Raman reports stabilized with a silica coating [66]. Two differently labeled SERS nanotags were injected into a mouse at three different injection sites and it was possible to detect all three using their SERS signature. Multiplexing was also used to study the fate of two different SERS, one functionalized with PEG and one without, where it was found that both the PEGylated and non-PEGylated nanotags accumulated in the liver to the same extent. In a similar study, the same group were able to carry out multiplex detection of ten different nanotags *in vivo*, spatially separated at different injection sites [67]. During this study, the simultaneous detection of five different tags was carried out to determine the accumulation of the nanotags within the liver. During this study, the potential application of the nanotags for diagnostics application at depth within tissue, was discussed for example, for the laparoscopic detection tumors, however, this was not demonstrated. However, in a recent publication using surface enhanced spatially offset Raman (SESORS) the detection of functionalized nanoparticles, SERS signals could be detected at a depth of between 15 and 25 mm in tissue [68]. This clearly demonstrates the great potential of SERS for targeted *in vivo* diagnostics at clinically relevant depths.

Another recent article by Kneipp et al. demonstrates the use of multiplexing within live cells [69]. Two different SERS particles, aggregates of metal nanoparticles functionalized with two different reporter molecules could be imaged within cells using SERS. The data obtained was analyzed using cluster methods and principal components analysis to detect the two SERS reporters within the live cells.

5 Conclusions and Future Outlook

SERS is an extremely sensitive and selective technique; however, one of its main advantages over other detection techniques is the ability to detect multiple analytes simultaneously. Although the potential to provide this type of multiplexed analysis is often discussed with reference to SERS there are actually few publications that successfully demonstrate this in practice. However, in recent years the multiplexing capability has been more widely explored by researchers within the field resulting in data to support this capability of the technique.

This chapter has demonstrated the advantages of using SERS as an analytical technique for the detection of dye-labeled oligonucleotides when compared to other commonly used techniques. The experimental procedures and design of DNA

probes which allows extremely sensitive and selective detection to be achievable have been outlined with an emphasis on the multiplexing capability. The range of labels available for SERS analysis, the ability to multiplex and subsequently successfully analyze multiplex data has been examined. These aspects have been discussed and indicate how SERRS can be obtained in a quantitative, multiplexed manner from meaningful targets. This is now leading to the development of quantitative methodologies for a range of specific targets using SERRS detection including proteins, DNA, and small molecules. The key feature of SERS which provides the largest advantage over competitive techniques is the multiplexing capability and this looks set to be exploited for in the future in terms of research and commercial applications. For instance, the use of SERRS detection in genuine molecular diagnostic assays is still in its infancy but shows excellent promise and a number of different avenues are currently under investigation by several groups to exploit SERRS as a highly informative, bioanalytical technique for quantitative, ultrasensitive analysis.

References

1. Fleischmann M, Hendra PJ, McQuillan AJ (1974) Raman spectra of pyridine adsorbed at a silver electrode. *Chem Phys Lett* 26(2):163–166
2. Jeanmarie DL, Van Duyne RP (1977) Surface Raman spectroelectrochemistry: part I. Heterocyclic, aromatic, and aliphatic amines adsorbed on the anodized silver electrode. *J Electroanal Chem* 84:1–20
3. Hildebrandt P, Stockburger M (1984) Surface-enhanced resonance Raman spectroscopy of Rhodamine 6G adsorbed on colloidal silver. *J Phys Chem* 88(24):5935–5944
4. Emory SR, Nie S (1997) Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. *Science* 275:1102–1106
5. Kneipp K, Wang Y, Kneipp H, Perelman LT, Itzkan I, Dasari RR, Feld M (1997) Single molecule detection using surface-enhanced Raman scattering (SERS). *Phys Rev Lett* 78:1667–1670
6. Munro CH, Smith WE, White PC (1995) Qualitative and semi-quantitative trace analysis of acidic monoazo dyes by surface enhanced resonance Raman scattering. *Analyst* 120:993–1003
7. Lee PC, Meisel D (1982) Adsorption and surface-enhanced Raman of silver and gold sols. *J Phys Chem* 86:3391–3395
8. Li H, Ying L, Green JJ, Balasubramanian S, Klenerman D (2003) Ultrasensitive coincidence fluorescence detection of single DNA molecules. *Anal Chem* 75:1664–1670
9. Rodger C, Smith WE, Dent D, Edmondson J (1996) Surface-enhanced resonance-Raman scattering: an informative probe of surfaces. *J Chem Soc Dalton* 5:791–799
10. Isola NR, Stokes DL, Vo-Dinh T (1998) Surface enhanced Raman gene probe for HIV detection. *Anal Chem* 70:1352–1356
11. Graham D, Brown R, Smith WE (2001) SERRS detection of PNA and DNA labelled with a specifically designed benzotriazole azo dye. *Chem Commun* 11:1002–1003
12. Brown R, Smith WE, Graham D (2003) Synthesis of a benzotriazole azo dye phosphoramidite for labeling of oligonucleotides. *Tetrahedron Lett* 44(7):1339–1342
13. Albrecht MG, Creighton JA (1977) Anomalously intense Raman spectra of pyridine at a silver electrode. *J Am Chem Soc* 99:5215–5217

14. Stacy AM, Van Duyne RP (1983) Surface enhanced Raman and resonance Raman spectroscopy in a non-aqueous electrochemical environment: tris (2,2'-bipyridine)ruthenium(II) adsorbed on silver from acetonitrile. *Chem Phys Lett* 102:365–370
15. Campion A, Kambhampati P (1998) Surface-enhanced Raman scattering. *Chem Soc Rev* 4:241–250
16. Schultz G, Janik-Czachor M, Van Duyne RP (1981) Surface enhanced Raman spectroscopy: a re-examination of the role of surface roughness and electrochemical anodization. *Surf Sci* 104:419–434
17. Jiang X, Campion A (1987) Chemical effects in surface-enhanced Raman scattering: pyridine chemisorbed on silver adatoms on Rh (100). *Chem Phys Lett* 140(1):95–100
18. Creighton JA, Blatchford CG, Albrecht MG (1979) Plasma resonance enhancement of Raman scattering by pyridine adsorbed on silver or gold sol particles of size comparable to the excitation wavelength. *J Chem Soc Faraday Trans II* 75:790–798
19. Blatchford CG, Campbell JR, Creighton JA (1982) Plasma resonance – enhanced Raman scattering by absorbates on gold colloids: the effects of aggregation. *Surf Sci* 120(2):435–455
20. Kneipp K, Dasari RR, Wang Y (1994) Near-infrared surface-enhanced Raman scattering (NIR SERS) on colloidal silver and gold. *Appl Spectrosc* 48(8):951–955
21. Grabar KC, Freeman RG, Hommer MB, Natan MJ (1995) Preparation and characterization of au colloid monolayers. *Anal Chem* 67(4):735–743
22. Grabar KC, Brown KR, Keating CD, Stranick SJ, Tang S-L, Natan MJ (1997) Nanoscale characterization of gold colloid monolayers: a comparison of four techniques. *Anal Chem* 69(3):471–477
23. Curtis CC, Duff DG, Edwards PP, Jefferson DA, Johnson BFG, Kirkland AI, Wallace AS (1988) Preparation and structural characterization of an unprotected copper sol. *J Phys Chem* 92:2270–2275
24. Huang HH, Yan FQ, Kek YM, Chew CH, Xu GQ, Ji W, Oh PS, Tang SH (1997) Synthesis characterization, and nonlinear optical properties of copper nanoparticles. *Langmuir* 13:172–175
25. Volkan M, Stokes DL, Vo-Dinh T (2000) Surface-enhanced Raman of dopamine and neurotransmitters using sol–gel substrates and polymer-coated fiber-optic probes. *Appl Spectrosc* 54(12):1842–1848
26. Laserna JJ, Campiglia AD, Winefordner JD (1988) Surface-enhanced Raman spectrometry on a silver-coated filter paper substrate. *Anal Chim Acta* 208:21–30
27. Ruperez A, Laserna JJ (1994) Surface-enhanced Raman spectrometry on a silver substrate prepared by the nitric acid etching method. *Anal Chim Acta* 291:147–153
28. Bello JM, Stokes DL, Vo-Dinh T (1989) Titanium dioxide based substrate for optical monitors in surface-enhanced Raman scattering analysis. *Anal Chem* 61:1779–1783
29. Walsh RJ, Chumanov G (2001) Silver coated porous alumina as a new substrate for surface-enhanced Raman scattering. *Appl Spectrosc* 55(12):1695–1700
30. Bell SEJ, Spencer SJ (2001) Disposable, stable media for reproducible surface-enhanced Raman spectroscopy. *Analyst* 126:1–3
31. Bharathi S, Fishelson N, Lev O (1999) Direct synthesis and characterization of gold and other noble metal nanodispersions in sol–gel-derived organically modified silicates. *Langmuir* 15:1929–1937
32. Saegmueller B, Brehm G, Schneider S (2000) In situ generated photolytic silver in a gelatin matrix: an approach for high-throughput SERS spectroscopy applying microtiter plates. *Appl Spectrosc* 54(12):1849–1856
33. Perez R, Ruperez A, Laserna JJ (1998) Evaluation of silver substrates for surface-enhanced Raman detection of drugs banned in sport practices. *Anal Chim Acta* 376:255–263
34. Norrod KL, Sudnik LM, Rousell D, Rowlen KL (1997) Quantitative comparison of five SERS substrates: sensitivity and limit of detection. *Appl Spectrosc* 51(7):994–1001
35. McKenzie F, Faulds K, Graham D (2010) Mixed metal nanoparticle assembly and the effect on surface-enhanced Raman scattering. *Nanoscale* 2:78–80

36. Stokes RJ, Macaskill A, Lundahl PJ, Smith WE, Faulds K, Graham D (2007) Quantitative enhanced Raman scattering of labelled DNA from gold and silver nanoparticles. *Small* 3(9):1593–1604
37. Munro CH, Smith WE, Garner M, Clarkson J, White PC (1995) Characterization of the surface of a citrate-reduced colloid optimized for use as a substrate for surface-enhanced resonance Raman scattering. *Langmuir* 11:3712–3720
38. Chow MK, Zukoski CF (1994) Gold sol formation mechanisms: role of colloidal stability. *J Colloid Interface Sci* 165:97–109
39. Biggs S, Mulvaney P, Zukoski CF, Greiser F (1994) Study of anion adsorption at the gold-aqueous solution interface by atomic force microscopy. *J Am Chem Soc* 116:9150–9157
40. MacAskill A, Chernonosov A, Koval V, Lukyanets E, Fedorova O, Smith WE, Faulds K, Graham D (2007) Quantitative surface-enhanced resonance Raman scattering of phthalocyanine labelled oligonucleotides. *Nucleic Acids Res* 35:e42
41. Faulds K, Fruk L, Robson DC, Thompson DG, Enright A, Smith WE, Graham D (2006) A new approach for DNA detection by SERRS. *Faraday Discuss* 132:261–268
42. Faulds K, Smith WE, Graham D (2004) Evaluation of surface enhanced resonance Raman scattering (SERRS) for highly sensitive and quantitative DNA analysis. *Anal Chem* 76:412–417
43. Basu HS, Marton LJ (1987) The interaction of spermine and pentamines with DNA. *Biochem J* 244:243–246
44. Graham D, Smith WE, Linacre AMT, Munro CH, Watson ND, White PC (1997) Selective detection of deoxyribonucleic acid at ultra low concentrations by SERRS. *Anal Chem* 69(22):4703–4707
45. Faulds K, MacKenzie F, Graham D (2007) Evaluation of the number of modified bases required for quantitative SERRS from labelled DNA. *Analyst* 132:1100–1102
46. Faulds K, Barbagallo RP, Keer JT, Smith WE, Graham D (2004) SERRS as a more sensitive technique for the detection of labelled oligonucleotides compared to fluorescence. *Analyst* 129:567–568
47. Graham D, Mallinder BJ, Smith WE (2000) Surface-enhanced resonance Raman scattering as a novel method of DNA discrimination. *Angew Chem Int Ed* 39(6):1061–1063
48. Frances T, Docherty FT, Clark M, McNay G, Graham D, Smith WE (2004) Multiple labelled nanoparticles for bio detection. *Faraday Discuss* 126:281–288
49. Faulds K, Mackenzie F, Smith WE, Graham D (2007) Quantitative simultaneous multianalyte detection of DNA by dual- wavelength surface-enhanced resonance Raman scattering. *Angew Chem Int Ed* 46(11):1829–1831
50. Brereton R (2003) *Chemometrics: data analysis for the laboratory and chemical plant*. Wiley, Chichester
51. Manly BFJ (1994) *Multivariate statistical methods: a primer*. Chapman & Hall/CRC Press, New York
52. Faulds K, Jarvis R, Smith WE, Graham D, Goodacre R (2008) Multiplexed detection of six labelled oligonucleotides using surface enhanced resonance Raman scattering (SERRS). *Analyst* 2008(133):1505–1512
53. Graham D, Mallinder BJ, Whitcombe D, Smith WE (2001) Surface enhanced resonance Raman scattering (SERRS) – a first example of its use in multiplex genotyping. *ChemPhysChem* 2(12):746–748
54. Graham D, Mallinder DJ, Whitcombe D, Watson ND, Smith WE (2002) Single multiplex genotyping by surface-enhanced resonance Raman scattering. *Anal Chem* 74:1069–1074
55. MacAskill A, Crawford D, Graham D, Faulds K (2009) DNA sequence detection using surface enhanced resonance Raman spectroscopy in a homogeneous multiplexed assay. *Anal Chem* 81(19):8134–8140
56. Allain LR, Vo-Dinh T (2002) Surface-enhanced Raman scattering detection of the breast cancer susceptibility gene BRCA1 using a silver-coated microarray platform. *Anal Chim Acta* 469:149–154

57. Mustafa C, Stokes D, Allain LR, Vo-Dinh T (2003) Surface-enhanced Raman scattering substrate based on a self-assembled monolayer for use in gene diagnostics. *Anal Chem* 75:6196–6201
58. Wang H-N, Vo-Dinh T (2009) Multiplex detection of breast cancer biomarkers using plasmonic molecular sentinel nanoprobe. *Nanotechnology* 20:065101 (6 pp)
59. Lowe AJ, Huh YS, Strckland AD, Erickson D, Batt CA (2010) Multiplex single nucleotide polymorphism genotyping utilizing ligase detection reaction coupled surface enhanced Raman spectroscopy. *Anal Chem* 82(13):5810–5814
60. Cao YC, Jin R, Mirkin CA (2002) Nanoparticles with Raman spectroscopic fingerprints for DNA and RNA detection. *Science* 297(5586):1536–1540
61. Sun L, Yu C, Irudayaraj J (2007) Surface-enhanced Raman scattering based nonfluorescent probe for multiplex DNA detection. *Anal Chem* 79:3981–3988
62. Sun L, Yu C, Irudayaraj J (2008) Raman multiplexers for alternative gene splicing. *Anal Chem* 80:3342–3349
63. Sun L, Irudayaraj J (2009) PCR-free quantification of multiple splice variants in a cancer gene by surface-enhanced Raman spectroscopy. *J Phys Chem B* 113:14021–14025
64. Graham D, Thompson DG, Smith WE, Faulds K (2008) Control of enhanced Raman scattering using a DNA-based assembly process of dye-coded nanoparticles. *Nat Nanotechnol* 3(9):548–551
65. Qian X, Peng X-H, Ansari DO, Yin-Goen Q, Chen GZ, Shin DM, Yang L, Young AN, Wang MD, Nie S (2008) In vivo tumor targeting and spectroscopic detection with surface-enhanced Raman nanoparticle tags. *Nature Biotechnol* 26:83–90
66. Keren S, Zavaleta C, Cheng Z, de la Zerda A, Gheysens O, Ghambhir SS (2008) Noninvasive molecular imaging of small living subjects using Raman spectroscopy. *Proc Natl Acad Sci USA* 105:5844–5849
67. Zavaleta CL, Smith BR, Walton I, Doering W, Davis G, Shojaei B, Natan MJ, Ghambhir SS (2009) Multiplexed imaging of surface enhanced Raman scattering nanotags in living mice using noninvasive Raman spectroscopy. *Proc Natl Acad Sci USA* 106(32):13511–13516
68. Stone N, Faulds K, Graham D, Matousek P (2010) Deep-SERRS: demonstration of deep Raman spectroscopy for non-invasive detection of conjugated SERRS nanoparticles buried within 25 mm of mammalian tissue. *Anal Chem* 82:3969–3973
69. Matschulat A, Drescher D, Kneipp J (2010) Surface-enhanced Raman scattering hybrid nanoprobe multiplexing and imaging in biological systems. *ACS Nano* 4(6):3259–3269