Tailor-made Surface Enhanced Raman Scattering (SERS) substrates: An alternative biological probe

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Abstract

In much of today's biological research, fluorescent probes have emerged as the standard for many biological assays; however, Raman spectroscopy presents an interesting non-invasive, label-free option. Though typically a weak interaction, Surface-enhanced Raman scattering (SERS) has emerged as surface sensitive optical phenomenon which results in greatly enhanced Raman signals of molecules that are absorbed on rough and nanostructured metallic surfaces. With reported enhancement factors as high as 10^{14} and 10^{15}, this technique has the ability to allow one to detect single molecules in addition to trace amounts of absorbed material. In this paper I will discuss the origins of the observed Raman signal enhancement, the nature of so-called hot spots and materials and techniques used in the fabrication of SERS substrates, in addition to ellucidating some of the current applications of this specialized Raman technique.

Contents

1 Introduction

In much of today's biological research, fluorescent probes have emerged as the standard for many biological assays. Though these probes are often versatile, customizable, and potentially quantitative, problems such as auto-fluorescence from the sample, photo-bleaching of the fluorophore, fluorophore size, and incomplete substrate labelling can often pose as major obstacles when studying biological systems, especially *in vivo*.[1–3] To make matters worse, these probes are often not of use in the detection of trace amounts of material.

Alternatively, Raman spectroscopy is a very powerful analytic technique which uses bond polarizability to provide information about molecular structure (Fig. 1). Raman spectroscopy is highly sensitive and can be used to detect biomolecules within cells. Furthermore, because of the weak Raman scattering of water, biological samples can be analyzed without the need for exogenous labels and little to no sample preparation; however, general Raman spectroscopy is limited by the size of the Raman cross-section.[4] Analytes present at trace amounts exhibit very small Raman cross-sections, thus, in order to reliably analyze these materials, more specialized techniques must be employed.

Surface-enhanced Raman scattering (SERS) is a surface sensitive optical phenomenon which results in greatly enhanced Raman signals of molecules that are absorbed on rough and nanostructured metallic surfaces.[5] SERS measurements are non-destructive, often provide narrow spectral bands, require little to no sample preparation and may be conducted in air, vacuum, or solution.[6] With reported enhancement factors as high as 10^{14} and 10^{15}, this technique has the ability to allow one to detect single molecules in addition to trace amounts of absorbed material.[7,8] It is these advantages which make SERS substrates an interesting choice for biological research.

In the following sections, we will discuss the origins of the observed Raman signal enhancement, the nature of so-called hot spots and materials and techniques used in the fabrication of SERS substrates. Following this discussion we will investigate current research in which various SERS substrates have been employed as alternatives to established labelling techniques.

2 Origins of Raman Enhancement

First witnessed by C.V. Raman and K.S. Krishnan in 1928, Raman scattering is an inelastic photon scattering process capable of probing molecular structure without exogenous labeling.[9] Though a powerful tool, Raman spectroscopy is often limited by its inefficiency; only approximately one out of every 10^{10} photons is inelastically scattered.[10] Thus, for Raman spectroscopy to be of use, it is necessary to somehow increase the frequency of these inelastic scattering events.

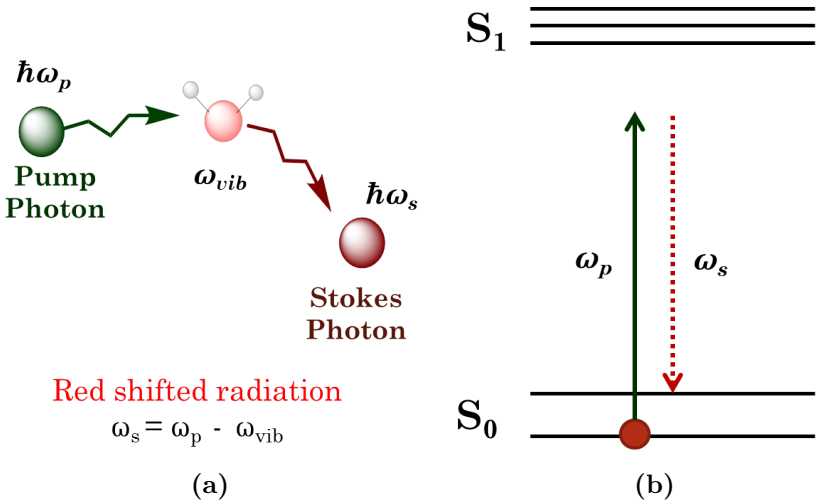

Figure 1: *Schematic of spontaneous Raman scattering* Raman scattering is a non-resonant, inelastic scattering process used to probe phonon modes. A pump photon is excited to a virtual state through interaction with a phonon. The resulting re-emitted Stokes photon is then red-shifted from the original pump photon.

In 1974, while studying the Raman spectra of pyridine absorbed on an electrochemically roughened silver electrode, Fleischmann *et. al* observed Raman scattering with intensities several orders of magnitude greater than previously anticipated.[11] Though this increased signal was initially attributed to the large surface area of the roughened electrode, later studies by Jeanmaire and Van Duyne and Albrecht and Creighton showed that the increase in surface area alone was not enough to account for this observed enhancement.[12,13] This phenomenon was later penned surface-enhanced Raman scattering (SERS). Though the exact mechanism is still a subject of debate within the community, two general factors are thought to be behind the increased effective SERS cross-section- a long-range electromagenetic (EM) effect and a short-range chemical (CHEM) effect.[10]

2.1 Chemical Enhancement

A minor contributor to the enhancement observed from SERS, the theory of chemical enhancement was introduced as a means of describing discrepancies between theoretical predictions of initial electromagnetic models ($\tilde{}10^4$) and empirically observed results ($\tilde{}10^6$).[10] Though specific mechanisms are still unknown, it is thought that active sites, such as crystal defects or adatoms, on the metal surface undergo a charge-transfer intermediate complex with absorbed molecules resulting in increased polarizability of the absorbate.[7,12] As this charge-transfer could only be possible when the analyte molecule and nanostructured metal surface are in close proximity, this is believed to be a short-range effect. Enhancement factors derived from these CHEM

effects are estimated to be between 10^1 - 10^2 times.[6]

2.2 Electromagnetic Enhancement

The dominant contributor to the enhancement factor of SERS measurements, the electromagnetic (EM) mechanism is based on amplified EM fields generated by localized surface plasmon resonance (LSPR) of nanoscale roughness features of the metal surface.[10,14] In general, flat surfaces of good conductors possess EM resonances, known as surface plasmons, whose frequency and parallel momentum obey the dispersion relationship below where $\varepsilon(\omega)$ and ε_0 refer to the dielectric functions of the conductor and ambient environment respectively.

$$k_{//}{}^2 = (\omega/c)^2 Re[\epsilon_0\epsilon(\epsilon_0 + \epsilon)^{-1}]$$

For this plasmon to be excited by a plane wave incident to the ambient, both the frequency and parallel momentum must be conserved; however, this is not generally possible in air or vacuum. As a result, these surface plasmons are typically confined to the metal surface, dissipating any energy as heat. This, however, is not the case for roughened surfaces.[5]

Though there is not one model which accurately describes all SERS features, there are a collection of theories which successfully describe many of these details. In general, these theories are based around the assumption that in SERS systems, plasmon-like EM resonances are coupled to EM plane waves. It is this coupling which is responsible for the excitation of plasmons, allowing at least partial radiation and thus localization of energy. The degree of observed enhancement is highly dependent on the topology of the surface as nanoscale features, such as sharp points and interparticle junctions, can create more intense EM fields. These theories predict that strong enhancements are generally observed when (a) the particle size is smaller than the wavelength, (b) the frequency of exciation or scattering is near the surface plasmon resonance condition and (b) the molecule is in close proximity to the surface.[5,10]

3 Substrate Fabrication

In general, SERS measurements are typically conducted on noble metals such as Au and Ag, which satisfy both the real and imaginary dielectric constant requirements for EM enhancement. Initial SERS experiments were carried out on electrochemically roughened metal electrodes; however, the lack of standardized roughness on the nanoscale meant that enhancement factors varied greatly between experiments.[11–13] Thus without some sort of internal standard, SERS on these substrates cannot be reliably used for quantitative chemical analyses. Advances in various nanofabrication techniques have resulted in the development of many new fabrication methods for these SERS substrates. Though some of these methods are more successful

than others, the goal is to create easy to make, inexpensive, standardized SERS substrates wtih nanoscale precision capable of routine SERS-based chemical analysis with minimal variation between experiments.

3.1 Colloidal Metal Nanoparticles

Colloidal metal nanoparticles are some of the most widely used SERS substrates. These long studied compounds are relatively easy to prepare, quite flexible in their use and can be tailor-made for specific applications.[15] Features such as shape and size can be easily manipulated and because of their liquid-like nature, colloidal substrates can be dispersed thoughout objects, even inside macromolecules and cells.[8,16–20]

The main downside of these substrates is the lack of uniformity. Factors such as distribution of shapes and aggregation influence the surface-enhancing properties of colloidal metal nanoparticle substrates. Though these colloids exhibit high SERS activities at their junctions, the randomness of the formation of these junctions can result in varying SERS enhancements.[6] In an attempt to regulate junction spacing and minimize such variations, some colloids have been self-assembled on planar platforms.[21,22]

3.2 Lithography

Lithography techniques, literally meaning "to write on stone", come in many different varieties and can be used to create reproducible, highly ordered SERS substrates consisting of surface-bound nanostructures. Two of the more commonly used lithography methods, electron beam (EBL) and photolithography, have different limits and resolutions; however, both techniques are based on the same five general fabrication steps - 1) resist addition, 2) exposure, 3) development, 4) metal deposition and 5) resist lift-off (Fig. 2). Photolithography, uses a UV light source to expose a resist layer through a pre-fabricated mask. This high throughput copying method is cheaper than many of the other lithography techniques and is thus well-suited for bulk substrate production; however, diffraction limited resolution and a small depth of focus limit the detail of the nanostructured pattern.[23] Alternatively, in EBL a focused, high energy electron beam is used to expose the resist.[24] This direct writing technique has a higher resolution and shorter turn around time than other lithography techniques; however, it is also more expensive and because of the serial nature of fabrication, not as well suited for large-scale production.

3.2.1 Nanosphere Lithography

Nanosphere lithography (NSL) presents an alternative method for the inexpensive, bulk production of surface-bound nanoparticle arrays. Rather than the typical resist layer of other methods, NSL utilizes a monodisperse colloidal suspension of nanospeheres of uniform diameter to form a crystalline deposition shadow mask. As the colloid solvent evaporates, nanospheres are

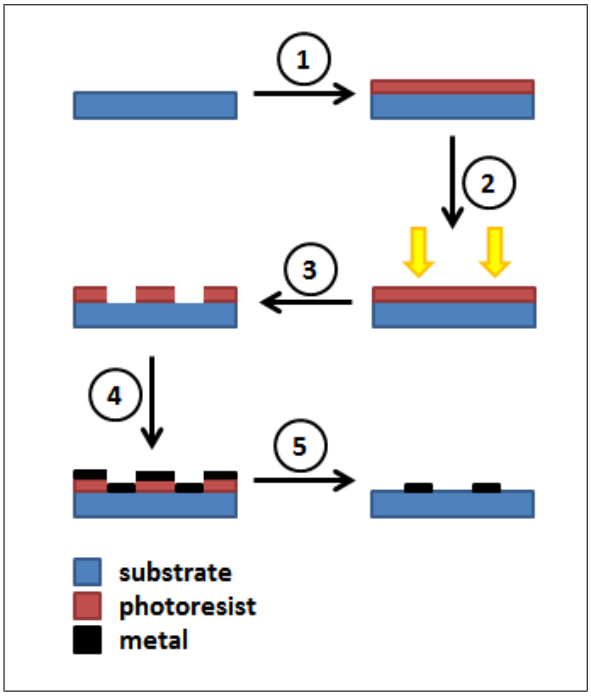

Figure 2: *Lithography fabrication steps* 1) Resist Addition - photoresist is deposited (spin-coated) onto substrate platform, 2) Exposure - an external stimulus (electron beam, x-ray, UV light, etc.) is used chemically change the resist layer in desired areas, 3) Development - exposed, chemically changed photoresist layers are removed, 4) Metal Deposition - various deposition methods may be used to deposit a thin metallic layer onto the sustrate and remaining resist layers, 5) Resist Lift-Off - remaining resist is removed leaving only the metal pattern directly deposited onto the substrate platform

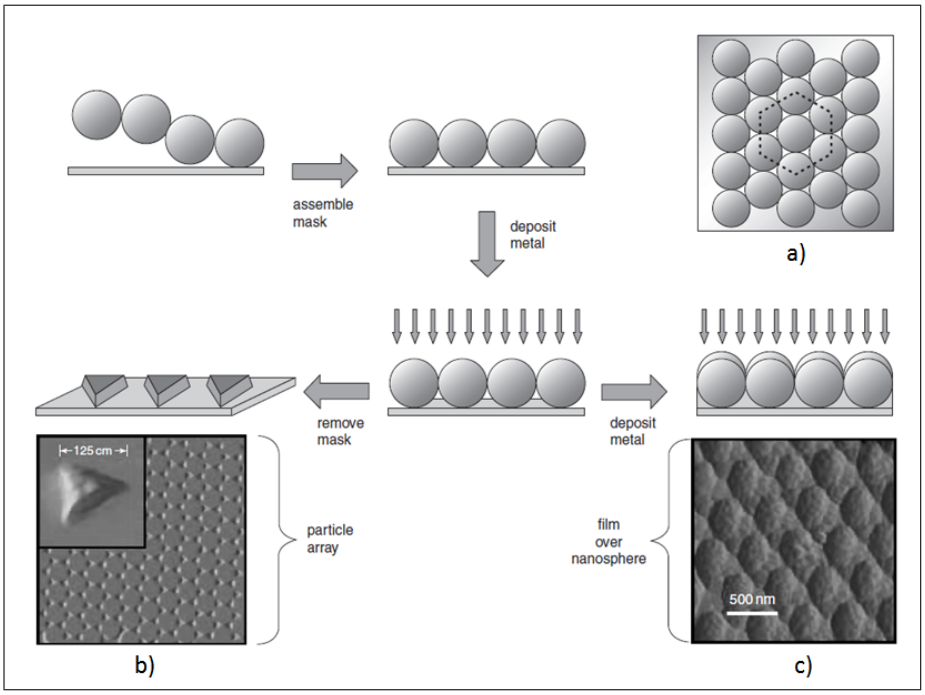

Figure 3: *Fabrication of NSL triangular particle array and metal film over nanosphere (MFON) substrates* Carboxylare modified polymer microspheres of uniform diameter are self assembled into a hexagonally closed-packed (a) crystal on a supporting substrate platform. Metal, typically Au or Ag, is then deposited through this nanosphere deposition mask. If nanospeheres are removed, an array of triangular shaped partcles remains (b). If >100 nm of metal is deposited, a continuous film covers the spheres, creating a MFON surface (c). Reproduced from ref. [10]. Copyright IEE 2005.

drawn together by capillary forces, self-assembling into a hexagonally close-packed (hcp) crystal structure when dry (Fig. 3a). After the deposition of a thin layer of metal (<100 nm) and removal of the nanosphere mask, what remains are surface-confined, uniformly spaced triangular structures of uniform thickness and size (Fig. 3b).

The crystalline nature of the nanosphere deposition mask is both the largest benefit and greatest drawback of this method. Because the nanospheres form a crystal, the resulting nanoparticle array is highly ordered, with easilly controlled shape, size, and interparticle spacing. Futhermore, the self-assembling nature of the crystal formation, eliminates an element of human error which could add to deviation between structures; however, all crystals are prone to some amount of defects. As is the case for all naturally occuring crystals, the nanosphere masks are prone to variety of defects including, but not limited to, dislocations, vacancies, and interstitial additions. Despite this drawback, with defect-free domain sizes typically

within the 10-100 μm range, this method is still quite reliable.[10]

3.3 Metal Film on Nanostructures

Another class of substrates consisting of surface-bound nanostructures are metal film on nanostructures (MFON) substrates. These robust SERS substrates are fabricated similarly to NSL substrates, the main difference being that instead of sonicating away the nanosphere crystalline layer and using the remaining triangular nanostructure array as the SERS substrate, the metal-coated nanospheres themselves are used to generate the SERS signal in these substrates. At thicknesses >100 nm, a continuous metallic film is deposited on top of the nanospheres (Fig. 3c). Optical properties are determined by the thickness of the metallic layer and the diameter of the spheres, both easily controlled parameters. Furthermore, despite the nonhomogeniety of the nanoscale roughness profile, the relative uniformity of the larger-scale templating has proven to be enough to generate a relatively narrow surface plasmon peak (FWHM ˜200 nm) and reproducible results.[10] It is these qualities which make MFON surfaces a sensible choice for the large-scale production of reproducible planar SERS surfaces used for routine SERS analysis.[25,26]

4 Applications

4.1 Single Molecule SERS

The ability to reliably and selectively detect single molecules in solution is of great interest in a variety of fields and is essential in the quest to fully understand many complicated biological systems. Though techniques such as two-photon excitation[27] and STED[28,29] microscopy currently allow for the non-invasive visualization of single molecules in solution, there are drawbacks to both. As each are fluorescence techniques, probe photobleaching is always a concern when imaging over an extended timeframe. Additionally, the need for confocal rastering can make dynammic imaging of fast moving molecules difficult. Lastly, unlike Raman, fluorescence measurements do not provide detailed molecular information. Single molecule SERS (SM-SERS) provides a label-free, Raman based alternative to these fluorescence methods.

When using near-infrared excitation for dyes absorbed onto colloidal silver, Kniepp and co-workers observed extraordinaryly large Raman cross-sections, as high as 10^{-16} cm^2/ molecule - an improvement of roughly 14 orders of magnitude.[30] With enhancement factors so high, it was hypothesized that this substrate could be used to perform single molecule measurements.[7,31] As a proof of concept, Kniepp and co-workers conducted near infrared SERS (NIR-SERS) experiments on single crystal violet molecules.[7] Using an aqueous colloidal silver solution, prepared using a standard citrate reduction, that had been activated with a 10^{-2} M concentration of NaCl to form small 100 - 150 nm sized silver clusters as the SERS substrates, they

were able to successfully detect single crystals.[32]

Because SERS is a Raman, not fluorescence, technique, they were able to take advantage of shorter vibration relaxation times, shorter integration times and avoid any photodecomposition of probed molecules. With a collection time of 1 s and intensity of roughly 2×10^5 W/cm^2 from a 830 nm Ar^+ laser, a clear Raman fingerprint was obtained of features between 700 and 1700 cm^{-1}. Furthermore, the average residence time of a particle in the probed volume was estimated to be between 10 and 20 seconds. As this as at least ten times longer than the collection time, it is probable that a single-molecule Raman spectrum is indeed generated by the same single molecule.

When determining the concentration of dye to use, it was important to find a balance between having a low enough concentration to reduce dye molecule aggregation but high enough to ensure that a single molecule was actually contained within the scattering volume. To determine the ideal concentration, statistical analyses were performed on plots of 100 SERS measurements for the Raman line at 1174 cm^{-1} at various dye concentrations (Fig. 4). At an average concentration of 0.6 dye molecules/ volume, SERS measurements best fit a poisson distribution. Three distinct peaks describe the probability of finding 0, 1, 2, or 3 moledules in the scattering volume during the actual measurement (Fig. 4c). Fig. 4b shows that increasing the dye concentration by a factor of 10 changes the statistics of the SERS signal entirely. This small change was enough to make the characteristic Poisson distribution vanish, the SERS signal becoming more Gaussian. Despite this small concentration window, it is important to note that the well-separated, "quantized" signals for 1, 2, or 3 molecules suggests that the enhancement mechanism(s) are mostly unaffected by the small variance in silver particle cluster shape and size (˜10-40 nm).[7]

After the success of this proof of concept, one of the next logical steps was to try to apply this technique to biological samples. In 1999, Xu *et al.* did just that by applying this SMSERS system to single hemoglobin (Hb) molecules.[8] Though the same NaCl activated silver colloid was used, Raman spectra were obtained from a polymer coated Si wafer immobilized droplet of the Hb/Ag solution. Additionally, to minimize heating and prevent possible photochemical effects, a lower incident laser power, in the μW range, was used. As a result, a longer collection time of 30 s was required. Despite these changes, vibrational Raman spectra were obtained for the single hemoglobin molecules, proving that it is indeed possible to conduct SMSERS on biological samples. It is, however, worthwhile to note that this new methodology would not be suitable for *in vivo* samples as the immobilization process would disrupt the cellular environment and the extended collection time would make imaging of dynamically changing systems unreliable.

One of the main drawbacks of SMSERS is the potential for temporal fluc-

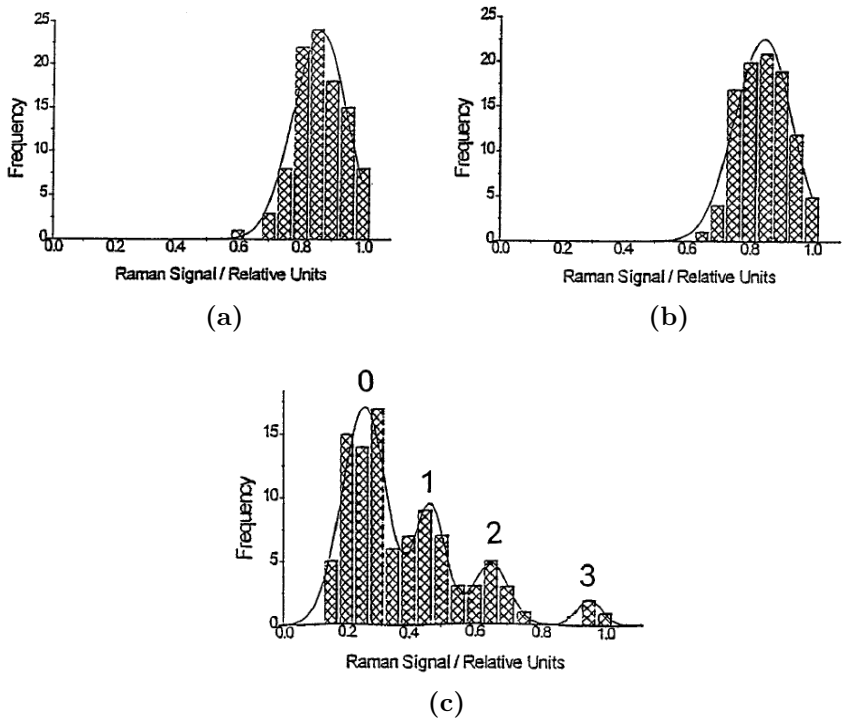

Figure 4: *Statistical analyses of SERS measurements* (*a*) 100 spontaneous Raman measurements at 1030 cm^{-1} of 10^{14} methanol molecules fit to Gaussian distribution. (*b*) 100 SERS measurements at 1174 cm^{-1} of 6 crystal violet molecules in scattering volume fit to Gaussian distribution. (*c*) 100 SERS measurements at 1174 cm^{-1} for an average of 0.6 crystal violet molecules in the probed volume. Solid line corresponds to Poisson distribution fit of data. Peaks correspond to probability of finding 0, 1, 2, or 3 molecules, respectively, in the scattering volume. Reproduced from ref. [7]. Copyright APS 1997.

tuations in collected spectra. Though they were not reported in the study conducted by Kniepp *et al.*, these fluctuations have been reported in several studies and are similar to effects well known from single-molecule fluorescence studies.[8,31,33] Two time series of SMSERS measurements of Hb can be seen in Fig. 5 (C1-C6 and D1-D6). Whereas the spectrum of a dense layer of Hg/Ag aggregates is stable over time (Fig. 5B), this is not the case for the time series. In these time series, intense peaks appear and dissappear at will (C2-C4) and sometimes broaden (D1-D3) with no discernable pattern, making it difficult to know what is real and what is artifact without a previous reference.[8] Though the source of these temporal fluctuations is not yet known for sure, their presence signals a departure from time-invarient ensemble averages seen in normal Raman spectroscopy, suggesting that single molecule detection has indeed been reached. This being said, it is possible that these fluctuations could be attributed to the extended collection time, conformational changes, photochemical effects or a combination of any of these factors. Further investigation would be required to pinpoint the exact source.

4.2 Label-free Detection of Mismatches in DNA

Since the success of the Human Genome Project and subsequent advances in sequencing techniques genetic mapping has become an integral part of biological research.[34] The ability to identify of specific DNA sequences and easily recognize single nucleotide polymorphisms (SNPs), amongst other things, has changed the way we do medical research and is essential for understanding the cause of many inherited diseases. Though there are established fluoresence and label-free (MALDI-TOF) techniques for the identification of short DNA sequences, SERS provides a unique, highly sensitive, low cost alternative to more conventional methods.[35–37]

S.E.J. Bell and co-workers demonstrated that it was possible to produce reproducible SERS spectra of unthiolated DNA.[38] Bell *et al.* were not the first to propose the use of SERS for this purpose, previous approaches involved the detection of SERS labels rather than DNA itself or the use of thiolated DNA sequences. Though the thiolation helped to promote surface binding, the specific binding of DNA via the sulfur group gave the DNA a more tilted orientation which in general impacted recognition of various bases differently and specifically resulted in more dominant Raman signals from adenine.[39]

As a first step, 30-mer single-stranded DNA sequences were adsorbed onto colloidal silver nanoparticles and aggregated with $MgSO_4$ rather than NaCl as Cl^- ions would displace DNA from the surface (Fig. 6). As seen in Fig. 7c, this yielded a strong SERS signal and unlike the thiolated sample (7a) showed no preference for adenine or any other nucleobase.[38] After first taking SERS spectra of each of the nucleobases individually, they were then able to use spectra differences to both detect and identify SNPs. Fig. 8 displays the SERS spectra of two similar DNA sequences differing only by an A $\Rightarrow$ G

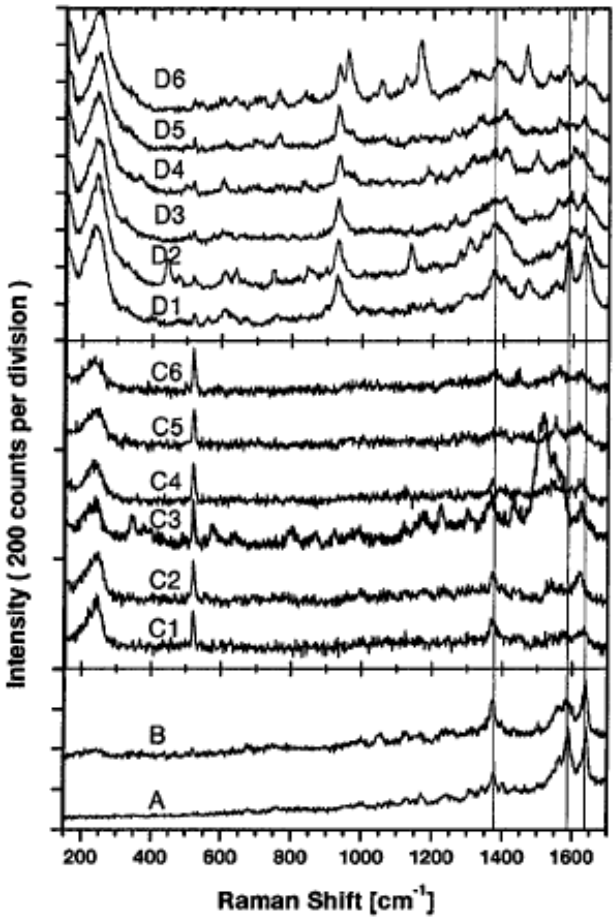

Figure 5: *SMSERS temporal fluctuations* "Normal" confocal Raman spectra of crystalline met-Hb (*A*) and a dense layer of Hb/Ag aggregates (*B*), and two time series of active sites obtained at single molelecule detection concentrations (*C1-C6 and D1-D6*). All spectra were measured with the same collection time (30 s) and efficiency though incident laser power was 1 mW in *A* and 1 μW in *B-D*. Reproduced from ref. [8]. Copyright APS 1999.

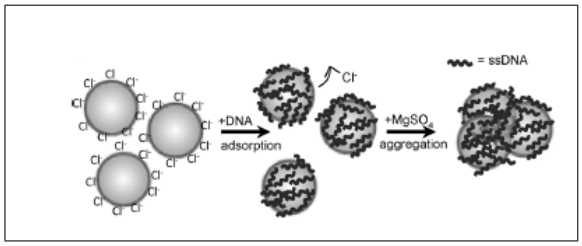

Figure 6: *Interaction between DNA and metallic nanoparticles in unthiolated SERS method.* DNA is first adsorbed onto the surface through its nucleobases, displacing Cl^- molecules in the process. DNA coated particles are then aggregated using a salt that does not displace DNA from the surface (e.g. $MgSO_4$). Reproduced from ref. [20]. Copyright Wiley-VCH 2012.

polymorphism. Though it is difficult to see any differences between spectra by eye, digitally subtracting one from the other give the difference spectrum containing only positive and negative feature change (Fig. 8c). In this way, comparing the peaks present in this difference spectrum made assignment of polymorphism bases simple and straightforward. Later studies showed that this method could also be applied to double-stranded DNA samples.[20]

The ease with which SNPs can be identifed in combination with other factors such as ease of assay set-up and low cost, make SERS a viable option for mismatch identification; however, it would not be advised to use SERS as opposed to common fluorescence techniques for detailed sequencing of DNA strands. The power of this technique comes from the the ability to compare the difference spectrum to known spectra of individual bases. When sequencing a whole chain, this reference is no longer viable. Though SERS could be useful for determining composition of the chain, order would be much more difficult to ascertain from the Raman spectrum alone.

5 Conclusions

Surface-enhanced spectroscopy presents a versatile, inexpensive, non-invasive label-free option to conventional fluorescence techniques. With reported enhancement factors as high as 10^{14} and 10^{15}, this technique has the ability to allow one to detect single molecules in addition to trace amounts of absorbed material. With a variety of fabrication methods and substrate materials and advances in nanotechnology, SERS substrates have the ability to be tailored to the specific needs of a given experiment. Given time and more research into the specific mechanisms behind this phenomenon, the sky is the limit in regards to the application of this technique.

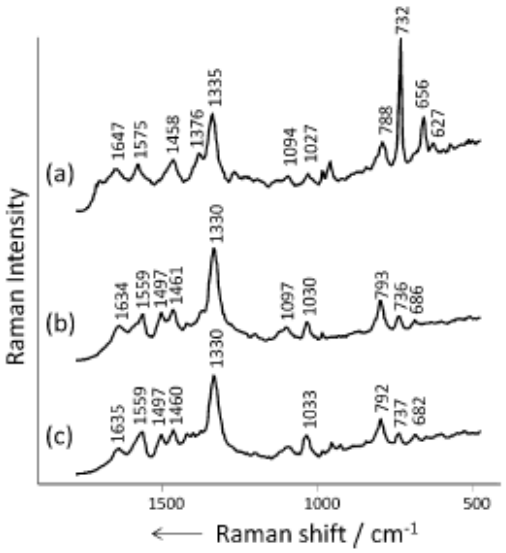

Figure 7: SERS spectra of 10^{-5} M solutions of (a) thermally treated thiolated DNA sample, (b) untreated, thiolated DNA sample, and (c) unthiolated DNA sample recorded on citrate-reduced Ag colloids aggregated with 0.1 M $MgSO_4$. Reproduced from ref. [38]. Copyright Wiley-VCH 2011.

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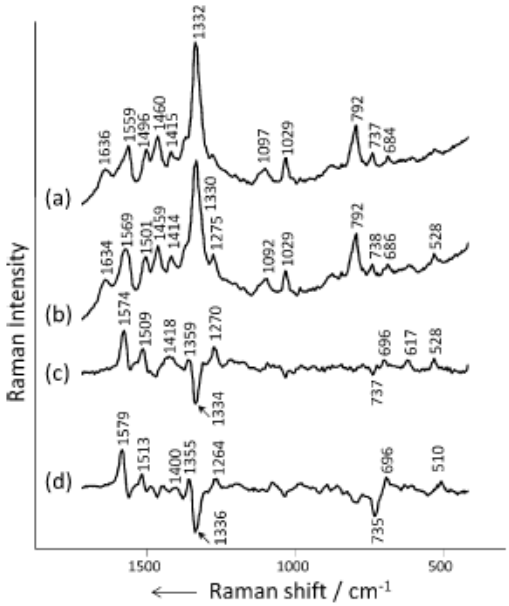

Figure 8: SERS spectra of two DNA sequences with an A (a) to G (b) polymorphism. (c) Difference spectrum of (b-a) shown with thrice extended intensity. (d) Model difference spectrum of dGMP minus poly A. dGMP is used rather than poly G as poly G forms secondary structures which interfere with SERS measurements. All SERS spectra utilized NH_2OH-reduced Ag colloids aggregated with 0.1 M $MgSO_4$. Reproduced from ref. [38]. Copyright Wiley-VCH 2011.

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