ADVANCES IN SENSORS:
REVIEWS

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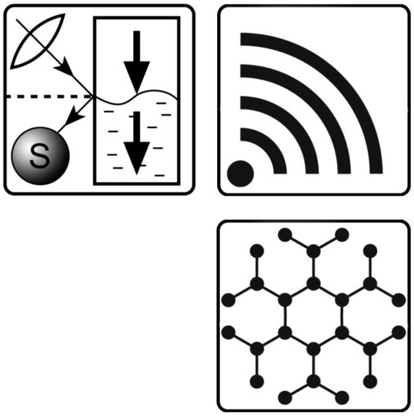
Sergey Y. Yurish
Editor

Sensors and Applications in Measuring and Automation Control Systems



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Sensors and Applications in Measuring and Automation Control Systems

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Chapter 9

Surface-Enhanced Raman Scattering: A Novel Tool for Biomedical Applications

Sudhir Cherukulappurath

9.1. Introduction

Surface-enhanced Raman scattering (SERS) is a powerful non-invasive spectroscopic technique that has recently gained lot of interest in molecular detection and identification owing to its high sensitivity and portability. Several applications that require biomolecular detection such as in medical biotechnology and pharmaceutical studies are now turning towards SERS as a reliable means to identify molecules. In Raman scattering, incident lights interact is made to interact with the analyte molecules resulting in a radiative scattering of the photons with not only the incident frequency (called elastic Rayleigh scattering) but also slightly shifted frequencies (termed as inelastic Raman scattering). The frequencies of Raman scattered photons can be smaller (Stokes shift) or greater (anti-Stokes shift) than that of the incident excitation light. As the probability of Raman scattering is very low (of the order of $10^{-6)}$, it is essential to have either a large concentration of molecules or a highly intense laser light for excitation in order to detect the Raman signal and proper chemical identification. However, using SERS, there is a huge enhancement of the scattered light that can be easily detected.

The first observation of enhanced Raman signals was reported in 1974 by Fleischmann et al. while studying the pyridine molecules adsorbed onto silver electrodes [1]. An explanation to the phenomenon was later given by Jeanmaire and Van Duyne in 1977 [2]. This phenomenon, later termed as SERS, is now a widely-used method in applications involving biomolecular detection of low concentration. SERS has now developed into a mature field and with the advent of state-of-the-art nanofabrication techniques, single-molecule Raman spectroscopy has been possible [3-4]. The application of SERS has moved from physics to material science, chemistry, environmental studies and more recently to biomedical applications. There are several reviews on SERS in general which form useful guide to the advancements in the field [5-10]. This chapter, an extension of

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the review paper [11] attempts to highlight the developments in SERS research with special reference to biomedical applications.

The first part of the chapter deals with the theory of SERS and discusses the possible mechanisms for the observed enhancement of Raman signals in SERS. Next section deals with a brief review of different substrates reported for SERS applications. SERS based biomolecular detection is currently a hot topic owing to its potential applications and will be discussed in the successive section. The sections after will deal with biomedical applications of SERS, glucose sensors based on SERS including DNA/RNA detection, immunoglobin protein detection and other relevant topics. This chapter will conclude with the basic challenges and future prospects.

9.2. Mechanism of SERS

After the first observation of enhancements in Raman scattered light by Fleischmann et al., experiments by Van Duyne group revealed that surface enhancements were due to the chemical adsorption of the molecules onto rough silver surfaces. The drastic increase in the Raman intensity of adsorbed molecules was attributed to increased surface electromagnetic fields on the metal surface. At the same time, Albrecht and Creighton had already published a report on the observation of intense Raman signals from pyridine on silver electrodes [12]. It was argued that a broadening mechanism of the excited states of the adsorbed molecules due to the presence of metal surface was responsible for the enhancements and the role of surface plasmons was speculated. Since then there has been several explanations of which some of them are based on experimental observations.

Large enhancement of Raman signals from molecules in the vicinity of metal surfaces forms the basis of SERS. While the explanation given to the first observation of Raman enhancement was based on electrochemical changes to the molecule on adhesion to the metal surface [1], now it is largely believed that surface plasmons on the metal surface has a big role [2, 13-15]. The two primary mechanisms responsible for observing SERS are (1) enhancement of local electromagnetic field due to surface plasmons and (2) chemical enhancement attributed to charge transfer mechanism.

Surface plasmons are periodic electromagnetic oscillations of the conduction electrons on a metal surface [16]. Photons can interact with surface plasmons leading to interesting effects. The field of study of surface plasmons, called plasmonics, is now very established branch of nanophotonics [17]. Noble metals such as Au, Ag are some of the most popular plasmonic metals that are used in plasmonic applications. The conduction electrons in the surface of plasmonic metals can oscillate with the same frequency as the incoming photons for a certain band of frequencies. When the frequency of the incident light is beyond a threshold frequency, these conduction electrons can no longer match the drive frequency and will tend to slow down. This usually happens for high frequency ultraviolet light and for the same reason, most plasmonic effects are pronounced in the visible part of the electromagnetic spectrum. Metal nanoparticles (usually with sizes lower than the incident wavelength) such as nanospheres have a definite geometry and thus confines the conduction electrons inside this boundary. The resonance frequency of these 'plasma' electrons largely depend on the material properties such as dielectric function of the metal as well its surrounding region as well as its geometry. This resonance is often called localized surface plasmon resonance (LSPR) and plays a crucial role in surface-enhanced processes such as SERS. The oscillating dipole nature of the plasmons create a secondary field around the metal particle often termed as 'local field' (Fig. 9.1). This in turn leads to enhanced scattering, absorption and extinction of the incident light by the plasmonic metal particle.

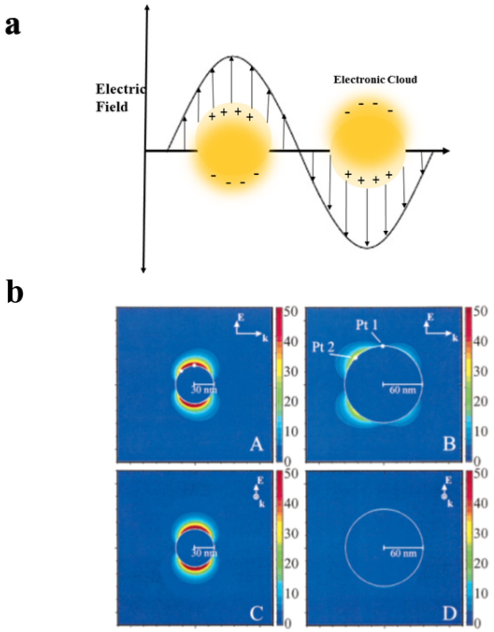


Fig. 9.1. Localized surface plasmons. (a) Schematic of localized surface plasmon oscillations on excitation with light. The electron cloud oscillates with the external electric field thereby creating a polarization of charges; (b) Simulated image of the electric field around nanoparticles of different sizes (30 nm and 60 nm radius). Reprinted with permission from [23]. Copyright (2016) American Chemical Society.

As the momentum of surface plasmons is higher than that of free photons, in order to excite and couple surface plasmons on a thin metal film with light, special schemes need to be adopted. However, this condition is not required for LSPR excitation and hence is widely used for SERS. Since most SERS platforms are based on metal nanoparticles rather than thin films, only LSPR will be discussed here.

It is now widely accepted that apart from the physical electromagnetic enhancement of the local field, a chemical enhancement process also contributes to SERS [18-20]. Several reports on experimental observation of resonant Raman scattering on molecules adsorbed onto a metal surface suggest that there is indeed a broadening of the electronic states of the adsorbate and new intermediate levels are formed due to this interaction of the analyte with the metal surface [21-22]. This 'charge transfer mechanism' is responsible to an enhancement in the scattering. Although small (of the order of 10^3), chemical enhancement do contribute to the total enhancement in SERS. In the chemical enhancement theory, it is hypothesized that the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) tends to broaden out thereby bringing them closer to the Fermi energy level (Fig. 9.2). This facilitates charge transfer from either the molecule to the metal or vice versa when excited with light. As a result, the polarizability of the molecule is enhanced up to 1000-fold when the excitation photons are in resonance with the charge transfer energy bands.

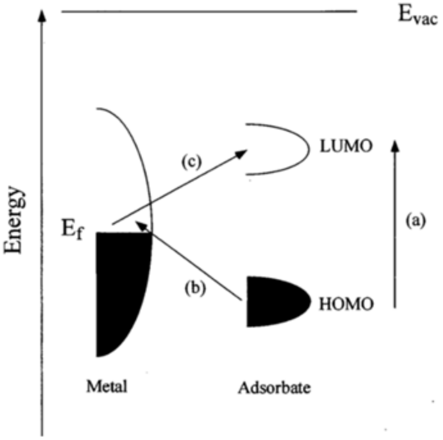


Fig. 9.2. Orbital energy diagram of a molecule adsorbed to metal surface. HOMO and LUMO levels are broadened due to the interaction thereby allowing charge transfer excitations. Reproduced from [19] with permission of The Royal Society of Chemistry.

On the other hand, electromagnetic enhancements due to surface plasmon excitations can be of several orders of magnitude larger than the chemical enhancement mechanism discussed above. At plasmonic resonance, the local electromagnetic field around the metal surface is locally enhanced thereby increasing the Raman signals [23-24]. This enhancement mechanism can be easily visualized in a classical physics point of view. The free-electron theory of metals is a good approximation in plasmonic models and can be used to explain the theory of surface plasmons without resorting to quantum mechanical treatments. The free-conduction electrons in the metal surface gets perturbed by the external electric field (usually light). This brings changes in the probability densities of the electronic wavefunction resulting in a change in the dipole moment, **P**. This induced dipole moment is directly proportional to the external electric field **E** through the constant called polarizibility α:

$$\boldsymbol{P} = \alpha \boldsymbol{E} \tag{9.1}$$

It should be noted these quantities are tensors in 3D space. The local electric field interacts with the polarizability of the molecule in the vicinity of the field. This interaction leads to the inelastic scattering of incident photons collected as Raman spectrum.

Since the electric field intensity around a plasmonic nanoparticle is enhanced and the intensity of the scattered photons is related to the square of the incident intensity the overall SERS intensity is related to the induced field through [10, 25]:

$$I_{SERS} \approx |\boldsymbol{E}(\omega_{inc})|^4, \tag{9.2}$$

where I_{SERS} is the SERS intensity and ω_{inc} represents the frequency of incident excitation.

Hence if there is a 100-fold increase in the local electric field the Raman intensity will be increased by a factor of 10^8. This factor is termed 'enhancement factor' (EF) and is often quoted in SERS experiments. EF is usually determined by taking the ratio of Raman intensities with and without the plasmonic field normalized to the number of molecules on the surface.

In experiments this can obtained by comparing the SERS intensity with the Raman intensity of bulk molecules after normalizing for the number of molecules.

$$EF = \frac{I_{SERS}/N_{surf}}{I_{bulk}/N_{vol}}, \tag{9.3}$$

where I_{bulk} represents the intensity of Raman spectrum in bulk sample while N_{surf} and N_{vol} are the respective number density of molecules.

The intensity of SERS signals decay with distance from the metallic surface. This is expected as surface plasmon fields responsible for the enhancement of Raman signals are evanescent. The molecule does not have to be in direct contact with the metal surface but need to be in the vicinity of the plasmonic field which is usually few nm from the metal surface. As the plasmonic field decays exponentially from the surface, it is necessary that the analyte molecules are close enough, usually few nanometers, to the metal surface in order to obtain SERS signal. Experimental evidences for this statement has been reported in which spacer layers of varying thickness was used between the molecules and the metal surface [26].

The experimental set up for SERS measurements can be very similar to conventional Raman systems. The excitation light, usually a low power laser, is focused onto the nanoparticle substrate which also contains the analyte molecules. The analyte molecules are either attached to the metal surface via chemical adsorption, covalent bonds or through a partition layer system. For resonant excitation, the wavelength of laser excitation should overlap with the plasmonic resonance of the nanoparticles. This optimizes the scattering process and increases the signal-to-noise ratio. The scattered signal is detected using a spectrometer system as in regular Raman spectroscopy.

It is interesting to note that SERS spectrum of a molecule can be different from the Raman signal. For example, in SERS, the intensity of higher frequency vibrations tends to lower. Overtones and overlapped bands are not observed in SERS. The resonances can be broadened and even slightly shifted when the molecule is adhered to the metal surface. Certain selection rules are either relaxed or altered and there seems to be a depolarization of the scattered light unlike bulk Raman spectra. However, the prominent peaks of Raman scattering are obtained as in conventional Raman spectra.

9.3. Plasmonic Nanostructures for SERS

The substrates used for SERS plays a vital role in determining the quality and quantity of Raman signals measured from the analyte molecules. The first reported observation of intense Raman scattering was on pyridine molecules adsorbed on to a simple silver electrode prepared by electrodeposition process. The inherent random roughness on the silver surface contributed to the enhanced Raman signals. Since then several different geometries of metallic nanoparticles have been studied for SERS, Ag and Au being the most popular metals used in these substrates. Single metal nanoparticles, by themselves can be used to as Raman enhancers but the most effective way is to couple nanoparticles to create stronger and larger number of electromagnetic ‘hotspots’. The strong coupling of local electromagnetic field of the metal nanoparticles gives rise to intense enhancements of the local fields forming the ‘hotspots’. Molecules that are in the vicinity of such coupled fields tend to show large enhancements of Raman signals.

Recent advances in nanofabrication technology has contributed to the ability of creating special geometries that can give rise to large EFs. For example, the popular metal film-over-nanospheres (FONs) that are fabricated through nanosphere lithography (NSL) process have been reported to present strong hot spots capable of observing SERS from single molecules [27-30. Van Duyne and coworkers have reported several measurements based on Ag-FONs and Au-FONs SERS substrates [31-32]. Although easy to fabricate, FON substrates presents spatial inhomogenieties, which puts other structures that show similar trend includes silica coated metallic star nanoparticles [33-34], dimers [35], nanoparticle clusters [36], shell-isolated nanoparticles (SHINERS) [37-38], Ag cage structures [39], mushrooms [40], ALD coated nanoparticles [41] and nanowire structures [42]. Some of the most popular SERS nanostructures are shown in Fig. 9.3.

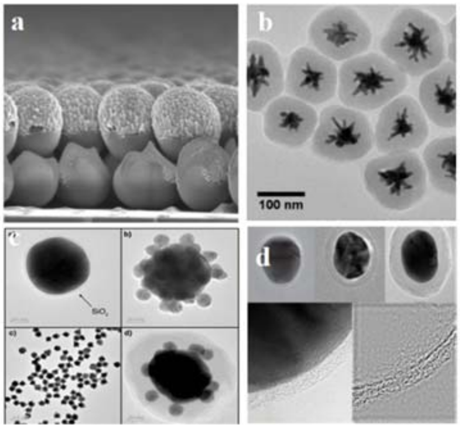


Fig. 9.3. Different nanostructures used for SERS (a) Metal film over nanostructures (FON). SEM image of AgFONs. Reprinted with permission from [29]. Copyright (2016). American Chemical Society (b) Silica coated nanostars Reprinted with permission from ([33]). Copyright (2016). American Chemical Society (c) 3D self-assembled plasmonic superstructures. From [36] with permission from John Wiley and Sons. (d) Silica shell isolated nanoparticles (SHINERS). Reprinted by permission from Macmillan Publishers Ltd: Nature [38], Copyright (2016).

It has been known that rough surfaces and random cluster of nanoparticles can give SERS signals, but a more efficient way to yield high EFs will be to properly engineer the size and distribution of the nanoparticles such that their plasmonic resonances match with the excitation light wavelength. In this regard, dimer plasmonic nanostructures are of particular interest as SERS substrates owing to the fact that a strong electromagnetic hotspot is formed at the nanogap between the two nanostructures. For example, the metal over FON structure mentioned earlier presents such highly localized hot spots thus becoming a popular substrate for SERS applications.

Tip-enhanced Raman scattering (TERS) is now becoming a hot research branch of SERS owing to its high-resolution capability and several papers on TERS for biomedical application are available [43-46]. Here, a metal coated tip is used as probe and it is illuminated with laser light to induce surface plasmon fields at the tip. The tip can then be raster scanned over the molecules under study and the scattered signals after interaction with the molecules are collected for detection. Raman-images of single molecules with super-resolution can be achieved using TERS microscopy. However, there are certain drawbacks of this techniques which includes cumbersome measurement set-up, poor reproducibility and low signal-to-noise ratio that need to be addressed.

Although Ag and Au are the most widely used for SERS, other metals such as Al, Cu, alkali metals (Li, Na, K, and Cs), Pt, Ga, In and some alloys have been tried and tested. Owing to its plasmonic resonances in the UV, Aluminum is found to be effective for UV-based SERS measurements. However, high reactivity (including large susceptibility to oxidation) and cost has restricted their use as SERS substrates.

9.4. SERS Based Biomolecular Detection

SERS has now become a well-developed and mature technique for the detection of biomolecules offering good sensitivity as well as selectivity. SERS based sensors have been reported for diagnosis and treatment of cancer, Alzheimer's and Parkinson's diseases. Large molecule sensing by SERS has shown reasonable interest owing to greater sensitivity and cost-effectiveness. In the following section, a review of some popular SERS based detection schemes are presented.

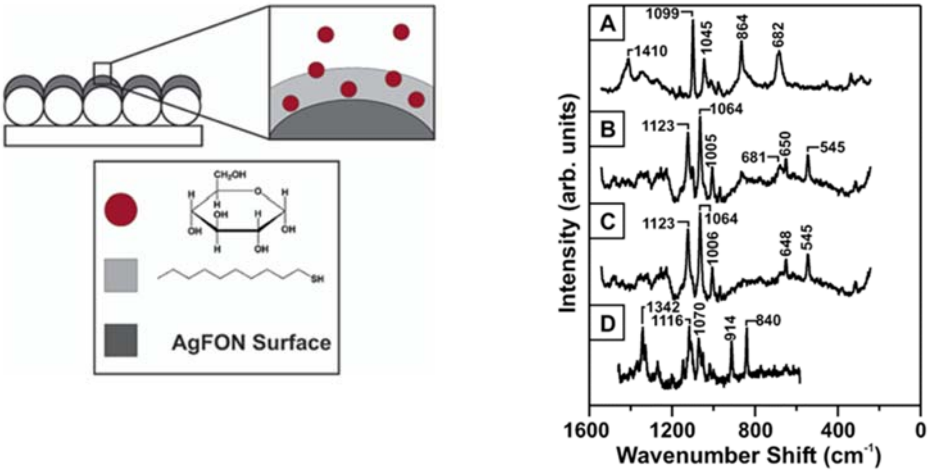
9.4.1. SERS Based Biosensing

Detection of biomolecules and bio organisms have been of great interest owing to their importance in biomedical field. In particular label-free detection of microorganisms and pathogens using SERS provide significant advantages over methods such as fluorescent marking. For example, by proper detection and study of certain pathogens, it is possible to develop pharmaceutical drugs that can be effectively used to eliminate them. In this regard, SERS has been developed as a tool for effective detection even for small concentrations of the molecules. Van Duyne el at. reported a rapid detection technique for anthrax biomarkers using SERS [47]. Calcium dipicolinate (CaDPA), considered to be a biomarker for bacillus spores, was detected using SERS based on Ag-FON samples. Spore concentrations of the order of 10^{-12} M was reported to be detected. Following this there has been an exponential growth in the number of publications on bio-detection based on SERS. Recently, several reports on detection of whole microorganisms based on SERS substrates have been published. For example, Boardman et al. have demonstrated the rapid detection of bacteria directly from blood using a novel technique based on SERS [48]. They used Au nanoparticles embedded in SiO_2 as substrate and were able to detect 17 different bacterial species separated from blood samples. In another recent report, E-coli detection was achieved by in-situ coating of the bacterial cell wall with Ag nanoparticles in water followed by SERS measurements [49]. This method offers several advantages such as high sensitivity, reduced measurement times, high portability and lower reaction volumes over conventional label-free bio-detection methods. An interesting work includes the development of lab-on-a-chip device based SERS data base for the differentiation of six mycobacteria including both tuberculosis and non-tuberculosis strains [50]. Such developments clearly aim at utilizing the potential of SERS in medical applications in the future.

9.4.2. Glucose Sensing

In recent years, there has been a concerning level of increase in the number of diabetic patients globally. The failure of insulin response mechanism in such patients cause fluctuations in glucose levels leading to further health related issues. Blood glucose levels are often monitored in diabetic patients usually by finger pricking method. Researchers have been working towards developing non-invasive glucose detection methods aiming at minimizing trauma to the patients. Raman spectroscopic method can detect glucose levels in vitro but requires strong and long laser exposures. This process can be made more efficient using SERS based detection. Ag FON (Silver film-over- nanostructures) have been successfully employed as SERS substrates for the purpose by Van Duyne, et. al [29-30]. Since glucose is not readily adsorbed on to silver film, a special partition layer is created in order to bring the glucose molecules in close vicinity of the metal surface as represented in Fig. 9.4. To achieve this, a self-assembled monolayer (SAM) of 1-decanethiol and mercapto-hexanol (DT/MH) is formed over the metal surface. This assisted in creating a glucose concentration gradient which was then detected using SERS. Several different partition layers were studied by the group but only these straight alkanethiols were found to be effective. Using this technique physiologically relevant glucose levels were detected, thus proving the utility in medical applications.

A further improvement was achieved using spatially offset Raman spectroscopy (SORS) was obtained by the same group. In this method, the scattered light is collected from different regions that are offset from the laser excitation point thereby providing an improved depth in resolution. Combining this with SERS bring a powerful tool called surface enhanced SORS (SESORS) for bio-detection. Van Duyne group successfully used this technique by injecting sensors and monitoring glucose levels through living rat´s skin [51-52]. It has been reported that this method will be a significant tool in biomedical applications in the future.



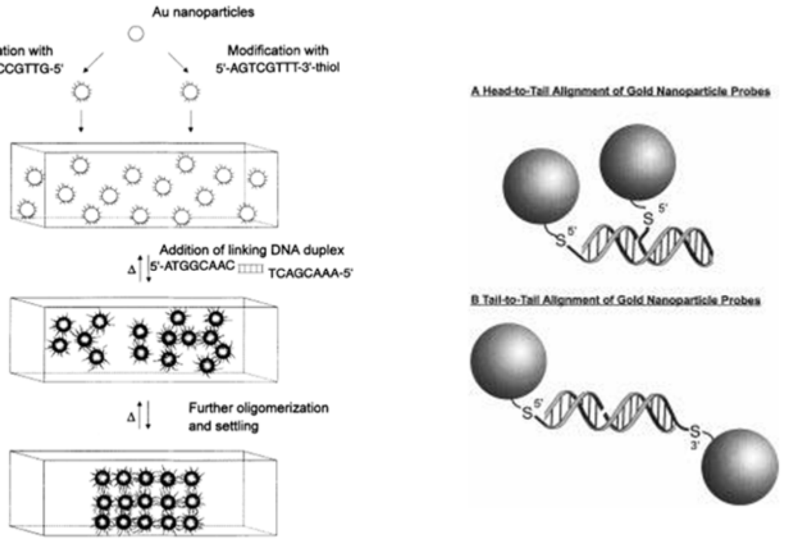
(a) (b)

Fig. 9.4. Glucose sensing. (a) Scheme for glucose sensing using AgFON substrates. Glucose is partitioned into an alkanethiol monolayer adsorbed on the silver film substrate. (b) SERS spectra from glucose molecules. A and B represents spectra obtained without and with glucose in the partition layer respectively. C is the residual glucose spectrum obtained by subtracting A from B. D represents pure Raman spectrum of glucose for comparison. Reprinted with permission from [31]. Copyright (2016) American Chemical Society.

9.4.3. SERS Markers for DNA/RNA Detection

Recent developments in nanotechnology have facilitated sensitive and selective detection of nucleic acids. This has in turn revolutionized modern biomedical analysis as well as diagnostic tools. SERS based detection of nucleic acids offers several advantages over conventional methods such as fluorescent spectroscopy. It also forms a complementary analysis tool to other sophisticated techniques like NMR and mass spectroscopy. SERS presents better sensitivity with lower limits of detection and greater spatial resolution. Moreover, undesirable effects such as photobleaching and quenching can be reduced to a great extent. In particular, by using excitation wavelengths that spectrally match the electronic absorption bands of the biomolecules, it is possible to improve the efficiency of scattering process. This technique, often termed surface enhanced resonance Raman scattering (SERRS), has now become a potential tool of DNA detection [14].

Earlier methods of DNA detection involved immobilizing the molecules to silver or gold nanoparticles along with a Raman reporter (Fig. 9.5). In order to achieve this, surface functionalization methods were developed in order to attach the DNA strands onto the metal surface, followed by the assembly of the nanoparticles [53-54]. 13 nm Au nanoparticles were linked to oligonucleotides that were functionalized with a thiol group at their tail end. Two non-complimentary oligonucleotide solutions so prepared are then mixed together. Due to their non-complementary nature, there is no reaction. Additional linking of DNA duplex to this components and oligomerization results in the assembly of DNA-linked nanoparticles.



(a) (b)

Fig. 9.5. (a) Schematic of the DNA-based nanoparticle assembly [53]. Different schemes for attaching DNA to gold nanoparticles. Reprinted with permission from [54]. Copyright (2016) American Chemical Society.

A multiplexed detection of DNA and RNA using gold nanoparticle probes that were labeled with oligonucleotides and Raman markers was reported [55]. Three component sandwich assay system was utilized in their method (Fig. 9.6). The nanoparticles were attached with cyanine3 (Cy3) thiol-capped oligonucleotides for monitoring different DNA strands. Here, Cy3 was chosen as the Raman tag as it easily hybridizes with the specific DNA strand under investigation. To further enhance the Raman signals silver hydroquinine was passed through forming silver nanoparticles along the Cy3 strands. Several different strands of DNA and RNA were detected using this method with a detection limit of 20M.

To further improve the detection mechanism, some groups have developed techniques where in a fluorescent marker molecule was also attached apart from Raman active reporter. This combined assay system provided better information regarding the DNA. For example, Fang et al. used Rhodamine-B as both Raman tag as well as fluorescent marker [56]. Single strands of DNA were detected by Fabris et al. by first hybridizing DNA with peptide nucleic acid (PNA) which was then immobilized on Ag nanoparticles that were attached with Rhodamine-6G [57]. The detection limit of this method was reported to be of the order of pM concentration (Fig. 9.7).

Multiplexed DNA detection could be achieved by using several different dyes that were excited with a single wavelength [58]. A different approach of using two different wavelengths that matched the electronic absorption of a particular oligonucleotide was also reported by the same group [59].

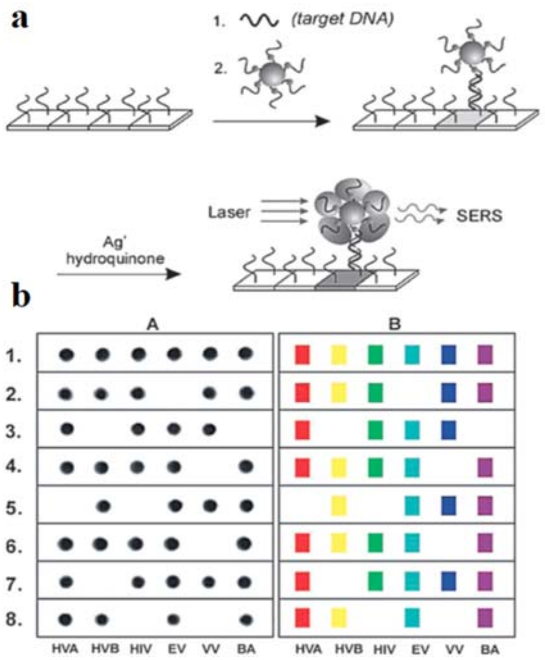


Fig. 9.6. SERRS based DNA detection using gold nanoparticles that are functionalized with dye-labelled oligonucleotide followed by a silver staining.
From [55]. Reprinted with permission from AAAS.

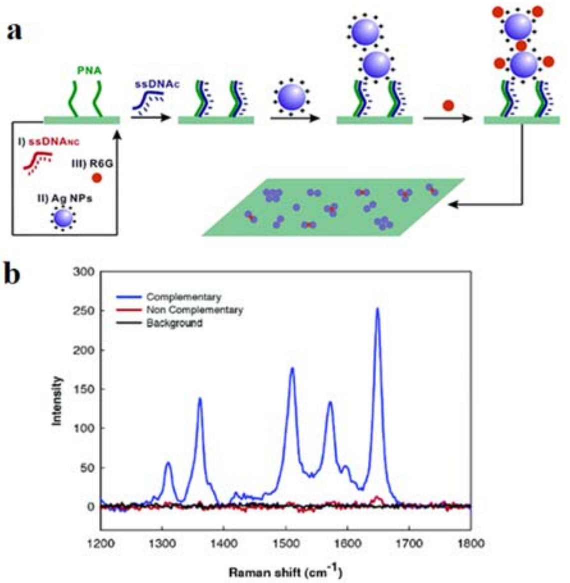


Fig. 9.7. (a) Scheme adopted by Fabris *et al.* for the detection of hybridized DNA. Peptide nucleic acid was used for hybridization and immobilized on AG colloids with Rhodamine-6G; (b) Averaged SERS signals from PNA hybridized DNA. Reprinted (adapted) with permission from [57]. Copyright (2016) American Chemical Society.

Another powerful and unique approach is DNA- based self-assembly of plasmonic nanoparticles to enhance Raman scattering. Originally developed by Mirkin, et al. [53-54], this method has been reported for sandwich assay with silver nanoparticles that were coated with oligonucleotides and Raman marker molecules [60]. Graham, et al. presented a controlled aggregation of DNA coated silver nanoparticles through a target-dependent sequence specific DNA hybridization assay. Maximum enhancement of Raman signals was obtained by cleverly placing the Raman scattering molecules in the interstices of the assembled metal nanoparticles.

Label-free approaches for SERS based DNA detection has gained considerable interest and simple mononucleotide detection have been reported. Bell, et al. demonstrated SERS detection of adenine, guanine, thymine, cytosine, and uracil using citrate-reduced silver colloids that were aggregated with $MgSO_4$ [61]. As in the previous method, the analyte mononucleotide can get in the hotspots of the aggregated nanoparticles thereby achieving maximum enhancement of Raman scattered light (Fig. 9.8). SERS from 2‘-deoxyadenosine 5‘-monophosphate (dAMP) attached to Ag colloids were obtained using this technique.

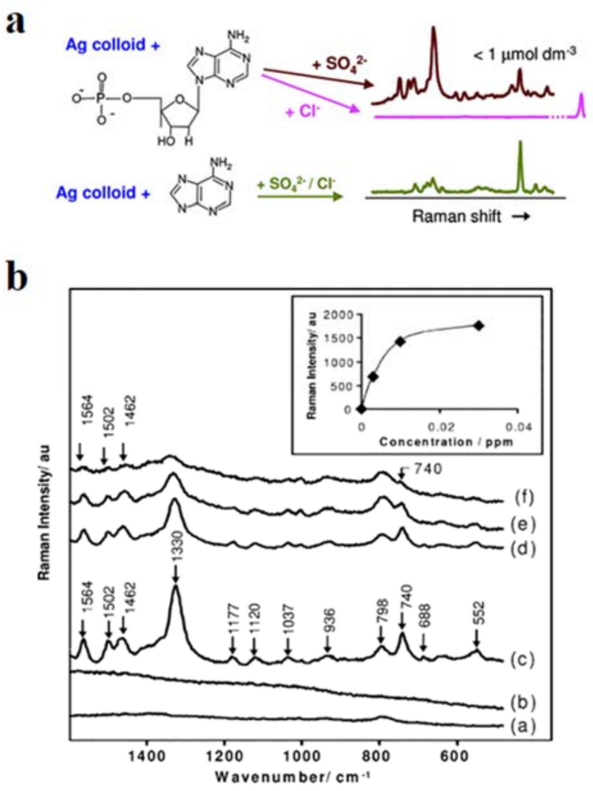


Fig. 9.8. Method for obtaining SERS spectra from DNA/RNA mononucleotide by aggregating citrate reduced Mg colloids with $MgSO_4$. SERS signal of 2‘-deoxyadenosine 5‘-monophosphate (dAMP) for different concentrations obtained using this method. Reprinted (adapted) with permission from [61]. Copyright (2016) American Chemical Society.

This method was also extended to single base nucleotide mismatch detections in short DNA strands [62]. Single base sensitivity of DNA bases using similar methods have been reported by several groups. Detection of DNA hybridization using label-free methods is useful in forensics and genetic studies. Barhoumi and Halas have demonstrated label-free detection of DNA in hybridized state using the plasmonic properties of Au nanoshells [63]. These Au nanoshells comprise of silica core with a thin film of Au. The dominant adenine peak at 736 cm^{-1} is removed and replaced with its isomer 2-aminopurine (Fig. 9.9). This aminopurine substituted DNA is then adsorbed onto the Au nanoshells using a thiol moiety on its ends. The ratio of intensity of peaks of adenine (at 736 cm^{-1}) and 2-aminopurine (at 807 cm^{-1}) gives a quantitative degree of hybridization.

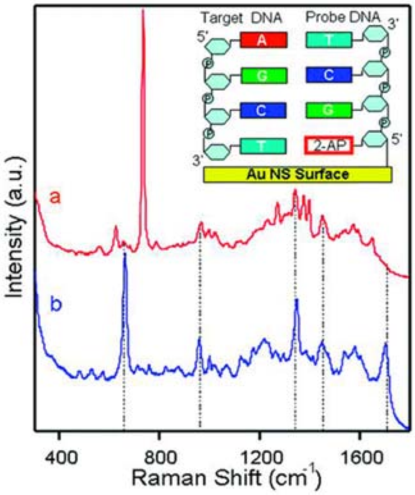


Fig. 9.9. (a) Au nanoshell based SERS spectra for a DNA sequence (a) ST20N1, containing adenine bases, and (b) ST20N2, without the adenine bases. Inset shows the schematic of DNA hybridization. Reprinted (adapted) with permission from [62]. Copyright (2016) American Chemical Society.

Improvements in label-free detection can be achieved by proper control of the plasmonic nanoparticle assembly. Dielectrophoresis has been used for assembly of nanoparticles that have DNA bases attached to them. Adenine molecules adsorbed onto Au nanoparticles were detected using a dynamic dielectrophoresis-enabled assembly of metal nanoparticles in the form of pearl chains with nanometer-sized gaps. As electrophoretic forces overcome diffusion this approach provides a rapid detection scheme with good sensitivity. Low molecular concentrations in the pM range was detected [64-65]. Magnetophoresis of magnetically active SERS nano-reporters (or plasmonically active magnetic nanoparticles) also provides a way to overcome the diffusion-limited assembly on substrates.

The number of reports on SERS based DNA/RNA detection has increased exponentially in the last few years clearly points towards the tremendous potential of the technique and the promises it holds in biomolecular detection.

9.4.4. Immunoglobin Protein Detection Based on SERS

Understanding of biomolecular process in living organisms is crucial not only in modern biology but also in medical science. In particular detection of protein plays an important role in disease diagnosis and cure. Future drug discovery is dependent on protein sensing and analysis. State-of-the-art protein detection includes immunoassay tests, fluorescence readout and microscopic methods. Raman microscopy has been used for study of proteins and their interactions [66-69]. However, conventional Raman spectroscopy suffers from low scattering cross sections, high fluorescent background and the necessity to have larger quantities of samples. Recently surface plasmon-based biosensing has gained reasonable interest owing to its improved sensitivity and portability. Indeed, owing to its advantages, SERS provide an interesting alternative to the above techniques.

Different approaches have been adopted for protein detection using SERS. The most straightforward way is the direct detection of protein molecules by collecting the SERS signal. Amino acids, the building blocks of proteins as well as smaller peptide groups have been well characterized using SERS [70-73]. In these studies, Ag colloids were used as SERS substrates and several homodipeptides that were adsorbed onto the Ag colloids were analyzed. This also gave better insights towards the orientation of adsorbed aminoacids. Stewart, et al. studied peptides and aminoacids adsorbed onto electrochemically prepared silver surface [74] while Hu, et al. used silver colloid to obtain SERS from lysosomes [75]. Water soluble proteins and dipeptides were studied by Chumanev, et al. [76]. Several other studies on small protein SERS were also reported [77-79]. Ozaki's group have studied enzymes such as lysozyme, ribonuclease B, avidin, catalase, hemoglobin, and cytochrome using SERS (Fig. 9.10). The enzymes were adsorbed onto colloidal silver after mixing acidifed sulphate, which enhanced the detection limits [80].

Large protein molecules can often show complicated SERs signal that makes identification difficult. In such cases, the complete pattern of SERS peaks is taken and analyzed instead of looking for single vibrational signatures. In addition to the direct (intrinsic) SERS measurements of proteins, it is possible to add reporter molecules to the proteins and then measure SERS (extrinsic). Some of the most common SERS reporter molecules include 5,50-dithiobis(succinimidyl-2-nitrobenzoate) (DSNB) with a peak at 1336 cm-1 shift [81], 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with a peak at 1342 cm-1 shift [82], 4-mercaptobenzoic acid (MBA) with 1585 cm-1 peak [83], 4-nitrobenzenethiol (4-NBT) at 1336 cm-1 shift , 2-methoxybenzenethiol (2-MeOBT) with intensity monitored at 1037 cm-1 shift, 3-methoxybenzenethiol (3-MeOBT) with intensity monitored at 992 cm-1 shift, and 2-napthalenethiol (NT) with intensity monitored at 1384 cm-1 shift [84].

Sandwich immunoassay is a very common way for protein detection. An immunoassay based SERS study was first reported by Tarcha et al. where they measured SERS spectra from immunoassay of thyroid stimulating hormone (TSH) [85]. Grubisha et al. used a novel reagent consisting of reagent consists of gold nanoparticles that were modified to integrate bioselective species (e.g., antibodies) with molecular labels for the generation of strong, biolyte-selective SERS signals [86]. Gold-coated glass substrates are functionalized with the target antibody and it is then exposed to the solution containing the corresponding antigens. A sandwich complex assay is formed when Raman-labelled metal colloidal solution is added. Detection of femtomolar concentration of prostate-specific antigen (PSA) using SERS was reported by authors (Fig. 9.11). This method allows in vitro early diagnosis for certain cancers in a very short time interval.

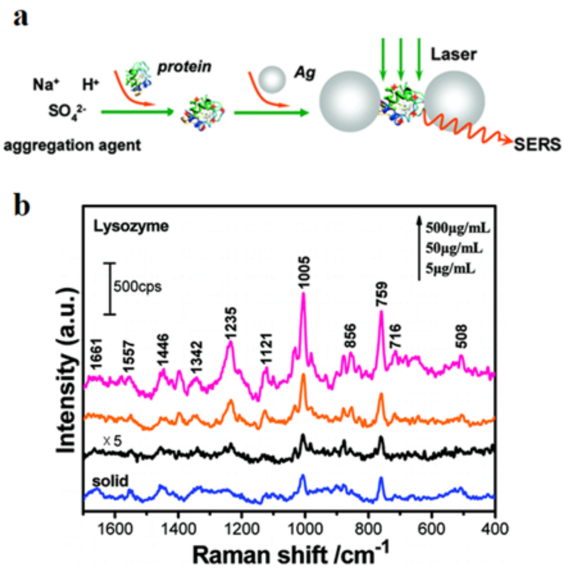


Fig. 9.10. (a) Schematic of the protocol used in the aggregation of Ag colloids for label-free protein detection. (b) SERS spectra from catalyze and control experiment spectra. Reprinted (adapted) with permission from [80]. Copyright (2016) American Chemical Society.

Raman markers were used for the detection of thrombin at subpicomolar concentrations using a protein-protein recognition system containing gold nanoparticles that were capped with a bifunctional molecule [87]. This molecule is capable of forming a covalent link with the aromatic residues of the protein moiety. Certain vibration bands of this link could be enhanced by the gold nanoparticle thereby detecting thrombin. A detection limit of 10-13 M was reported by the authors using this method. In fact, gold nanoparticles play a vital role in SERS detection systems and an extensive review of SERS nanoparticles for medical applications can be seen in reference [88]. In another interesting work, SERS based microscopy was used to image the selective localization of PSA in a prostate tissue. Gold nanostar particles were conjugated to an antibody against the tumor suppressor and white light immunization and scanning gave the Raman image of the PSA localization [89-90]. Histopathological analysis requires the localization of certain tissues using immunohistochemistry. In this work, gold nanostars that were fabricated using colloidal chemistry methods were conjugated with tumor suppressor p63, a p53 homologue. A white light source was illuminated onto the tissue for imaging. The image obtained when overlapped with the false color SERS image shows the presence of basal cells of the benign prostate. This demonstration of protein detection using SERS imaging shows the potential of this method to become a medical tool for early diagnosis of several diseases including cancer.

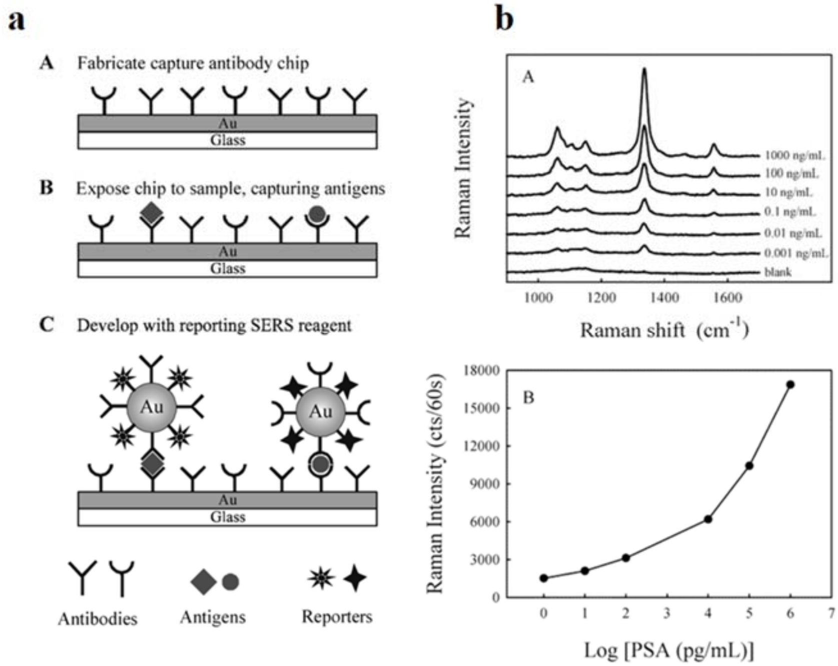


Fig. 9.11. Femtomolar detection of PSA. (a) Schematic of the steps involved in the method. (b) Evolution of SERS signal from PSA immunoassay for different concentrations and the dose-response curve for free PSA in human Serum. Reprinted with permission from [86]. Copyright (2016) American Chemical Society.

9.5. Conclusion and Future Prospects

Since the discovery of SERS, there has been tremendous increase in the number of publications in the field. Several different applications based on SERS have been demonstrated thereby bringing it to the scientific limelight in recent years. In the last decade or so, SERS has slowly developed into a useful spectroscopic tool that has potential applications in physical, chemical material as well as life sciences. In particular, the biomedical applications of SERS have motivated several researchers to develop very efficient sensing platforms based on SERS. The enhancement of Raman scattering by plasmonic fields is now well understood and researchers are now moving forward with engineering more efficient nanostructures to improve the sensitivity along with reduced fabrication costs. It is now widely believed that SERS mechanism has two contributions: the major one being electromagnetic and a minor chemical enhancement. Plasmonic field contributions plays a major role in the electromagnetic enhancement of Raman scattering process. Thus, it becomes essential to wisely engineer the plasmonic nanostructures to obtain optimum enhancements. As plasmonic fields are confined to the surface, the signal enhancement decays exponentially with the distance from the surface and hence termed evanescent. It is then imperative to have the analyte molecules very close to the nanoparticle surface to be in the vicinity of the electromagnetic field. Chemical enhancements, on the other hand, are not well understood due to the difficulty in theoretical as well as experimental observations. A theoretical description of chemical enhancement will require accurate knowledge of the vibrational and electronic states of the analyte molecule that is adsorbed onto the metal surface. A substantial amount of work, both theoretical as well as experimental, need to be carried out in order to fully understand the mechanism.

It should be mentioned that the recent developments in nanofabrication methods have contributed largely for the advancement of SERS based applications. One of the major routes of fabrication of SERS substrates is the bottom up colloidal synthesis of nanoparticles. It is now possible to obtain shape as well as size sensitive structures that can be tuned for the experimental requirements such as excitation wavelength. However, there are some drawbacks of this method based on colloidal chemistry. Firstly, inhomogeneity of the nanoparticles obtained can be an issue in quantitative studies. Different regions of the sample can present different enhancements due to structural inhomogeneity. Moreover, colloidal purity can sometimes be questionable and may require further chemical analysis. Unknown compositions in the structures can lead to spurious Raman signals that interfere with those of the studied molecules.

On the other hand, top-to-bottom approaches have some advantageous over colloidal fabrication methods. There is better control over size, shape and distribution of the nanoparticles. Good reproducibility of the shape and size of the nanostructures is possible using the top-to-bottom approach. State-of-the-art nanofabrication techniques such as electron beam lithography, nanosphere lithography, focused ion beam milling and optical lithography have been made use of for fabricating interesting nanoparticle geometries that is unachievable through colloidal chemistry synthesis. However higher cost of fabrication and longer preparation times are disadvantageous for several real-life applications.

It is clear that conventional Raman spectroscopy is now slowly being replaced by SERS in most applications. This is particularly true for biomedical applications such as disease diagnosis and bio-detection. SERS spectroscopy provides valuable information about biomolecules in living organisms and their interactions with greater sensitivity. The number of publications based on biomedical application of SERS has seen a tremendous increase in the recent years. It is now possible to use SERS for the detection of DNA/RNA and proteins. Label-free SERS in vivo as well as in vitro provide vital information on the chemical composition of these biomolecules without additional markers that can impede certain natural processes. Qualitative as well as quantitative data can be elucidated for SERS signals which can help in characterizing the molecules and the system in a complete fashion. Raman signals from single DNA as well as hybridized strands is now achievable using label-free SERRS techniques [63, 91-92]. Early stage detection of certain cancers such as prostate cancer is now possible with the help of SERS studies. Prostate cancer marker PSA can be detected in the human serum. Reports on the detection of breast cancer cell biomarkers based on SERS have been published. SERS has also been applied in other biomedical test such as the detection of calcium ions. SERS imaging forms a powerful tool for visualizing bio medically relevant processes. For example, SERS microscopy has helped in understanding protein localization in tissues that are cancerous. In a recent report, an intraoperative tumor resection based on SERS imaging in live rats was reported. These works provide promise for advanced and accurate tumor imaging and resection.

Nevertheless, there are few challenges that need to be addressed for direct application of SERS in practical biomedical applications. Fabrication of reliable and cost-effective substrates is still in the optimization stage. Although current developments in nanofabrication technology has contributed to the advancement of novel SERS substrates, it is yet not clear if ideal SERS platforms have been developed.

On the other hand, researchers are exploring the possibility of using materials beyond Ag and Au. One novel material that is now becoming a strong candidate for new generation SERS is graphene. But large-scale fabrication of graphene substrates is still a challenge. Other factors such as environmental issues of nanotechnology, cellular contamination, ethical issues are being discussed and the outcomes are not known for now. These issues need to be addressed in the near future so as to plan a viable and sustainable technology.

In conclusion, SERS is has developed into a modern technique that holds tremendous potential for biomedical applications. In the coming years, it is believed that SERS will be used in real-world applications such as disease diagnostics and biosensing and will provide valuable information of biochemical processes. SERS can provide vital information that are cannot be obtained from conventional analytical methods such as fluorescence. SERs will become portable and cost-effective tool for pharmacological applications such as drug discovery and disease diagnostics.

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