

Review article

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Fundamentals and applications of SERS-based bioanalytical sensing

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Abstract: Plasmonics is an emerging field that examines the interaction between light and metallic nanostructures at the metal-dielectric interface. Surface-enhanced Raman scattering (SERS) is a powerful analytical technique that uses plasmonics to obtain detailed chemical information of molecules or molecular assemblies adsorbed or attached to nanostructured metallic surfaces. For bioanalytical applications, these surfaces are engineered to optimize for high enhancement factors and molecular specificity. In this review we focus on the fabrication of SERS substrates and their use for bioanalytical applications. We review the fundamental mechanisms of SERS and parameters governing SERS enhancement. We also discuss developments in the field of novel SERS substrates. This includes the use of different materials, sizes, shapes, and architectures to achieve high sensitivity and specificity as well as tunability or flexibility. Different fundamental approaches are discussed, such as label-free and functional assays. In addition, we highlight recent relevant advances for bioanalytical SERS applied to small molecules, proteins, DNA, and biologically relevant nanoparticles. Subsequently, we discuss the importance of data analysis and signal detection schemes to achieve smaller instruments with low cost for SERS-based point-of-care technology developments. Finally, we review the main advantages and challenges of SERS-based biosensing and provide a brief outlook.

Keywords: Raman; surface-enhanced Raman spectroscopy; plasmonics; analytical biosensors.

1 Introduction

Surface plasmons (SPs) are the collective excitation of free conductive electrons excited by electromagnetic radiation at the metal-dielectric interface [1]. They are supported by noble metal thin films or nanoparticle (NP) surfaces. The study of the interaction between light and metallic nanostructures is a rapidly emerging research area known as plasmonics [2–5]. Targeted engineering of plasmonic nanostructures gives us the ability to control and manipulate visible light at the nanometer scale [6–8] for applications that can make a real-world impact such as integration and miniaturization of electronics, photonic interconnects, or sensitive analytical devices.

There are two types of SPs: (i) propagating and (ii) nonpropagating [1, 9]. Propagating SPs are called surface plasmon polaritons (SPPs) generated on noble (such as Au or Ag) metallic thin films 10–200 nm in thickness. Nonpropagating SPs, on the other hand, are called localized surface plasmon resonances (LSPRs) and are generated on the surface of NPs 10–200 nm in size or created by nanosphere lithography [9, 10]. The electromagnetic field that is generated for SPPs on the noble metallic thin film propagates 10–100 μm in the x and y directions and 200–300 nm in z direction along the metal-dielectric interface. The propagation distance depends on the type of metal, film thicknesses, and surface roughness [5, 10, 11].

The effect of an electric field created by LSPR excitation on a molecule can be understood if we look first at a simplified structure such as a sphere and consider a molecule at a distance d from the surface of the sphere. In this case, the electric field created outside the sphere by the electrostatic dipole inside the sphere will decay by $1/(r+d)^3$, where r is the radius of the sphere. The surface-enhanced Raman spectroscopy (SERS) intensity, on the other hand, will decay with $1/(r+d)^{12}$, which indicates that the highest intensity is obtained for a molecule at the surface and the intensity will decay very fast as the molecule is moved away from the surface of the sphere. It is, however, important to note that “long-range” effects (for molecules at 10 nm or more away from the metallic surface of the sphere) can be observed as well [12, 13].

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The plasmonic properties of metallic NPs include the resonance frequency of the SPs and magnitude of the electromagnetic field generated at the surface. These properties are strongly dependent on their type, size, shape, and composition and the dielectric environment [14–17].

Several types of plasmonic devices have been developed such as plasmonic filters [6], wave-guides [6–8], and nanoscopic light sources [9]. Due to the ability to tune their response to incident light, plasmonic nanostructures have also been used in biomedical applications [18]. Several studies show the use of plasmonic nanostructures in biophysical research [19, 20], biomedical imaging and sensing [21, 22], medical diagnostics [23], and cancer therapy [24, 25]. In addition, the research that explores the combination of plasmonics with chemistry, chemoplasmonics [26–28], is another rapidly growing field. Chemical modifications made on the surface of the plasmonic structure may impart chemical specificity as well as higher sensitivity for improved analytical capabilities in applications based on SERS [29], LSPR spectroscopy [30], or surface plasmon resonance (SPR) spectroscopy [31, 32].

The electromagnetic fields generated by SPs and localized SPs at the surface of the metal will interact with the incoming photons and also with the Raman emitted photons to provide significant enhancement of the Raman scattered photons (electromagnetic enhancement). If the molecule is chemically bound to the surface of the metal, additional enhancement can be observed that is generated by the charge transfer between the metal and the molecule (chemical enhancement). These processes are forming the basis of SERS and will be discussed in greater detail below.

SERS is a powerful technique that uses the enhancement of the Raman signal of molecules situated in the near vicinity of metallic nanostructures to obtain detailed information regarding the identity of those molecules [33–36], with sensitivities down to single-molecule level [37–39]. The enhancement of the Raman signal is based on two mechanisms: electromagnetic enhancement [40, 41] and chemical enhancement [42, 43]. Electromagnetic enhancement is due to the excitation of the SPs of noble metal nanostructures. When a Raman scattering molecule is subjected to intense electromagnetic fields generated on the metal surfaces, the higher electric field intensity results in stronger polarization of the molecule, and thus the higher induced dipole moment is obtained. This is directly related to the intensity of Raman scattered light [44]. Electromagnetic enhancement is considered the major component (enhancement contribution of 10^4 – 10^7) of the enhancement mechanism [44]. Chemical enhancement is due to charge transfer between metal and adsorbed

molecules on plasmonic nanostructures. The contribution of chemical enhancement is smaller (a factor of 10 – 10^2), and its magnitude depends on the chemical structure of the molecule [44–47].

Since electromagnetic enhancement is the major contributing mechanism, research focuses on targeted engineering of novel plasmonic structures to obtain high enhancement factors while maintaining reproducibility across the substrates. Plasmonic properties can be tuned by changing physical properties such as size [48–52], shape and type [53–61], composition [62–64], and dimensionality (2D and 3D) [65–73] of the plasmonic nanostructures. When the physical characteristics of nanostructures are tuned, the resonance frequency (or wavelength) of the SPs is changed. Higher SERS enhancement factors are obtained when the wavelength of the SPs of the nanostructure (λ_{sp}) is located between the excitation wavelength (λ_{exc}) and the wavelength of Raman signal (λ_{rs}). Theoretical and experimental results demonstrated that the maximum enhancement occurs when the λ_{sp} is equal to the average of the λ_{exc} and the λ_{rs} , that is, when $\lambda_{sp} = 1/2(\lambda_{exc} + \lambda_{rs})$ [44, 74–77]. Tuning the physical properties of nanostructures also changes the magnitude of the electromagnetic field generated on the surface when exposed to monochromatic light. The intensity and distribution of the electromagnetic field generated on the plasmonic nanostructures determines to a great extent the SERS enhancement factor, which is directly proportional to the fourth power of electromagnetic field intensity generated on the plasmonic nanostructures [10, 78]. Thus, one of the main tasks in engineering plasmonic structures is generating intense electric fields on their surface. Electrodynamics calculations can help estimate the resonant frequency and electric field intensity of the SPs generated by light in nanostructures of various geometries. Discrete dipole approximation (DDA) [10, 79] and finite-difference time-domain [80] methods are commonly used for such theoretical calculations. As an example, Figure 1 shows the electrodynamic calculations of plasmonic properties of silver nanoparticles (AgNPs) having different shapes using DDA method to estimate the enhancement factor [10]. The extinction wavelengths and the intensity and distribution of electric fields (E) on the surfaces are simulated. Figure 1B–D show contours of $|E|^2$ around three of the particles for wavelengths corresponding to λ_{max} and for polarizations that lead to the largest $|E|^2$.

So far we discussed how physical properties of metallic nanostructures could affect their plasmonic properties and therefore their SERS enhancement factor. There are, however, other parameters influencing SERS, such as

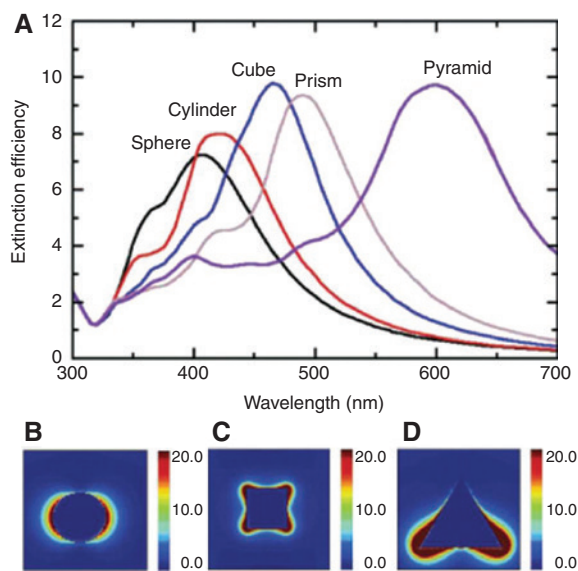


Figure 1: Simulations of extinction efficiencies and intensity contours. (A) Simulation of extinction efficiency of the silver nanoparticles having different shapes and $|E|^2$ contours for a (B) sphere, (C) cube, and (D) pyramid, plotted for wavelengths corresponding to the plasmon peak in (A), with peak $|E|^2$ values of 54, 745, and 9770, respectively [10]. These results show that the highest enhancement factor (10^8) is obtained when the shape of the nanoparticles is pyramid-shaped due to the approximation of the enhancement factor as $|E|^4$. Reproduced with permission from Ref. [10].

molecule-substrate distance, type of structures (colloids and solid substrates), and aggregation status. Molecules must be covalently bound or in close vicinity (in the range of a few nanometers) to the substrate in order to obtain significant Raman enhancement. As the distance between molecule and substrate decreases, larger enhancement is obtained [81, 82]. There are two types of SERS substrates mostly used in SERS experiments: colloidal suspensions (NPs) and solid substrates. Colloidal suspensions are common due to the ease of preparation and relatively high enhancement factors. Molecules or molecular structures must be bound to or in the vicinity of noble metal nanostructures, in the range of 1–4 nm, for a significant SERS enhancement. Due to the fact that this distance is influenced by the nature of the interactions between molecules and nanostructured surfaces, the charge properties of molecules and molecular structures play an important role in the performance of SERS-based Raman measurements. When colloidal noble metal NPs are used, the surface charge of NPs and the charge of molecules must be carefully considered [83, 84]. The SERS activity is stronger when the detected molecules possess the opposite charge of the interacting colloidal NPs. This is due to the induced aggregation caused by the reduced zeta potential of NPs [84]. When NPs aggregate, SERS activity

also increases. Controlling this aggregation helps generate higher SERS enhancement due to the increased possibility of “hot-spot” formation [85, 86]. One must consider, however, that very large aggregates diminish the effective formation of SPs due to the deformations and dampening of the electron cloud in the aggregate and therefore generate poor SERS activity. Small-sized aggregates composed of 200–300 AGNPs generally seem to achieve the largest enhancement factors [87].

Another important consideration is related to the availability and cost of optical components and detectors, which makes the visible and near-infrared (NIR) ranges more accessible. Yet another factor in the wavelength selection (for SERS applications only) is the position of the plasmon resonance absorption band. The maximum SERS signal is obtained when the laser wavelength is tuned to be slightly blue-shifted compared to the plasmonic resonance [88]. In practice, it is easier to tune the plasmonic resonance through material and structure modifications. As such, numerous and diverse plasmonic nanostructures have been fabricated.

2 Review of SERS substrates

2.1 Materials for SERS substrates

Most metals, including Al and Cu, exhibit plasmonic properties in the UV region and can therefore be used as SERS substrates [13, 89]. Although Cu- and Al-based nanostructures are cheaper than the other metals, easy oxidation and relatively low enhancement factor are serious disadvantages. Up to date, plasmonic nanostructures based on Au and Ag are most commonly used due to their higher enhancement factors and availability of plasmonic resonances in the visible and NIR regions [88]. However, the tunability range of the plasmonic resonance is wider for Ag (300–1200 nm) than for Au (500–1200 nm). The intensity/magnitude of SPs generated on the nanostructures is directly proportional to the quality factor $Q = w (d\epsilon_r/dw) / 2(\epsilon_i)^2$, where w is the excitation frequency, and ϵ_r and ϵ_i are the real and imaginary components of the metal dielectric function, both of which vary with the excitation wavelength of light. Ag has the largest Q across the spectrum of the SPs and therefore the largest enhancement factor [12, 90]. Another advantage of Ag is that it has a lower cost compared to Au. Ag is therefore an excellent choice for analytical SERS measurements due to its relatively low cost, wide tunability range, and high enhancement factor.

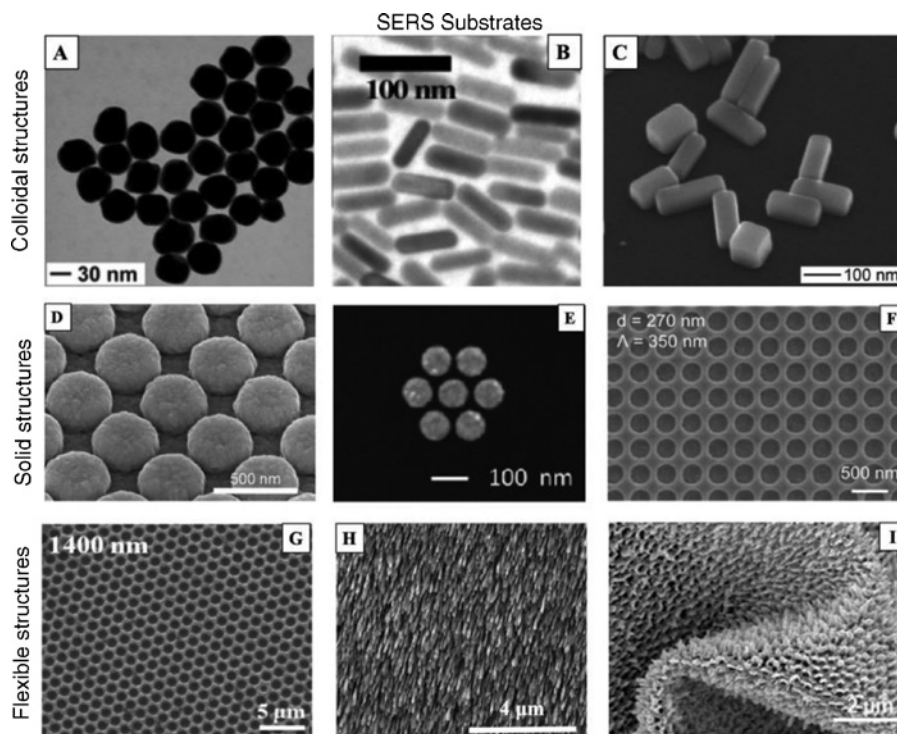


Figure 2: SEM images of different types of SERS substrates. (A) Spherical gold nanoparticles [50], (B) gold nanorods [91], (C) silver nanobar [92], (D) silver plasmonic nanodome array [93], (E) gold nanocluster [94], (F) gold nanoholes [67], (G) silver nanovoids [73], (H) silver nanocolumnar film [95], and (I) silver nano-pillars [96]. Reproduced with permission from Refs. [50, 67, 73, 91–96].

In addition to the specific materials used in analytical SERS, the physical characteristics of the material structures, such as size, shape, type (colloidal, 2D, and 3D), and composition, are also extremely important. Briefly, the SERS substrates can be divided into three main groups as colloidal, solid, and flexible structures. The detailed information regarding types of SERS substrates are given below. Figure 2 shows scanning electron microscopy (SEM) images of some SERS substrates fabricated in the literature using different methods.

2.2 Colloidal structures

Colloidal suspensions of NPs are widely used for SERS enhancement due to easy preparation and tunability of the plasmonic resonance. Tunability of the plasmonic resonance is generally achieved by changing size, shape, type, or composition of the colloidal structures, as discussed earlier. The SERS activity of spherical gold nanoparticles (AuNPs) and AgNPs improve by increasing the size. Spheres [50, 97], nanorods [56, 61, 91], nanoplates [60], nanowire [98], nanobars [92], and nanorice [92] have been successfully prepared and shown to have different SERS properties.

Hollow AuNPs (30 nm) were also prepared and utilized for pH measurements. The authors demonstrate in this article that SERS activity of hollow NPs is nearly 10 times higher compared to standard AgNPs [99]. A SERS active structure composed of a biocompatible dendrimer- and peptide-encapsulated few-atom Ag nanoclusters for the measurements of single molecules via anti-Stokes Raman spectroscopy was also demonstrated [100].

Bimetallic NPs can be fabricated via different methods [101–107]. The most common method is wet chemical synthesis [62–64]. The bimetallic NPs can be prepared homogeneously with the reduction of two metal ion alloys. They can also be prepared heterogeneously by following reduction of two metal ions called core-shell NPs. Material composition can also tune colloidal NPs SERS enhancement. Various metal alloys are achievable, such as AuAg, CuAu, and AuFe. SERS activity is strongly dependent on the composition and ratio of the bimetallic alloy [108–110]. Although there are many factors, Au-coated AgNPs (Ag@Au) or Ag-coated AuNPs (Au@Ag) [111–113] were found to have greater SERS activity than their single metal counterparts. Dielectric core-metal shell NPs have also been used for SERS application [114–116]. For this type of core-shell NPs, core size, type of metal shell, and shell thickness are the critical factors for SERS activity [114–116].

While colloidal NP suspensions can be physically tuned to achieve higher enhancement factors, the distance of the analyte to the metallic structure also plays a significant role. This distance depends on the nature of analyte-metal interaction. Direct chemisorption of the analyte to the metal is generally preferred as it yields a shorter distance. Physisorption plays a significant role as well, and it depends on the charge of the analyte relative to the charge of the NPs. To obtain homogenous and stable NPs, they must carry charges. Depending on the preparation methods, negatively or positively charged NPs can be obtained. When the charges are opposite, analytes interact with the NPs via attractive forces and induce the aggregation of NPs by reducing the zeta potential of the NPs. The aggregates result in a superior SERS enhancement. SPs of aggregates also shift to longer wavelengths with the broad spectrum which is critical for different excitation laser lines [117].

2.3 Solid structures

Two- and three-dimensional plasmonic nanostructures have been fabricated and widely used in SERS studies. Two-dimensional plasmonic nanostructures are thinly patterned substrates. They can be fabricated by assembly of NPs or vapor deposition of metal on a substrate to obtain thin film plasmonic nanostructures. Inter-particle distance, orientation, type, and size of NPs in the assembled NPs are critical factors for SERS performance [85, 118, 119]. Roughness and thickness of the film and type of the metals are the influencing parameters for SERS when thin films are used as SERS substrate [120]. There are only a few reports regarding 2D SERS substrates due to their poor SERS activity. Three-dimensional nanostructures are plasmonic surfaces with more physical depth. Nanoholes, nanovoids, Nanodomes, nanoclusters, and nanoarrays are 3D nanostructures, which have been successfully fabricated. Nanoholes can be prepared using electron beam lithography (EBL) [67, 71], focused ion beam [68], or soft lithography [66, 69, 121]. Nanovoid arrays are prepared using porous anodic alumina [122] or the combination of nanosphere lithography and electrochemical deposition technique [65, 72, 123]. Plasmonic properties were tuned by changing the diameter and periodicity of the nanoholes to obtain maximum SERS enhancement [67, 68, 71]. Changing the diameter and height in nanovoids tunes their plasmonic properties [65, 72]. Nanoholes and nanovoids show around 10^4 – 10^6 SERS enhancement [66–69, 71]. Au and Ag nanodomes were fabricated using nanoreplica

molding [124, 125]. Depending on the inter-dome spacing, SERS enhancement factor of Ag and Au nanodomes were 8.51×10^7 and 1.37×10^8 , respectively. Some plasmonic nanoclusters fabricated using EBL have their plasmonic properties tuned by cluster size, geometry, and inter-particle spacing [94]. Ag nanorod arrays are uniform, reproducible, and large-area substrates with high SERS enhancement and are fabricated by oblique angle vapor deposition (OAD) [126]. The diversity in fabrication methods is driven by the wide array of potential applications. Nanoporous Au was also fabricated as a highly active, tunable, stable, biocompatible, and reusable SERS substrate. The largest enhancement was obtained when the nanofoams with average pore widths of 250 nm were used for 632.8 nm excitation [127]. More recently, significant attention was dedicated towards the fabrication of graphene-based SERS substrate and their analytical applications [128–130]. The results demonstrate that graphene-based SERS substrate can be used for the detection of chemical and biomolecules with high sensitivity and quantitative analysis.

2.4 Flexible structures

Flexible SERS substrates have potential applications in low-cost embedded and integrated sensors for medical, environmental, and industrial markets [131]. These are mechanically flexible, low-cost, reproducible, and sensitive and can be manufactured using various advanced methods [73, 95, 96, 132–141] to have large areas. Their plasmonic properties can be tuned by changing shape, size, or morphology of nanostructures and also by mechanically bending, stretching, and twisting. Flexible SERS substrates have been fabricated out of paper and polymers [131]. Electrospinning was used to obtain flexible SERS substrates with 10^9 enhancement by assembling AgNPs on poly(vinyl alcohol) [132]. Gold nanodimers were prepared on a stretchable elastomeric silicon rubber, and the SERS performance was tuned by changing the interparticle gap between nanorod dimers using mechanical strain [133]. Large-area flexible SERS substrate arrays (including pillar, nib, ellipsoidal cylinder, and triangular tip) have been also fabricated on poly(dimethylsiloxane) (PDMS) surfaces using shadow mask assisted evaporation. SERS performance was tuned by changing the morphology of the array, with the largest enhancement obtained using a triangular tip [96]. Silver nanocolumnar films were deposited on a flexible PDMS and polyethylene terephthalate using OAD [95], and the SERS performance changed with mechanical (tensile/bending) strain [95]. Sand paper was

used as template for the deposition of silver to obtain SERS substrate to use for the detection of pesticides on difference surfaces [134].

A simple method consisting of a combination of soft lithography and nanosphere lithography was used to fabricate large-area, tunable, and mechanically flexible plasmonic nanostructures [73]. Soft lithographic methods that use elastomers such as PDMS offer increased parallelism, simplicity, and flexibility. Nanosphere lithography, on the other hand, uses small spherical particles to obtain a template for lithography. In this, spherical sulfate latex particles with different diameters were deposited on a regular glass slide. PDMS elastomer was poured on the deposited latex particles and cured to obtain bowl-shaped nanovoids. The Ag layer (60 nm) was sputtered on the PDMS with and without Cr (5 nm) to obtain flexible plasmonic nanostructures. The plasmonic properties of these nanostructures were tuned by changing the size of the latex particles. Larger particles had larger diameter and deeper nanovoids, and smaller particles had smaller diameter and shallower nanovoids. Maximum enhancement factors (1.31×10^6 and 1.42×10^6) were obtained for nanostructures coated with a Ag layer having 1400 nm diameter (for 785 nm laser excitation) and 800 nm diameter (for 633 nm laser excitation) [73].

3 Functional and label-free assays

SERS has some advantages over other types of assays for detecting molecules of interest. High enhancement of the Raman signal is inherently built into the assay, allowing for easier detection of low concentrations. Multiplexing is another advantage due to the Raman peaks allowing for easier distinction of different molecules. The simple spectroscopic detection mechanism is low cost and reliable. Extremely small distances between the analyte and the resonating structures are needed to achieve surface enhancement and make useful assays. This distance depends on the mechanism used to bring the analyte close to the metallic surface. Direct chemisorption of molecules to the metal yields a shorter distance and allows for specific binding. Physisorption, on the other hand, depends on the charge of the analyte relative to the charge of the NPs and can also be used to build assays.

Next, we will review how SERS assays are built by binding the analyte to the surface using antibodies, aptamers, another compound, or sometimes no functionalization at all. Label-free assays, which allow for direct

measurement of the analyte, are also gaining popularity and will be discussed as well.

3.1 Functional assays

Detecting an analyte in a system often requires specific capturing of the molecule onto a substrate. This can be achieved via functionalization, which uses a specific capturing molecule. The capturing molecule is usually an antibody, peptide, or nucleic acid sequence that has high binding affinity to the molecule of interest. A marker may be added to the other end of the binding molecule in the form of a label. This is the basic principle behind immuno-assays such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay. The substrates that these assays use are designed to hold the capturing molecule but do not play a role in the detection mechanism. In contrast, SERS-based assays are built on metallic substrates that actively enhance the Raman signal and therefore are part of the overall detection process.

The capturing molecules in SERS assays are most often made from antibodies or aptamers. Aptamers are small nucleic acid molecules made from DNA or RNA that form structures capable of specifically bringing proteins to cellular targets. Aptamers are used to detect a variety of molecules [142–147]. Antibodies or immunoglobulins are large proteins which specifically attach to antigens or targets such as bacteria. Antibodies are the most common connector molecule in a SERS system. There are reported limits of detection (LODs) in the femtomolar range for prostate-specific antigen (PSA) in serum [148]. Antibodies have been used in a multi-analyte system where they have maintained sensitivity and specificity [149]. Antibody-based SERS assays have also been used for *in vivo* tumor detection in live animals [150]. There is, however, a significant disadvantage of antibodies when it comes to direct detection of the analyte because their relatively large size sets a large distance between the analyte and the substrate. There are other capturing molecules that are gaining some attention such as enzymes, molecularly imprinted polymers, and affimers [151–153]. While they hold promise for functional SERS assays, they have yet to gain momentum for both research and real-world applications.

While functionalization offers specific detection of molecules of interest, non-functionalized assays involve binding directly to the surface of the metal. The lack of capturing molecules removes a potentially expensive isolation step in the assay, and it enables the analyte to be

physically closer to the enhancing field, thereby allowing for stronger enhancement.

3.2 Label-free assays

Label-free SERS assays directly measure the SERS spectrum of the analyte. This can be difficult to achieve, especially because of the distance added by larger capturing molecules. Since they are smaller than antibodies, aptamer-based assays have a greater possibility for label-free detection. For example, aptamer-based detection of coagulation protein α -thrombin has been demonstrated for concentrations as low as 100 pM [144] and shows Raman peaks that can be used for the development of similar assays [145, 146]. However, not all label-free assays need to be functionalized. The development of label-free, non-functionalized assays is promising [154]. Even in unpurified samples, single picomolar concentrations can be measured [155]. There is a variety of detected molecules, including TNT [155], neurotransmitters dopamine and serotonin [156], and incubated *Escherichia coli* [157]. Label-free SERS assays have also been developed using PDMS in an integrated microfluidic device for biomolecular detection [158]. Stuart et al. also detected glucose molecules using “molecular combs” to slow down the diffusion near SERS substrates [159].

3.3 SERS nanotags

In labeled assays, a nanotag is used as the reporter molecule. The tags are chosen to have specific, unique, and strong SERS signals. Preference is given to tags that exhibit peaks outside the fingerprint Raman region of biological molecules, such as nitriles, alkynes, or diynes. However, tags that exhibit strong SERS spectra in the fingerprint region can be used as well. Tens of SERS nanotags can be used in a multiplexed assay [160, 161]. Fluorescence assays also work by attaching a specific binding molecule to the detectable particle. However, unlike fluorescent assays, SERS nanotags can be excited by any wavelength, and, even though their fluorescence may decrease (if the tags exhibit fluorescence), their SERS intensities do not decrease with laser exposure. This lengthens the period of detection and simplifies the excitation conditions, while maintaining a multiplexing ability unparalleled in other methods.

There are several studies focusing on the development of novel SERS nanotags for the specific detection of biological molecules [150, 160–169]. Such assays have

been developed to target cancer cells [164], other cancer biomarkers [166], and proteins [163]. SERS assays containing nanotags consist of three components: (1) SERS substrates such as AuNPs or AgNPs to enhance the signal, (2) Raman active molecules/reporters to obtain unique spectrum, and (3) an attachment molecule allowing for bio-specificity. The attachment usually involves coated glass or polymer beads, which makes easy surface chemistry for the attachment of different targeting molecules. SERS nanotag assays have been mostly prepared as core-shell NPs such as silver core-glass shell [160], gold core-silica shell [162], or gold core-silver shell [166, 167] for use in biosensing [150, 168, 169].

3.4 Peak/frequency shift based assays

A great deal of information is embedded in a SERS spectrum. Changes in certain peak intensities are directly proportional to the concentration of the analyte. Changes in the frequency of a certain vibrational peak sometimes occur when materials are in a certain state of stress or strain, making the development of a stress-sensitive nanomechanical biosensor possible [170–172]. This method provides a novel biosensing approach with high selectivity and possibility for label-free biomolecule detection. When binding occurs between targeting agents and binding molecules, a stress on the bond causes small frequency shifts that may be detected with high-resolution spectroscopy [170]. This approach may be applied to protein assay where a frequency shift upon the binding of the analyte to the antibody is measured and quantified.

4 Bioanalytical applications of SERS

4.1 SERS of small molecules

Current analytical methods for detection and quantification of small molecules include mass spectrometry, chromatographic-based techniques, and immunochemical methods (Figure 3). SERS promises to be a viable alternative due to its multiplexing ability, potential for high sensitivity and specificity, capability of rapid measurements, and possibility to be integrated in small packages for measurements in the field or at the point of care.

SERS is particularly well suited to detect small molecules because of the close proximity of the analyte to the plasmonic structure. There are several categories of assays

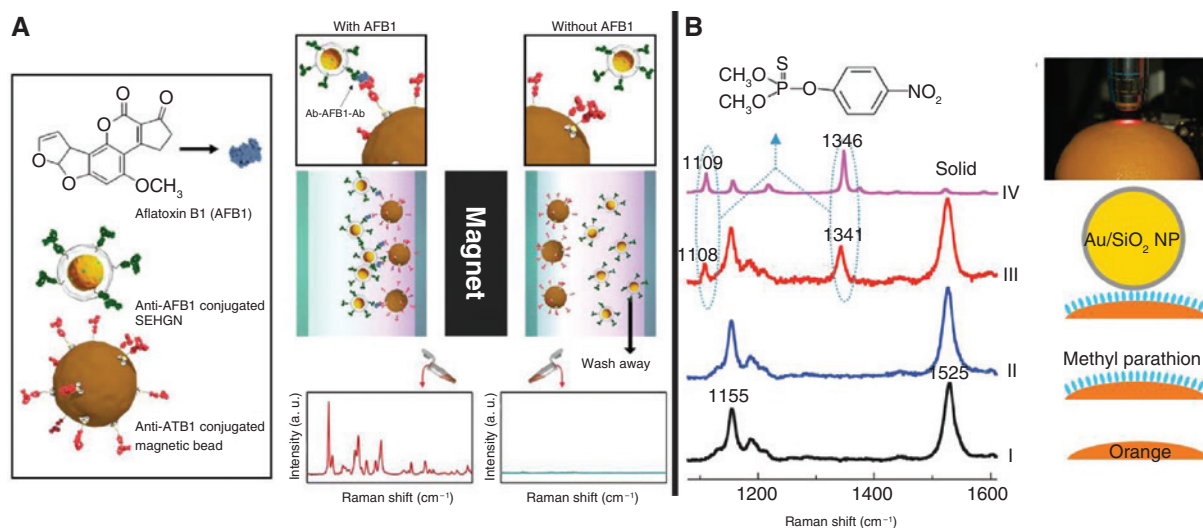


Figure 3: Small molecule detection has various approaches. (A) Ko uses a two-part colloid to enhance the SERS signal with Au and isolate the particles using magnetic beads to detect Aflatoxin B1 [173] and (B) Li places the Au shell directly on an orange peel to enhance the signal of pesticide [174]. Reproduced with permission from Refs. [173, 174].

being developed for SERS on small molecules. Monitoring certain components of food is important from a regulatory and public health standpoint. Several types of dangerous ingredients can be found in food such as excessive additives, mycotoxin, and pesticides. Regulated food additives which are only safe at low levels need to be inspected, including some coloring agents and antimicrobials. A dangerous chemical called melamine is illegally added to food to artificially enhance levels of protein. Fungi can grow on food and produce mycotoxins, which can cause nerve damage when ingested by humans. Similarly, pesticides can remain on foods and are toxic to humans. There are also many non-food applications of SERS. Environmental monitoring of organic pollutants in soil and water that can leak into the food chain or disrupt an ecosystem should be performed regularly for public safety. In addition, SERS assays to test narcotics have been developed for rapid testing.

Antimicrobials and colorants are added into some processed foods for preservation and visual appeal. For example, the adulterant melamine is illegally added to increase the appearance of protein. At high levels, these food additives are dangerous and toxic. The food and drink colorant azorubine or E 122 found in beverages is another example of one such additive, and SERS was used to quantify the levels with no sample preparation [175]. Various prohibited colorants, such as amaranth, erythrosine, lemon yellow, and sunset yellow, are also good candidates for SERS [176]. Sudan I dye is a class three carcinogen that can be found in culinary spices and can be detected even in a chemically complex sample [177]. SERS

detection of colorant is also possible in non-food samples, such as in textiles [178]. Various plasticizers can be found in orange juice using SERS at around seven orders of magnitude lower than FDA limits [179]. Adulterants can be detected using SERS [180] in wheat gluten, chicken feed, cakes, and noodles [181]. Melamine can be found in liquid milk [182, 183], milk powder [184], and infant formula [185] at very low levels.

Toxins from fungi called mycotoxins also appear in food and can cause harmful side effects such as nerve damage. Four major aflatoxins, a type of mycotoxin, which appear in food are B₁, B₂, G₁, and G₂. They can be detected at low concentrations in solution [124] and in ground maize [186] using SERS. Quantitation has been developed for at least B₁ [173]. Mycotoxin citrinin, which is produced by several fungi species, can also be detected in trace amounts [187]. There are severe consequences for mycotoxin consumption for both humans and domesticated animals.

Pesticides are also dangerous toxins found in food. They can readily be found on the surface of fresh fruits and vegetables. A SERS measurement on fruit can nondestructively detect various pesticides [174, 188, 189], making a good candidate for a high throughput assay. Quantification is possible under controlled conditions [190]. Assays also exist to detect the insecticide methyl-parathion [191] and ferbam fungicide [192]. In addition to food, pesticides can leak into the ecosystem.

Organic pollutants in the water and soil can leak into the food supply or adversely affect an ecosystem. They are found in rural and urban environments and are associated with elevated cancer rates [193]. Detection is important

for environmental monitoring. Some of these present a challenge because they have an unusually low Raman cross section and require special enhancement materials, but many assays have been developed for the diverse array of analytes. An enhancement substrate consisting of an Au-coated TiO_2 nanotube array can be used to detect benzenethiol, 1-naphthyl-amine, and pyridine [194]. A reusable substrate of Au-coated TiO_2 was developed to measure an array of pollutants, herbicides, and pesticides [195]. A substrate made from ZnO, reduced graphene oxide, and Au NPs was developed to detect Rhodamine 6G [196]. Pentachlorophenol, diethylhexyl phthalate, and trinitrotoluene can be measured using Ag and carbon-coated Fe_3O_4 microspheres [197].

Narcotics and controlled substances are another good candidate for SERS [198]. A quick and simple technique for rapid detection of active ingredients in pills or powders would be a great tool for law enforcement. It also allows for an alternative identification technique to HPLC/mass spectroscopy (MS). Assays have been developed for detecting amphetamine in 26 collected XTC tablets with a good LOD [199]. Dihydrocodeine, doxepine, citalopram, trimipramine, carbamazepine, and methadone can be detected at 1 mg/sample from blood or urine [200]. In addition to narcotics, doping in athletics calls for quick and simple testing. Doping drugs, such as clenbuterol, salbutamol, and terbutaline, can be detected using SERS and AuNPs [201].

4.2 SERS of proteins

The accurate, sensitive, and rapid identification and characterization of proteins is critically important in both clinical and industrial applications. They can exist as enzymes or hormones and be involved in transport mechanisms. Protein detection is generally divided into two types of approaches. The first type is an immunoassay-based method employing antibody-antigen interaction based on fluorescence measurements [202]. However, the broad emission spectra of the dyes makes multiplexing a challenge, and the detection limits are higher due to photobleaching [203]. The second type of approach is MS after separation and purification [204, 205]. Although MS-based detection is sensitive and reliable, the high cost, time requirement, and need for skilled labor to interpret the data are drawbacks. Identification of biologically related molecules and structures using SERS is more attractive due to the “finger printing” property and the limited influence of water on the signal. Spectra with peaks of narrow bandwidth create unique fingerprints

and allow for greater multiplexing and specificity. The limited influence of water allows for detection in aquatic solutions and of minimally processed biological samples.

Detection and identification of proteins using SERS can be specific or non-specific. Specific detection utilizes targeting agents like antibodies or aptamers to capture the specific proteins. Non-specific detection uses the intrinsic spectra of proteins. Several reports have been published regarding the detection and identification of proteins and protein mixtures in the literature using different approaches, which have been classified and schematically illustrated in Figure 4.

Specific SERS detection uses targeting agents to capture the proteins of interest. SERS spectra are then obtained from either labels or by monitoring the peak shift at a specific wavenumber due to the structural deformation on the bond as a result of antibody-antigen binding event. When Raman reporter molecules and targeting agents are used, the approach can be described as specific and labeled SERS. However, when only capturing agents are used, the method can be described as specific and label-free for the protein detection and identification. Raman reporter molecules have become increasingly popular for SERS-based immunoassays. In those studies, the Raman reporter molecules/dyes are covalently bound to the metallic NP with the capturing agents. When the binding between proteins and targeting agents occurs, a change in the SERS spectra obtained from the dyes indicate the presence of certain molecules. Dye-functionalized NP probes were used for specific protein-binding, and SERS was used to probe for protein-small molecule and protein-protein interactions [206]. Silver staining was performed to obtain higher SERS signal that allows to obtain lower LOD. A novel SERS-based immunoassay method for the detection of PSA was reported. In this study, 30 nm AuNPs were used as SERS substrate and also to bind the Raman reporter and bioselective targeting agent. The results demonstrated that PSA can be detected as low as ≈ 1 pg/ml and ≈ 4 pg/ml in human serum and bovine serum albumin, respectively [148]. A SERS-based immunoassay was developed for the detection of hepatitis B virus (HBV) surface antigen using AuNPs modified with mercapto benzoic acid (MBA-Raman reporter) with a specific antibody for the HBV targeting agent. Silver staining was also used to enhance the SERS signal to lower the LOD to 0.5 $\mu\text{g/ml}$ [209]. Ag/ SiO_2 core-shell Raman tags were prepared and used for the simple, fast, and inexpensive detection of human α -fetoprotein, which is a tumor marker used for the diagnosis of hepatocellular carcinoma, with the LOD of 11.5 pg/ml [210]. Surface-enhanced resonance Raman scattering (SERRS) was also used for immunoassay-based protein detection.

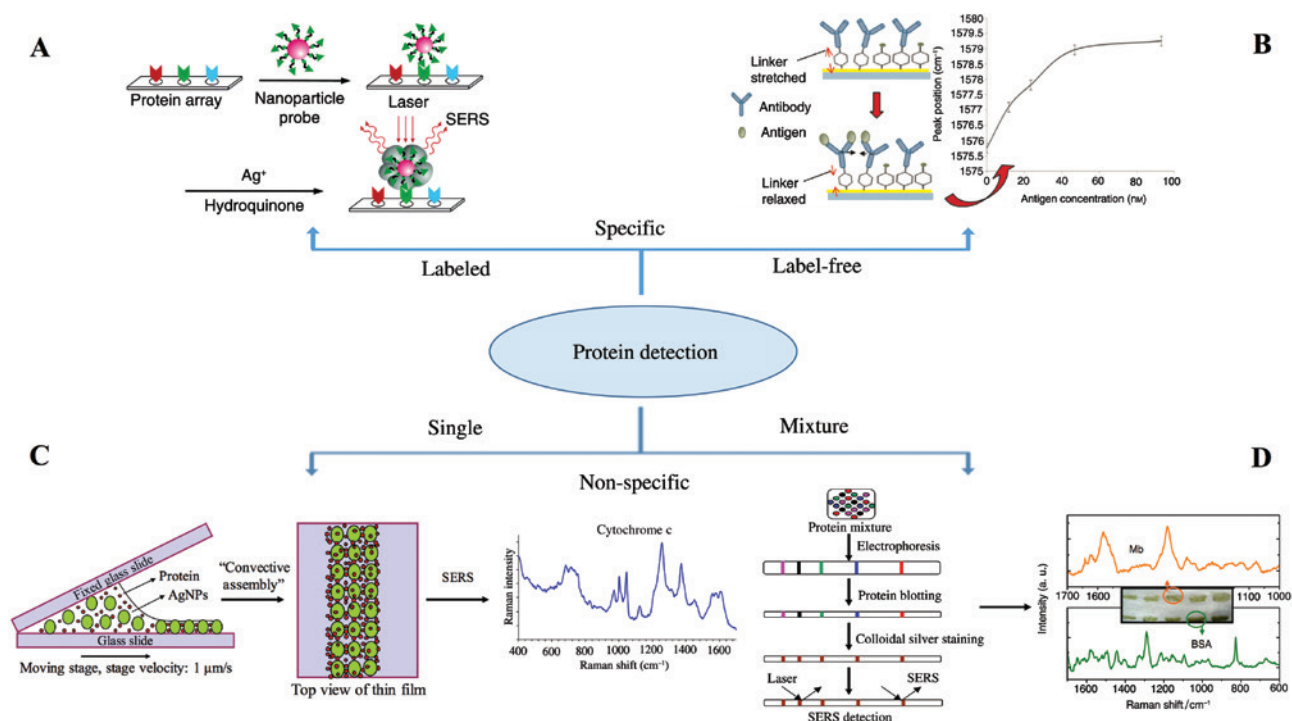


Figure 4: Approaches for SERS-based protein detection. (A) Dye-functionalized nanoparticle probes for detection of SERS-based protein-small molecule and protein-protein interactions [206], (B) capturing agents based on the frequency shift upon the binding of molecules to the antibody [170], (C) convective assembly method used for the controlled aggregation of proteins and AgNPs to obtain rich and reproducible SERS spectra for the label-free protein detection [207], and (D) an approach combining the separation of proteins using PAGE and transferring the protein spots onto cellulose membrane (western-blotting) and detecting with SERS after staining with colloidal AgNPs [208]. Reproduced with permission from Refs. [170, 206–208].

For the first time, a SERRS-based immunoassay on the bottom of a microtiter plate was reported [211]. In this study, fluorescein isothiocyanate was used as a Raman probe and was compared to ELISA. The SERS method and ELISA provided similar LODs of 0.2 ng/ml. The results demonstrated that the proposed SERRS-based immunoassay may have great potential as a high-sensitivity and high-throughput immunoassay. SERS peak shift was also used for specific label-free protein detection [170–172]. The reports describing this approach measure Raman frequency shifts of capturing agents upon chemical binding to molecules of interest. Peak shifts obtained in this way can be used for quantitative analysis of binding, due to the fact that the frequency shift is directly proportional to the analyte concentration. A novel protocol based on SERS-based immunoassay for detection of protein-protein and protein-ligand interactions has been reported [212]. Such work has great potential for high-sensitivity and high-throughput chip-based protein measurements.

Non-specific label-free protein detection uses the intrinsic spectra of proteins. Colloidal NPs and metallic nanostructures have been employed as substrates for the label-free SERS detection of protein. Molecules or molecular structures must be on surfaces or in close

vicinity to the surface of noble metal nanostructures for a satisfactory SERS enhancement. The charge properties of molecules and molecular structures are important for the performance of these measurements because they determine the distance between molecules and nanostructured surfaces. When colloidal noble metal NPs are used, the surface charge properties of the NPs and molecules must be carefully considered [83, 84]. The SERS activity of molecules that possess the opposite charge of the colloidal NPs is superior due to the induced aggregation caused by the reduced zeta potential on the NPs [83]. Controlled aggregation may also help to increase the reproducibility and intensity of the SERS spectra when colloidal NPs such as AuNPs or AgNPs are used as substrates. Proteins can carry varying amounts of charge depending on the pH of environment, so the charge properties of NPs must also be considered. There are several reports describing label-free protein detection using colloidal NPs, especially AgNPs due to their superior plasmonic properties. Proteins carrying a heme group were particularly well characterized with SERS [83, 213].

One way to obtain reproducible and sensitive SERS spectra from proteins for label-free detection is by inducing controlled aggregation of NP-protein mixtures. Acidified

sulfate has been used as an aggregation agent to induce interactions between AgNPs and proteins and obtain sensitive and reproducible label-free detection. This protocol allows for simple, sensitive, and reproducible label-free detection proteins with LODs as low as 50 ng/ml [214].

A layer-by-layer technique was also demonstrated for highly sensitive and reproducible protein detection. In this case, the protein is assembled between two layers of NPs for label-free detection [215]. Another study demonstrated that convective assembly can be used for controlled aggregation of proteins and AgNPs to obtain rich and reproducible SERS spectra for label-free protein detection and identification. This approach demonstrated a LOD of 0.5 $\mu\text{g/ml}$ [207]. A novel method based on the aggregation of suspended droplet of mixture containing AgNPs and proteins from a hydrophobic surface was reported for the label-free detection of proteins with a LOD down to 0.05 $\mu\text{g/ml}$ for the model proteins [216]. A simple sample preparation method for sensitive (LOD 0.5 $\mu\text{g/ml}$) and reproducible label-free detection of proteins based on the self-assembly of AgNPs and proteins on hydrophobic surfaces was also demonstrated [217].

Two-dimensional solid metallic nanostructures can be used for protein detection to eliminate the influence of charge properties and background interference on SERS spectra. However, the SERS spectra are obtained only from proteins touching the SERS active metallic structures which typically provide poor and irreproducible spectra. On the other hand, well-defined 3D metallic nanostructures exhibit large enhancement factors reproducible across large areas and therefore more likely to obtain rich, strong, and reproducible SERS spectra of proteins with the elimination of background interference and charge properties. Recently, well-defined 3D plasmonic nanostructures were fabricated using a combination of soft lithography and nanosphere lithography. These structures were then used for label-free protein detection with no background [218]. Other well-defined structures such as arrays of gold concave nanocubes on a PDMS film were also used for label-free protein detection [219].

SERS can be also used for the detection of proteins in a mixture [220, 221]. An approach combining the separation of proteins using polyacrylamide gel electrophoresis (PAGE) and transferring the protein spots onto a cellulose membrane (western blotting) and detecting with SERS after staining with colloidal AgNPs was reported [208]. Label-free detection of proteins can be performed with differential separation from their mixtures after a convective assembly process. Binary and ternary proteins were mixed with AgNPs and assembled using convective assembly into ordered structures. The spectra acquired from the

different assembled area indicated that the proteins were differentially distributed [222].

4.3 SERS-based DNA detection

Technologies for detection of DNA are important in several fields. Medicine uses bioanalytical chemistry for disease diagnosis, detection of gene mutation, and identification of bacteria and viruses. The current gold standard for detection of DNA is polymerase chain reaction (PCR), which has single DNA sensitivity. However, the PCR method is still labor-intensive and time-consuming and needs qualified scientists as well as requires expensive instrumentation [223]. The development of methods for rapid, easy-to-use, and cost-effective DNA detection is crucial, especially for point-of-care diagnostics. SERS-based DNA detection is an emerging research field due to the several advantages compared to other detection methods such as PCR and fluorescence-based microarrays. The narrow peaks and the larger number of reporter molecules makes SERS a better candidate for multiplex detection. Simple sample preparation and relatively low cost and labor are also advantages of SERS for DNA detection compared to other methods [223]. SERS-based DNA detection can be label free or can use exogenous labels (Figure 5).

Research focusing on label-free SERS detection of DNA is limited due to the poor interaction of negatively charged NPs and DNA. In addition, different DNA molecules present extremely similar SERS spectra which are dominated by the vibrational modes of adenine. There are few reports of label-free SERS of DNA using SiO_2/Au core-shell nanostructures. DNA was incubated with the SERS substrates, and spectra were obtained. This study demonstrated that this approach can be successful in obtaining high-quality and reproducible SERS spectra of single-stranded and double stranded DNA molecules [228]. Positively charged AgNPs were successfully synthesized and used for label-free detection of negatively charged DNA. This was achieved with a phosphate backbone that helps increase the interaction of DNA and AgNPs and allows to obtain more intense and reproducible SERS spectra at nanogram level by inducing aggregation [229]. Iodide-modified AgNPs were also used for sensitive and reproducible label-free detection of single- and double-stranded DNA in aqueous solution by inducing interaction between AgNPs and DNA [224].

SERS DNA detection with labels can be done with either a sandwich or hairpin approach. The sandwich approach uses target DNA-Raman reporter molecules

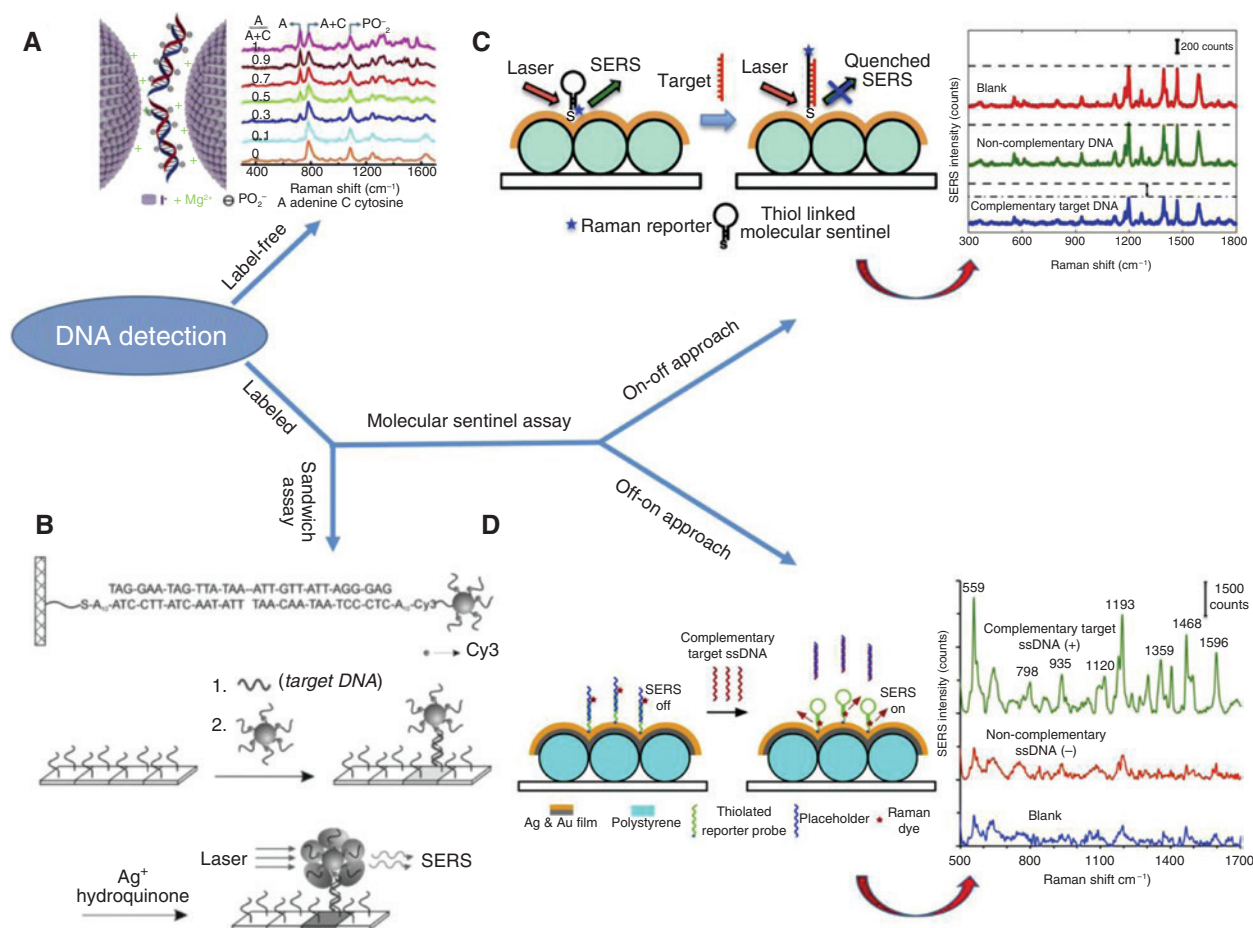


Figure 5: Approaches for SERS-based DNA detection. (A) Label-free DNA detection using iodide-modified AgNPs [224], (B) sandwich assay for the detection of DNA using AuNPs probes labeled with oligonucleotides [225], (C) Raman dye labelled hairpin-DNA probes for the detection of DNA based on the on-off approach [226]. (D) DNA detection based on the off-on approach. [227]. Reproduced with permission from Refs. [224–227].

[225, 230–240]. The first report of SERS DNA and RNA detection using sandwich structures was published by Mirkin and co-workers. In this study, AuNPs probes labeled with oligonucleotides and Raman active dyes were used. The SERS signal was obtained by forming sandwich structures of microarray DNA-target DNA-AuNPs probe. Multiplex DNA detection using different Raman active dyes can be achieved using this approach with a 20 fM LOD [225]. A similar approach was used for the detection of BRCA1 breast cancer gene. ssDNA, which is the complementary of target BRCA1 gene, was assembled on a silver-coated SERS substrate. Raman active and dye-labeled BRCA1 ssDNA was incubated with the substrate for hybridization. AgNPs were used to increase the SERS signal coming from dye. The strongest SERS signal was obtained when the target DNA bound the SERS substrate [230]. Nonfluorescent Raman tags can also be used as labels. Both DNA probing sequence and Raman tags were covalently attached to the AuNPs and

used for the multiplexed detection of target DNA [231] and for gene splicing [232]. Multiplexed DNA detection for different strain of *E. coli* was achieved with SERRS using NPs modified with six different DNA sequences and Raman dyes [233]. A magnetic capture-based SERS assay for DNA detection was developed using Au-coated paramagnetic NPs modified with probe DNA for target DNA. RNA genomes of the Rift Valley fever virus and West Nile virus were successfully detected by using malachite green and erythrosine B Raman dyes, respectively [234]. Novel SERS substrates of Ag nanorice antennas on Au triangle nanoarrays were fabricated for the detection of the HBV DNA as sandwich assay with a LOD of 50 aM [235]. Ag@SiO₂ core-shell nano-SERS-tags were prepared for the detection of specific DNA targets based on sandwich hybridization assays. AgNPs served as SERS substrates with a label to probe the target DNA. The multiplexing capability was successfully tested using four different SERS tags and showed excellent potential [236].

Raman dye-labeled hairpin DNA probes are similar to molecular beacons used in fluorescence-based detection. Tuan Vo-Dinh and coworkers have developed a method called molecular sentinel [226, 227, 241–244]. The sensing mechanism of this approach is based on structural changes of DNA probes upon hybridization with the target DNA that results in a change of the SERS signal. There are two approaches (on-off and off-on), depending on the SERS signal change upon hybridization with the target DNA. In the first approach, Raman dye labelled hairpin-DNA probes are attached to the plasmonic NPs or nanostructures to form a stem-loop configuration. This is called a “closed state”. At this state, intense SERS signals are obtained due to touching the Raman labels to the SERS active substrates. This mode is called “signal on”. Upon the hybridization of the target DNA with this surface, the stem-loop configuration is disrupted and becomes open state so that Raman labels separate from the SERS active surface and results in decreasing SERS scattering intensity. This mode is called “signal off”. “Off-on” is the opposite phenomenon of the “on-off” approach. At the first open state stage, there is no SERS signal. Upon the hybridization of target DNA with this surface, the stem-loop configuration is obtained to become a closed state such that Raman labels are getting closer to the SERS active surface, resulting in higher SERS scattering intensity. One group detected a gene sequence of human immunodeficiency virus (HIV) using AgNP-molecular sentinel probes based on on-off approach with SERS [241]. Another report showed the multiplexed detection of breast cancer marker genes using SERS-based molecular sentinel technology and the on-off approach. The results demonstrated that SERS-based molecular sentinel techniques can be used for multiplexed DNA detection [242]. Molecular sentinel probes can be also immobilized on a solid structure. Development of rapid, cost-effective biosensors for DNA detection was achieved using SERS-based molecular sentinel technology. In this case, the probes were attached to a metal film over nanosphere and used for the detection of a common inflammation biomarker [226]. Another similar study demonstrated the detection of a DNA sequence of the Ki-67 gene (which is a breast cancer biomarker) using metal-coated triangular-shaped nanowire SERS substrate [243]. Sensitive, reproducible, and multiplex DNA detection using SERS was also performed using a molecular beacon [244]. The molecular beacon probes were successfully immobilized onto AuNPs attached on the surface of silicon nanowire arrays. In the absence of target DNA, the SERS signal was obtained due to the close distance between Raman active dyes and metallic AuNPs. In the presence of target

DNA, the stem-loop configuration is disrupted due to the hybridization. Thus, in this “on-off” approach, dye molecules separate from the AuNPs and lead to weaker SERS intensities. Another “off-on” approach used a novel DNA bioassay based on bimetallic nanowave chips. Using this approach, specific oligonucleotide sequences of the dengue virus 4 were detected [227].

4.4 Detection of other biologically relevant nanoparticles

SERS is capable of detecting biologically relevant NPs, such as exosomes and viruses (Figure 6). Exosomes are a class of extracellular vesicles that are approximately 30–200 nm diameter. Until recently, they were thought to be part of a mechanism used by cells to dispose of waste. Now it is believed that exosomes play a role in intercellular communication. Understanding their composition is crucial in elucidating their biological function. Exosomes from stressed or abnormal cells are secreted at different rates and with different contents. Since exosomes are found in most body fluids, they offer an opportunity for non-invasive diagnostic for diseases such as cancer. Another potential candidate for SERS-based assays, which appears in body fluids, are viruses. HIV, influenza, and many other viruses have had severe effects on humans on both population and individual level. Whole viruses are 20–300 nm long, making them good candidates for SERS detection.

The biomolecular diversity of exosomes can be observed using SERS [247]. As the exosome solution dries, the exosomes burst. Spectra taken while an exosome solution is drying provides data on the membrane and then the contents [245]. When exosomes from healthy and tumorous colon cells were concentrated, the exosomes from tumorous cells showed an identifiably stronger RNA signal in a SERS spectrum, while exosomes from healthy cells showed a stronger lipid spectrum [248]. Exosomes from hypoxic ovarian tumor cells have different biomarkers from normal tumor cells [249]. Exosomes may play a role in cancer signaling, allowing cells to send a command for senescence when treatment starts, and thus increasing the resistance. SERS of exosomes thus shows potential for both cancer diagnosis and research for the mechanisms by which tumors respond to their environment.

While there are many ways to detect viruses with SERS, including using proteins, DNA, or RNA, whole viruses can be detected as well. In 2005, a sandwich immunoassay was developed to detect feline calicivirus with a limit of 10^6 viruses/ml [250]. The sensitivity of SERS, using

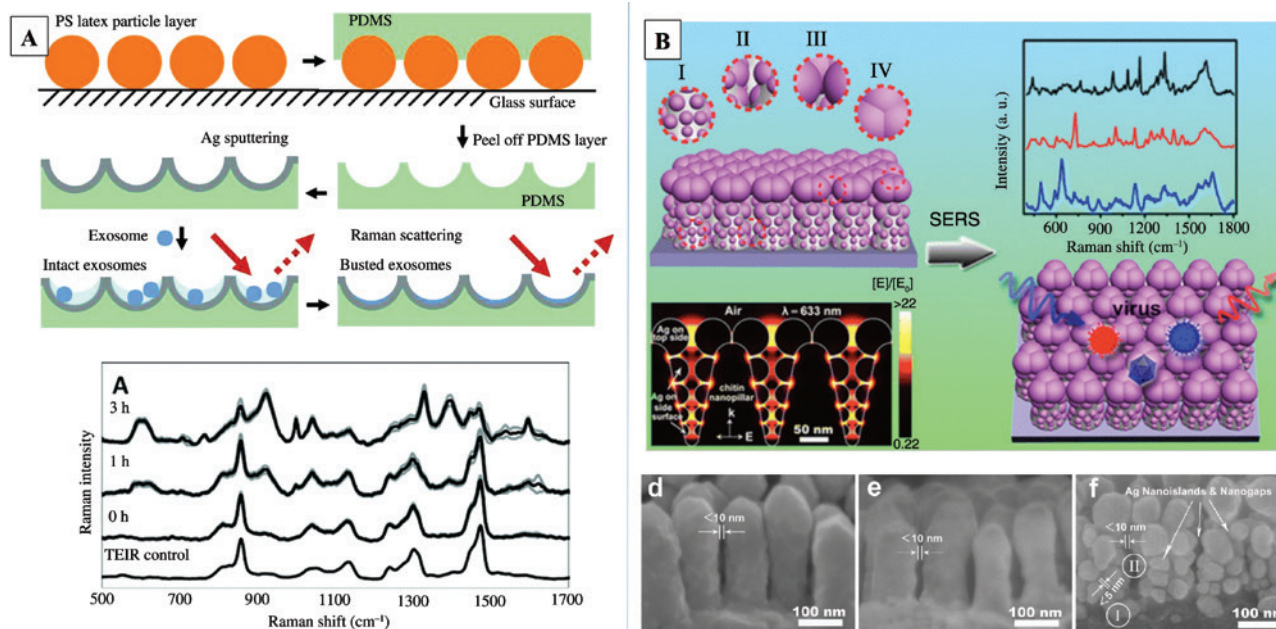


Figure 6: Detection of biologically-relevant nanoparticles. (A) Ag/PDMS SERS substrate is used to detect purified exosomes. During the drying process, they burst and new spectral peaks become visible as the contents become exposed [245]. (B) A SERS assay to detect animal viruses uses Ag-coated chitin biomimetic scaffolding from cicada wings [246]. Reproduced with permission from Refs. [245, 246].

tip enhancement, can detect a single tobacco mosaic virus [251]. Ag nanorod arrays have been used to detect adenovirus, rhinovirus, and HIV virus [252], respiratory syncytial virus (RSV), HIV, and rotavirus [253] and used to differentiate between strains of RSV [254]. Innovative detection mechanisms, such as using cicada nanopillar arrays as a substrate scaffold [246], are being developed. Commercially available substrates detect bovine papular stomatitis, pseudocowpox, and Yaba monkey tumor virus without the need for reagents or labels and can be used to identify an unknown parapoxvirus [255]. While virus detection through SERS is moving to development using antigens [256] or nucleic acid, specific and sensitive whole virus detection is possible.

5 Conclusions and outlook

We presented a review of current literature related to the use of SERS in bioanalytical applications. We first introduced the fundamentals of plasmonics and SERS, including a phenomenological description of the mechanisms leading to the enhancement of the Raman signal of molecules located in close proximity to metallic nanostructures. We then discussed materials available for plasmonics as well as various types of structures that can be fabricated to generate large SERS enhancement factors. A review of potential assays and their classification is

presented, followed by specific examples of assay developments and analytical measurements of different classes of molecules, ranging from small molecules to proteins and DNA, and finally to small particles such as exosomes and viruses.

While the examples presented in this review show potential for analytical measurements, there are still significant problems that need to be addressed before SERS can become a mainstream tool beyond the research laboratory. One important point to discuss is the spatial reproducibility of SERS substrates that determines the consistency for both inter- and intra-sample measurement. Most reports demonstrating excellent reproducibility also show a lower enhancement factor. This is due to the absence of highly efficient hot spots that are associated with extremely high enhancement factors. However, the need for lower LODs in certain applications means that the availability of high-density homogeneous hot spots in those situations may be beneficial, especially for analyte concentrations below approximately 10–50 pM. The dilemma is how to still achieve analytical-quality measurements when large fluctuations in SERS signal exist not only in different points along the substrate but sometimes also in the same spot, which are characteristic for single molecules. Performing measurements for longer periods of time to average all fluctuations is one potential solution. Another way to improve the statistics in the measurement is by illuminating the sample with a larger laser

spot and collecting the signal from this area. Yet another potential solution is by scanning the excitation beam and collecting the signal over a large area. Quantification may be achieved in this case by averaging the intensity of the SERS signal in each pixel. For very low concentrations, however, where single molecules are expected in each pixel, quantification may be achieved by counting and plotting the number of pixels that exhibit a SERS signal as a function of concentration.

While improving the SERS substrates is one important area of future research, effective combination of plasmonics with chemistry, which we call here chemoplasmonics, for targeted analyte detection is another area of importance. In addition, improvements in the signal detection by the development of better detectors and more efficient spectrometers will also play a significant role in the improvement of the sensitivity of bioanalytical SERS instrumentation. Given that the sample volume that is needed for SERS measurements is in the picoliter range, the combination of SERS with microfluidics will also likely become a major component in future developments. Yet another area of future research is related to the development of *in situ* bioanalytical assays.

Overall, bioanalytical SERS holds great promise to be used for applications beyond the laboratory. Due to their relatively low cost, easier sample preparation, and smaller sample volumes, SERS assays may become accessible and inexpensive enough to be an important tool in testing analytes in low resource settings or at the point of care.

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