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Plasmonics-Based Nanostructures for Surface-Enhanced Raman Scattering Bioanalysis

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Summary

Surface-enhanced Raman scattering (SERS) spectroscopy is a plasmonics-based spectroscopic technique that combines modern laser spectroscopy with unique optical properties of metallic nanostructures, resulting in strongly increased Raman signals when molecules are adsorbed on or near nanometer-size structures of special metals such as gold, silver, and transition metals. This chapter provides a synopsis of the development and application of SERS-active metallic nanostructures, especially for the analysis of biologically relevant compounds. Some highlights of this chapter include reports of SERS as an immunoassay readout method, SERS gene nanoprobes, near-field scanning optical microscopy SERS probes, SERS as a tool for single-molecule detection, and SERS nanoprobes for cellular studies.

Key Words: Surface-enhanced Raman scattering; genomics; single-molecule detection; near-field scanning optical microscopy; plasmonics; bioanalysis.

1. Introduction

Raman spectroscopy is based on vibrational transitions that yield very narrow spectral features that are characteristic of the investigated sample. Thus, it has long been regarded as a valuable tool for the identification of chemical and biological samples as well as the elucidation of molecular structure, surface processes, and interface reactions. Despite such advantages, Raman scattering suffers the disadvantage of extremely poor efficiency. Compared to luminescence-based processes, Raman spectroscopy has an inherently small cross-section (e.g., 10^{-30} cm^2/molecule), thus precluding the possibility of analyte detection at low concentration levels without special enhancement processes. Some modes of signal enhancement have included resonance Raman scattering and nonlinear processes such as coherent anti-Stokes Raman scattering.

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However, the need for high-power, multiple-wavelength excitation sources has limited the widespread use of these techniques.

Nevertheless, there has been a renewed interest in Raman techniques in the past two decades owing to the discovery of the surface-enhanced Raman scattering (SERS) effect, which results from the adsorption of molecules on specially nanotextured metallic surfaces. This large enhancement was first reported in 1974 by Fleischmann et al. ***(1)***, who observed the effect for pyridine molecules adsorbed on electrochemically roughened silver electrodes. It was initially believed that the enhancement resulted from the increased surface area produced by the electrochemical roughening, giving rise to increased probed sample density. The teams of Jeanmaire and Van Duyne ***(2)*** and Albrecht and Creighton ***(3)*** later confirmed the enhancement (up to 10^8) but attributed the effect to more complex surface enhancement processes, which continue to be the subject of intense theoretical studies. More recent reports have cited SERS enhancements from 10^{13} to 10^{15}, thus demonstrating the potential for single-molecule detection with SERS ***(4–9)***.

By the mid-1980s, because of the aggressive development of SERS substrates and application to a wide range of chemicals, the potential of SERS as a routine analytical technique was recognized. The SERS technique has since continued to receive increased interest, as evidenced by the large numbers of articles and review articles ***(10–21)***. Furthermore, the scope of SERS has been extended to include other surface-enhanced spectroscopies such as surface-enhanced second-harmonic generation ***(22)*** and surface-enhanced hyper-Raman scattering ***(23)***.

The SERS effect is based on a combination of several processes, involving electromagnetic enhancement and chemical enhancement ***(20)***. Electromagnetic enhancement involves plasmonics, which is related to enhanced electromagnetic properties of metallic nanostructures. The term *plasmonics* is derived from "plasmons," which are the quanta associated with longitudinal waves propagating in matter through the collective motion of large numbers of electrons. Incident light irradiating these surfaces excites conduction electrons in the metal and induces excitation of surface plasmons leading to enormous electromagnetic enhancement of spectral signatures (such as SERS and surface-enhanced fluorescence) for ultrasensitive detection of biomolecules.

A host of biological compounds (e.g., proteins, amino acids, lipids, fats, fatty acids, DNA, RNA, antibodies, enzymes) has been studied via SERS. Extensive progress in the development of dependable SERS substrates over the past few decades has promoted the application of SERS in the rapidly expanding field of biotechnology, as demonstrated in several excellent reviews ***(24–28)***. This chapter provides a synopsis of the development of plasmonics-based nanostructures for SERS analysis of biologically relevant compounds.

Some highlights of this chapter include reports of SERS as an immunoassay readout method, SERS gene nanoprobes, near-field scanning optical microscopy (NSOM) SERS probes, SERS as a tool for single-molecule detection, and SERS nanoprobes for cellular studies.

2. Methods

2.1. Development of SERS-Active Metal Electrodes

Electrochemically roughened electrodes were the first media with which the SERS effect was observed ***(1)***. Observation of this effect resulted in further inaugural studies to confirm it and to establish enhancement factors ***(2,3,14)***. Although several metals have been investigated for SERS activity in electrochemical cells ***(29–33)***, silver has been the most commonly used. During electrochemical preparation, silver at the electrode surface is first oxidized by the reaction $Ag \rightarrow Ag^+ + e^-$; then, elemental silver is redeposited in the ensuing reduction process, $Ag^+ + e^- \rightarrow Ag$. This oxidation-reduction procedure generally produces protrusions on the electrode surface in a size range of 25 to 500 nm. Strong SERS signals appear only after several electrochemical oxidation-reduction cycles, often referred to as "activation cycles."

Transition metals such as bare Pt, Ru, Rh, Pd, Fe, Co, and Ni electrodes have also been investigated as SERS substrates ***(34–36)***. It has been found that transition metals exhibit surface enhancement factors ranging from one to four orders of magnitude, depending on the nature of the metal and the surface morphology. In one study, photoalterations of the copper electrode resulted in a further 10-fold increase in SERS ***(37)***.

2.2. Development of SERS-Active Metal Nanoparticle Colloids

SERS-active suspensions of elemental metal colloids or nanoparticles of various sizes can be chemically formed in solution. Hence, they can be readily used in suspension for *in situ* solution SERS measurements. Alternatively, they can be immobilized on various solid media for use as surface-based SERS substrates. As with roughened metal electrodes, silver is the most commonly used material. Silver colloids can easily be prepared by reducing a solution of $AgNO_3$ with ice-cold $NaBH_4$ ***(38–40)***, trisodium citrate ***(41–43)***, or hydrogen peroxide under basic conditions ***(44)***. Other more innovative techniques that reduce the need for wet chemistry have been demonstrated. For example, Ahern and Garrell ***(45)*** described a unique *in situ* photoreduction method to produce photocolloids in solutions, and another innovative method involved laser ablation of colloids from silver foils into aqueous solutions ***(46)***.

Gold colloids have also been investigated as SERS-active media. Because gold is virtually bioinert, it may prove to be a valuable material for biomedical applications of SERS. Furthermore, gold produces large SERS enhancement

factors when near-infrared (IR) excitation sources are used. Near-IR excitation radiation is particularly useful in biomedical studies because it allows greater penetration depths in tissues while causing less fluorescence background relative to visible radiation. Gold sols have been used for the SERS detection of various dyes ***(41,47)***. In particular, Kneipp et al. ***(7)*** have observed extremely large enhancement factors (up to 10^{14}) for dyes adsorbed on gold colloids when using near-IR excitation.

2.3. Development of Solid SERS Substrates Based on Metallic Nanostructures

In addition to immobilized colloids, researchers have developed a variety of solid surface–based SERS substrates that are produced entirely from solid materials, as depicted schematically in **Fig. 1**. In contrast to immobilized colloids, the solid SERS-based probes described subsequently exhibit a high degree of reproducibility. In addition, nanoshells with a dielectric core and a metallic shell (e.g., Au/SiO_2), or a metallic core and a dielectric shell (e.g., Au core/SiO_2 shell), or a metallic core and a metallic shell (e.g., Au core/Ag shell, Ag core/Ag shell, and Ag core/Au shell), provide a tunable geometry in which the magnitude of the local electromagnetic field at the nanoparticle surface can be precisely controlled ***(48–51)***.

2.3.1. Metal Nanoparticle Island Films

Metallic nanostructured SERS substrates based on metal island films (**Fig. 1A**) are among the most easily prepared surface-based media, granted the availability of a vacuum evaporation system. Such systems are commonly equipped with crystal microbalances for monitoring metal film thickness. Metal island films can be produced by depositing a thin (<10 nm) layer of a metal directly onto a smooth solid base support via sputter deposition ***(40)*** or vacuum evaporation. At such a small thickness, the metal layer forms as aggregated, isolated metal islands, the size and shape of which can be influenced largely by the metal thickness, deposition rate, geometry, and temperature, as well as postdeposition annealing. To optimize the production of SERS-active metal island films, various diagnostic techniques have been used, including optical absorption, atomic force microscopy, SERS, or combinations thereof ***(52,53)***. More recently, silver island films have been characterized by combining NSOM with Raman spectroscopy, thereby achieving <70-nm resolution ***(54)***. A disadvantage of metal island films is that they are easily disturbed by solvents encountered in typical biomedical analyses ***(55,56)***. To minimize this disadvantage, buffer metal layers, (3-mercaptopropyl)-trimethoxysilane layers, and organometallic paint layers have been applied to glass supports to stabilize gold island films ***(57)***.

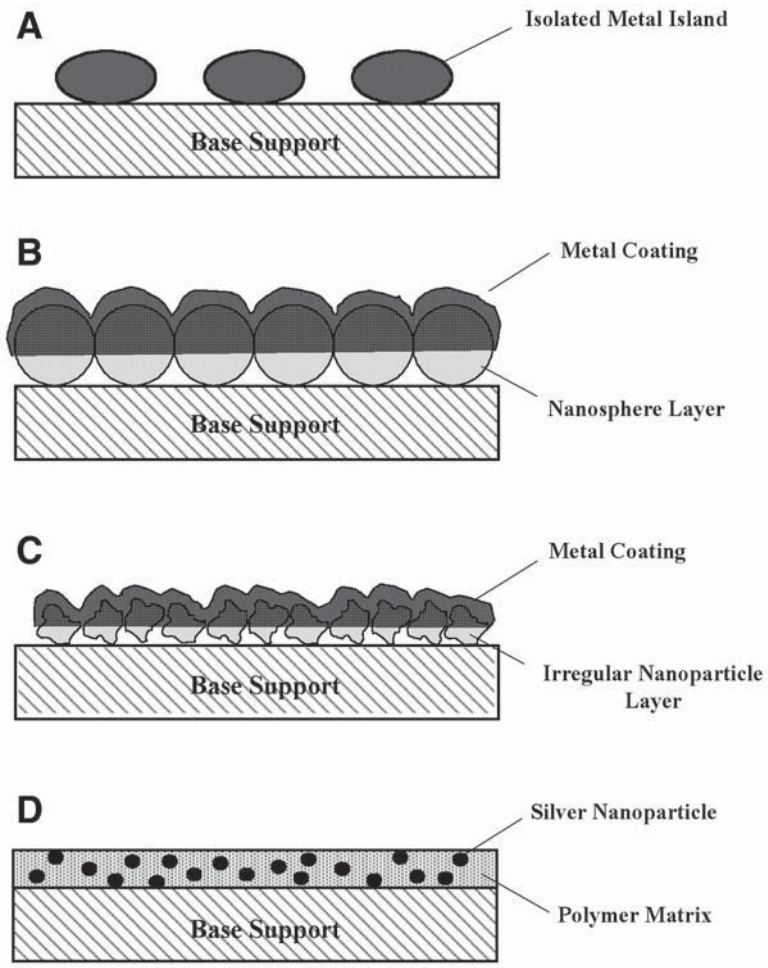

Fig. 1. Various types of SERS-active metallic nanostructures: **(A)** metal island films; **(B)** metal-coated nanospheres (seminanoshells); **(C)** metal-coated random nanostructures; **(D)** polymer coatings embedded with metal nanoparticles.

2.3.2. Metal-Coated Nanosphere Substrates

In our laboratory, we have developed a very dependable solid surface–based SERS substrate technology that can be generally described as metal-coated dielectric nanospheres (**Fig. 1B**) supported by various planar support media. Nanospheres within a specific size range (e.g., 50–500 nm) are spin coated on a solid support in order to produce the roughness required to induce the SERS effect. The resulting nanostructured plate is then coated with a layer of silver (50–150 nm), which provides the conduction electrons required for the surface

plasmon mechanisms. All factors of surface morphology are easily controlled, enabling high batch-to-batch reproducibility. An additional advantage is that the relatively thick layer of silver is less vulnerable to air oxidation than silver islands. Furthermore, the surface is highly resistant to disturbance by sample solvents, making this type of SERS substrate very practical for biomedical applications. Teflon and latex are particularly well suited for SERS substrates because they are commercially available in a wide variety of sizes, which can be selected for optimal enhancement.

Preparation of a nanosphere substrate is relatively easy. A 50-µL aliquot of a suspension of nanospheres is deposited evenly over the surface of a dielectric planar support medium, such as filter paper, cellulosic membrane, glass, or quartz ***(58–62)***. Using a photoresist to produce uniform monolayer coverage, the substrate is then spun at 800 to 2000 rpm for about 20 s. The spheres adhere to the glass surface, providing uniform coverage. The nanosphere-coated support is then placed in a vacuum evaporator, where silver is deposited on the roughened surface at a rate of 0.15 to 0.2 nm/s.

2.3.3. Metal-Coated Nanoparticle Substrates

Nanoparticles with irregular shapes (**Fig. 1C**) can also be used in place of regularly shaped nanospheres in the production of dependable, cost-effective SERS substrates. Dielectric nanoparticle materials investigated in our laboratory have included alumina ***(63)***, titanium dioxide ***(64)***, and fumed silica ***(65)***. The production of irregular nanoparticle-based substrates is achieved with an ease equivalent to that for nanosphere-based substrates described in **Subheading 2.3.2.** Generally, 5 to 10% (w/v) aqueous suspensions of the nanoparticles are spin coated onto solid support media, then coated by 75 to 150 nm of silver via vacuum evaporation. As an alternative to the vacuum evaporation process, other groups have investigated silver coating via chemical processes ***(66,67)***. Nevertheless, substrates prepared via vacuum evaporation yield exceptional reproducibility.

Alumina-based substrates produced by vacuum evaporation have proven to be among the most dependable, with a batch-to-batch variability of typically <10% for induced signals of selected model compounds. The surface of an alumina-based substrate consists of randomly distributed surface agglomerates and protrusions in the 10- to 100-nm range. These structures produce large electromagnetic fields on the surface when the incident photon energy is in resonance with the localized surface plasmons. Alumina-based substrates have numerous applications ***(68–75)***.

Silver-coated titanium dioxide ***(64)*** and fumed silica ***(65)*** surfaces also provide efficient SERS-active substrates. Titanium dioxide provides the necessary nanosize surface roughness for the SERS effect; the nominal particle

diameter of TiO_2 used in our probes is 0.2 μm. When coated with 50 to 100 nm of silver, this type of probe can yield exceptional SERS enhancement with limits of detection of various compounds in the parts-per-billion range. Fumed silica substrates have allowed trace detection of polynuclear aromatic hydrocarbons and pesticides ***(64,76)***.

2.3.4. SERS Substrates Based on Metal-Coated Quartz Posts

Silver-coated, regularly spaced submicron posts formed in quartz substrates have proven to be a dependable, yet labor-intensive, SERS substrate. Lithographic techniques have been used to control the surface roughness to a degree suitable for testing the electromagnetic model of SERS ***(77,78)***. Although these surfaces produce a Raman enhancement on the order of 10^7, they are difficult to produce with a large surface area. However, an alternative etching procedure for producing quartz posts overcomes this limitation by using an island film as an etching mask on an SiO_2 substrate ***(79–83)***. The preparation of SiO_2 prolate posts is a multistep operation, as depicted in **Fig. 2**. A 500-nm layer of SiO_2 is first thermally evaporated onto a fused quartz base support at a rate of 0.1 to 0.2 nm/s. The resulting thermally deposited crystalline quartz is annealed at 950°C to the fused quartz for 45 min. A 5-nm silver layer is then evaporated onto the thermal SiO_2 layer, and the substrate is flash heated (500°C) for 20 s, causing the thin silver layer to bead up in small globules. These isolated silver globules act as etch masks when the substrate is subsequently etched for 30 to 60 min in a CHF_3 plasma. This etching produces submicron prolate SiO_2 posts under the silver globules. Since fused quartz is etched much more slowly than is thermally deposited quartz, the fused quartz base survives the etching process. The posts are then cleaned to remove the silver etch mask and coated with either a continuous 80-nm silver layer ***(83)*** or a discontinuous layer with the silver deposition restricted to the tips of the quartz posts ***(80,81)***. A disadvantage of quartz post–based substrates is the difficult, time-consuming etching procedure required for post fabrication. Nevertheless, optimized quartz posts can serve as the base for a reusable SERS substrate, provided that the silver coating is replaced between uses.

2.3.5. SERS Substrates Based on Metal Nanoparticle-Embedded Media

Silver nanoparticles embedded in various solid porous media (**Fig. 1D**) have recently been investigated as stable SERS substrates with the potential for selective detection. The *in situ* production of these nanoparticles in solid matrices provides several advantages. For example, a solid matrix not only spatially stabilizes but also physically protects the colloids. In addition, the porosity of such materials as sol-gels, cellulose acetate gels, and polycarbonate films permits interaction of analyte compounds with the embedded metal nanoparticles.

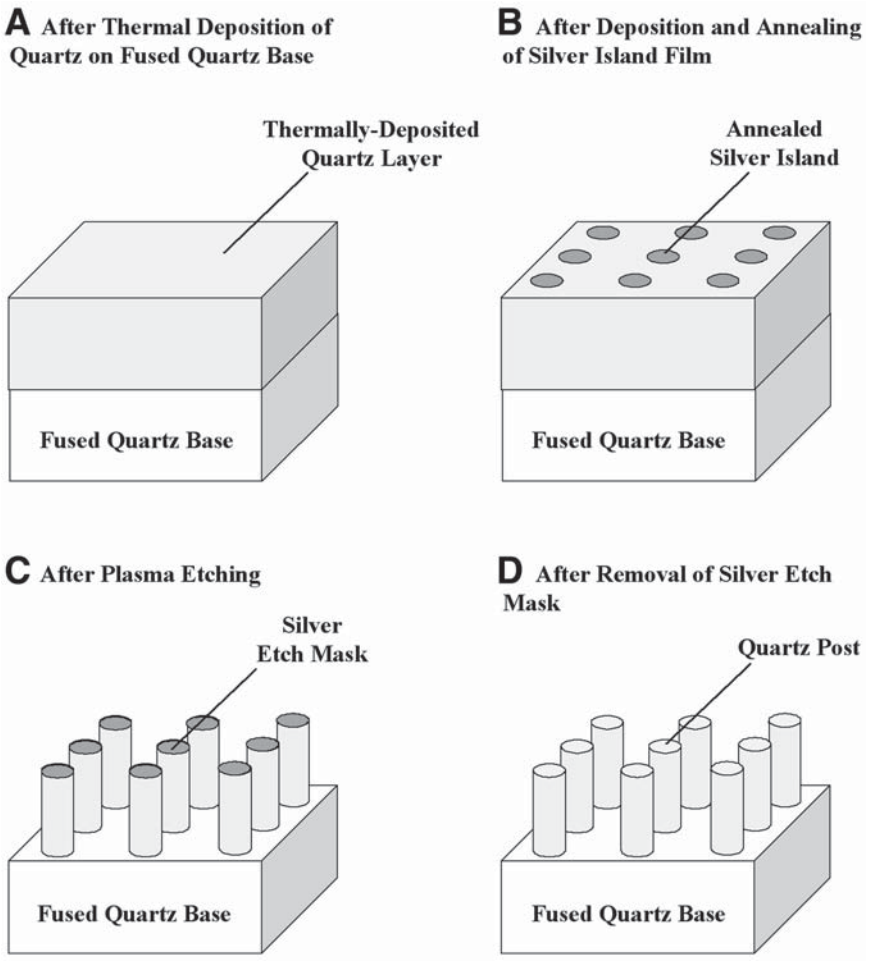

Fig. 2. Schematic diagram of production of SERS-active quartz post: **(A)** after thermal deposition of quartz on fused quartz base; **(B)** after deposition and annealing of silver island film; **(C)** after plasma etching; **(D)** after removal of silver etch mask.

Furthermore, control of pore size and matrix polarity through simple chemical means (particularly when using the sol-gel technique) can impart selectivity. Finally, the chemical processes generally used to prepare such substrates make the SERS technique accessible for general analytical laboratories.

A silver nanoparticle-embedded sol-gel substrate produced through the chemical reduction of silver halide particles distributed in the sol-gel matrix has been reported for the detection of neurotransmitters and dopamine ***(84,85)***. *In situ* precipitation of silver chloride nanoparticles via reaction of silver nitrate

with trichloroacetic acid throughout the sol-gel matrix was performed before curing. Immediately prior to use, the silver chloride particles were reduced to elemental silver nanoparticles with $FeSO_4 \cdot 7H_2O$. An advantage of this technique is that the sol-gel can be stored for long periods in the nonreduced form, precluding vulnerability to air oxidation.

2.4. Overcoatings on SERS Substrates

2.4.1. General Organic, Metallic, and Dielectric Overcoatings

Polymer coatings have been applied to a variety of solid surface–based SERS substrates. In one study, a relatively thick layer (approx 10 μm) of poly-(vinylpyrrolidone) (PVP) was applied to alumina-based silver SERS substrates ***(86)***. In addition to prolonging shelf life, this procedure demonstrated the preservation of surface features vital to the SERS effect by offering resistance to physical disturbance. Furthermore, chemical selectivity was imparted via selective permeability, particularly for compounds having hydrogen-bonding properties. The procedure involved simply dipping the bare silver SERS probes into a 5% (w/v) methanolic solution of the polymer, followed by room temperature curing on a level surface for approx 30 min. PVP coatings have also been applied to silver island–based SERS probes for selective detection of airborne chemicals ***(87,88)***. In these studies ***(87,88)***, the coating was observed to preserve the delicate silver island films over a 20-d period.

A combination of polybenzimidazol and mercaptobenzimidazol has been applied to SERS-active, nitric acid–etched copper foils ***(89)***. This coating was determined to inhibit corrosion and thus prolong the shelf life of the SERS-active substrate. In addition, treatment of silver SERS substrates with various thiols can provide long-term stability, even for substrates stored in water for 1 mo ***(90)***. To promote enhanced adsorption of metal ions (Cu^{+2}, Pb^{+2}, Cd^{+2}), 4-(2-pyridylazo) resorcinol coatings have been applied to SERS-active silver ***(91)***. As an example of the enhanced selectivity offered by reactive coatings in the analysis of complex biological matrices, bilirubin and salicylate have been detected at below normal therapeutic levels via direct SERS analysis of whole blood ***(92)***. In that study, application of the coating, diazonium, precluded the need for a time-consuming and possibly risky preparation of a less complex serum sample. A reactive coating has also been proposed for the one-step detection of the illicit drugs amphetamine and methamphetamine ***(93)***.

Extensive efforts have been devoted to the development of coatings based on inorganic materials. For example, transition metals (Pd, Rh, Pt, Ir, Ru) have been applied to gold substrates as pinhole-free, ultrathin (two to three monolayers) films to enhance adsorption of carbon monoxide and nitric oxide while preserving the SERS-inducing properties of the underlying gold substrates

(94,95). The ultrathin coatings were achieved through a special electrodeposition process. SiO_2-coated silver island–based substrates have also been reported as viable SERS substrates ***(96–98)***. These dielectric-coated substrates have made possible the quantitative detection of dyes adsorbed from solutions. Furthermore, they have been used in fundamental analyte adsorption kinetics studies. SiO_2-coated substrates could prove especially useful in SERS-based biosensors because of the well-defined hydrophilic and stabilizing properties of the coating. Such substrates could be resistant to oxidation and physical damage, hence both prolonging shelf life and offering the potential for reuse.

2.4.2. Self-Assembled Monolayer Overcoatings

Self-assembled monolayers (SAMs) have proven to be a valuable factor in SERS substrate development and theoretical applications of SERS. For example, they have been used to immobilize SERS-active metal colloids on planar supports in a highly ordered fashion ***(99–102)***. In fact, specific colloidal patterns have been prepared by microcontact printing of SAMs on planar surfaces before exposure to SERS-active colloids ***(101)***. SAMs have also been used for the production of UV-induced SERS-active photopatterns on silver films coated with *p*-nitrophenol ***(103)***. In more fundamental studies, the inherent SERS signals of SAMs have been used to evaluate the surface uniformity of SERS probe surfaces, including electrochemically roughened gold electrodes and immobilized gold colloids ***(104)***. The application of SAMs to SERS substrate surfaces also allows selective detection. For example, SAMs of mercaptoalkalinic acids on colloidal silver substrates have been used for the SERS detection of selectively adsorbed cytochrome-c ***(105)*** and have formed the basis for anchoring capture DNA probes for SERS-active hybridization platforms ***(106)***. Monolayers of cysteamine on silver SERS substrates have also been reported ***(107,108)***. Such monolayers could promote the selective adsorption of proteins.

2.4.3. Bioreceptor Monolayer Overcoating

The potential use of SERS in biodiagnostic tests has been demonstrated through the use of immobilized monolayers of bioreceptors, including oligonucleotides and antibodies. The use of surface-enhanced Raman gene (SERG) probes for medical diagnostics ***(109–113)*** is covered in greater detail in **Subheading 4.** In the production of SERG probes, SERS-active dye labels can be attached to oligonucleotide primers used in polymerase chain reaction (PCR) amplification of specific target DNA sequences. Following PCR, the resulting labeled amplicons from the targeted DNA region of interest can be denatured, allowing hybridization of specific, single-stranded, labeled DNA to oligonucleotide capture probes, which can be immobilized on solid supports.

Contact with SERS-active media permits subsequent detection of the SERG probe. This method combines the high sensitivity of the SERS technique with the inherent molecular specificity offered by DNA sequence hybridization.

Antibody monolayers have also been the basis of SERS detection with molecular selectivity ***(114,115)***, thus demonstrating the potential for SERS in immunoassays. In one study, Ni et al. ***(115)*** demonstrated the simultaneous detection of two antigens in a single sandwich immunoassay using two reporter molecules. Capture antibodies selective for the target antigens were bound to gold colloids. The capture antibody–coated colloids were then exposed to a mixture of target antigens. Finally, reporter antibodies specific to the immobilized target antigens were immobilized on the colloid via interaction with the target antigens. SERS-active markers on the reporter antibodies permitted ultrasensitive detection of the immobilized antigens. Each antigen was assigned a different marker, yet both reporters could be detected in a single measurement because of minimal overlap of the respective Raman spectra. In another study, Dou et al. ***(114)*** demonstrated the potential for a SERS-based immunoassay with no need for reporter molecules. Instead, they monitored the native SERS signatures of anti–mouse IgG antibodies adsorbed on gold nanoparticles. Because of the structure-specific nature of Raman scattering, they were able to directly confirm conjugation with antigens through the observation of changes in relative intensities of spectral features of the IgG spectrum. Neither reporter probes nor rinsing steps to remove unbound antigens were required in this assay.

3. SERS as an Immunoassay Readout Method

Immunoassays have received considerable interest for more than a decade. For example, in 1989, Rohr et al. ***(116)*** reported on an early example of a SERS-based immunoassay. The same year, Grabbe and Buck ***(117)*** reported on SERS studies of native human Ig-G. More recently, SERS signatures of native antibodies have been the basis for a simplified immunoassay in which conjugation of the antibody with a target antigen was confirmed through observance of alterations in the SERS spectrum for the native antibody ***(114)***.

As an alternative, extremely sensitive detection can be achieved with reporter antibody probes tagged with intensely SERS-active compounds or with enzymes that react with substrates to yield SERS-active products. These methods often involve sandwich immunoassay techniques, which increase the number of required steps but offer the advantages of excellent sensitivity and the potential for "label multiplexing." For example, Ni et al. ***(115)*** recently reported the simultaneous detection of two types of antigens in a single assay by using two reporter antibodies. The labels of the reporter probes yielded SERS spectral features with minimal overlap, permitting exploitation of a label multiplex advantage. In a sandwich assay format, one set of antibodies

immobilized the target antigens on gold colloids while the reporter antibody probes were subsequently immobilized at allosteric sites of the target antigens.

In other studies, reporter antibodies have been tagged with the enzyme peroxidase ***(118,119)***. Once the peroxidase-labeled antigen was immobilized on the target, it could be exposed to the substrates, *o*-phenylenediamine and hydrogen peroxide. A sustained reaction of the enzyme with a multitude of substrate molecules yielded an extremely high yield of the SERS-active product, azoaniline. This method has thus far been limited to sandwich immunoassay formats. For example, in a study to detect membrane-bound enzymes in cells, cells were first exposed to primary antibodies specific to the membrane-bound enzymes, then exposed to a peroxidase-tagged reporter antibody specific to the primary antibody ***(118)***. A format that uses a capture antibody to immobilize a target antigen, and a reporter antibody to bind to a different antigenic site of the immobilized target antigen, has also been reported ***(119)***.

Recently, successful detection of immunoglobulins using gold nanoshells was achieved in saline, serum, and whole blood ***(120)***. This system constitutes a simple immunoassay capable of detecting subnanogram-per-milliliter quantities of various analytes in different media within 10 to 30 min. Innovative results have also been reported by Mulvaney et al. ***(121)***, who prepared "glass-coated, analyte-tagged" nanoparticles for use in multiplexed bioassays. Recently, Doering and Ni ***(122)*** reported an improved class of core-shell colloidal nanoparticles that are highly efficient for SERS and suitable for multiplexed detection and spectroscopy at the single-particle level. The core-shell structure contains a metallic core for optical enhancement, a reporter molecule for spectroscopic signature, and an encapsulating silica shell for protection and conjugation. With nearly optimized gold cores and silica shells, the core-shell nanoparticles are stable in both aqueous electrolytes and organic solvents, yielding intense single-particle SERS spectra ***(122)***.

4. SERS Gene Probes

There has been a great deal of interest in the development of optical techniques for genomics analysis such as nonradioactive DNA probes for use in biomedical diagnostics, pathogen detection, gene identification, gene mapping, and DNA sequencing. Hybridization of a nucleic acid probe to DNA biotargets (e.g., gene sequences, bacteria, viral DNA) permits a very high degree of accuracy in identifying DNA sequences complementary to that probe. The possibility of using SERS for low-level detection of DNA bases and oligonucleotides was demonstrated in several studies in the 1980s ***(123–126)***. More recently, the possibility of using Raman and/or SERS labels for extremely sensitive detection of DNA has been demonstrated. For example, Graham et al. ***(113)*** have claimed adequate sensitivity for single-molecule DNA detection via sur-

face-enhanced resonance Raman scattering (SERRS). With the use of labeled DNA as gene probes, the SERS technique has been recently applied to the detection of DNA fragments of the human immunodeficiency virus ***(112)*** as well as B-cell lymphoma 2 gene ***(110)***.

A critical aspect of sequencing the entire human genome involves defining and identifying large insert clones of DNA corresponding to specific regions of the human genome. An approach that facilitates large-scale genomic sequencing involves developing maps of human chromosomes into maps based on large-insert bacterial clones, such as bacterial artificial chromosomes (BACs) ***(127–129)***. A time-saving method for detecting multiple BAC clone–labeled probes simultaneously would be especially appealing for the BAC approach to genome sequencing and mapping. The SERS technique can provide this label-multiplex capability. The SERS gene probes described in this section preclude the need for radioactive labels and have great potential to provide sensitivity, selectivity, and label multiplexing for DNA sequencing as well as clinical assays.

A detection system used for recording the SERS spectrum of individual spots is illustrated in **Fig. 3**. An individual spot could correspond to an individual microdot on a hybridization platform. This system can readily be assembled using commercially available or off-the-shelf components. A focused, low-power laser beam is used to excite an individual spot on the hybridization platform. In our studies, a helium-neon laser is used to provide 632.8-nm excitation with approx 5 mW of power. A bandpass filter is used to isolate the 632.8-nm line prior to sample excitation. A signal collection optical module is used to collect the SERS signal at 180° with respect to propagation of the incident laser beam. The collection module includes a Raman holographic filter, which rejects the Rayleigh scattered radiation before it enters the collection fiber. Finally, the collection fiber is coupled to a spectrograph (Instruments S4 Inc., Edison, NJ, HR-320) which is equipped with a Princeton, Trenton, NJ, RE-ICCD-576S detection system. The signal collection module can be coupled directly to the spectrograph, bypassing the optical fiber. However, the optical fiber greatly simplifies measurements and improves reproducibility by minimizing critical optical alignment steps.

Because the SERS gene probe hybridization platform consists of a two-dimensional (2D) array of DNA hybridization spots, a method for recording signals from all spots simultaneously would be highly advantageous, reducing analysis time as well as precluding the need for platform scanning. For analysis of SERS gene probe–based assays, this feat can be accomplished through multispectral imaging (MSI). The concept of MSI is illustrated in **Fig. 4**. With conventional imaging, the optical emission from every pixel of an image is without the capability for tunable wavelength selection (**Fig. 4A**). With con-

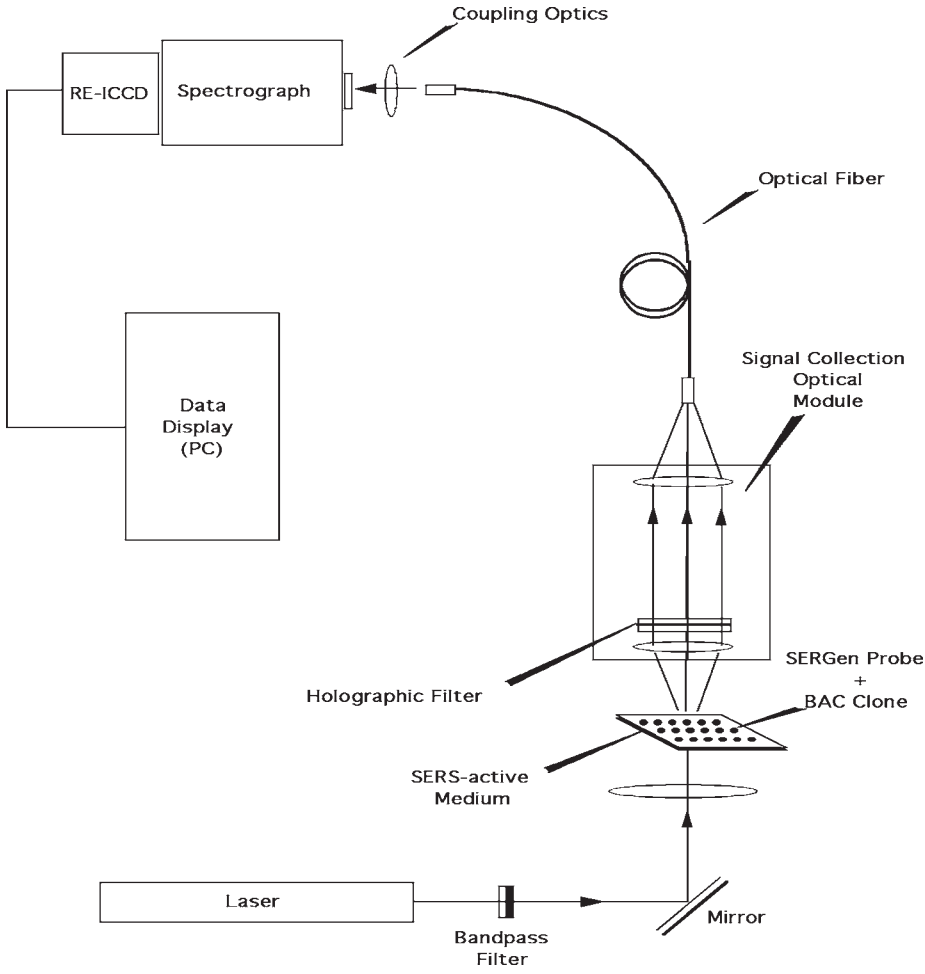

Fig. 3. Instrumental setup for spectral acquisition from individual spots on a bio-assay platform. RE-ICCD, red-enhanced intensified charge-coupled device.

ventional spectroscopy, the signal at every wavelength within a spectral range can be recorded, but for only a single analyte spot. This condition is the basis for the single-point analysis system described above in the previous paragraph. The MSI concept (**Fig. 4C**) combines these two recording modalities, thereby allowing the acquisition of a Raman spectrum for every hybridization spot on the assay platform, provided that the entire platform can be included in the instrument's field of view. Critical to the success of this concept is an imaging spectrometer (**Fig. 5**). In our studies, a rapid-scanning solid-state device, an acoustooptic tunable filter (AOTF), is used for tunable wavelength selection

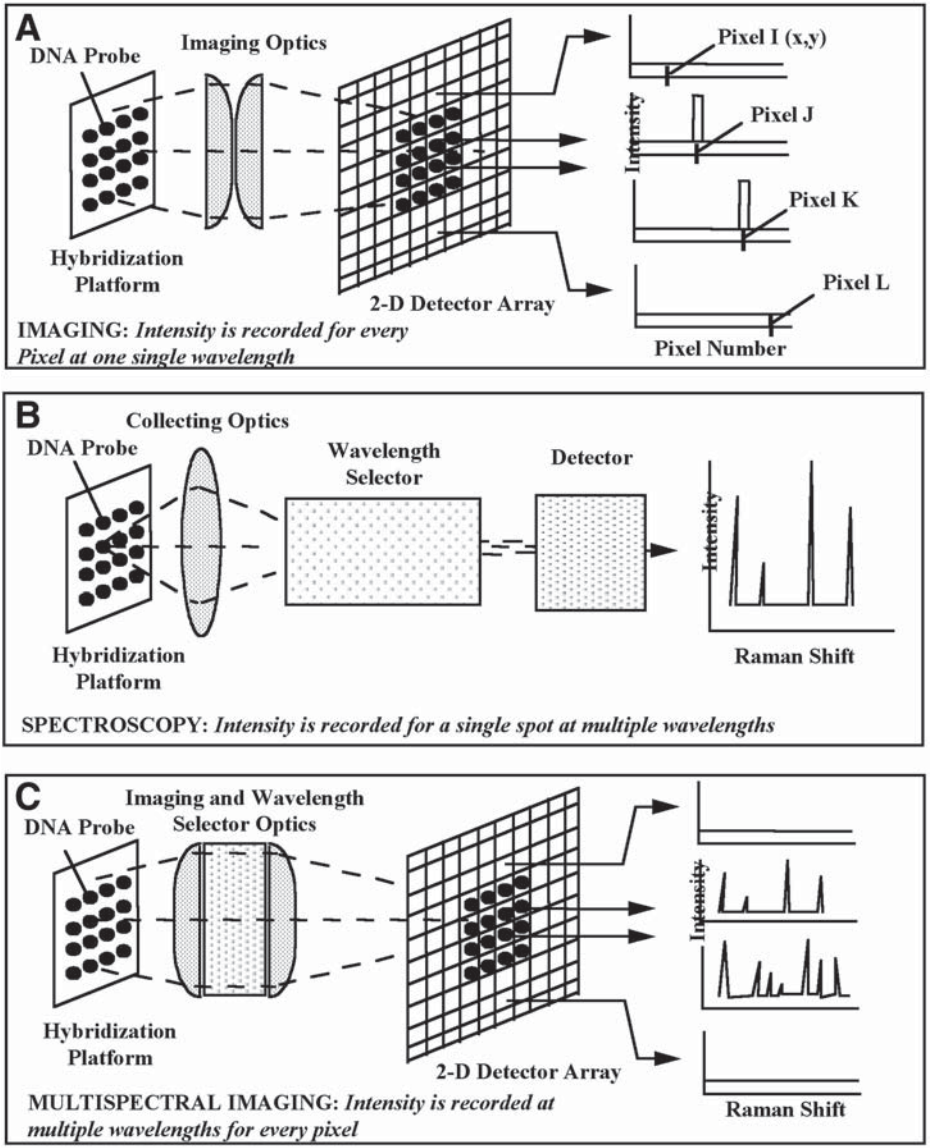

Fig. 4. Various modes of data acquisition from bioassay platforms: **(A)** imaging; **(B)** spectroscopy; **(C)** MSI.

with image-preserving capability. This compact solid-state device has an effective wavelength range of 450 to 700 nm with a spectral resolution of 2 Å and a diffraction efficiency of 70%. Wavelength tuning is achieved simply by supplying the AOTF with a tunable radiofrequency signal.

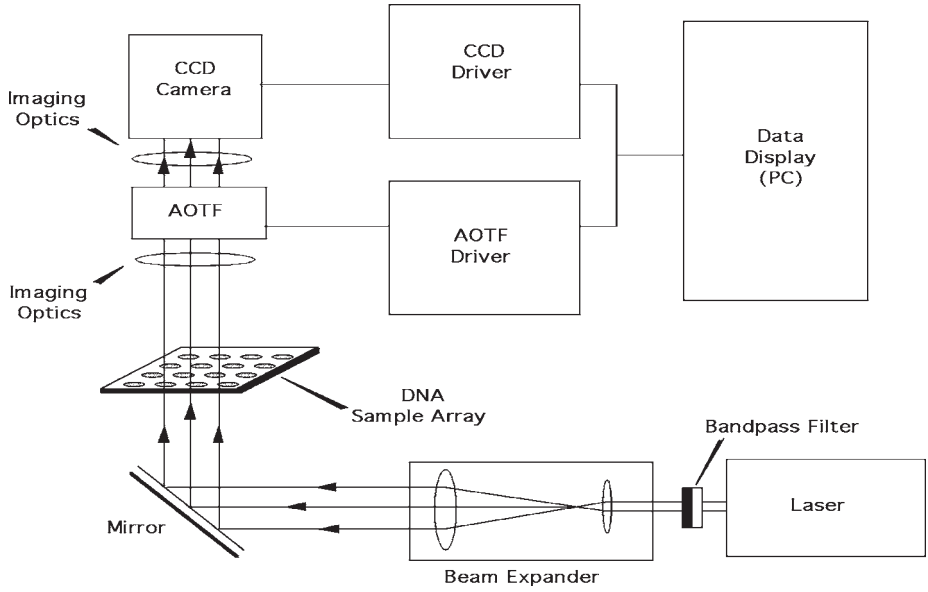

Fig. 5. Schematic diagram of an instrument for MSI of bioassay platforms. CCD, charge-coupled device.

We have recently developed in our laboratory a SERS-active DNA hybridization platform that greatly enhances the utility of the SERS gene probe technology ***(106)***. In previous work, hybridization of labeled DNA probes was performed on conventional commercially available platforms (e.g., DNA-bind plates), followed by SERS activation of the platform ***(24,112)***. To make the SERS gene probe technology more practical, we have developed an inherently SERS-active hybridization platform ***(106)***. No SERS activation of the assay is required following the hybridization step. The new DNA assay platform is an array of oligonucleotide capture probes immobilized directly on a silver island–based substrate prepared on glass. The capture probes were anchored directly to the silver surface via reaction with an SAM of alkyl mercaptans. The coupling approach involved esterification under mild conditions of a carboxylic acid (immobilized mercaptoundecanoic acid) with a labile group; an *N*-hydroxysuccinimide (NHS) derivative; and further reaction with a 5'-amine-labeled DNA capture probe, producing a stable amide ***(130)***. Our recent study demonstrated the potential of this technology in cancer gene detection (*BRCA1* and *BAX* genes) ***(106)***.

5. NSOM SERS Probes

NSOM is a methodology for obtaining subwavelength resolution (20–200 nm) with the spectroscopic information afforded by conventional optical spectro-

scopic techniques ***(131,132)***. The high spatial resolution of NSOM permits spectroscopic studies of individual biomolecules in high-density systems such as encountered in in vivo experiments ***(8,133,134)***. In this respect, NSOM combined with a Raman microscope is particularly attractive. So far, this novel scanning probe technique has been restricted to fluorescence and luminescence spectroscopy. With the use of SERS and tapered single-mode fiber probes, near-field Raman spectra have been obtained from single silver nanoparticles with excitation intensities as low as 10 nW ***(135)***. Recently, the combination of NSOM and SERS to obtain spectral, spatial, and chemical information of molecular adsorbates with subwavelength lateral resolution was reported ***(8,9)***. Near-field SERS spectra of cresyl fast violet and Rhodamine 6G on silver substrates have been obtained. Spectra from as few as 300 molecules or $<10^{-2}$ monolayers adsorbed on about 10 silver nanoparticles can be recorded.

6. SERS as a Tool for Detection of Single-Molecules

There have been several reports of single-molecule detection using SERS in recent years ***(4–7,113,136,137)***. A critical factor in these milestone studies has been the development of exceptional SERS substrates. Most of the reports of single-molecule SERS detection have involved the use of metal colloids in suspensions. For example, Kneipp et al. demonstrated single-molecule detection of crystal violet in both silver ***(5,6)*** and colloid suspensions ***(7)***. In these studies, an effective cross-section of approx 10^{-16} cm^2/molecule was observed, corresponding to a 10^{14} enhancement factor. The gold nanoparticles were commercially available but required proper agglomeration through the addition of sodium chloride. These results are promising because these cyanine dyes could be used as bioassay markers.

In some cases, bioassay markers can be resonance enhanced in addition to benefiting from SERS. For example, a DNA marker, 2,5,1',3',7',9'-hexachloro-6-carboxyfluorescein (HEX), has been used to achieve single-molecule detection via SERRS in a silver colloid suspension ***(113)***. The HEX signature was observed for 8×10^{-13} *M* DNA, which corresponded to less than 1 molecule per probed volume at any time required for measurement. Single-molecule detection has also been demonstrated on planar surface–based substrates. For example, enhancements to factors of 10^{14}–10^{15} have been observed for Rhodamine-6G molecules adsorbed on silver colloids that had been immobilized on a polylysine-coated glass surface ***(4)***. Similarly, 10^{14}–10^{15} factor enhancements have been reported for hemoglobin molecules adsorbed on silver nanoparticles immobilized on a polymer-coated silicon wafer ***(136)***. Researchers in this study reported, however, that single-molecule detection was observed only for hemoglobin molecules situated between and adsorbed to two silver nanoparticles.

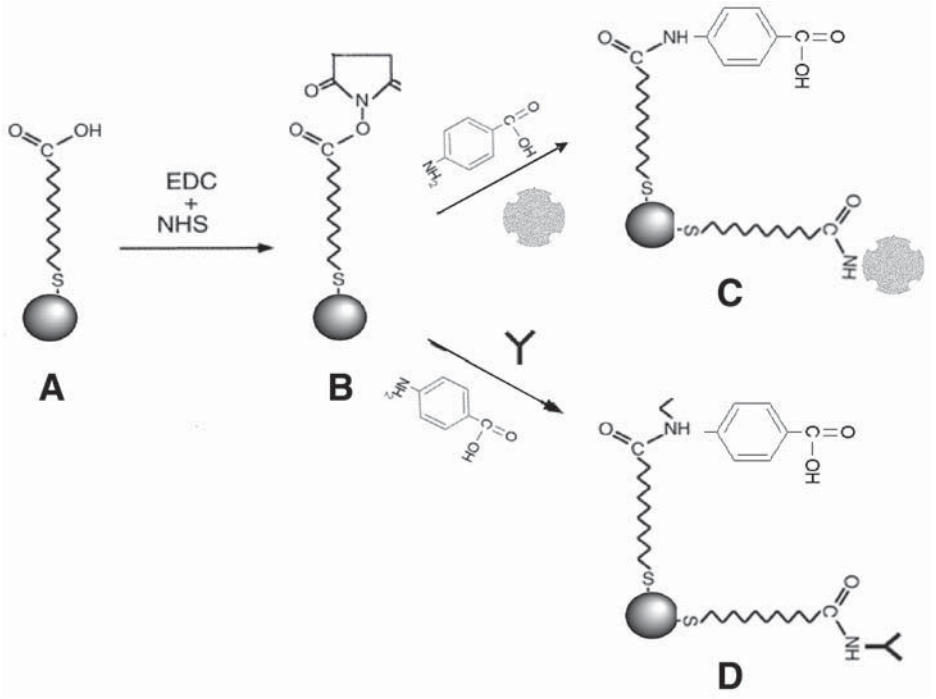

Fig. 6. Chemistry of dual labeling of SERS-active metal nanoparticles. **(A)** Formation of SAM of 11-mercaptoundecanoic acid (MUA) on a metal nanoparticle surface; **(B)** activation of MUA carboxylic acid groups by the carbodiimide, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), and formation of NHS ester by N-hydroxysuccinimide (NHS); **(C,D)** subsequent reaction of activated intermediate with a combination of Raman marker and either streptavidin or amino group–containing biomolecules (e.g., antibodies).

7. SERS Nanoprobes for Intracellular Analysis

Direct observation of single molecules and single molecular events inside living cells could dramatically improve our understanding of basic cellular processes (e.g., signal transduction and gene transcription) as well as our knowledge of the intracellular transport and fate of therapeutic agents (e.g., antisense RNA and gene therapy vectors) ***(106,138)***. Therefore, it is extremely important to develop techniques to measure the reactions of individual molecules in living cells with spatiotemporal resolution ***(139)***.

In our laboratory, we are developing a strategy for the targeted delivery of SERS active metal colloids into single cells ***(140)***. **Figure 6** illustrates the surface chemistry involved in the process. The covalent attachment of a Raman-active marker (e.g., *p*-amino benzoic acid [PABA]) and a universal linker (e.g., streptavidin) onto alkanethiol-modified gold surfaces is achieved by

forming amide bonds via an *N*-hydroxysulfosuccinimide ester intermediate. The free carboxylic acid groups of PABA remain available for derivatization with many amino group-containing biomolecules, and the remaining unoccupied binding sites of streptavidin also allow for ready association with a variety of commercially available biotinylated biomolecules (e.g., antibodies, peptides, and nucleotides) via (strept)avidin-biotin interaction. Thus, the dual labeling on the same SERS-active metal nanoparticles provides a generic format for the functionalization of silver/gold nanoparticles. Considering the high sensitivity of SERS and the great flexibility of the surface modification schemes, the methodology is believed to be able to find wide application in numerous biomolecular systems, especially inside single living cells.

The feasibility of measuring SERS of native constituents with a single viable cell using 60-nm gold particles has been demonstrated recently ***(139)***, in which SERS mapping over a cell monolayer with 1-μm lateral resolution shows different Raman spectra at almost all places, reflecting the highly inhomogeneous chemical organization of the cells. To identify cellular constituents at the single molecule level within single cells, it is clear that many parameters such as excitation wavelength, nanoparticle size, and state of aggregation still need to be optimized.

8. Conclusion

The development of dependable SERS-active metallic nanostructures has spurred renewed interest in Raman scattering as a practical analytical tool in bioanalysis. Because of the mainly electromagnetic origin of the SERS enhancement, it may be possible to achieve a strong SERS effect for each molecule. Until such developments were achieved, there was little interest in Raman scattering–based analysis because the cross-section of normal Raman scattering is minuscule (10^{-30} cm^2/molecule) relative to the cross-sections available through other molecular spectroscopies, particularly luminescence. In the past, high laser powers were used to compensate for this shortcoming. However, this procedure had limited effectiveness, largely because it induced photodecomposition on probed molecules, particularly at trace-level concentrations. By contrast, SERS-inducing media enable trace-level detection with relatively low laser powers.

Both liquid- and solid-based SERS-inducing media are now being used for the detection of biologically significant compounds; moreover, innovative SERS-based biomedical applications such as gene probes are being developed. The development of such substrates and their use in practical analytical applications has required a triumph over the daunting challenge of producing nanoscale structures in reproducible and cost-effective ways. A significant portion of recent efforts has been devoted to investigating SERS coatings to

improve selectivity, longevity, and ruggedness. It is expected that the current development of SERS-active nanostructures will continue to lead to new exciting opportunities in biological applications such as *in situ* cellular monitoring and sensing.

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