Surface-Enhanced Raman Spectroscopy-Active Substrates: Adapting the Shape of Plasmonic Nanoparticles for Different Biological Applications

Elina A. Vitol1,∗,†, Gary Friedman2, and Yury Gogotsi1, ∗

1 Department of Materials Science and Engineering and A.J. Drexel Nanotechnology Institute, Drexel University, Philadelphia, PA 19104, USA
2 Department of Electrical and Computer Engineering and A.J. Drexel Nanotechnology Institute, Drexel University, Philadelphia, PA 19104, USA

We discuss the relationship between the shape of plasmonic nanoparticles and the biological surface-enhanced Raman spectroscopy (SERS) applications which they can enable. As a step forward in developing SERS-active substrates adapted to a particular application, we demonstrate that a modification of the widely used protocol for the sodium citrate mediated reduction of chloroauric acid, which is typically employed only for obtaining spherical gold nanoparticles, can yield flat polygonal nanoparticles at room temperature and a decreased amount of the reducing agent. The significant advantage of the described approach is that it allows for synthesis of nanoparticles with different geometries using a well-established synthesis protocol without the need for any additional chemicals or special synthesis apparatus. By contrasting spherical and anisotropically shaped nanoparticles, we demonstrate that multifaceted nanoparticles with sharp edges are better suitable for SERS analysis of low concentration analytes requiring strong SERS enhancement. On the other hand, gold nanoparticles with isotropic shapes, while giving a smaller enhancement, can provide a more reproducible SERS signal. This is important for analytical applications of complex biological systems where large SERS enhancement may not always be required, whereas data reproducibility and minimal false positive rate are imperative. Using a SERS-active substrate comprising isotropically shaped gold nanoparticles, we demonstrate the differences between Gram-negative (E. coli) and Gram-positive (S. aureus) bacteria, attributable to the outer membrane and peptidoglycan layer, with the level of detail which has not been previously reported with optical spectroscopic techniques.

Keywords: SERS, Gold Nanoparticles, Synthesis, Cells, Bacteria, Sensors.

1. INTRODUCTION
Surface-enhanced Raman spectroscopy (SERS) allows for high sensitivity molecular detection with down to single molecule resolution.1–3 It is established that the main mechanism governing Raman enhancement in SERS is related to plasmonic amplification of electromagnetic field on metal nanoparticles with certain geometrical and material properties.2,4 Therefore, the size, shape and structure of nanoparticles are crucial for achieving the desired Raman scattering amplification at a given excitation wavelength. The problem, however, arises when a question about the reproducibility of SERS spectra is raised. Unlike in regular Raman spectroscopy, where the spectral bands of the same material can be reliably reproduced in different laboratories, SERS features are very much dependent on the substrate, which provides the signal enhancement.3 The ability to control the shape of nanoparticles along with their collective geometry on a substrate is a key requirement for engineering SERS-active substrates with optimal trade-off between the signal enhancement and data reproducibility.

Historically, one of the most widely accepted routes to forming a SERS substrate is to air dry a drop of colloidal gold or silver nanoparticles and then collect multiple spectra until a so-called hot-spot with the strongest signal is found. While this approach is acceptable for detecting low
concentration analytes, which has been a primary application of SERS since its discovery in 1974, it has several disadvantages that may obscure the capabilities of SERS methods. First, the aggregates of nanoparticles formed in this manner have random geometry. Second, this method is difficult to adapt for analysis of extended complex objects such as cell membranes or cell organelles which are much larger than the source of plasmonic enhancement. Looking at this kind of subjects through the prism of SERS, while relying on random aggregated particles, would provide very limited information about their chemical composition and structure since only certain areas of the sample would coincide with the hot spots. An alternative way is to move the hot-spot itself which represents the principle of tip-enhanced Raman spectroscopy. Deckert-Gaudig et al. have recently utilized this method for studying the membrane structure of Halobacterium Salinarum.

The spectral reproducibility in SERS is directly related to the reproducibility of collective nanoparticle geometry which translates to the fact that SERS-active substrates with controlled organization of plasmonic particles are the best candidates for obtaining reproducible data. This can be achieved by creating plasmonic structures with controlled configurations of spherical nanoparticles. As an example, we have previously developed SERS-active nanopipettes with the tip functionalized with gold nanoparticles. Using this probe, we demonstrated well reproducible SERS fingerprints of cell nucleus and cytoplasmic compartments with controlled configurations of spherical nanoparticles. In order to obtain gold nanoparticles with different shapes, we show that smaller isotropically shaped nanoparticles are the best candidates for obtaining reproducible data. An alternative way is to move the hot-spot itself which represents the principle of tip-enhanced Raman spectroscopy. Deckert-Gaudig et al. have recently utilized this method for studying the membrane structure of Halobacterium Salinarum.

Biocompatibility and relative ease of making and using aqueous colloids of gold nanoparticles have made them the most commonly used SERS-active carriers for biological applications. Despite the fact that the synthesis of gold nanoparticles appears to be one of the most widely explored areas in nanotechnology, to the best of our knowledge, there have not been any reports on a single-step synthesis protocol which allows for obtaining nanoparticles with different shapes serving the needs of surface-enhanced Raman spectroscopy. For biological applications, this ranges from single molecule detection to analysis of single living cells.

One of the widely employed protocols for synthesizing colloidal gold nanoparticles is based on the method proposed by Turkevich et al. in 1951. It relies on the reduction of tetrachloroauric acid HAuCl₄ by trisodium citrate at 100 °C. Typically, this method is used for synthesizing nanoparticles with close-to-spherical shapes and the diameters on the order of 10–20 nm, 20 ± 0.15 nm according to the original protocol. It has also been demonstrated that larger equiaxial (close to spherical shape) nanoparticles can be obtained by decreasing the amount of sodium citrate. Also, a number of synthesis protocols for obtaining polygonal plasmonic nanoparticles have been reported in the literature. Photoinduced transformation of silver nanospheres to nanoprisms was performed by exposing the nanospheres to broadband light source. Silver nanoplates were synthesized using silver nitrate reduction in the presence of ethyltrimeethylammonium bromide. Shankar et al. have studied the formation of flat polygonal particles using citric acid as a reducing agent. Using biological reagents, such as bacteria and lemongrass extracts, for synthesis of polydisperse polygonal nanoparticles has been shown as an attractive alternative to these methods. For SERS applications, however, the discussed approaches have the disadvantage of introducing strong background signal from either solvents or by-products of the synthesis reaction.

In this work, we show that gold nanoparticles with both isotropic and anisotropic shapes can be obtained by the well-known method of sodium citrate reduction of tetrachloroauric acid which is typically used only for making spherical particles with diameters below 100 nm. The ultimate goal of this work is to develop SERS-active substrates suitable for studying biological samples ranging from low concentration single analyte solutions to complex biological systems such as cell membranes. Herein, it will be shown that SERS-active substrates fabricated using flat polygonal nanoparticles with sharp edges are best suitable for detection of low-concentration analytes, as demonstrated by studying the SERS spectra of glycine. Further, we show that smaller isotropically shaped nanoparticles that yield a weaker but more uniform SERS enhancement, when deposited on a substrate with controlled inter-particle distance, are instrumental for studying complex extended objects such as, for example, the outer membranes of prokaryotic cells. The results demonstrate significant differences in SERS fingerprints of Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli) bacteria with the level of detail which have not been previously reported.

2. MATERIALS AND METHODS

Trisodium citrate, ethanol, glycine and sodium hydroxide were purchased from Sigma Aldrich. HAuCl₄ was purchased from AlfaAesar.

2.1. Synthesis of Gold Nanoparticles

In order to obtain gold nanoparticles with different shapes and sizes, the synthesis temperature and the amount of sodium citrate used for HAuCl₄ reduction were varied as described below. All glassware was washed with aqua regia and thoroughly rinsed with the deionized water.

- 10 ml of 1 mM HAuCl₄ were mixed with 0.2 ml of 1 wt% of trisodium citrate (Na₃C₆H₅O₇·2H₂O) aqueous solution at room temperature and left for 24 h stirring slowly. The final solution was light orange in color.
10 ml of 1 mM HAuCl₄ were mixed with 0.5 ml of 1 wt% of sodium citrate aqueous solution at room temperature with slow stirring. The mixture turned blue 5 min after mixing and after 30 min continued stirring it changed the color to dark pink.

10 ml of 1 mM HAuCl₄ were brought to boiling and then 0.2 ml of 1 wt% of sodium citrate aqueous solution was added with vigorous stirring. The mixture became red 5 minutes after the addition of the reducing agent.

10 ml of 1 mM HAuCl₄ were brought to boiling and then 0.5 ml of 1 wt% of sodium citrate aqueous solution was added with vigorous stirring. The mixture became deep red in color almost immediately after the addition of the reducing agent.

2.2. Fabrication of SERS Substrates

The substrates were fabricated according to the previously published protocol using all four types of the gold nanoparticles synthesized for this study. Briefly, 1 × 1 cm pieces of microscope glass slides were washed with the mixture of 210 ml of 95% ethanol mixed with 35 g NaOH dissolved in 140 ml MilliQ water. Then the slides were thoroughly rinsed with MilliQ water, dried, and dip coated with 0.001% poly-L-lysine and dried at room temperature for 24 h. PLL coating enables the electrostatic binding of negatively charged gold nanoparticles and positively charged amine groups of PLL. In the last step, the slides were coated with gold nanoparticles by dipping them in the gold colloid for 3 h.

2.3. Characterization of Nanoparticles

SEM images of nanoparticles were collected with the field emission scanning electron microscope Zeiss Supra 50VP (Carl Zeiss SMT AG, Germany). The microscope was operated in high vacuum mode. For imaging, gold nanoparticles were deposited on a standard aluminum SEM stub. The images were collected at 5 kV accelerating voltage. The absorption spectra of nanoparticle colloids were acquired using a UV-VIS spectrophotometer (Thermo Scientific, Evolution 600). Zeta potential of gold nanoparticles was measured using a Zetaziser Nano ZS (Malvern Instruments, UK). For all preparations, the characteristic zeta potential was about −40 mV.

2.4. SERS Measurements

Raman spectroscopy analysis was performed using a micro-Raman spectrometer (RM 1000, Renishaw, UK) equipped with a diode laser operating at 785 nm wavelength (1200 lines/mm grating) with nominal power of 100 mW. The laser source was focused on the sample through a 50×, 0.75 NA objective and the scattered signal was acquired through the same objective. The signal was collected with the high sensitivity ultra-low noise RenCam CCD detector. The acquisition time was 30 s. Data analysis was performed using the Renishaw WiRe 2.0 software.

2.5. Bacterial Culture

Escherichia coli, K-12 strain, (E. coli) and Staphylococcus aureus (S. aureus) were purchased from American Type Culture Collection (ATCC, Manassas, VA). For SERS measurements, the bacteria were suspended in 0.25 ml of phosphate buffer saline at the concentration of approximately 10⁵ cells/ml.

3. RESULTS AND DISCUSSION

3.1. Characterization of Nanoparticles

The absorption spectra of the nanoparticle colloids and their corresponding photographs are shown in Figure 1. The designations of nanoparticle types are based on the synthesis protocol. Samples 1 and 2 correspond to nanoparticles produced at the room temperature with 1:50 and 1:20 ratios between the reducing agent (trisodium citrate) and 1 mM HAuCl₄, respectively. Samples 3 and 4 were synthesized with the same respective ratios of the reagents at 100 °C. The absorption spectra demonstrate that nanoparticles produced at room temperature using the lowest amount of sodium citrate are polydisperse as indicated by very broad plasmon absorption band (trace 1). This observation is further confirmed by SEM analysis (Fig. 2, panel 1).

The mechanism of anisotropic nanoparticle growth is likely to be attributed to the presence of Cl⁻ ions. Studying the role of different halide ions (F⁻, Cl⁻, Br⁻ and I⁻) in synthesis of polygonal gold nanoparticles, Shankar et al. have shown that Cl⁻ has the largest effect on inhibiting the gold crystal growth along (111) direction thus promoting the formation of flat nanoparticles. A more detailed study of the nanoparticles’ growth is beyond the scope of this work and would be a subject of a separate study. Kimling et al. have also shown that sodium citrate reduction method can yield flat gold nanoparticles at room temperature under UV illumination. Our results show that the polygonal gold particles can be obtained just
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Figure 2. SEM micrographs of the nanoparticles synthesized at room temperature (1, 2) and at 100 °C (3, 4) with 1:50 (samples 1 and 3) and 1:20 (samples 2 and 4) ratios between the reducing agent and HAuCl₄.

by using sodium citrate when performing the synthesis at the room temperature (23 °C) without the need for any additional reagents or activation conditions. The shape of the nanoparticles can be tailored by controlling the synthesis temperature and a larger number of flat polygons with respect to more spherical particles can be obtained at lower sodium citrate concentrations. The latter can be seen from comparing subsets 1 and 2 in Figure 2. A larger proportion of close-to-spherical nanoparticles with respect to polygonal ones in the type 2 colloid indicates that the crystal growth is defined by the relative amount of sodium citrate. SEM analysis shows that the colloids synthesized at 100 °C did not contain any flat polygonal nanoparticles (Fig. 2, subsets 3 and 4). Further purification and separation of particles can be achieved by centrifugation, as typically done with gold nanorods.²²

These findings are important not just in view of broadening the applications of the Turkevich method, but also for opening the opportunities for synthesizing multifaceted flat nanoparticles without using natural reducing agents which could contaminate the nanoparticles by various organic by-products. The latter is especially important in SERS when the nanoparticles are intended for high sensitivity measurements were the presence of any contaminants is likely to result in spurious Raman bands and obscure the actual analytical signal. The SERS functionality of the synthesized nanoparticles is of primary interest for our work, so we will now consider their applications.

3.2. Spherical and Anisotropic Gold Particles as SERS-Active Substrates for Detection of Low Concentration Analyte

SERS spectra of low concentration (1 mM) aqueous solution of glycine, which is undetectable by regular Raman spectroscopy, obtained with all 4 types of the nanoparticles are shown in Figure 3. For each sample type, the measurements were repeated at least 10 times. The SERS-active substrates were prepared as described in Methods. Glycine was chosen for this study as a simplest amino acid with well-characterized Raman scattering properties. The SERS spectra were acquired in liquid after the glycine solution was placed on the substrate. The most pronounced spectral band which appears in the SERS spectra of glycine for each type of substrate is at about 735 cm⁻¹. This band is characteristic for NH₂ functional group of glycine. Podstawka et al. have previously shown that enhancement of the NH₂ bands in the SERS spectra of glycine obtained on gold substrates can be attributed to the preferential adsorption of this group onto gold.²³ The differences between glycine spectra measured with different types of SERS-active substrates are likely to be due to variations in electromagnetic field enhancement on these substrates. The results of modelling electromagnetic field distribution on polygonal nanoparticles have shown that the strongest electromagnetic field is localized at the edges of nanoparticles.²⁴ This phenomenon can be directly related to the fact that the substrates containing flat polygonal nanoparticles (type 1) yield the SERS spectra of glycine with the most pronounced bands (Fig. 3, trace 1).

3.3. SERS Analysis of Wall Structure for Gram-Positive and Gram-Negative Bacteria

The SERS-active substrates based on gold nanoparticles with close-to-spherical shapes (type 4) were applied for investigating the SERS fingerprints of S. aureus and E. coli as representatives of Gram-positive and Gram-negative bacteria. This type of nanoparticles (type 4 according to the synthesis protocol) was selected due to their uniform size distribution, which translates to improved reproducibility of SERS spectra obtained with them. While type 1
multifaceted polygonal nanoparticles yield stronger SERS enhancement, as described above, it is important to note that in many applications it is more advantageous to use nanoparticles which provide weaker but more reproducible enhancement. A SERS-active substrate with uniformly spaced 50 nm gold particles yields well reproducible spectra of biological molecules with inherently complex composition, such as calcium second messengers. Moreover, it eliminates the need for the so called “activation” when smaller 10–20 nm colloidal nanoparticles are aggregated prior to SERS measurements. Although the formation of large aggregates can yield stronger SERS enhancement, it can also negatively affect the spectral data reproducibility due to the non-uniform distribution of SERS hot spots on such aggregates. It has been estimated that hot spots occupy less than 1% of the total substrate area available for analyte molecule adsorption.

Here, *Escherichia coli* and *Staphylococcus aureus* bacteria were analyzed. To illustrate the data reproducibility, Figure 4 shows multiple representative spectra collected for each type of bacteria. It can be clearly seen that the SERS fingerprints of these bacteria are very distinct. The differences between SERS spectra of Gram-positive and Gram-negative bacteria (in our case, *S. aureus* and *E. coli*, respectively) can be primarily attributed to the difference in their wall structure.

Gram-negative bacteria contain an additional outer membrane surrounding the peptidoglycan layer and the underlying plasma membrane with only the latter two being present in Gram-positive bacteria types. Since here the intact bacteria are lying on a SERS substrate rather than being impregnated with nanoparticles, we are primarily probing the Raman signature of the lipid-rich outer membrane in case of *E. coli* and for *S. aureus* we are looking at the structure of peptidoglycan layer. As seen in Figure 4, the SERS spectrum of *E. coli* contains 5 strong bands at 717, 914, 1068, 1112 and 1218 cm\(^{-1}\). The C—N and C—C stretching in the lipid layer exhibit themselves in the 717 and 1068 cm\(^{-1}\) bands and have been shown before.

Prominent bands at 914 and 1112 cm\(^{-1}\) are likely due to skeletal modes in proteins. For *S. aureus*, there is a larger number of weak yet consistently appearing bands with narrow peaks at 926 and 1565 cm\(^{-1}\) and multiple bands in the 900–1200 cm\(^{-1}\) region. The domination of protein and carbohydrate-attributable bands is indicative of the SERS spectrum being characteristic for the “exposed” peptidoglycan layer in *S. aureus* which is covered with the outer membrane in *E. coli*.

It is crucial to note that the spectral differences observed in this work are very pronounced compared to the previously published data when the spectra of several types of bacteria share a large number of characteristic bands, without major differences between Gram-positive and Gram-negative bacteria. Bacterial SERS spectra have been studied using multiple excitation wavelengths, ranging from 488 to 785 nm, and various substrates have been employed for this purpose, including aggregated silver nanoparticles and even nanoparticles synthesized on bacteria itself. However, given the complexity and the size of bacteria with respect to the plasmonic nanoparticles, we hypothesize that regularly structured SERS-active substrates are most suitable for analyzing this kind of a subject. In other words, the “hot spots” which are currently considered to be responsible for large SERS enhancement on randomly aggregated gold and silver nanoparticles are instrumental for analyzing ultralow concentration analytes where the spectral reproducibility might be, in some cases, given a lower priority over the fact of the spectral detection. Recently, the methods for guiding a molecule adsorption onto a specific hot spot have been proposed which opens the possibility for studying multicomponent single molecule solutions with SERS. For complex systems such as bacteria and especially eukaryotic cells, it appears to be practical to use SERS-active substrates with controlled geometry of plasmonic elements which are not necessarily capable of distinguishing single molecule events but can yield reproducible spectra, where the reproducibility is within the margins defined by the SERS mechanism which does not exclude the possibility of spectral flickering and occasional modulations of intensity in certain bands.

![Figure 4](image-url). SERS fingerprints of (a) *E. coli* and (b) *S. aureus*. The spectra were measured with a self-assembled monolayer of type (4) gold nanoparticles (50 nm diameter) on a glass substrate coated with poly-L-lysine. Multiple spectra were acquired across the substrate and the results clearly demonstrate that the data is well reproducible. Distinct characteristic spectral features are attributed to the difference in the outer wall composition and structure of Gram-positive and Gram-negative bacteria.
4. CONCLUSIONS

The geometry of gold nanoparticles used for making SERS-active substrates should be taken into account in view of different types of biological applications. As a step forward in developing SERS substrates tailored to a particular application, we demonstrate that a modification of the widely used protocol for the sodium citrate reduction of chloroauric acid, which is typically employed only for obtaining spherical nanoparticles, can yield flat polygonal nanoparticles at room temperature and a decreased amount of the reducing agent. Flat multifaceted plasmonic nanoparticles with sharp edges are particularly important for SERS analysis of very dilute analytes where strong SERS enhancement is critical. On the other hand, gold nanoparticles with isotropic shapes, while giving a lower enhancement, provide a more reproducible SERS signal that is important in analytical applications that need minimal false positive rate, such as detection and identification of Gram-positive and Gram-negative bacteria. The significant advantage of the described approach is that it allows for synthesis of nanoparticles with different geometries using a well-established synthesis protocol without the need for any additional chemicals or special synthesis apparatus. Using a SERS-active substrate comprising isotropically shaped gold nanoparticles, we demonstrated the differences between \textit{E. coli} and \textit{S. aureus}, attributable to the outer membrane and peptidoglycan layer, with the level of detail which has not been previously reported with optical spectroscopic techniques.

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References and Notes

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