Experimental parameters influencing surface-enhanced Raman scattering of bacteria

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Abstract. Surface-enhanced Raman scattering (SERS) is a powerful technique for the analysis of a variety of molecules and molecular structures. Due to its great complexity, the acquisition of detailed molecular information from biological organizations such as bacteria is still a challenging task. SERS can provide valuable information once silver or gold surfaces can be brought in close contact with the biological organization. Because several experimental parameters can affect SERS spectra of bacteria, the experimental conditions must be well defined for comparable and reproducible results. The influence of experimental parameters, such as the type of noble metal, size, and aggregation properties of nanoparticles, and the wavelength of the laser light on the SERS of *E. coli* and *B. megaterium* are examined. It is demonstrated that the impact of these parameters could be enormous and a standard protocol must be developed depending on the goal of the study. © *2007 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2798640]

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1 Introduction

In recent years, there has been considerable interest in the application of surface-enhanced Raman scattering (SERS) to investigate biological structures. This is mostly due to its immunity to water, which is an essential component of biological samples, and the simplicity of the sample preparation. When the analyte of interest is in solution, the choice is almost always a gold or silver colloidal suspension for the SERS experiments. Along with the type of colloidal suspension, other experimental parameters, such as the type, size, and aggregation properties of nanoparticles, and wavelength of the light source ought to be studied for optimum results.[1]

A number of small molecules,[2] biomolecules,[3–5] and living cells[6–9] are studied using SERS. Information regarding the study of biological mass structures such as whole bacterium cells is still limited and more research must be devoted to understand the fundamentals of the SERS of biological structures. In all reported bacterial SERS studies, a gold or silver colloidal suspension was employed. The SERS of photosynthetic bacterium was reported by Holt and Cotton.[10] Since this first report, several groups have demonstrated the feasibility of acquiring and using the SERS spectra for identification and classification.[11–19] Efrima and Bronk, Zeiri et al., and Zeiri and Efrima added the bacteria into a solution where silver was reduced to nanoparticles.[11–13] Thus, they successfully covered the bacterial surface with silver nanoparticles. They also successfully introduced silver nanoparticles inside the bacterium and demonstrated that the SERS spectra obtained from the bacterium cell wall and inside the bacterium were different.[11] In their study, they used a 532-nm laser light to collect the SERS scattering. In another study by Sengupta, Laucks, and Davis, Sengupta, Mujacic, and Davis, and Laucks et al., several aspects of the SERS of bacteria and pollens were explored.[14–16] Their sample preparation was similar to Zeiri et al., where they placed the bacteria in colloidal solution until the colloids were adsorbed on the bacterial wall, before SERS acquisition by employing a laser at 514-nm wavelength. They successfully studied the influence of colloid reproducibility, particle size, and aggregation on SERS spectra in solution. They reported that bacterial SERS spectra evolve over time and stabilize within a few hours. Jarvis and Goodacre, and Jarvis, Booker, and Goodacre used silver colloids and 532-, and 785-nm wavelength laser light in separate studies.[17,18] They prepared samples by simply mixing the bacteria with the silver colloidal suspension and acquired the SERS spectra after the sample was dried at room temperature. Premasiri et al. attached the gold nanoparticles on silica particles before mixing with bacterial cells.[19]

One of the greatest advantages of using SERS for identification and classification of bacteria is the speed. Previous studies have clearly demonstrated that SERS spectra of bacteria can be used for fast identification and classification.[11–19] However, to achieve this goal, it is necessary to understand all the parameters establishing a concrete protocol for reproducible SERS spectra. Once the goal is to identify the bacteria fast, solid consideration must be given to sample preparation. We have chosen to prepare samples by simple mixing in a

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way that is similar to the one reported by Jarvis et al. In this respect, we have examined several parameters influencing the SERS of bacteria by simple mixing. The size of a bacterial cell is enormous compared to average-size biomolecules. Thus, it is important to understand the behavior of nanoparticles and bacteria cells when they are in the sample. In the case of a molecule, mixing causes the molecules to be adsorbed on nanoparticles if the sizes of the molecules are much smaller than the size of the nanoparticles. However, for a biochemical mass such as a bacterium, nanoparticles are mostly adsorbed on the bacterial cell wall, depending on the size of the nanoparticle. Thus, the obtained bacterial sample would be quite different from the molecular sample.

Previously, we studied the effect of increased concentration of the silver colloidal suspension concentration, surface charge, and pH on bacterial SERS, and found that these factors have an enormous impact on SERS spectra of bacteria.[20] This study as an extension of our previous study explores the influence of experimental parameters such as the type of noble metal, size, and aggregation characteristics of nanoparticles, along with laser wavelength on SERS spectra of bacteria. Two model bacteria, *E. coli* and *B. megaterium*, were chosen for the study.

2 Materials and Methods

2.1 *Chemicals*

$AgNO_3$ (99.5%) was purchased from Fluka (Seelze, Germany). Sodium citrate (99%) and nutrient agar were purchased from Merck (Darmstadt, Germany). $HAuCl_4 3H_2O$ was purchased from Alfa Aesar (Karlsruhe, Germany). All chemicals were used as received without further purification.

2.2 *Preparation of Bacteria Samples*

For SERS measurements, both bacteria were obtained from our microorganism collection (Yeditepe University, Genetics and Bioengineering Department) and were verified by Sherlock Microbial Identification System version 4.5 (MIDI, Newark, Delaware) before use. The bacteria were grown axenically and aerobically at 37°C on 20-mL nutrient agar and collected with sterile plastic inoculating loops from solid culture plates. The samples were collected after the bacteria were cultured 12 h in all experiments. The collected samples were added into 1-mL deionized water, vortexed, and centrifuged for 5 min at 7500 rpm. The supernatant was discarded. This procedure was repeated three times. 5 μL of each washed bacterium was added into a 100-μL silver colloid suspension. Then, it was mixed with a vortex to create a mixture as homogenous as possible. 5 μL of this mixture was placed onto a glass slide or CaF_2 and dried at room temperature for about 15 min before analysis.

2.3 *Preparation of Silver Colloids*

Ag colloid was prepared by the method reported by Lee and Meisel.[21] Briefly, 90-mg AgNO3 was dissolved in 500-ml water. This solution was heated to a boil. 10-ml aliquot of 1% sodium citrate was added into the solution and kept boiling until the volume reached half of the initial volume. The maximum of its absorption was recorded at 420 nm.

Fig. 1 SEM image of silver nanoparticles synthesized with citrate reduction method.

2.4 *Preparation of Gold Colloids*

Gold nanoparticles were prepared by the citrate reduction of $HAuCl_4 \cdot 3H_2O$ at six different sizes (16, 25, 41, 72, 98, and 147 nm) by adding different volumes of the citrate solution.[22–24] To obtain 16-nm gold nanoparticles, 1.00 mL of the 1% citrate stock solution was added into 50 ml of $HAuCl_4 \cdot 3H_2O$ solution (0.01%). To synthesize 25-, 41-, 72-, 98-, and 147-nm gold nanoparticles, 0.75, 0.50, 0.30, 0.21, and 0.16 mL of 1% citrate stock solution was added into 50 mL of $HAuCl_4.3H_2O$ solution, respectively. The $HAuCl_4 \cdot 3H_2O$ solution was heated until it boiled, then the citrate solution was added. The final solution was kept boiling for 15 min.

2.5 *Raman Instrumentation*

All measurements were performed using a completely automated Renishaw InVia Reflex Raman Microscopy System (Renishaw Plc., New Mills, Wotton-under-Edge Gloucestershire, United Kingdom) equipped with an 830-nm diode and 514-nm argon-ion lasers. The laser power was in the range of 0.2 to 6 mW, and the exposure time was 10 sec for a 830-nm diode laser and 120 sec for a 514-nm wavelength laser. A 50× objective was used. The wavelength of the instrument was automatically calibrated using an internal silicon wafer, and the spectrum was centered at 520 cm^{-1}.

2.6 *Scanning Electron Microscope*

The prepared gold and silver nanoparticles and bacterial samples were spotted and dried on a scanning electron microscope (SEM) specimen stub. A Karl Zeiss EVO 40 model SEM instrument was used. The accelerating voltage was in the range of 5 to 10 kV.

3 Results and Discussion

Figure 1 shows the SEM image of silver nanoparticles synthesized with the citrate reduction method, which produces mostly spherical and a few of rod-shaped silver nanoparticles in the range of 40 to 60 nm. Morphological SEM study of the gold colloids synthesized by citrate reduction revealed that the gold nanoparticles had spherical shapes and were reasonably uniform, with an average aspect ratio of 1:1, as seen in Fig. 2 compared to the silver nanoparticles. Figure 2(a) shows the

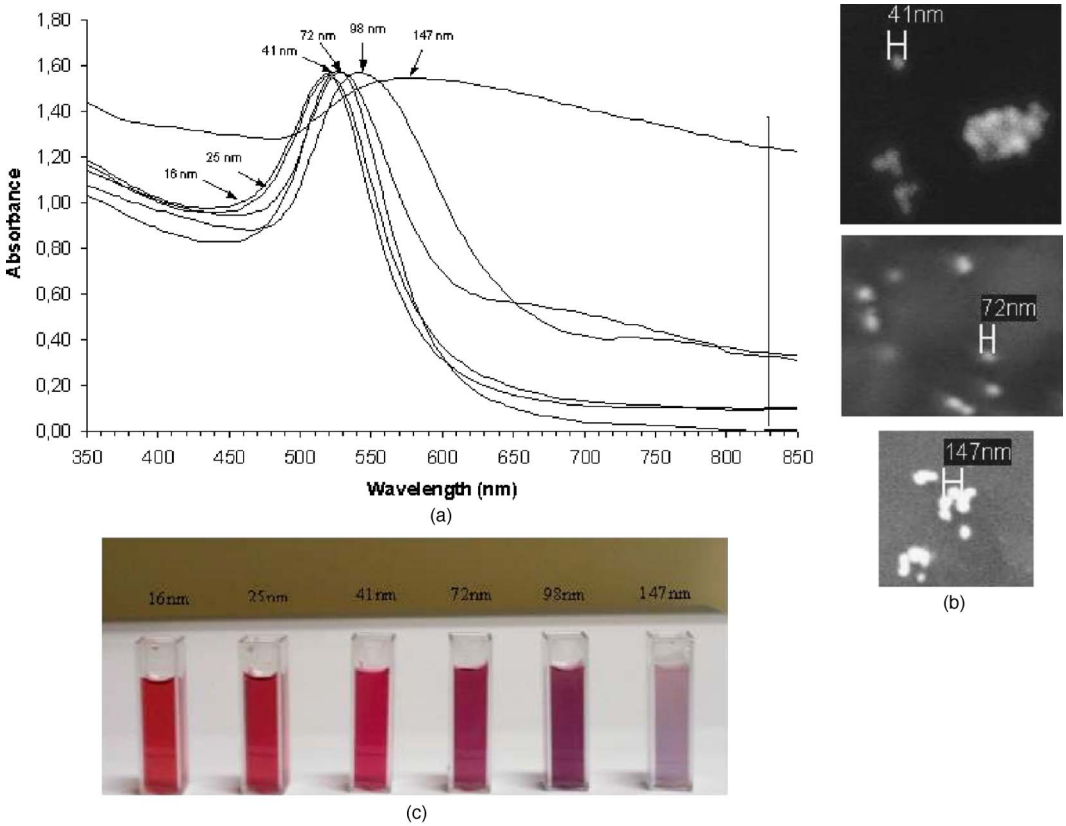


Fig. 2 (a) UV-visible absorption changes of colloidal gold (16, 25, 41, 72, 98, and 147 nm). (b) SEM images of selected sizes of gold nanoparticles. (c) Color change of gold colloidal suspension as the size changes (color online only).

shift of maximum absorption from 520 nm to 575 nm as the particle size increases. Figure 2(b) shows the SEM images of selected sizes of gold nanoparticles at 41-, 72-, and 147-nm average sizes as examples. Figure 2(c) shows the shift of the resonance wavelength toward the longer wavelengths with the particle size increasing as the color changes.

Figure 3 shows the aggregated silver nanoparticles upon mixing with an *E. coli* sample. A certain degree of aggregation is preferred because Raman scattering enhancement is limited for single silver nanoparticles. Indeed, a tremendous enhancement with the use of a NIR laser wavelength on silver nanoparticle aggregates was reported.[25] The citrate-reduced gold and silver nanoparticles stay well dispersed in the suspension, and they only form small aggregates upon evaporation of water when spotted on a surface such as glass and mica, or upon addition of an ionic compound such as NaCl into the colloidal suspension.[26,27] In this study, the sample was prepared by simple mixing of bacteria and colloidal suspension and drying the sample at room temperature. Although the drying can cause some aggregation, inspection of Fig. 3 reveals that mixing a bacterium sample generates a higher degree of aggregation. This aggregation tendency can be explained with the change of surface charge properties of the nanoparticles due to the presence of metabolites and the remnants of the culture media during the washing procedure in the bacterial sample.

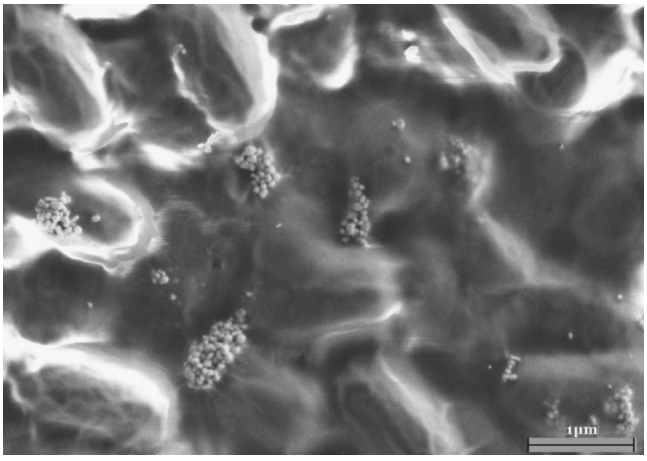


Fig. 3 SEM image of *E. coli* sample prepared with silver nanoparticles.

The wavelength of the laser light can be an important factor, depending on the gold or silver colloids used in the SERS experiments. A wavelength in the visible region could be a proper selection with the use of the silver colloid, because the surface plasmon excitation takes place in the visible region, while a wavelength in the NIR region could be suitable with the gold colloid due to the shift of surface plasmon excitation to the longer wavelengths. The surface plasmon resonance of the silver and gold nanoparticles shift to the longer wavelengths as they form aggregates. The relationship between aggregation and SERS enhancement was studied for gold and

silver nanoparticles in detail.[25,26] The presence of a threshold size for both gold and silver nanoparticle aggregates was reported for SERS enhancement. After a certain size of aggregate, the enhancement in Raman scattering is not significant for small molecules.[25,27–29] The unusual enhancement in Raman scattering with silver or gold clusters can be explained with the overlapping surface plasmons of individual nanoparticles at the particle cross sections.[25–28] Although the bacterial cell is much larger than a molecule, and the interaction is only possible for silver or gold nanoparticles and their aggregates through the bacterial cell wall, a significant improvement in spectral quality and reproducibility is observed with the use of silver colloid, along with a laser wavelength in the NIR region for bacterial SERS.[20] The improvement observed in the quality of SERS spectra in this study can also be attributed to the collective contribution of surface plasmons of nanoparticles forming the aggregates. The other contributing factor to the quality of the SERS spectra could be the improved penetration depth with the use of NIR laser light compared to laser wavelength in the visible region. The sample is prepared by casting the mixture on a glass or CaF_2 slide. A multilayer bacteria-nanoparticle-aggregate mixture is evident, and it is very possible that silver nanoparticles and their aggregates are trapped among the bacteria cells. While the laser light travels deep into the sample, it increases the possibility of interacting with more nanoparticle-bacteria interfaces, which increases the number of spots to collect Raman scattering. Therefore, the use of a NIR laser wavelength and silver colloid results in good quality and more reproducible SERS spectra, compared to the wavelength in the visible region. Figures 4(a) and 4(b) show the SERS spectra of *E. coli* with the use of silver nanoparticles by employing a laser light at two different wavelengths, 514 and 830 nm, respectively. This comparison reveals that the quality of the SERS spectra obtained with both lasers on silver colloid is of good quality. However, the use of a 514-nm wavelength laser generates highly irreproducible SERS spectra from spot to spot and requires longer collection times.

Figure 4(b) shows the influence of the nanoparticle type on SERS spectra of *E. coli* with the use of a 514-nm wavelength laser. Because a 514-nm wavelength light is not a suitable resonance wavelength for the gold colloid, no enhancement was observed. On the contrary, the SERS spectra obtained with the use of silver colloid results with a good quality spectrum at this wavelength. Figure 5 shows the comparison of SERS spectra of *B. megaterium* with gold and silver nanoparticles with the use of an 830-nm wavelength laser. A colloidal suspension containing the 147-nm size nanoparticles was used for the preparation of the sample to compare with the silver colloidal suspension. The SERS spectra obtained with gold and silver colloids at 830 nm are comparable. The more features observed on the spectrum obtained with the use of the gold suspension can be explained by the closer match of surface plasmon resonance and the excitation wavelength.

To investigate the influence of the size of nanoparticles on bacterial SERS, gold nanoparticles in the range of 16 to 147 nm were synthesized and employed. The initial concentration of gold salt used for the synthesis of the gold colloid remained the same, and only the concentration of citrate solution was varied. Thus, the concentration of gold nanoparticles in the colloidal suspension decreases with the increased

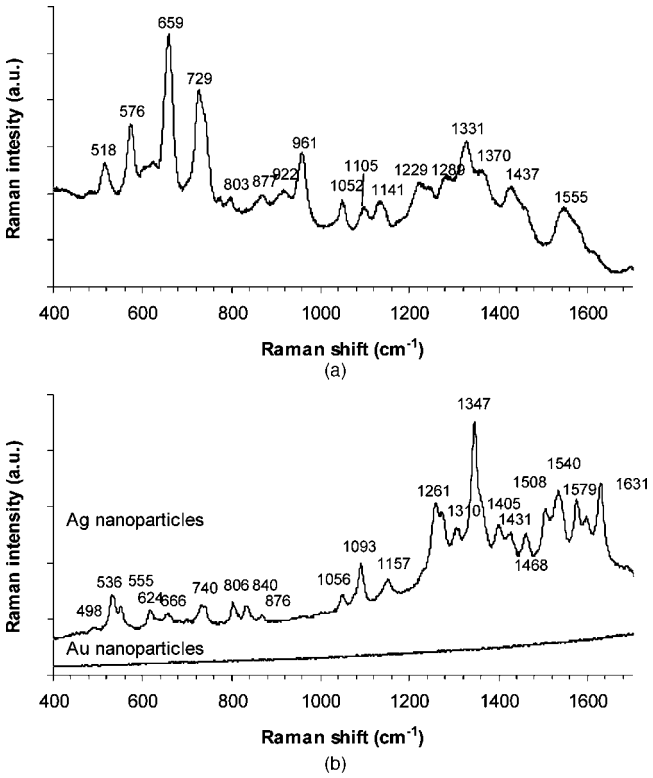


Fig. 4 SERS spectra of *E. coli* with (a) silver nanoparticles at 830-nm wavelength, and (b) gold and silver nanoparticles at 514-nm wavelength.

particle size. Because the improvement in the SERS spectra quality is related to the particle size and the degree of aggregation, the behavior of gold nanoparticles when mixed with bacteria was investigated with a SEM. Figures 6(a)–6(c) show the SEM images of three sizes of gold nanoparticles mixed with bacterial samples. It was observed that mixing of the bacteria and colloid suspension caused the formation of aggregates to a certain degree. The SEM images indicated that the number of nanoparticles in the aggregates was increased as the size of the gold nanoparticles increased. The average number of nanoparticles in the aggregates was estimated to be in the range of 10 to 25 [Fig. 6(a)], 20 to 35 [Fig. 6(b)], and

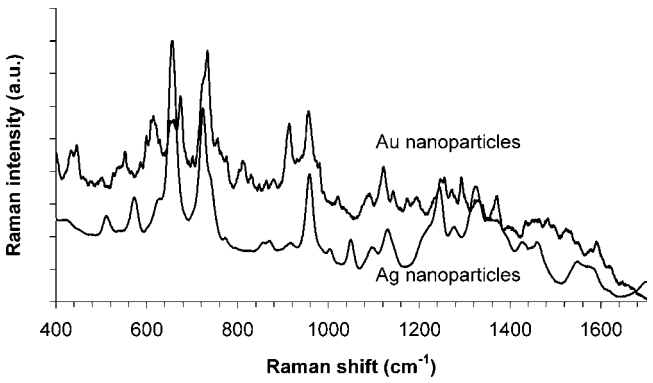
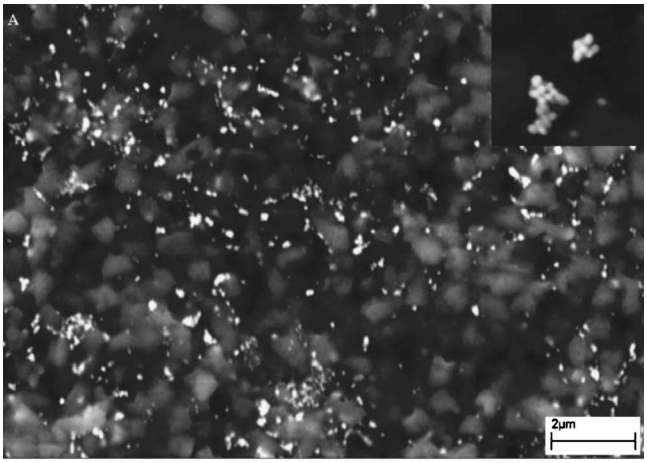
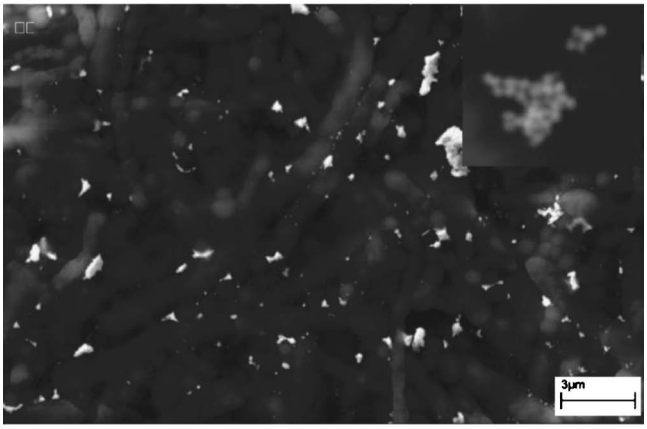


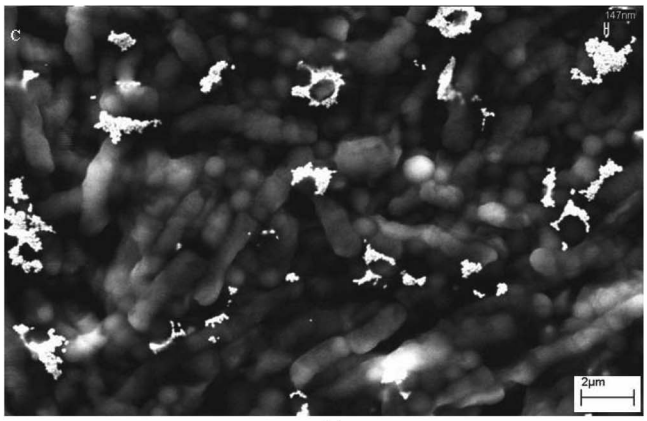
Fig. 5 SERS spectra of *B. megaterium* with gold and silver nanoparticles using a laser at 830-nm wavelength.



(a)



(b)



(c)

Fig. 6 SEM images of *E. coli* and *B. megaterium* sample prepared using (a) *E. coli* sample prepared with 25-nm gold nanoparticles, (b) *B. megaterium* sample prepared with 72, and (c) *B. megaterium* sample prepared with 147-nm gold nanoparticles.

30 to 50 [Fig. 6(c)] per aggregates, for 25-, 72-, and 147-nm sizes of gold nanoparticles, respectively. Because the number of nanoparticles is smaller in a colloidal suspension with a larger nanoparticle size than in a colloidal suspension with a smaller nanoparticle size, the number of aggregates appears to be smaller in a bacterial sample prepared with a colloid containing a larger size of gold nanoparticles. Thus, the mean distance between the aggregates becomes greater as the size of nanoparticles is increased. The smaller sizes of gold nanoparticles form smaller aggregates, and it tends to reside less in the number of gold nanoparticles in each aggregate compared to the aggregates formed from larger gold nanoparticles. Although it is a fact that the enhancement of Raman scattering is directly proportional to the size of the gold nanoparticles,[30] the presence of aggregates in the sample suggests that aggregation can also be a contributing factor for the improvement in the SERS spectra seen in Fig. 7. It should be noted that the aggregation behavior of silver and gold nanoparticles seems independent of the type of bacteria and does not lead to an extensive aggregation.

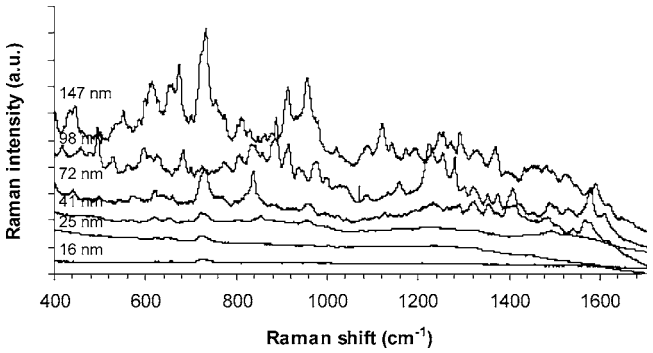


Fig. 7 Influence of gold nanoparticle size on SERS spectrum of *B. megaterium*.

4 Conclusions

The type, size, and aggregation properties of nanoparticles, along with the laser wavelength, are important factors. The results indicate that a laser wavelength in the NIR region is a more suitable choice for both silver and gold colloids for bacterial SERS. The size and aggregation properties of colloids are the other factors influencing the SERS spectra, and a size greater than 72-nm for gold nanoparticles could be a more proper choice. The experimental factors investigated in this study could have enormous impact on bacterial SERS, and they must be taken into serious consideration while planning experiments.

Acknowledgments

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