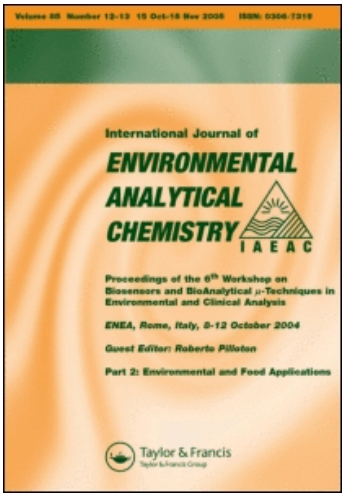

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Towards single-microorganism detection using surface-enhanced Raman spectroscopy

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Towards single-microorganism detection using surface-enhanced Raman spectroscopy

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The identification and discrimination of microorganisms is important not only for clinical reasons but also for pharmaceutical clean room production and food-processing technology. Vibrational spectroscopy such as IR, Raman, and surface-enhanced Raman scattering (SERS) can provide a rapid 'fingerprint' on the chemical structure of molecules and is used to obtain a 'fingerprint' from microorganisms as well. Because of the requirement that a single bacterium cell and noble metal nanoparticles must be in close contact and the lack of a significant physical support to hold nanoparticles around the single bacterium cell, the acquisition of SERS spectra for a single bacterium using colloidal nanoparticles could be a challenging task. The feasibility of SERS for identification down to a single bacterium is investigated. A Gram-negative bacterium, *Escherichia coli*, is chosen as a model for the investigation. Because the adsorption of silver nanoparticles onto the bacterial cell is an exclusive way for locating nanoparticles close to the bacterium cell, the absorption characteristics of silver nanoparticles with different surface charges are investigated. It is demonstrated that the citrate-reduced colloidal silver solution generates more reproducible SERS spectra. It is found that *E. coli* cells aggregate upon mixing with silver colloidal solution, and this may provide an additional benefit in locating the bacterial cell under a light microscope. It is also found that a laser wavelength in the UV region could be a better choice for the study due to the shallow penetration depth. It is finally shown that it is possible to obtain SERS spectra from a single cell down to a few bacterial cells, depending on the aggregation properties of bacterial cells for identification and discrimination.

Keywords: Microorganism; Detection; SERS; Raman; Nanoparticles

1. Introduction

Detection and identification of pathogenic microorganisms continue to be a challenge in medical, environmental, and pharmaceutical samples, for example. Once a pathogenic microorganism enters the food chain or the environment through terrorism or another route, it may cause great damage to human health

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and the environment. For microorganisms that cannot be easily isolated and cultured, there is a need for an analytical technique that can analyse a single microorganism cell in a short time frame. Due to its 'fingerprinting' property, Raman spectroscopy can provide very specific information about a sample's chemical content in a few seconds and has been recently shown to be an emerging technique for fast and reliable microorganism detection [1–8]. For the theory and applications of Raman spectroscopy, many fine books are available [9]. Although Raman spectroscopy has several advantages such as its immunity to water and easy sample preparation over IR, it suffers from a weak scattering and high fluorescence background, especially with biological samples. The weak scattering phenomenon of Raman spectroscopy can be overcome using noble metal surfaces such as gold, silver, and copper to enhance the scattering, which reaches the sensitivity of fluorescence under the certain experimental conditions. The use of noble metal surface or colloidal particles also quenches fluorescence background. With the introduction of the Raman microscopy system, it is now possible to focus the laser beam down to micrometre sizes and collect chemical information from very small spots on the sample. The Raman microscopy system enables even single microorganisms to be targeted [10, 11]. The use of SERS was reported for bulk microorganism identification and classification [12–18]. These studies had shown that identification of a microorganism based on its 'fingerprint' SERS spectra could be successfully accomplished once a standard protocol was established.

When a silver or gold colloidal solution is mixed with a bacterial sample, a non-uniform mixture is obtained. Depending on the size of the nanoparticles, some are adsorbed on the bacterial cell wall, and some are arbitrarily distributed in the sample. The bacterial cells also provide physical support for each other to hold the larger nanoparticles around themselves. When the bacterial cell number is limited in the sample, the nanoparticles lack this physical support, and only adsorbed nanoparticles remain on the bacterial cell wall. Because of the proximity to the molecular structure and size requirement of the silver or gold nanoparticles in the SERS experiments, there must be enough adsorbed nanoparticles in close contact with the bacterial cell wall. This study is undertaken to clarify whether a simple mixing procedure can be achieved to place enough silver nanoparticles on the bacterial cell wall for SERS enhancement and whether the SERS spectra obtained from such a sample can be used for identification and discrimination. Efrima and Bronk, and Sengupta *et al.* investigated the adsorption of silver nanoparticles onto the bacterial cell wall and obtained the SERS spectra from bulk samples [12, 16]. However, they did not attempt to collect SERS spectra from the single bacterial cell or bacterial aggregates. In addition, their sample preparation was quite different from our sample preparation. Efrima and Bronk added bacteria into the solution in which Ag^{+} ions were reduced, thus covering the bacterial cell wall with silver nanoparticles [12]. Sengupta *et al.* simply added the bacteria into a silver colloidal solution and waited for the adsorption of silver nanoparticles onto the bacterial cell wall [16]. This study differs from other studies on sample preparation and takes advantage of the Raman microscopy system to focus the laser beam onto a single bacterial cell or aggregates composed of a few bacterial cells.

2. Experimental

2.1 *Chemicals*

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

2.2 *Preparation of bacteria samples*

Escherichia coli (35218 ATCC) used in this study were obtained from our microorganism collection (Yeditepe University, Genetics and Bioengineering Department). They were verified by Microbial Identification System (MIDI) before use and were grown axenically and aerobically for 24 h at 37°C on 20 mL of nutrient agar. The bacteria were collected with sterile plastic inoculating loops from solid culture plate. The collected samples were added into 1 mL of d.i. water, vortexed, and centrifuged for 5 min at 7500 rpm. The supernantent was discarded. This procedure was repeated three times. The 5 μL of solution of each washed bacterium was added into a 100 μL silver colloidal solution. Then, the solution was mixed with a vortex to yield a homegenous mixture. A 5 μL solution of this mixture was placed onto a CaF_2 slide and dried at room temperature for 15–30 min before analysis. For single bacterial cell analysis, a dilute bacterial solution was mixed with silver colloidal solution before being spread on a CaF_2 slide. Then, the sample was dried at room temperature before analysis.

2.3 *Preparation of silver colloids*

Ag colloid was prepared by the method reported by Lee [19]. Briefly, 90 mg of $AgNO_3$ was dissolved in 500 mL of water. This solution was heated to boiling point. A 10 mL aliquot of 1% sodium citrate was added into the solution and kept boiling until the volume reached half the initial volume. The maximum absorption was recorded at 420 nm.

Sodium-borohydride-reduced silver nanoparticles were prepared using Creighton's method [20] with some modifications. One hundred millilitres of 6 mM $AgNO_3$ and 300 mL of 5 mM sodium borohydride were prepared and cooled in an ice bath for 30 min. The ice-cold $AgNO_3$ was added into the ice-cold solution of sodium borohydride drop by drop while stirring the mixture. The colour of the solution turned yellowish. The maximum absorption was recorded at 395 nm.

2.4 *Raman instrumentation*

All measurements were performed using a completely automated Renishaw InVia Reflex Raman microscopy system (Renishaw Plc., New Mills, Wotton-under-Edge, UK) equipped with an 830 nm diode and 514.5 Argon-ion lasers. The laser power was in the range of 0.2–6 mW, and the exposure time was 10 s. A 50× objective was used.

The wavelength of the instrument was automatically calibrated using an internal silicon wafer, and the spectrum was centred at 520 cm^{-1}. For bulk-bacterial-sample experiments, a CaF_2 slide was used, and for single-cell experiments, a glass slide was used. Because of the great penetration depth of laser light at 830 nm, a 514.5 nm argon-ion laser was used to acquire the single bacterial cell SERS spectra while the bacteria cells were on the glass slide. All spectra except those for the single-cell experiments were taken with an 830 nm wavelength laser.

3. Results and discussion

Figure 1(a) and (b) shows the SEM images of bacteria simply mixed with silver colloidal nanoparticles. Because the SEM resolution is limited and does not show the silver nanoparticles smaller than 3–5 nm, a transmission electron microscopy (TEM) image was necessary to be able to see the adsorbed nanoparticles on the bacterial cell wall. A TEM image *E. coli* cell was obtained by Sengupta *et al.* after the adsorption of silver nanoparticles on the bacterial cell wall suspended in a silver colloidal solution [21]. They also studied the adsorption dynamics of silver nanoparticles on the bacterial cell wall and found that the quality of the SERS spectra increased with time due to the increased number of nanoparticles on the cell wall. Because it is evident from this study that the smaller silver nanoparticles are adsorbed to the bacterial cell wall, we did not obtain a TEM image. However, we focused on obtaining a quality and reproducible SERS spectra from the single or aggregates of bacteria. Because the nature of the interaction between the nanoparticles and the bacterial cell wall determines the density and proximity of the nanoparticles to the molecular structures on the cell wall, the SERS fingerprint will also be influenced. The ionization status of the functional groups on the wall structure of the bacterial cell that may affect this condition determines the degree of accumulation of the silver nanoparticles on the cell wall. Obtaining a rich fingerprint spectrum from a single bacterium cell strongly depends on the coverage of silver nanoparticles on the bacterial cell wall. Thus, the interaction of colloidal nanoparticles with different surface charge densities (polarities) with bacterial cell wall

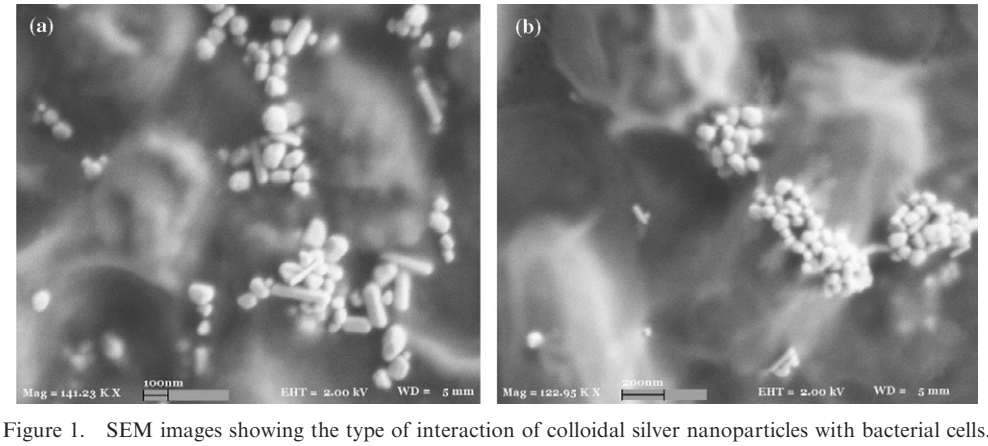

Figure 1. SEM images showing the type of interaction of colloidal silver nanoparticles with bacterial cells.

was first investigated. Figure 2 shows the SERS spectra of *E. coli* obtained with reduced silver colloidal nanoparticles with borohydride (Creighton's method) (a and b) and reduced citrate (Lee's method) (c and d), respectively. The nanoparticles synthesized using Creighton's method have a less negative charge density than with Lee's method [22, 23]. Thus, the nanoparticles synthesized with each method are expected to interact with bacterial cell wall at different strengths. Although this difference is observable between the SERS spectra obtained with the silver nanoparticles synthesized by two different methods, the use of silver nanoparticles synthesized by Creighton's method generates more irreproducible SERS spectra. Figure 2 compares the SERS spectra of *E. coli* acquired with silver colloidal suspensions synthesized with Creighton's method (the spectra (a) and (b) obtained with different batches) and Lee's method (the spectra (c) and (d) obtained with different batches). It may be concluded that the silver nanoparticles synthesized by Creighton's method interact with the bacterial cell wall to a much greater degree and generate more detailed SERS spectra, while the use of the silver nanoparticles synthesized using Lee's method generates more reproducible SERS spectra. Because the reproducibility is a more serious matter in bacterial SERS, the use of nanoparticles synthesized by Lee's method is preferred when attempting to collect SERS spectra from a single bacterial cell or aggregates.

The Raman microscopy system used in this study is equipped with two lasers, a diode laser at 830 nm and an Ar+ laser at 514 nm. Our previous studies showed that a diode laser at 830 nm was a more appropriate choice for bulk samples prepared with silver nanoparticles [24]. This is due to the greater penetration depth and better resonance characteristics of the aggregated silver nanoparticles at NIR wavelength regions. In bulk samples, as the light penetrates into the sample, it meets more silver nanoparticle-bacterial cell wall interfaces, thus generating more reproducible SERS spectra. However, the 514 nm laser provided better results for the single bacterial cell study as shown here. We believe this is mostly due to the shallower penetration depth at this wavelength compared with a wavelength in NIR region when the laser beam is focused on a single bacterial cell or bacterial aggregates. The stability and performance of silver colloidal particles in the bacterial sample were tested at 830 nm wavelength

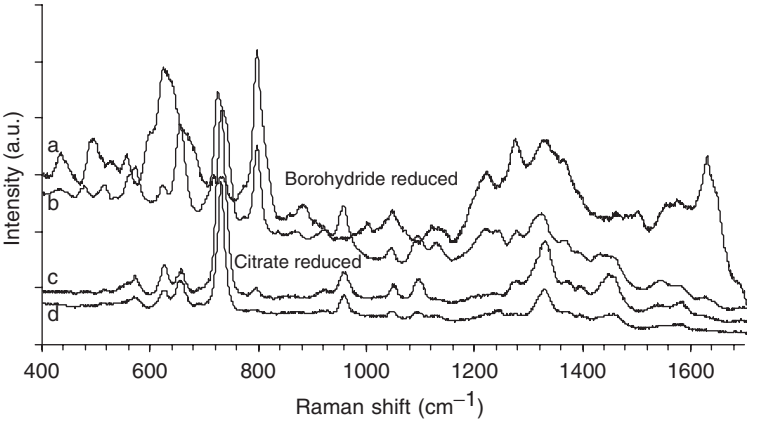

Figure 2. SERS spectra of *E. coli* acquired using borohydride- and citrate-reduced silver nanoparticles.

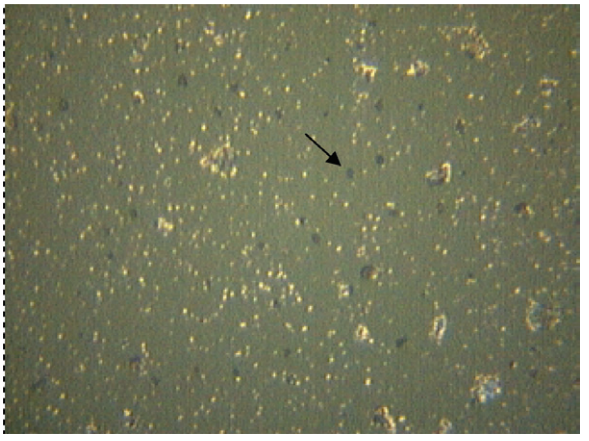

Figure 3. White-light images of aggregated *E. coli* cells (dark spots) and silver nanoparticle aggregates (bright spots) under a 50× objective.

because the variation from spot to spot and sample to sample in the SERS spectra when using a 514 nm wavelength laser is enormous for bulk samples [24].

This diluted bacterial solution was mixed with the silver colloidal solution and spotted on a glass slide. Figure 3 shows the bacterial aggregates and the silver nanoparticle aggregates under the microscope objective. The bright spots shown in figure 3 are the silver nanoparticle aggregates, and the brightness is the result of the excited surface plasmons of nanoparticles. This phenomenon has helped us to locate the bacterial cell(s). Although it was possible to locate the single bacterial cell under the light microscope before mixing with silver nanoparticles, this was not possible after mixing, and only small aggregates of bacterial cells with a few of them were observed. This could be due to the aggregation tendency of bacteria under unfavourable conditions.

The SERS spectra from these aggregated bacteria cells were sucessfully acquired. Figure 4(a) shows the SERS spectrum from a bulk bacterial sample. When a diluted bacterial solution was mixed with a silver colloidal solution, it was observed that *E. coli* cells were aggregated possibly due to the unfavourable condition in the final mixture. Thus, it was not possible to locate a single *E. coli* cell under the microscope objective. However, the sizes of *E. coli* aggregates suggested that there were only a few *E. coli* cells. Figure 4(b) shows the SERS spectrum obtained from such an aggregate. The collection of SERS spectra from these aggregates in 10 s indicates that the silver nanoparticles are in the close vicinity of bacterial cells. The silver nanoparticles could be both adsorbed on the *E. coli* cell wall and trapped among the aggregated *E. coli* cells. These results show that simple mixing can be sufficient for this type of study. In addition, the aggregation of bacteria after mixing with silver colloidal solution can be beneficial when analysing the real samples because the mixing procedure may force bacteria cells to aggregate. This may be helpful to locate the bacterial aggregates along with surface plasmons of aggregated silver nanoparticles. The comparison of both SERS spectra from bulk and aggregates reveals that the SERS spectra obtained from one to a few aggregated *E. coli* cells can be used for identification and discrimination.

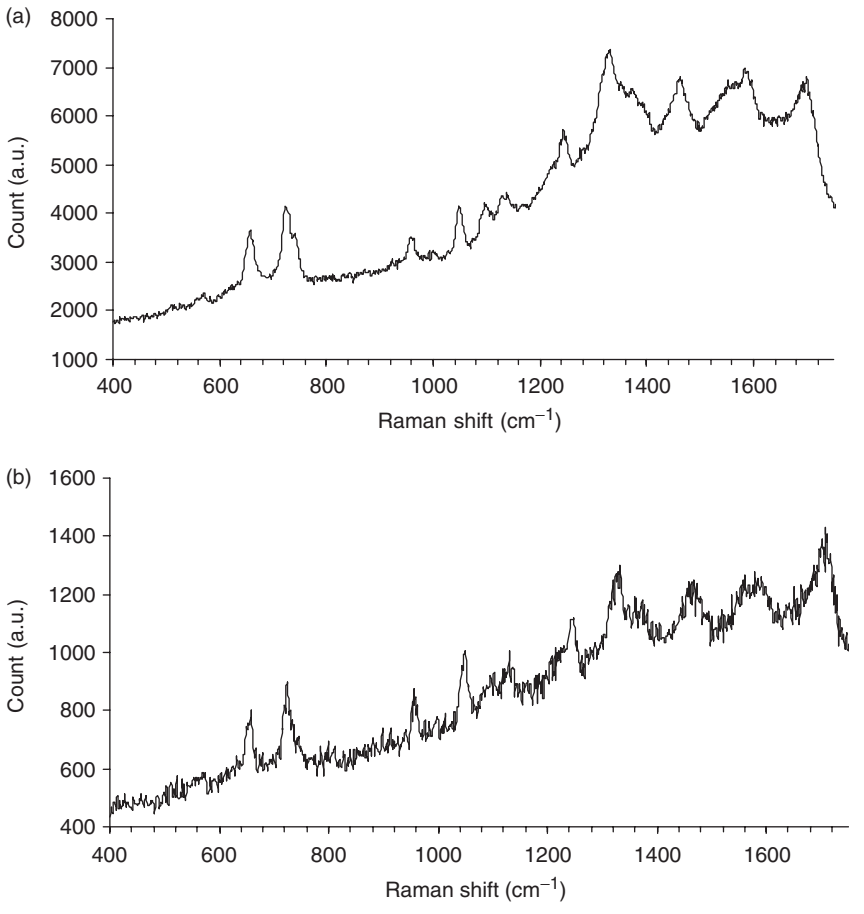

Figure 4. SERS spectra of *E. coli*; acquired from (b) a mass sample and (a) an aggregate.

4. Conclusions

It is possible to obtain a SERS spectrum from a limited number of bacterial cells, and the SERS spectra can be used for identification and discrimination once a single bacterium or aggregates of a few bacterial cells are located under the microscope objective. The surface plasmons of aggregated silver nanoparticles under white light help to locate the bacterium or its small aggregates. The strength of the interaction of the bacterial cell wall and silver nanoparticles plays a significant role to acquire a good-quality and reproducible SERS spectrum. Thus, the silver nanoparticles synthesized by Lee's method are a better choice. The aggregation of bacterial cells after mixing with colloidal silver can provide additional benefits to easily locate the bacterial aggregates from real samples under a light microscope. Our efforts to understand the behaviour of bacterial cells after mixing with colloidal solutions will continue in future studies to determine whether this property can be utilized for real-life samples.

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