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SERS study of bacteria using biosynthesized silver nanoparticles as SERS substrate

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Surface-enhanced Raman scattering (SERS) spectroscopy has great advantage as a spectroscopic analytical tool due to the large enhancement of weak Raman signal and thereby facilitate suitable identification of chemical and biological systems. SERS can be utilized as a powerful tool to identify pathogens like bacteria. However, it is difficult to fabricate homogeneous SERS active substrate in biosensing application to obtain uniform, stable and highly reproducible SERS signal. In this paper, we have used biosynthesized silver nanoparticles as SERS active substrate to identify bacteria with different cell wall compositions. The silver nanoparticles were synthesized using the leaves extract of *Neolamarckia cadamba* as the source for reducing and stabilizing agent. The pathogen detection by SERS technique using biosynthesized silver nanoparticles represents a novel approach for rapid microbial diagnostics, where SERS can be directly applied on clinical sample rather than pure cultured bacteria.

Key words: Bacteria, Spectroscopy, Raman scattering, Surface-enhanced Raman scattering, Silver nanoparticles, Biosynthesis.

1. Introduction

Surface-enhanced Raman scattering (SERS) phenomenon deals with the increase of the weak Raman scattering intensity by molecules in the presence of nanostructured metallic surface especially gold and silver.[1-3] Over the last forty years, it has developed into a versatile spectroscopic and analytical technique due to the rapid progress of nanoscience and nanotechnology. SERS is known as one of the most sensitive spectroscopic tools for highly sensitive and selective detection of chemical and biological system. The advent of SERS has opened up a promising way to overcome the low sensitivity of traditional Raman spectroscopy. The versatility of SERS technique can be exploited from diverse applications ranging from plasmonics to diagnostics.[4,5] SERS can be utilized as a versatile tool to determine molecular structure and also provides ultrasensitive detection limits, including single molecule sensitivity.[6] It has been used to detect pathogens which include bacteria and viruses.[7,8] Ever since, Efrima et al. first described the SERS spectrum of a bacterial cell surface,[9] several groups have reported the use of SERS-based assays for pathogen detection.[7-13] However, most of the SERS-based assays face a disadvantageous challenge due to too much fluctuations of captured SERS signals. These large fluctuations occur mainly from the non-homogeneity in the SERS-active substrates and due to inconsistent binding between the bacterial cell surface and the SERS substrate. Metal colloids and nanostructures, which can be used as SERS active substrate, have poor biocompatibility. Therefore, it is necessary to develop new novel biocompatible and homogeneous substrates for Raman enhancement in case of biological molecules especially for rapid pathogen detection.

The development of eco-friendly cost-effective protocol for the green synthesis of nanomaterials using microorganisms, like bacteria, viruses, fungi as well as plant and animal based products is an important aspect of nanotechnology.[14] Currently, there is growing need to develop eco-friendly synthetic protocols of various nanoparticles of different shapes and sizes for avoiding the adverse effects in comparison to traditional chemical methods. The biosynthesis of metal nanoparticles can provide a cost effective eco-friendly alternative to the most commonly used chemical routes due to its biocompatibility, simplicity and low cost. Several groups have used plant and fruit extracts to synthesize both silver and gold nanoparticles of various sizes and shapes.[15-19].There is much scope of further improvement in the synthesis of nanoparticles by biogenic methods using different biological resources as the source of reducing and stabilizing agents and their potential applications especially in the biomedical field. Obviously, large number of researchers diverted their attention towards the use of biological systems for the synthesis of metal nanostructures. In this paper, we describe the biosynthesis of silver nanoparticles using leaf extract of *Neolamarckia cadamba* and its use as SERS active substrate to detect bacteria within a very short time of 1-5 s with a limit of detection (LOD) of 10^3 CFU/ml (CFU = colony-forming unit) for *E. coli*. Herein, we demonstrate a novel application of the SERS technique for rapid detection and quantification of bacteria based on our newly developed almost homogeneous SERS-active substrate giving highly reproducible, stable and uniform Raman signal with large enhancement factor and almost zero fluctuation. The fabricated SERS substrate is extremely stable under ambient atmospheric conditions up to three months.

2. Experimental

2.1 Chemicals and reagents.

Silver nitrate ($AgNO_3$, 99.9 % Merck), and Ammonia solution (NH_3, Rankem) were used. All chemicals were used as such without any further purification. All the solutions were prepared using Milli-Q water during the synthesis. All the apparatus were washed by aqua-regia, rinsed in distilled water, dried in hot air oven and then used for the experiments.

2.2 Preparation of the leaf broth.

The leaves of *Neolamarckia cadamba* were washed with plenty of double distilled water at the beginning and followed by Milli-Q water and cut into pieces. The extract was prepared by heating 60 g of leaves sample dispersed in 240 ml of Milli-Q water for 5 min in Erlenmeyer flask using water bath at ~100^{o}C. The broth was cooled to room temperature and filtered through eight fold muslin cloth to obtain clear filtrate of the extract.

2.3 Synthesis of Silver nanoparticles from leaves broth.

Biosynthesis of silver nanoparticles using leaves broth of *Neolamarckia cadamba* was carried out by addition of 5 ml of broth of leaf extract to a reaction mixture containing 500 ml $AgNO_3$ (1×10^{-3}M) and 2.5 ml (0.625%) ammonia solution. The silver nanoparticles formed from the leaves broth and supernatant were separated first by centrifugation at 11000 rpm for 25 min. It was followed by centrifugation of collected supernatant at 17000 rpm for 30 min and washing of the pellets with Milli-Q water. The pellets were stored and used for further characterization and application as SERS active substrate to detect bacteria.

2.4 Characterization of Silver nanoparticles.

UV-vis spectral analysis was done by using UV-Vis Spectrophotometer UV-1800 (Shimadzu). Transmission electron microscopic (TEM) analysis was performed on Ag nanoparticles sample in order to investigate the size distribution and morphology of the synthesized nanoparticles. A drop of nanoparticles colloidal solution was loaded on carbon standard TEM grids and allowed to dry inside a vacuum dryer. The grid containing silver nanoparticles was scanned by a Transmission Electron Microscope (TEM) operated at an accelerating voltage of 200 kV.

The stability of the biosynthesized silver nanoparticles was verified by zeta-potential measurement. The zeta potential was calculated from the electrophoretic mobility using the Smoluchowski equation with the help of commercial software using a dynamic light-scattering (DLS) set up (DLS; Model DLS-nano ZS, Zetasizer, Nanoseries, Malvern Instruments).

2.5 Preparation of bacteria samples and Raman instrumentation/data processing.

Two bacterial strains were used in our study: *Staphylococcus aureus* (25923 ATCC), *Escherichia coli* (35218 ATCC) were obtained from American Type Culture Collection (ATCC). The bacteria were grown in 10 ml nutrient broth for 24 hr and cultures are incubated at 25^{o}C with shaking in a gyratory shaker at 5000 rpm to an OD_{600} of 0.5; the end of the exponential growth phase to ensure consistent cellular morphology related to growth stage. They were then washed five times with ultra-pure water and re-suspended in 50 µl of water. For SERS experiments, 5-10 µl of bacteria suspension was placed on the biosynthesized SERS substrate which has been treated with oxygen plasma, dried in a

laminar-flow cabinet for 10 min, and then mounted with 0.1 % agarose gel to immobilize the bacterial samples relative to the SERS substrate.

SERS experiments were carried out on a LabRam I confocal microprobe Raman system (Horiba JobinYvon, France, Model No: HR 800) equipped with He-Ne laser operating at λ = 632.8 nm with a laser power 1.7 mW as the excitation source. The instrument was calibrated with a silicon wafer focused under the 50 × objective and collected as a static spectrum centered at 520 cm^{-1} for 1 sec. The low laser power density used here will prevent the adverse effects associated with laser illumination, like local heating, deformation of the Ag-nanoparticles and photo-oxidation. Raman signals were collected from the information-rich part of the spectrum between 400 and 1600 cm^{-1} using an integration time between 1 to 5 sec. The raw SERS data sets were further processed using algorithms developed in our laboratory to remove excess noise. Finally, the spectra were normalized so that the photon count of the highest peak at wavelength of 732 nm was set to 1. For each SERS substrate, overall 10 spectra were recorded and further analyzed. The enhancement factor or the SERS substrate enhancement factor (SSCE) was calculated from these spectra. Each SERS profile stands for the mean spectrum averaged from 10 samples.

3. Results and discussion

The UV visible spectrum reveals two absorbance peaks at 409 and 509 nm as shown in Fig. 1. The absorbance peak generally arises due to the excitation of localized surface plasmon oscillations of the conduction electrons in case of metal nanoparticles like gold and silver. A sharp peak at 409 nm corresponds to the typical transverse plasmon observed for spherical silver nanoparticles. On the other hand, appearance of a broad

absorption plasmon band ~ 509 nm can be explained by the formation of a chain like structure by the synthesized nanoparticles and presence of twinned defects inside the nanoparticles. This was further confirmed the TEM micrographs. Figs. 2 (a), (b), (c) & (d) show the lower and higher magnification TEM micrographs of the Ag nanoparticles. From the TEM images, it was clearly observed that the nanoparticles are monodispersed in nature and can form chain like structure. The particle size ranged between 15 to 20 nm. The TEM images also clearly exhibit the presence of twinned defects in the nanoparticles crystals, which are very common in face-centred cubic (fcc) nanocrystals.[20] Fig. 2 (e) illustrates the selected-area diffraction pattern (SAED) of the synthesized Ag nanoparticles. The presence of spots in the diffraction patterns clearly indicates that the biosynthesized Ag nanocrystals are single crystalline in nature. The stability of the biosynthesized silver nanoparticles was verified by zeta-potential measurement. We find negative zeta potentials of 50-60 mV for our Ag nanoparticles. These ranges of zeta-potentials are ideal for highly stable colloidal sols. ASTM defines colloids or nanoparticles with zeta potential values greater than 40 mV (negative or positive) will exhibit good stability. The as synthesized silver nanoparticles show good stability as evident from the zeta potential value mentioned above. There is no sign of aggregation of the synthesized Ag nanoparticles even after six months. It suggests that the biosynthesized Ag nanoparticles are extremely stable.

Typical demonstration for bacteria detection using our SERS system and subsequent data processing are shown in Fig. 3. The SERS spectrum generated by illuminating the whole bacterium as it interacts with the silver nanoparticles should reveal principally the molecular composition within ten nanometers of the outermost bacterial envelope.[21] It is

well known that the components and architecture of the bacterial envelope are very different between Gram-positive and Gram-negative bacteria. We anticipated that such structural differences should be clearly visible by SERS analysis as shown in Fig. 3 (a) & Fig. 3 (b). Each SERS profile stands for the mean spectrum averaged from 10 samples. The SERS data were collected between 1-5 second. Raman photon counts on the order tens of thousands clearly indicate that these spectra result from the SERS process rather than normal Raman scattering or Raman effect. We have estimated the Raman enhancement factor or SERS substrate enhancement factor (SSEF) for the SERS substrate developed by us. It was estimated both for *Staphylococcus aureus* and *E. coli* bacteria. The enhancement factor SSEF can be defined as the scattering intensity per molecule with and without SERS effect and can be written in the form of following formula which has been already cited in the literature.[22]

$$SSEF = I_{SERS} \cdot N_{Ref} / I_{Ref} \cdot N_{SERS}$$

In this formula, I_{SERS} corresponds to the enhanced Raman intensity of the bacteria on the SERS substrate (i.e., biosynthesized Ag nanoparticles), I_{Ref} corresponds to the spontaneous Raman scattering intensity from the bacteria suspension under the laser spot on the Si surface, N_{Ref} is the number of bacteria in case of normal Raman scattering and N_{SERS} is the number of bacteria isolates uniformly spreading on the SERS substrate under laser spot. The enhancement factor was calculated for 10 samples and found to be $(3 \pm 0.20) \times 10^7$ and $(5 \pm 0.40) \times 10^7$ for S. *aureus* and *E. coli* bacteria, respectively. Jamil et al.[23] verified the reproducibility of the SERS signal obtained from the SERS substrate by measuring the standard deviation as well as relative standard deviations. They tested this by measuring from 150 repeated measurements from different locations on the substrate

for ultra-trace detection of trinitrotoluene in the environment. The SERS substrate developed by us is almost homogeneous with a relative standard deviation value of 6.32 calculated from 50 repeated measurements from various locations on the SERS substrate. The enhancement factor was calculated several times to check the reproducibility and found to be highly reproducible. Although, it is well known from the literature that it is almost impossible to develop a highly reproducible SERS substrate by drop dried method, we have achieved this by obtaining highly reproducible SERS spectra from the SERS substrate obtained using biosynthesized Ag nanoparticles.

Although, chemical information can be obtained from these SERS spectra, they are difficult to understand. There could be any number of SERS-active vibrational modes present in biological samples such as bacteria. In this case, with previous information on the biochemistry of the pathogens to be investigated, it is possible to mark certain peaks tentatively. In addition to this, further problem arises due to the lack of proper database resource of SERS spectra, making the process of peak identification more time-consuming. Although, database resources for standard Raman spectra of biological samples like different bacteria are gradually becoming available, it is not always the situation in where the peaks illustrated in a Raman spectrum will also be observed in the SERS spectrum of the same sample.

The electromagnetic field coupling between the silver nanoparticles in the chain like structure as observed from the TEM image plays very important role which can enhance the electromagnetic enhancement and overall SERS intensity. The effective area in the nanoparticles junction will offer the largest SERS enhancement and the whole area of the gap region has enhanced uniformly. This fact results in a stronger SERS signal from our

SERS substrate as demonstrated here. The above results indicate that metal nanoparticles with well-defined facet just like the presence of twinned defects have high potential and advantages in the SERS-based biosensors.

For both gram positive bacteria (*Staphylococcus aureus*) and gram-negative bacteria (*E. coli*), the obtained SERS datasets were normalized by constant value of photon count of the highest peak at 732 cm^{-1}. In case of gram positive bacteria (*Staphylococcus aureus*), main peaks were emerged at 626, 732, 1330, and 1460 cm^{-1} as shown in Fig. 3a. The SERS spectrum is characterized by strong bands due to nucleic acid bases at 732 cm^{-1}(adenine), and 1330 cm^{-1} (adenine).[24] The bands at 1460 cm^{-1}, can be attributed to the CH_2 deformation mode of proteins, the carboxylate stretching vibration (νa COO^-) of Trp (Tryptophan) and the amide I vibration of peptide groups.[25] In case of gram negative bacteria (*Escherichia coli*), the SERS spectrum contains peaks at 655, 725, 960, 1095, 1130, 1330 and 1460 cm^{-1} as shown in Fig 3b. The observed SERS spectrum for gram-negative *Escherichia coli* is totally different from the gram-positive *Staphylococcus aureus* bacteria. The bacterial cell wall, made of components with similar molecular compositions may not have similar features in the SERS spectra. The most important features in the SERS spectra of Gram-positive and Gram-negative bacteria (Figs. 3a and b) are located at ~730 and 1330 cm^{-1} which were assigned to the purine ring breathing mode and the C–N stretching mode of the adenine part of the lipid layer components of the cell wall[7]. On the other hand, Jarvis et al.[7] attributed the 730 cm^{-1} peak to a glycosidic ring mode from the cell wall peptidoglycan building blocks, N-acetyl-D-glucosamine (NAG) and N-acetylmuramic acid (NAM). Furthermore, the signal of the 730 cm^{-1} peak in Gram-negative bacteria is smaller than that in Gram-positive bacteria, which can be

explained by the following facts: Just Gram-negative bacteria have a thick outer membrane; the thickness of the peptidoglycan layer of Gram-positive bacteria (10 to 20 layers) is larger than that of Gram-negative bacteria (one to three layers). This character also supports that the 730 cm^{-1} peak is dominated by the peptidoglycan layer. The SERS data represented in our study is uniform, stable and highly reproducible without any fluctuation. Therefore, one can potentially differentiate known or unknown pathogens rapidly within a few sec such as bacteria using the SERS spectra of bacterial cell wall as fingerprint.

We have also tested the stability of our fabricated SERS substrate by exposing them to atmospheric conditions and performing SERS experiments on bacteria using the same procedure as mentioned previously. We have tested three SERS substrates, by exposing them to air for 1 and 3 months as well as freshly fabricated SERS substrate. For each SERS substrate, an average spectrum was obtained from 10 individual spectra. All three substrates showed similar spectra with almost same enhancement factor. There is no connection between the SERS signal and the time of exposure to air. This clearly confirms that the biosynthesized Ag nanoparticles used as the SERS substrate is very stable under atmospheric conditions even after three months.

However, the SERS based detection method of bacteria can not differentiate one strain from another within bacterial species. This is the major limitation of our SERS based detection of bacteria. The SERS spectra described in this paper lacks the molecular level specificity compared to genome sequencing or mass spectrometric based proteomics analysis. Further improvement in instrumentation of micro-Raman system as well as data analysis may enhance the differentiation power. It is important to mention here that SERS

based pathogen detection method is especially useful for the analysis of slow-growing bacteria, which typically may take weeks during laboratory tests. Inspite of all these limitations, biosynthesized Ag nanoparticles obtained from leaves extract are expected as potential candidate for SERS activity due to the strong affinities of Ag nanoparticles towards the aromatic functional groups present in bacterial cell walls. The presence of different aromatic functional groups attached on the surface of biosynthesized Ag nanoparticles was further verified using FT-IR spectroscopy (Fig. 4).

The sensitivity of the SERS technique for rapid detection of bacteria has been investigated by analyzing the SERS data obtained using different concentrations of *E. coli*. Fig. 5 illustrates the SERS calibration curve obtained with SERS peak area or SERS intensity of the peak at 1330 cm^{-1} (C–N stretching mode) as a function of concentration of bacteria *E. coli*. The 1330 cm^{-1} peak became detectable at 10^3 CFU/ml of *E. coli* concentration. This is the LOD for our biocompatible SERS active Ag nanoparticles for *E. coli* bacteria. The SERS intensity increases with concentration of the bacterial solution, as it is exponentially correlated to the concentration of *E. coli* bacterial cells in the sample between 10^3 CFU/ml to 10^8 CFU/ml. Experiments were repeated five times with each bacterial concentration, and the standard errors of the mean for each concentration are also shown in Fig. 5. We hope that we can detect single bacterium using our SERS substrate in the near future. It is important to mention here that the aim of our SERS study using biosynthesized Ag nanoparticles was to develop SERS technique as a rapid fingerprinting method for the characterization of bacteria particularly *E. coli,* which is associated with urinary tract infection (UTI), a common disease among most people of all age groups in developed countries like India and China.

At present, we are involved in the SERS-based detection technique of mycobacterium groups with species such as *Mycobacterium tuberculosis*, which is known to be drug-resistive to most of the common drugs. We are also studying the effect of antibiotic exposure on drug-sensitive bacteria studied by SERS using our biosynthesized Ag nanoparticles. It is expected that our study will create breakthrough in overall understanding of microbial diagnostics.

4. Conclusions

We describe here the use of biosynthesized silver nanoparticles obtained from leaves extract as SERS active substrate to detect bacteria. The developed SERS substrate will provide highly reproducible, stable and uniform Raman signal with large enhancement factor and almost zero fluctuation. The enhancement factor was calculated for 10 samples and found to be $(3 \pm 0.20) \times 10^7$ and $(5 \pm 0.40) \times 10^7$ for S. *aureus* and *E. coli* bacteria respectively. The SERS substrate developed by us is almost homogeneous with a relative standard deviation value of 6.32 calculated from 50 repeated measurements from various locations on the SERS substrate. In addition to this, the fabricated SERS substrate was extremely stable even after three months. Using this almost homogeneous, stable SERS active substrate, we have differentiated Gram positive from Gram negative bacteria. The SERS data presented in our study is highly stable, uniform and reproducible, which shows the versatility of our SERS active substrate. The SERS based detection of bacteria using biosynthesized Ag nanoparticles is higly sensitive with a limit of detection of 10^3 CFU/ml for *E. coli.* SERS based pathogen detection method is especially useful for the analysis of slow-growing bacteria, which typically may take weeks during laboratory tests. The SERS spectra described in this paper lacks the molecular level specificity

compared to other common techniques. We expect that one can detect single bacterium using our biosynthesized SERS substrate in the near future.

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Figures

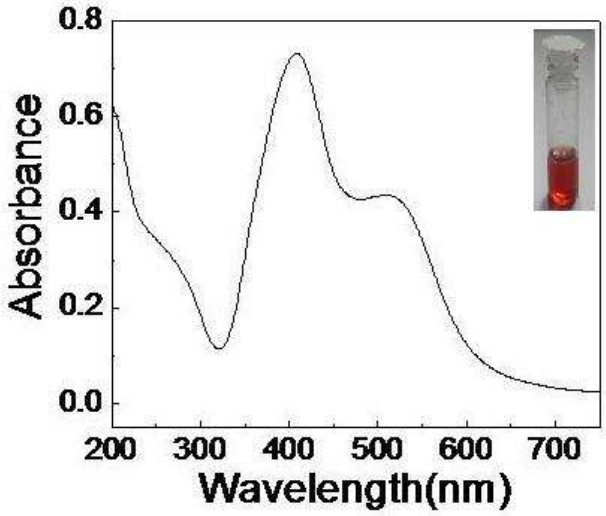

Fig. 1. UV-visible absorption spectra of Ag nanoparticles synthesized from flower extract.

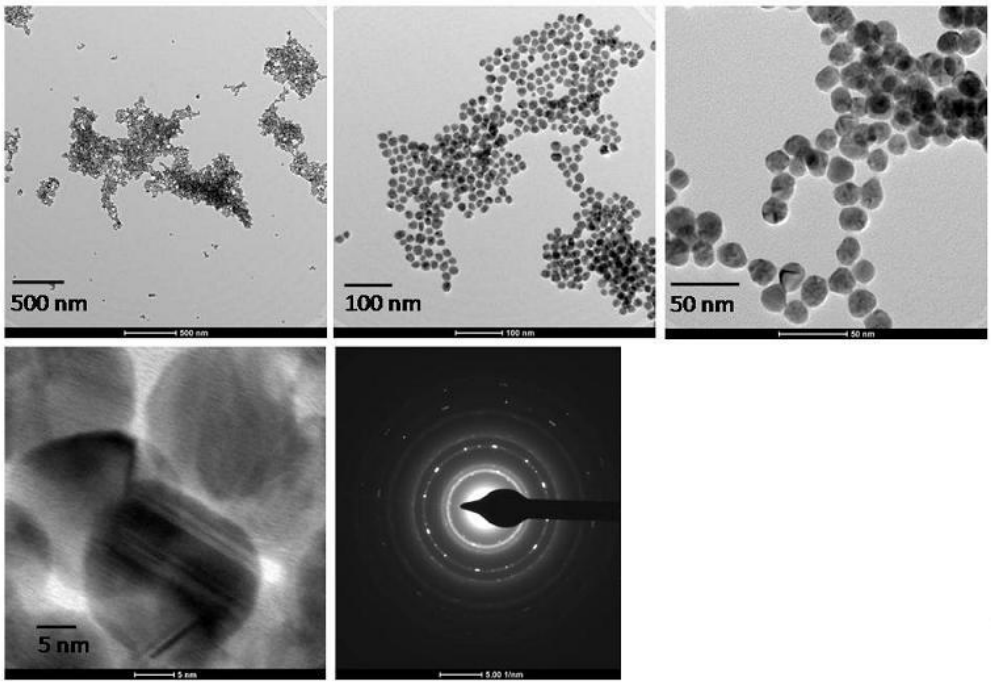

Fig. 2. Lower and higher magnification TEM images along with SAED pattern of the as synthesized Ag nanoparticles.

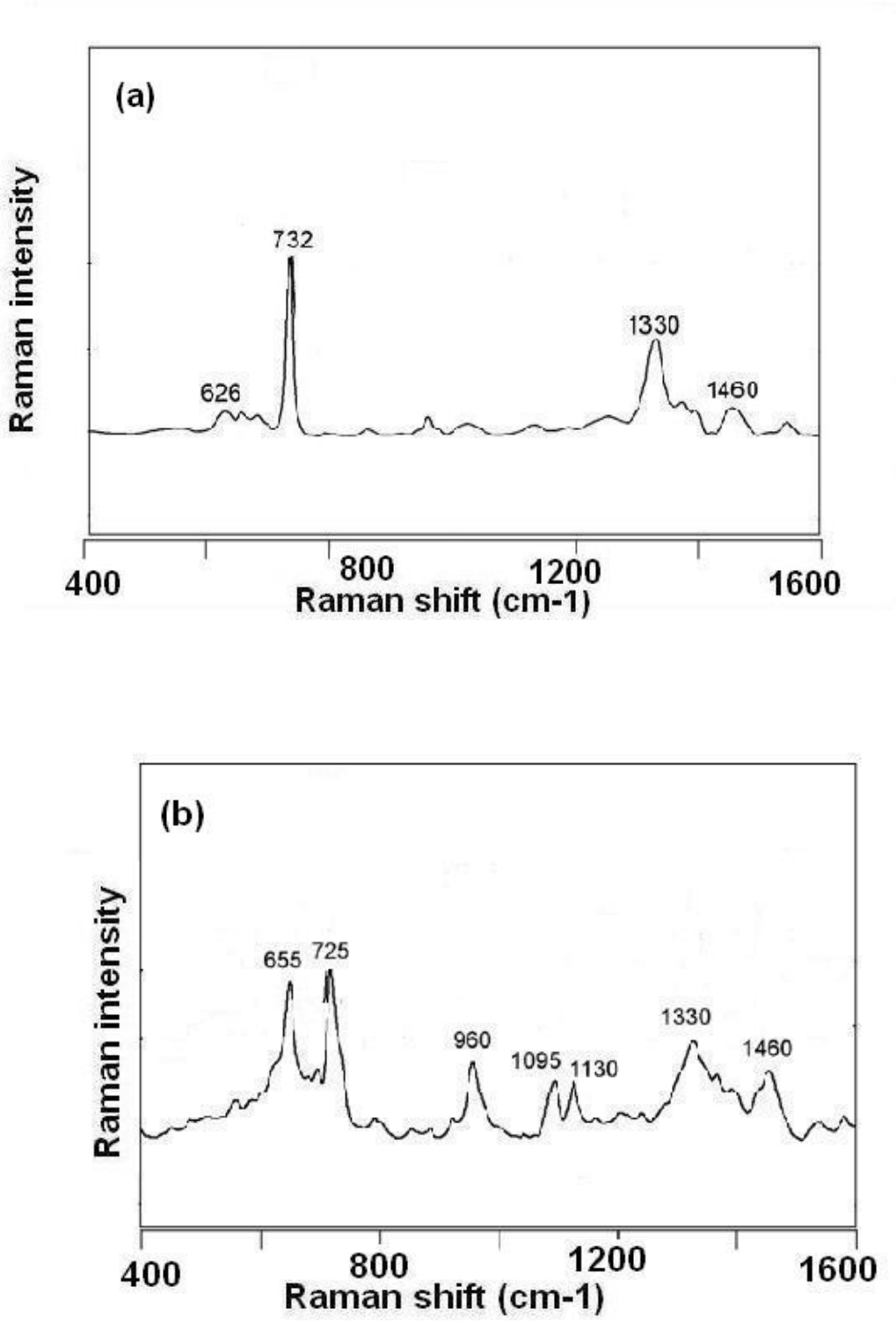

Fig. 3. SERS spectra of (a) gram-positive bacteria (*Staphylococcus aureus)* and (b) gram-negative bacteria (*E. coli*) using biosynthesized Ag as SERS substrate.

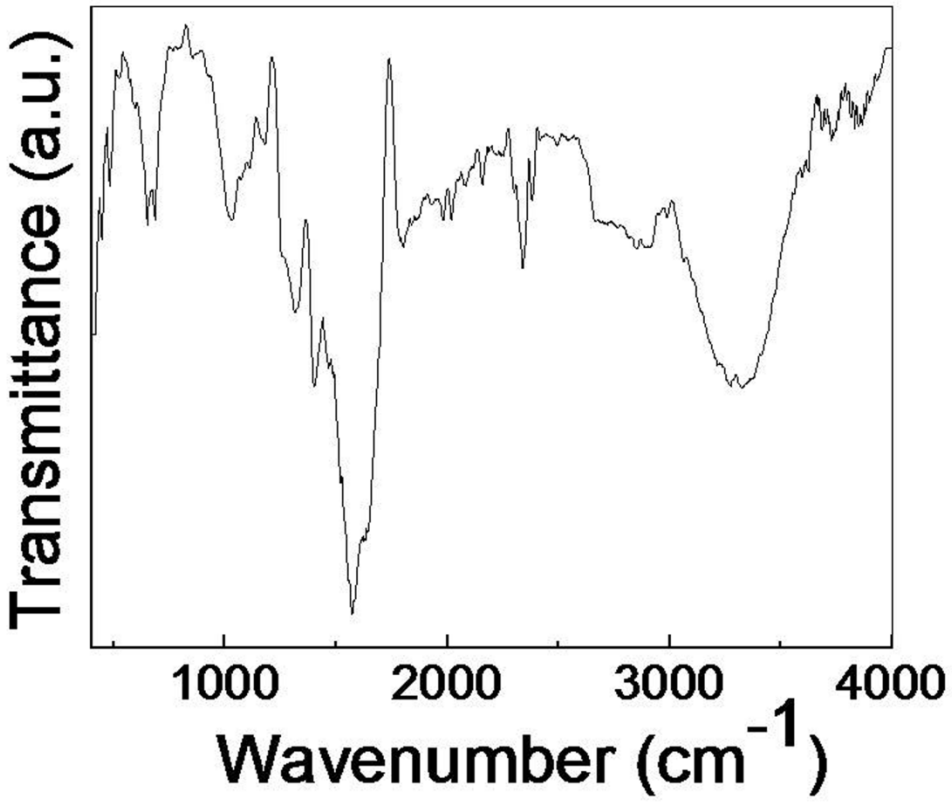

Fig. 4. FTIR spectrum of biosynthesized Ag nanoparticles.

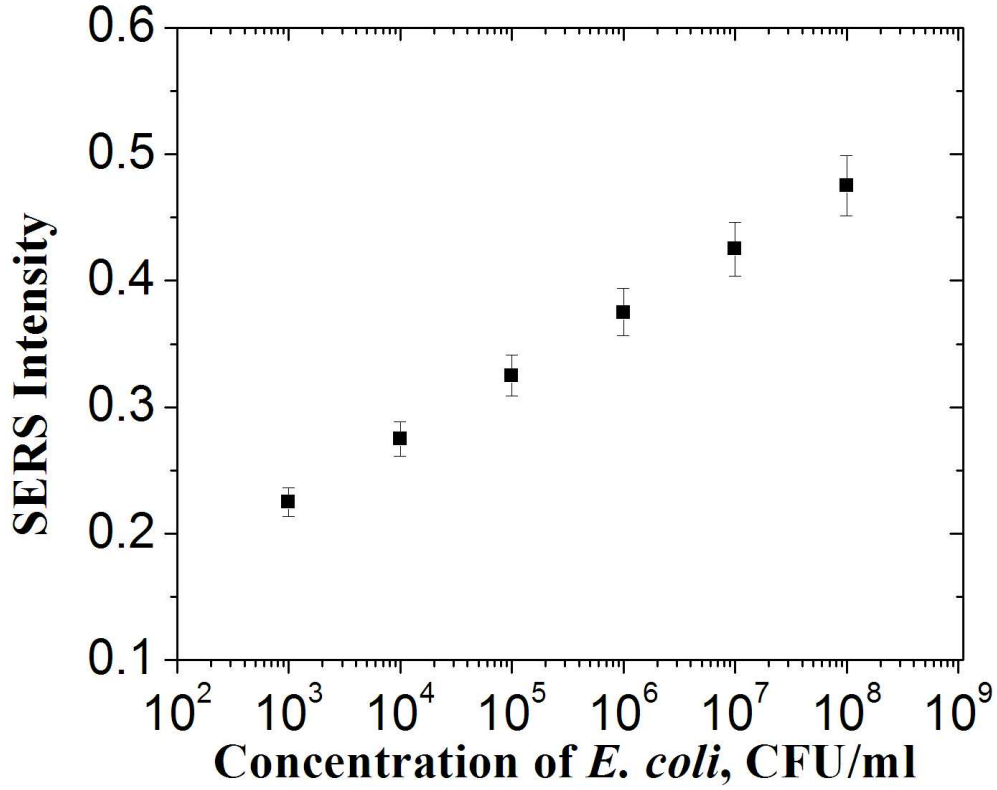

Fig. 5. The SERS calibration curve obtained with SERS peak area or SERS intensity of the peak at 1330 cm^{-1} (C–N stretching mode) as a function of concentration of bacteria *E. coli*.

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Graphical abstract

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