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Reproducible *E. coli* detection based on label-free SERS and mapping

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ABSTRACT

The biosensing for rapid detection of bacteria based on surface-enhanced Raman scattering (SERS) has been widely explored for recent years. It is still a challenge to achieve a high sensitive, reproducible label free detection method for bacteria. In this work, a label-free SERS detection method of *Escherichia coli* based on incubation with silver colloid was reported. Optimized incubation conditions including shaking speed, time and temperature were used to help construct a rapid SERS method for *E. coli* analysis. It was found that the enhancement of the Raman signal of *E. coli* could be achieved to 1.8×10^4 cps (counts per second) with high reproducibility. Three strains of *E. coli* DSM 1116/498/5695 could be successfully discriminated using such SERS method combining discriminant analysis. Finally, the lowest concentration of *E. coli* at 1×10^5 cell/mL can be detected by SERS mapping. Thus, our detection method offers higher sensitivity and reproducibility compared to previously reported label free simple-mixing methods, opening an avenue for developing various SERS-based biosensor.

1. Introduction

The need for rapid, highly sensitive, and versatile pathogenic detection is much crucial for food quality controls and human health care [1,2]. In consideration of the low infectious dose and high health risk of foodborne pathogenic *Escherichia coli* (*E. coli*) bacteria [3,4] such as *Shiga toxin-producing E. coli* (STEC), *Enterotoxigenic E. coli* (ETEC), a rapid, sensitive, and reliable detection method of *E. coli* is essential and highly desirable for preventing or eliminating potential infections. In the area of viral pathogen detection, the most conventional methods such as plating and culturing based techniques suffer from being time-consuming and a laborious enrichment process, requiring 6–24 h with an additional 1–3 days for confirmation by biochemical tests [5,6]. Biological analysis [7], such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) has been developed to identify and discriminate between bacteria at the strain level. However, these methodologies are impractical for rapid low-level bacteria detection due to lack of the sensitivity, specificity, cost, versatility, and portability.

A competitive candidate for rapid bacterial detection is surface-enhanced Raman scattering technique (SERS). With the integration of single-molecular sensitivity, narrow and intrinsic spectroscopic fingerprints, simple and fast preparation, and non-destructive data acquisition in aqueous solution, SERS has recently drawn considerable attention for biological applications, such as bacteria [8,9], cells [10], and tissues [11]. The extremely high sensitivity of SERS relies on the giant electromagnetic enhancement attributed to the electromagnetic field in the proximity of nanostructured noble metal (Au or Ag) surfaces [12]. Currently, the popular SERS based bacterial analysis was called label-free SERS bio-analysis, which usually employed Au NPs or Ag NPs colloidal or particle aggregates induced by inorganic salt by simply mixing with the bacterial cells [13–15]. Such simple mixing label-free SERS method requires for no specific detection label, which makes the detection easier and faster. However, the generated mixture solution is not always homogeneous [16], leading to uniform attachment of nanoparticles on the bacterial cell walls. The insufficient interaction of NPs with the bacteria could result in a very limited reproducibility of SERS spectra. Thus, it is very difficult to obtain reproducible and quantitative SERS results.

A major challenge to enable the transfer of SERS from research lab to practical applications is to overcome the poor reproducibility. The limitation can be improved either by concentrating the NPs [17] or constructing self-assembly NPs [18] on bacteria cell wall. However, all these methods have either poor reproducibility due to difficult control of NPs aggregation on the bacteria or a complicated synthesized procedure. Therefore, the main goal is to develop a rapid, simple and efficient method to make the NPs

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come into contact with the bacteria surface at as many points and close as possible for SERS application. Ag NPs has been widely used as an active-SERS substrate for bacteria detection due to their superior SERS enhancement [19]. Here, we report a label-free SERS detection method of *E. coli* based on their incubation with Ag NPs not just simply mixing to make a close and efficient attachment between NPs and bacteria. Optimized incubation conditions including shaking speed, time and temperature were investigated to achieve a better SERS effect. Three strains of *E. coli* (DSM 1116/498/5695) can be successfully characterized and detected on the basis of SERS spectra and discriminate analysis. In addition, the capability to detect different concentrations of *E. coli* by SERS mapping was evaluated.

2. Experimental

2.1. Chemicals and materials

37% hydrochloric acid (HCl), 65% nitric acid (HNO_3), sodium hydroxide (NaOH), hydroxylamine hydrochloride ($NH_2OH \cdot HCl$), silver nitrate ($AgNO_3$) were purchased from Sigma-Aldrich (Taufkirchen, Germany) and used without further purification. *E.coli* DSM 498/1116/5695 shock frozen strains and were purchased from DSM nutritional products (Grenzach, Germany). LB medium, and glass slides (26 mm × 76 mm × 1 mm) were purchased from Carl Roth (Karlsruhe, Germany). Activation of glass slides, cleaning steps and hydrophobic procedure were performed in plastic containers from Carl Roth. Hellmanex solution was purchased from Hellma (Muellheim, Germany). Ultrapure water (18.2 MΩ cm^{-1}) produced using a Millipore water purification system was used as solvent.

2.2. Silver nanoparticle (Ag NP) synthesis

The preparation of Ag NPs follows a modified procedure of Leopold and Lendl [20]. Briefly, 17 mg (0.1 mmol) of $AgNO_3$ was dissolved in Milli-Q water (10 mL). 100 mL 11.6 mg (0.17 mmol) $NH_2OH \cdot HCl$ solution containing 3.3 mL of NaOH (0.1 M) were prepared as the reducing agent and divided in 9 mL batches in centrifuge tubes. 1 mL of $AgNO_3$ solution was added to the 9 mL reducing agent in a flow rate of 0.67 mL s^{-1} without stirring. Finally, the centrifuge tube was inverted once to complete the Ag NPs synthesis. The size and morphology of silver nanoparticles was characterized using a UV–vis spectrometer and TEM. The yellow/greenish silver colloid was stored in the dark at 4 °C for further use.

2.3. Microorganism culture and preparation

2.3.1. E. coli cells culture

Three stock-frozen *E.coli* strains (DSM 498/1116/5695) were cultivated in LB medium (100 mL in 250 ml flasks) in a gyratory shaker at 100 rpm and 37 °C for 16 h. Then 10 mL *E. coli* cells suspension was harvested and washed twice with distilled water by centrifugation at 4500 rpm and 4 °C. Then the bacteria were stored in refrigerator at 4 °C for further use. Prior to analysis, the stock cell concentration of *E. coli* was determined by flow-cytometry using SYTO9.

2.3.2. Ag NPs-E. coli sample preparation

As shown in Fig. S1, the harvested bacteria cells above were further added to 10 mL prepared Ag NPs colloid and then vortexed to ensure the homogeneity of the mixture. Subsequently, the bacteria and Ag NPs mixtures of different batches were co-incubated in the gyratory shaker at different conditions as follows. The incubation conditions with shaking speed (0 rpm and 100 rpm), time (0 h, 0.5 h, 1.5 h, 3 h, 6 h, 24 h, 48 h) and temperature (4 °C, 25 °C, 37 °C, 45 °C) were investigated. The resulting hybrid sample is designated as AgNPs-*E.coli*. If not explicitly stated, only *E.coli* DSM 1116 was chosen as *E. coli* model to evaluate the optimized incubation condition.

2.4. Glass slides hydrophobic treatment

The preparation follows our previous work [21]. The normal glass slides were put in a chip reservoir containing 2% Hellmanex solution and left 1 h in ultrasonic bath to rinse. Subsequently, the slides were put on a shaker overnight (15 h) at room temperature. The treated slides were thoroughly washed five times with water and dried. The prepared glass slides were dipped in 200 mL of methanol/hydrochloric acid solution (1:1) for 1 h with shaking. Then the chips were washed with water five times. Subsequently, they were submerged in concentrated sulfuric acid and left shaking for 1 h [22]. The above slides cleaned by water were put in a 300 mL mixed methanol aqueous solution (1:1) under magnetic stirring, then 2 mL trimethoxy(propyl)silane and 3 mL 25% ammonia was added dropwise successively. The solution was stirred overnight. Finally, the chips were thoroughly washed in 300 mL absolute ethanol and dried under nitrogen flow for further use.

2.5. SERS measurements

SERS measurements were conducted with a Raman microscope (LabRAM HR, HORIBA Scientific, Japan).

2.5.1. SERS detection of AgNPs-E. coli in suspension

3 μL Ag NPs-*E. coli* suspension after incubation was pipetted onto the normal glass slides. If not explicitly stated, the concentration of *E.coli* was 1×10^8 cell/mL. The recording of the Raman spectra was started right after this sample preparation, using the 633 nm line of a He–Ne with 14 mW power at the sample and a 10 × objective. The Raman spectra were continuously (one spectrum every 40 s) collected with the auto repeat function until the droplet was dried. If not explicitly stated, the exposure time and the accumulation number were set with 1 s and 10 s, respectively, and the confocal slit width was 100 μm with a spectra region from 200 to 2000 cm^{-1}.

2.5.2. SERS mapping of AgNPs-E. coli on glass slides

A droplet of 3 μL AgNPs-*E.coli* suspension with different concentrations (1×10^5 cell/mL, 1×10^6 cell/mL, 1×10^7 cell/mL, 1×10^8 cell/mL) after optimized incubation condition was added on the hydrophobic glass slides, and then dried at room temperature. All SERS mapping data were obtained by using the 633-nm He–Ne laser line with 1.4 mW power and a 50 × objective. The area of each map was 40 μm × 40 μm with step size of 2 μm. In all, 441 Raman spectra were collected for each map. The exposure time and the accumulation number were 1 s and 5 s, respectively; the slit width was 100 μm, and the detecting spectra region was 50–2000 cm^{-1}.

2.6. Colloid diagnostics

All the UV–vis absorption spectra were recorded with a Specord Plus spectrometer (Analytik Jena, Jena, Germany). TEM images were taken with JEM 2010 (JEOL, Munich, Germany) with an accelerating voltage of 200 kV.

2.7. Data analysis

All the Raman spectra were recorded and processed with

LabSpec 5.0 from HORIBA Jobin Yvon and were exported to Origin 8.0 to plot. In order to calculate Raman peak intensity, a third-order polynomial equation was auto-applied in the intensities of each spectrum and then subtracted from gross spectrum.

3. Results and discussion

3.1. Optimization of Ag NPs-E. coli incubation condition

Ag NPs has been widely used as an active-SERS substrate for bacteria detection due to their superior SERS enhancement. In current bacterial label-free SERS experiments, suspensions of Ag NPs were employed by simply mixing with the bacterial cells to generate the SERS effect fast. However, such generated sample is not always a uniform mixture, resulting in a very limited reproducibility of bacterial SERS spectra. To improve the reproducibility and sensitivity of *E. coli* SERS spectra, the key possibility is that the bacterial surface should be brought into contact with as many silver nanoparticles as possible [18]. *E. coli* DSM 1116 was studied as model bacteria to investigate the optimized incubation condition. Firstly, we harvested *E. coli* cells from LB medium and washed. The *E. coli* cells (1×10^8 cell/mL) were added to Ag NPs colloid and then vortexed to ensure the homogeneity of the hybrid mixture. Subsequently, the mixing Ag NPs-*E. coli* solutions of different batches were co-incubated in the gyratory shaker at different conditions. Shaking speed (0 rpm and 100 rpm), temperature (4 °C, 25 °C, 37 °C, 45 °C) and incubation time (0 h, 0.5 h, 1.5 h, 3 h, 6 h, 24 h, 48 h) were investigated.

3.1.1. Effect of shaking status on SERS intensity

The Ag NPs-*E. coli* solutions were vortexed even and incubated overnight at still status (0 rpm) and shaking status (100 rpm) at 25 °C. The SERS spectra were recorded and shown in Fig. 1. The strongest Raman intensity of *E. coli* DSM 1116 at 732 cm^{-1} assigned to adenine was calculated. The intensity at shaking status was about 2-fold of that at still status. The reason may lie on that the bacterial cells and Ag NPs precipitated because of their gravity at still status. Instead the Ag NPs could well-distribute to the bacterial cell wall at shaking status and presented higher SERS enhancement.

3.1.2. Effect of incubation temperature on SERS intensity

Different temperature including 4 °C, 25 °C, 37 °C, and 45 °C were employed to incubate *E. coli* cells with Ag NPs colloid at 100 rpm. It can be seen in Fig. 2 that the Raman intensity at 732 cm^{-1} was climbing highest at 37 °C when bacteria showed their best biological activity, which indicated that the *E. coli* cells could have a more active interaction with more NPs.

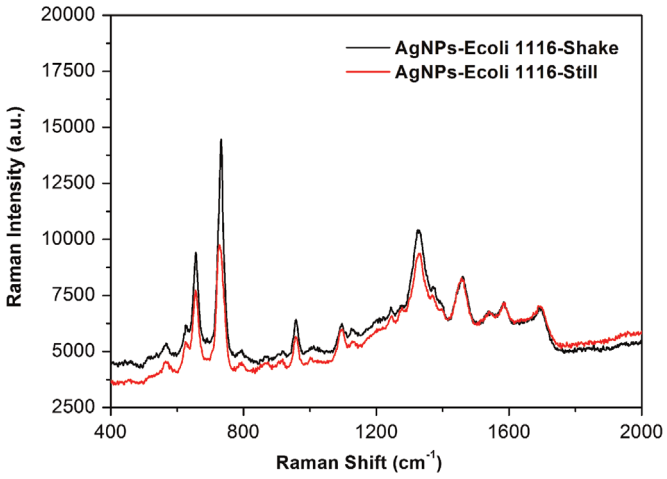

Fig. 1. The SERS spectra of Ag NPs-*E. coli* DSM 1116 at still and shaking status.

3.1.3. Effect of incubation time on SERS intensity

Based on the optimized parameters above, the Ag NPs-*E. coli* solutions were vortexed even and incubated for different time (0 h, 0.5 h, 1.5 h, 3 h, 6 h, 24 h, 48 h) with 100 rpm at 37 °C. The SERS spectra were continuously collected (Fig. 3A) with the incubation time accordingly. As it was reported, the SERS spectra were due to the interaction of Ag NPs with the molecule [23]. From Fig. 3B, it is shown that the Raman intensity at 732 cm^{-1} obtained the highest value at 3 h, which might attribute to the declining binding ability between the Ag NPs and dead cells on account of the toxicity of Ag NPs on *E. coli* cells [24] after incubation time of 3 h. Ag NPs could dissolve in the biological fluids with incubation time and the resulting Ag^+ could partly or even largely account for the toxicity of Ag NPs due to their affinities to protein thiols [25,26]. According to the research of Cui et al. [27], the intensity of band at 724 cm^{-1} decreased rapidly after the incubation time of 4 h. It was found that the number of viable *E. coli* cells was significant low at 4 h, demonstrating that more and more bacteria were killed with time. The SERS spectral changes with incubation time were due to the toxicity of Ag NPs and Ag^+ to bacteria cells. Our research was almost in consistent with the previous research. The slight differences might be due to different *E. coli* batches.

3.2. SERS detection at optimized incubation condition

The optimized incubation condition for Ag NPs- *E. coli* sample was found at 100 rpm, 3 h, 37 °C. The Raman results obtained by using this approach are highly reproducible and reliable. The reproducibility of this SERS approach for bacteria characterization is demonstrated by the analysis three different sample batches of 1×10^8 cell/mL *E. coli* DSM 1116 (as shown in Fig. 4A.). Compared with the simply mixing method, our method provided a higher SERS enhancement with 1.8×10^4 cps at 732 cm^{-1} (counts per second) (~3.5 fold) and much clearer peaks (Fig. S2 in Supporting information) with a better reproducibility (< 5% for 10 spectra). In addition, the availability of our method to detect *E. coli* in LB medium was also investigated as shown in Fig. 4B. The intensity of band at 732 cm^{-1} was 1350 cps, which indicated the our method was applicable.

The typical Raman peaks of *E. coli* DSM 1116 at 524, 568, 658, 732, 796, 874, 960, 1095, 1130, 1245, 1330, 1457, 1544, 1587, and 1698 cm^{-1} were observed. This shows the huge SERS enhancement effect on the components of cell wall of *E. coli*, such as polysaccharides, amino acids, nucleic acid, lipids and proteins, in accordance with the previous results [28] (see tentative assignment of peaks in Table S1). The obvious appearance of the strongest peak at 732 cm^{-1} and peak at 1130 cm^{-1} assigned to adenosine or adenosine monophosphate (AMP) indicated denatured DNA [29], which demonstrated the close binding between Ag NPs and cell wall of bacteria [30].

Interestingly, when Ag NPs-*E. coli* suspensions at optimized incubation condition were harvested, the color was discovered with an obvious change from yellow/green to dark green. UV–vis spectroscopy characterization and TEM examination were carried out to evaluate the Ag NPs-*E. coli* sample. As shown in Fig. 5A, no distinct absorption peaks can be observed from the original *E. coli* DSM 1116 solution. The original Ag NPs colloid displays as yellow/greenish suspension and has a narrow highest absorption peak at 396 nm. However, when *E. coli* cells were incubated in the Ag NPs colloid, the color of the Ag NPs-*E. coli* suspension was gradually changed to obvious dark green (see inset image in Fig. 5A) at our optimized incubation condition without any further variation. In

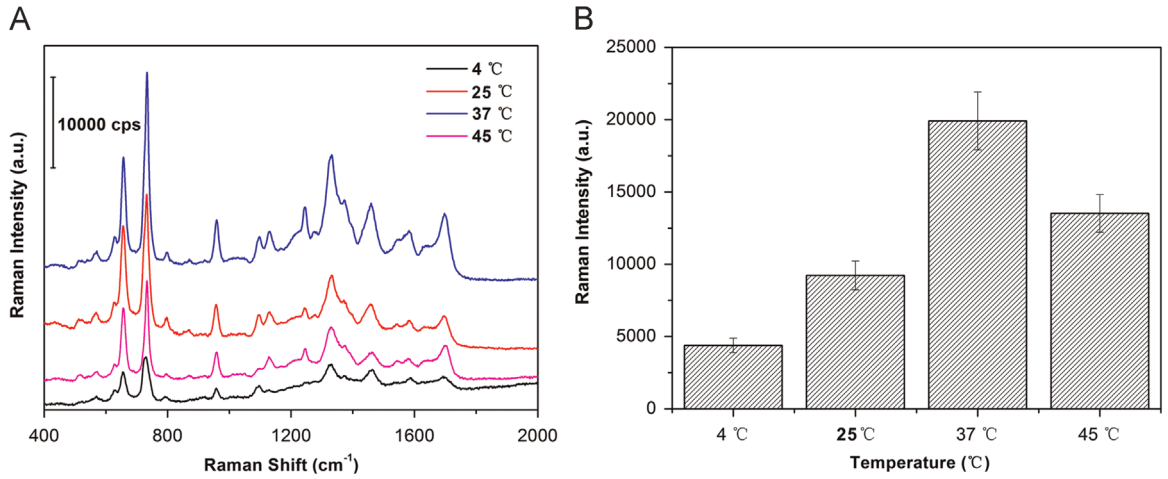

Fig. 2. (A) The SERS spectra of (B) the SERS intensity of AgNPs-*E. coli* DSM 1116 with different incubation temperature. Each sample was measured three times.

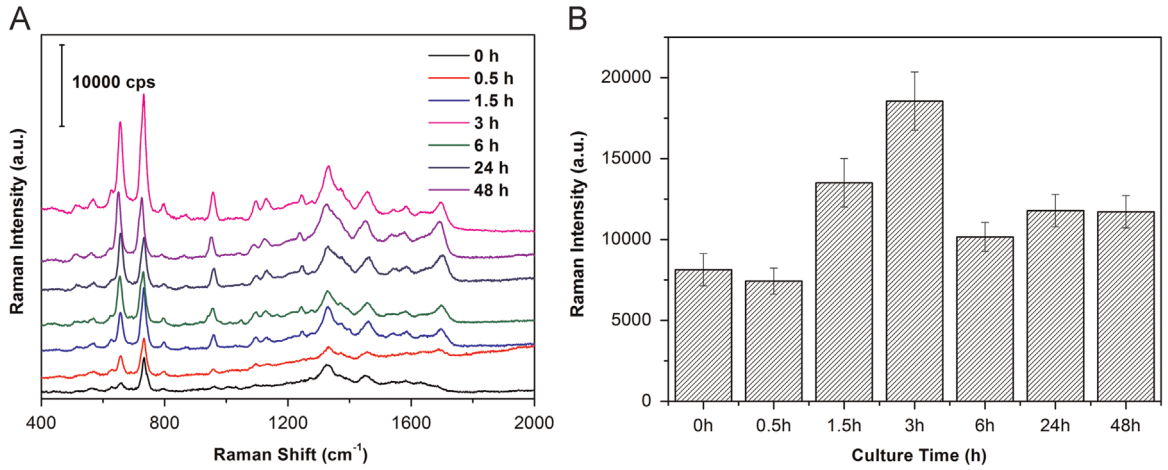

Fig. 3. (A) The SERS spectra of (B) the SERS intensity of AgNPs-*E. coli* DSM 1116 with different incubation time.

addition to a lower and broader absorption peak width ranging from 300 to 480 nm, a new broad band occurred and become wider with the increasing incubation time. The maximum of the new band at ~621 nm appeared at optimized incubation condition without any large changes, which indicated the Ag NPs may aggregate as many as possible on the cell wall (also can be seen in Fig. 5B.) and form the most stable complexity under this optimized incubation condition. Consequently, the cross section of the cell wall components can be efficiently amplified by electromagnetic field enhancement and the SERS signals of bacteria were great enhanced.

3.3. Discrimination of three E. coli strains by SERS

To investigate the potential of this strategy to discriminate *E. coli* on strain level by SERS, three strains of *E. coli* DSM (1116/498/

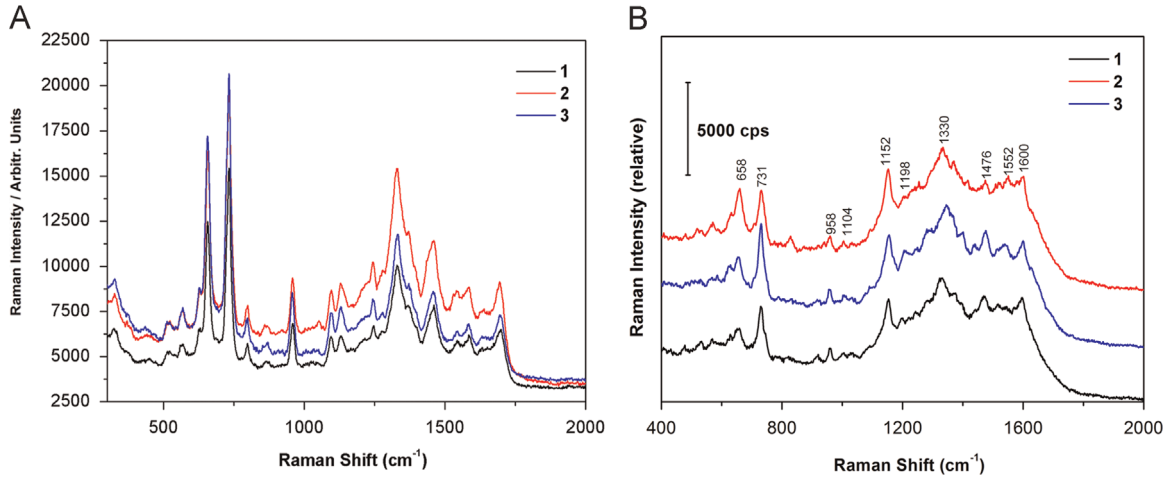

Fig. 4. The spectra of (A) Ag NPs-*E. coli* in water and (B) LB medium at optimized incubation condition.

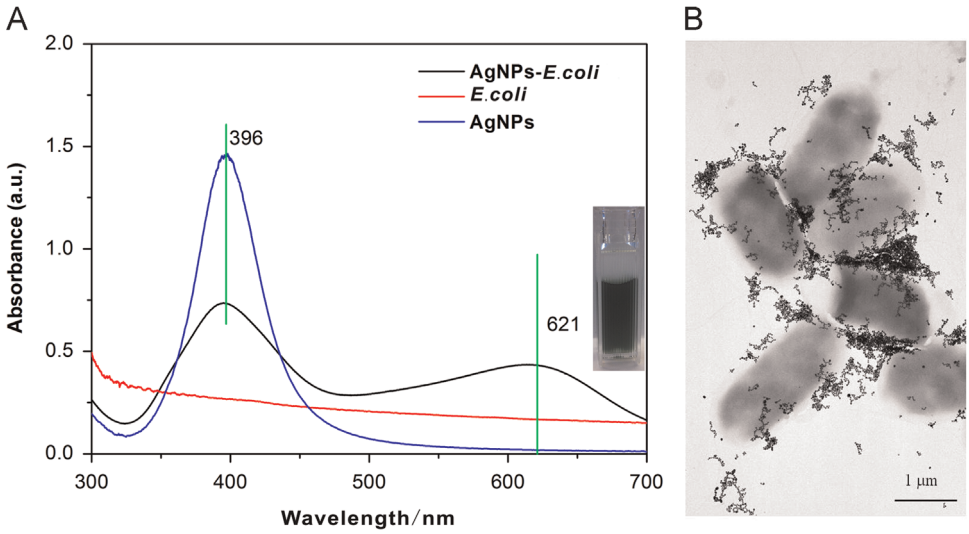

Fig. 5. (A) UV–vis spectra of Ag NPs, *E. coli*, and Ag NPs-*E. coli* (Inset image is the color of Ag NPs-*E. coli*) (B) TEM image of Ag NPs-*E. coli* sample. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

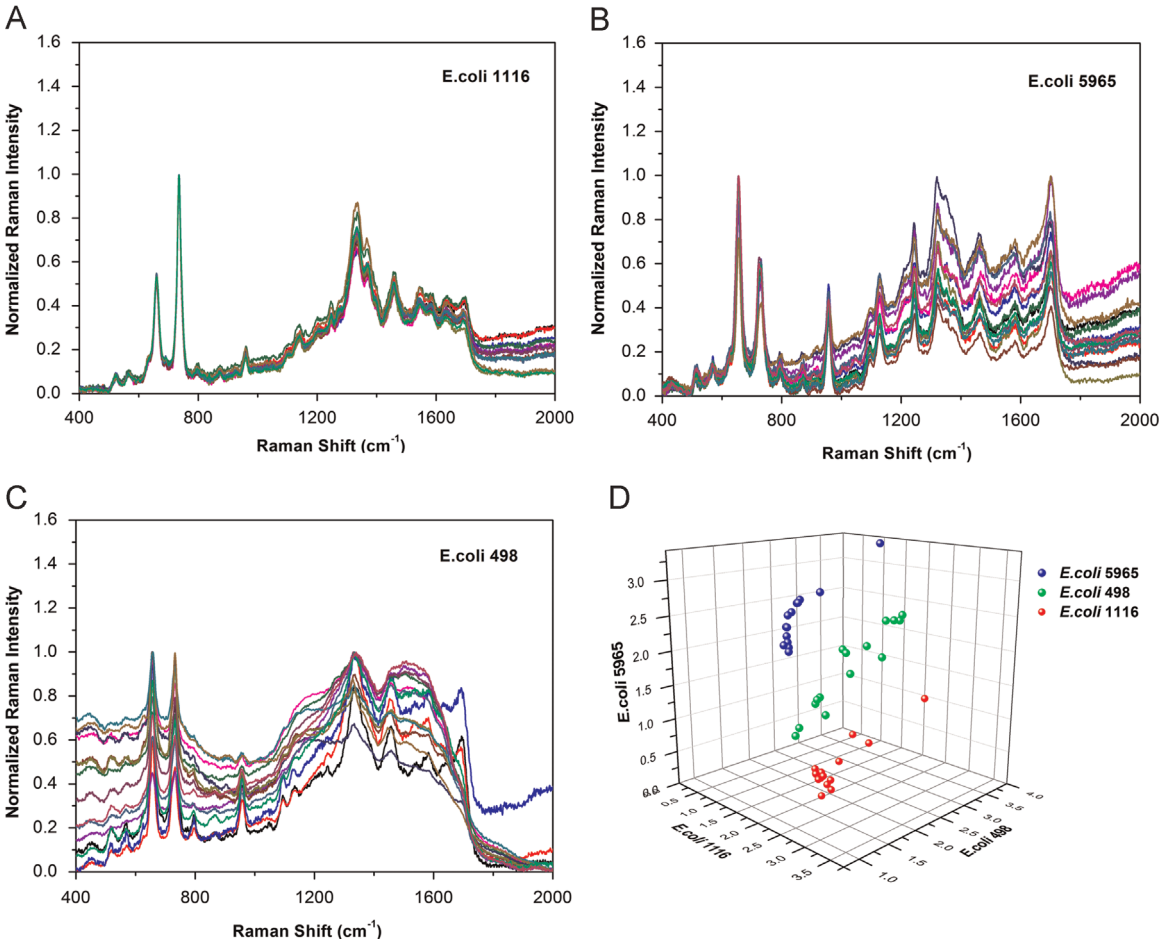

Fig. 6. (A–C) The SERS spectra of three strains of *E. coli* DSM 1116/5965/498 (D) 3D map of the classified results of three *E. coli* strains based on discriminate analysis. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

5695) were chosen. Such three kinds of Ag NPs-*E. coli* samples were harvested at the optimized incubation condition. The Raman spectra of three strains from 45 batches of samples (each strain was represented by 15 batches) were obtained and shown in Fig. 6. For clarity, all the SERS spectra have been normalized to a range from 0 to 1. A discriminate analysis (DA) was employed to help classify three strains of *E. coli*. In a 3D map of the results (Fig. 6D), red balls represent *E. coli* DSM 1116, green balls represent *E. coli* DSM 498, and blue balls represent *E. coli* DSM 5695. It can be seen that they are completely independent with each other in the space, demonstrating that our method combined DA could be used to discriminate the *E. coli* strains successfully.

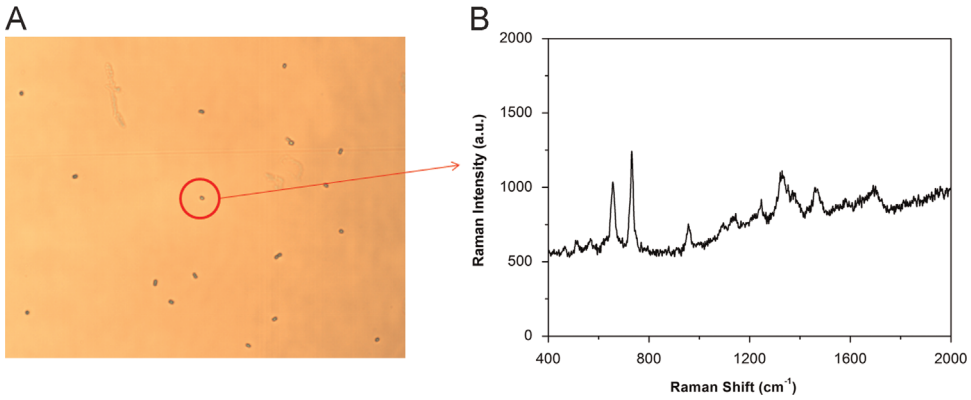

Fig. 7. Raman spectrum of single *E. coli* DSM 1116 cell on glass slide.

3.4. Detection of E. coli on a chip surface

Our method was not only used to detect *E. coli* in bulk liquid but also on surfaces. According to our previous study [21], hydrophobic glass slides possessing more homogeneity with higher coverage of bacteria than normal glass ones [31], which could increase the SERS signals due to its hydrophobic condensation effect [32], were applied in our method. A droplet of 3 μL AgNPs-*E. coli* DSM 1116 suspensions with different concentrations (1×10^5–1×10^8 cell/mL) after optimized condition incubation was pipetted on the hydrophobic glass slides, and then dried at room temperature. All SERS mapping were obtained with the area of

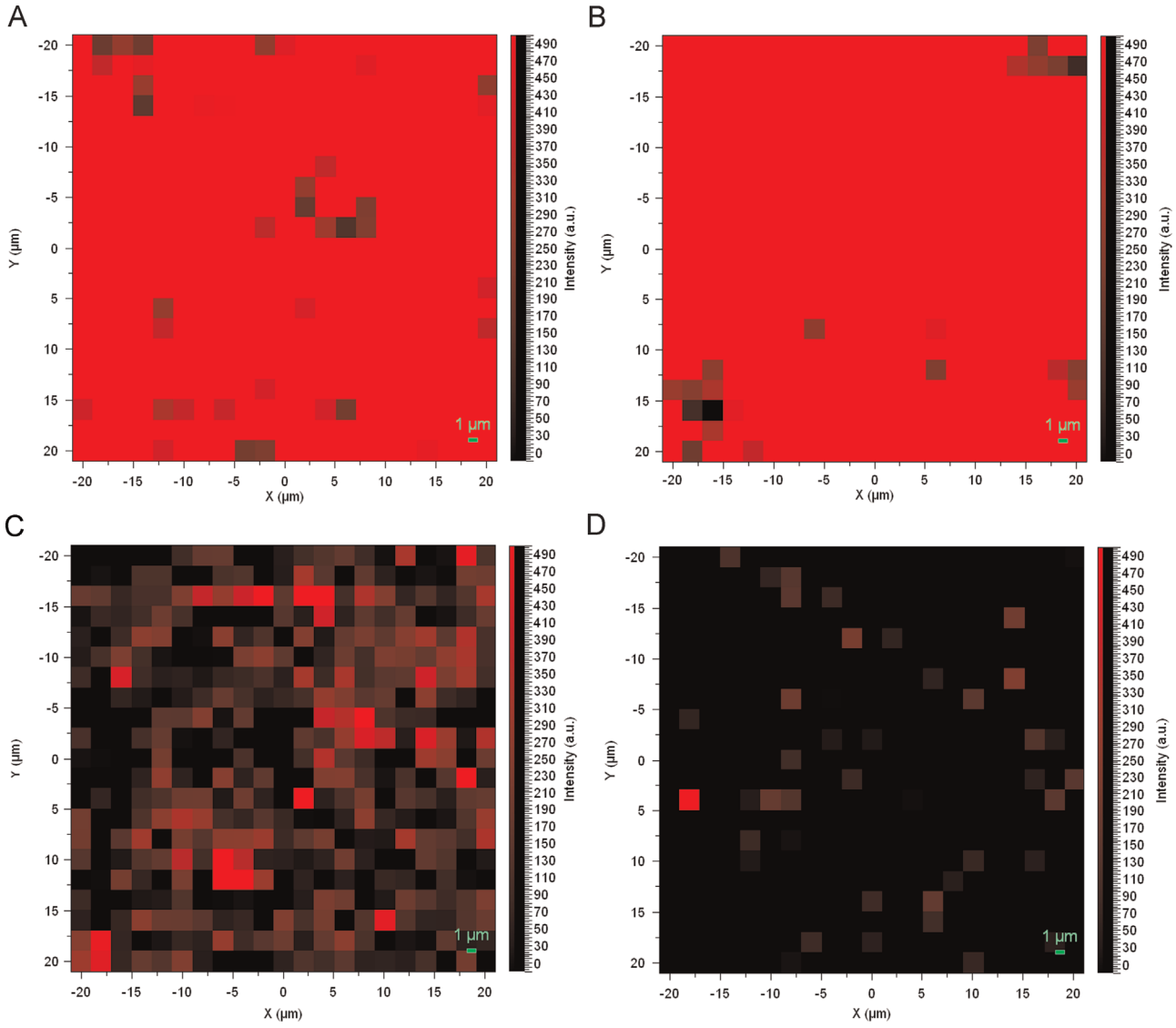

Fig. 8. SERS mapping of *E. coli* DSM 1116 with different concentrations (A) 1×10^8 cells/mL (B) 1×10^7 cells/mL (C) 1×10^6 cells/mL (D) 1×10^5 cells/mL, respectively. (Mapping area: $21 \times 21 = 441$ points, step size 2 μm). (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

40 μm × 40 μm and step size of 2 μm. In all, 441 Raman spectra were collected for each map.

The mapping of the surface detection is based on the SERS capacity of single bacterium cell as shown in Fig. 7. Each red spot represents the bacteria SERS signal detected, and the black spot means that no signal of bacteria was obtained. This spatially resolved mapping analysis of *E. coli* was used to detect different concentrations of *E. coli* DSM 1116 which could be seen in Fig. 8. The number of red dots, i.e., the number of bacteria bound to the surface, corresponds to the total concentration of bacteria. With the increasing of the red spots, the concentration of the bacteria was rising. However, when the concentration increased from 1×10^7 cells/mL to 1×10^8 cells/mL, the number of red spots did not increase correspondingly, which indicated *E. coli* DSM 1116 with 1×10^7 cells/mL already fully covered the slides and achieved a saturation of SERS signal. The dynamic range from 1×10^5 cells/mL to 1×10^7 cells/mL can be detected.

4. Conclusions

In this work, we presented a label-free SERS detection of *E. coli* based on incubation with silver colloid. Optimized incubation condition with shaking speed, time and temperature was found at 100 rpm, 3 h, 37 °C, respectively. An obvious color change occurred during the incubation. The closest attachment between the NPs and cells as many as possible was obtained at optimized incubation condition, which presented the strongest SERS enhancement. The strategy was successfully used to discriminate the three *E. coli* strains by statistical methods. Furthermore, different concentrations of *E. coli* could be evaluated by SERS mapping low to 1×10^5 cells/mL. Our detection method offers higher sensitivity and reproducibility compared to previously reported label free simple-mixing methods, opening an avenue for developing various SERS-based biosensor. The mechanism behind the color change during the interaction of NPs and bacteria will be further investigated.

Acknowledgments

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.09.006.

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