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Nanoparticles, Surface enhanced Raman Spectropic (SERS), Microorganism Identification

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Abstract

Anisotropic nanoparticles (i.e., silver nanocubes, gold nanorods) based SERS molecular probe were designed and fabricated for the rapid and specific detection of bacterial targets in a test-in-a-tube platform utilizing a novel dual-recognition mechanism. The probes were synthesized by covalently attaching Raman tags and bacteria-specific antibodies to the surface of silver nanocubes. Specific binding between the probes and bacterial targets ensured surface enhanced Raman spectroscopic (SERS) signatures of the targets to be observed alongside with the SERS signals of the Raman tags. The assessment through the dual signals (superimposed target and tag Raman signatures) established a specific recognition of the targets in a single step, no washing/separation steps were needed to separate target-bound probes from unbound ones, because unbound probes only yield tag signatures, and could easily be distinguished from the target-bound ones. The dual-recognition protocol implemented with a portable Raman spectrometer would become an easy-to-use, field-deployable spectroscopic sensor for onsite detection of pathogenic microorganisms.

Keywords: *Nanoparticles, Surface enhanced Raman Spectropic (SERS), Microorganism Identification*

Introduction

The presence of pathogenic bacteria in food and drinking water poses a threat to both public health and security. Approximately foodborne diseases cause 76 million illnesses and 5,000 deaths in the United States each year(Mead, Slutsker et al. 1999). To deal with this threat, the first step is to detect them at the earliest possible moment, preferably in-field. However, the conventional methods require series of enrichment and sample preparing steps which limit their in-field deplorability. API, as one of the most popular biochemical tests for bacterial identifications, requires a series of biochemical tests for organism culture. It takes several days to culture the cell, run the test and analyze the results(Jarvis and Goodacre 2008) in this process. Sandwich ELISA (Enzyme-linked immunosorbent assay), another widely used bacterial identification technique, also needs multiple washing steps to separate bound antibodies from unbound ones, which is also unfavorable for in field deployment. (Lam and Kostov 2009).

Raman spectroscopy has long been explored as a tool for biological targets detection and identification (Kneipp, Kneipp et al. 1999), especially with the development of surface enhanced Raman spectroscopy (SERS) that increases the sensitivity of Raman spectroscopy up to $10^{11\text{-}13}$ times(Doering and Nie 2002; Le Ru, Blackie et al. 2007). Noble metal nanoparticles (such as silver, gold etc.) were utilized as uniform, highly sensitive, and reproducible SERS substrates for pathogen biosensor applications(Tripp, Dluhy et al. 2008). Green *et al.*(Green, Chan et al. 2009) demonstrated that different species of *Listeria* could be differentiated based on their SERS spectral signatures analyzed with statistical multivariate discriminant method. With SERS and a novel barcode data processing procedure, Ziegler et al. (Patel, Premasiri et al. 2008) reported more than 50 bacteria could be differentiated. In general, direct differentiation/identification of bacterial targets through their unique Raman spectroscopic signatures require high-quality spectral data in conjunction with statistical analysis built upon known spectral fingerprints of bacterial species, which limit its field-deployability: high quality spectral data are difficult to acquire by a portable instrument, even with SERS; and the statistical recognition procedure won't work if the target to be detected is not in the existing spectral database.

An alternative strategy to utilize SERS for detection of biological targets was also explored by several groups, that is the approach of SERS-based molecular nanoprobes. (Porter, Lipert et al. 2008; Huang, Tay et al. 2009). In this approach nanoparticles functionalized with specific antibodies and Raman reporter molecules are deployed as molecular probes. These probes could specifically recognize bio-targets by antibody-antigen binding, and report these binding events through the Raman reporters. Their application bears great familiarity to fluorescence-labeled antibodies, with the advantage of broader reporter library (>1000 Raman-active molecules) and no photobleaching. However, in this approach, separation of target bound probes from unbound ones through multiple washing steps is still needed, which reduces its field deployability.

In this study we developed a ***dual-recognition mechanism*** utilizing SERS molecular probes to achieve target bacteria detection in one single step, which would be more suitable for in-field applications. As shown in Fig. 1, in the dual-recognition mechanism, Raman-labeled and functionalized anisotropic nanostructures (e.g., gold nanorods, silver nanocubes) are constructed as SERS nanoprobes that display specific label signatures (*probe signal*), and through covalently-bound antibodies they could bind to their target bacteria specifically**.** The antibody-antigen binding ensures that the target cells would attract enough nanoprobes to bind to them, and measureable SERS signals from the bacteria would be generated (non-target would NOT have enough nanoprobes bound to them, and their SERS signal would be non-

measurable). Observation of superimposed SERS signals of the probe and the target indicates the binding events, and subsequently definitely identifies the target in one single step; no washing or separation is needed. Furthermore, since the specificity of the target detection is provided by the antibodies, it is no longer critical to have high-quality spectra: as long as a few key signatures from the bacterial cells are recognized, a positive identification of the target can be reached. A portable Raman sensor thus becomes feasible under the dual-recognition scheme.

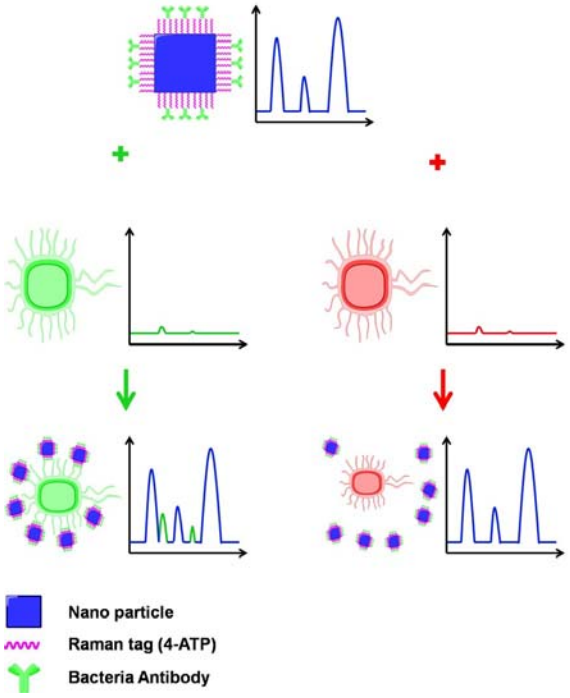

Fig. 1 Scheme of dual recognition bacterial detection SERS nano probe

MATERIALS AND METHODS

Anisotropic nanoparticle fabrication and functionalization

Gold nanorods (GNRs) were synthesized via seed-mediated growth method (Nikoobakht and El-Sayed 2003). Hexadecyltrimethylammoniumbromide (CTAB, 99%) and benzyldimethylammoniumchloride hydrate (BDAC, 99%), Sodium borohydride (99%), L-ascorbic acid, Gold (III) chloride hydrate (>99%) and Silver nitrate (>99%) were all purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Nanopure deionized and distilled water (18.2 MΩ) was used for all experiments. Gold nanorods were made through the seed-mediated growth. Gold nanoparticles with diameter around 4 nm were made as follows as seeds: 0.6mL, 0.01M freshly prepared, ice-cold NaBH4 solution was added to a mixture solution composed of 5 mL, 0.2 M CTAB, 0.25 mL, 0.01M HAuCl4 and 4.75 mL water. The solution was vigorously mixed for 2 min. The seeds were then kept at 27 °C for 3 hours to allow complete degradation of the remaining NaBH4, and were then used in subsequent nanorods growth: 5mL of 0.2 M CTAB solution and 5mL of 0.1 M BDAC solution were mixed to get bisurfactant solution. 80 µL of 0.01 M $AgNO_3$ and 0.5 mL of 0.01 M $HAuCl_4$ were added to the bisurfactant solution. After gentle mixing, 65 µL of 0.1 M ascorbic acid was added. The mixture

solution was mixed until color of the solution changed from yellow to colorless. After addition of 12 μL of seed solution, the growth solution was incubated at 27 °C overnight.

The GNRs were further functionalized by 4-aminothiophenol (4-ATP) as Raman tag and protein cross linker. Briefly, 4 mL of 3 nM gold nanorods were reacted with 0.5 mL of 10 mM 4-ATP dissolved in acidic water (pH = 2) under vigorously stirring at 60 ^{o}C for 3 h. The solution was then centrifuged and washed twice with 3 mM CTAB acidic aqueous solution (pH = 4). Finally, 4-ATP modified gold nanorods were redispersed in 2.5 mL of acidic water (pH = 4).

To covalently conjugate anti-bacteria antibody with GNRs, diazonium salt is needed to be synthesized from 4-ATP. 80 μL of as made GNR 4-ATP solution was reacted with 0.4 mL of 10^{-3} M sodium nitrite at pH 4 at 0 ^{o}C for 30 minutes(Bizzarri and Cannistraro 2007). Then the solution were neutralized by addition of 25μL of 1M sodium hydroxyl and 50μL 100mM phosphate buffer (pH = 7.4). Finally, anti-bacteria antibody was incubated with this diazonium salt solution at 0 ^{o}C for 4 hours giving antibody binding with GNR-4ATP.

Silver nanocubes were synthesized using the method suggested by Skrabalak and Xia (Skrabalak, Au et al. 2007). In four 20 mL glass reaction vials, 6 mL of ethylene glycol were added to each vial. Then these vials were incubated at 150 ^{o}C for 1 hour to evaporate the water in the system. After heating, 70, 80, 90 and 100 μL of 3 mM Na_2S in ethylene glycol were pipette to each vial. The mixture solution were heated for 8 to 10 minutes. Then 1.5 mL of 0.02 g/mL PVP (Polyvinylpyrrolidone) in ethylene glycol was added to each vial. Immediately, added 0.5 mL of 0.048 g/mL $AgNO_3$ in ethylene glycol. Finally, the whole mixture solutions were heating for up to 20 minutes until the solution becoming ochre-colored.

The reaction was quenched by putting the vials in water bath at room temperature. To rinse the nanocubes, twice of the volume of acetone were added to each vial spin down for 30 minutes at 2000*g*.Then particles were washed with deionized water for three times and finally resuspended in 4 mL of deionized water.

For 0.9 mL of as synthesized silver nanocubes, 0.1 mL of 10 mM 11-mercaptoundecaonic acid (MUDA) was added to the solution. Then the mixture solution was stirred at room temperature for 12 hours. To wash out the extra PVP and unbounded MUDA, the solution was spin down and washed with ethanol once and 10 mM phosphate buffer (pH = 7.4) for twice (Rycenga, Kim et al. 2009).

Well established EDC-NHS coupling protocol was used to conjugate anti-bacteria antibody to MUDA modified silver nanocubes.(Yu, Nakshatri et al. 2007) Briefly, 0.2 mL of as made MUDA modified silver nanocubes was treated with 50 μL a mixture of EDC (0.4 M) and NHS (0.1 M). 25 μL of 1 mg/mL anti-bacteria antibody was dded to the solution immediately. The mixture solution was then sonicated in ice water for 30 minutes. The unbounded antibody and coupling reagents were removed by centrifuge and resuspend in 10 mM phosphate buffer (pH = 7.4).

Bacteria cell culture and bacteria nanoprobe interaction

Two bacterial strains (*E. Coli* and *Listeria monocytogenes*) were grown in LB medium at 37 ^{o}C for 18 hours. The bacterial cells were then centrifuged and washed with PBS buffer for two times and finally redispersed in PBS buffer. The final bacterial cell concentration was determined by optical density (OD) measurement at 600 nm. The concentration of the bacterial cells was around 10^9 cfu/mL when the OD of the solution equaled to 1.

Certain concentration of bacteria was added to anti-bacterial antibody-conjugated nanoprobe solution. The mixture was incubated at room temperature for around 30 minutes. After incubation one drop of the mixture solution was placed on a mesh for TEM imaging using a 2007 JEOL 2100 200 kV STEM.

Raman Spectroscopic Measurement

Raman spectra were measured using a DXR Raman microscope (Thermo Scientific, Inc., Madison, WI) with 780 nm excitation and 14 mW laser power. Around 5 µL of the solution was spotted on gold coated glass slide and spectra of the droplet were measured before it was dried.

RESULTS AND DISCUSSION

Raman characterization of gold nano probe modification

With the help of thiol groups, a layer of 4-ATP molecules were anchored on the surface of gold nanorods after incubation with GNRs solution (Frey, Stadler et al. 2001). SERS was used to verify the modification procedure. As illustrated in fig 2, band at 1074 cm^{-1} is the stretching vibration of C-S bond and band at 1578 cm^{-1} is the C-C stretching vibration of benzene ring in 4-ATP (Zheng, Zhou et al. 2003). The appearance of these bands, instead of original CTAB bands, in SERS spectrum indicates successfully substitution of CTAB with 4-ATP on the gold surface.

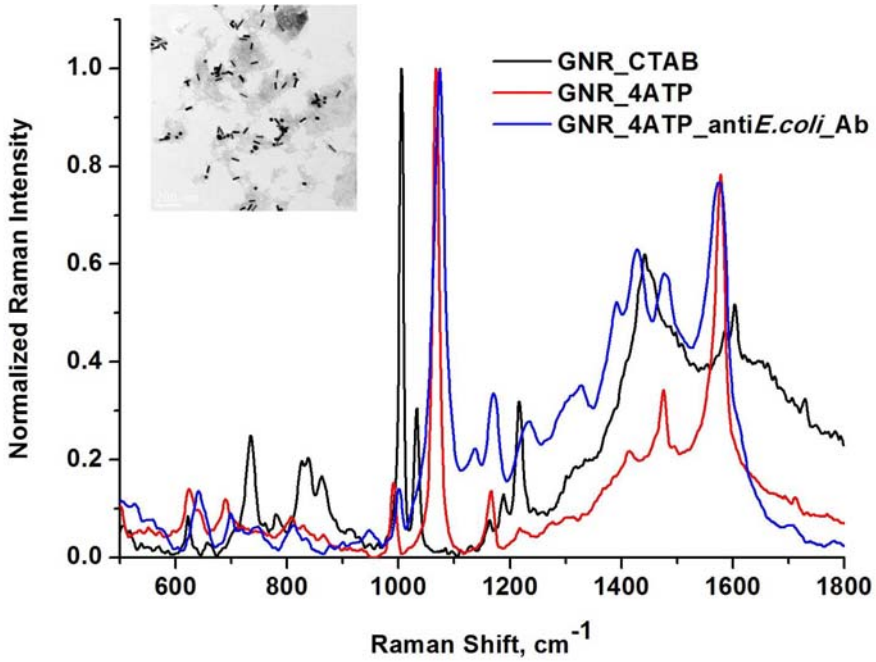

Fig 2. SERS spectra of gold nanorods covered with CTAB (black), 4-ATP (red) and 4-ATP and anti-*E. coli* antibody (blue). TEM image is GNRs modified by 4ATP and anti-*E. coli* antibody.

After react with nitrite ions in acid condition, diazonium salt was produced, which subsequently reacted with histidine in protein. Peak at 1391 cm^{-1} is assigned as N=N stretching vibration of diazonium salt. This new peak in SERS spectrum after protein conjugation proves the formation of diazonium bond (Jiao, Niu et al. 2005) (Fig 2). However it is difficult to identify peaks related to protein after antibody conjugation. One possible reason is that the concentration of antibodies on the surfaces of the nanoparticles is still too low to be detected. To detect protein by SERS, either high concentration of protein (Drachev, Thoreson et al. 2004) or extrinsic Raman label (Porter, Lipert et al. 2008) were needed.

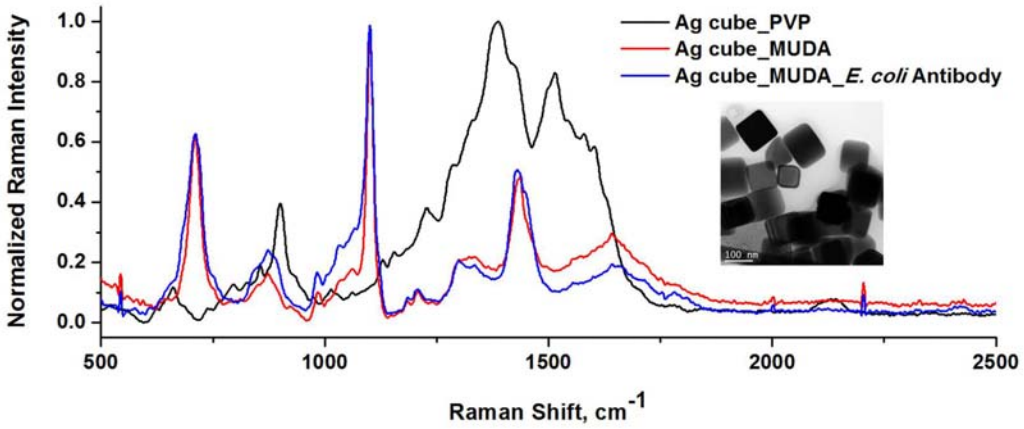

Fig 3. SERS spectra of silver nanocubes covered with PVP (black), MUDA (red) and MUDA and anti-*E. coli* antibody (blue). TEM image is silver nanocubes modified by MUDA and anti-*E. coli* antibody.

SERS spectra of silver nanocubes covered with PVP, MUDA and MUDA linked with *E. coli* antibody were shown in Fig 3. Replacement of PVP from surface of silver nanocubes with MUDA significantly altered the SERS signature of the nanocubes. However, similar as observed for GNRs, the attachment of antibodies to the MUDA-modified silver nanocubes did not introduce identifiable signatures in the Raman spectra of the silver nanocubes, due to weak signals resulted from the low concentration of antibodies on the nanocube surfaces.

Dual recognition to detect target bacteria

Fig 4 shows the TEM images of anti-*E. coli* antibody coated GNRs binding with *E. coli* (Fig 4A) and *Listeria monocytogenes* (Fig 4B). Binding between *E. coli* cells and the nanoprobes could be identified in TEM image, although the bacteria were not fully covered by nanoprobes. Further optimization of the binding conditions is needed in future work. For the *Listeria* which is a non-target for the nanoprobes, there appeared to be no binding of the nanoprobes to the *Listeria monocytogenes* cells.

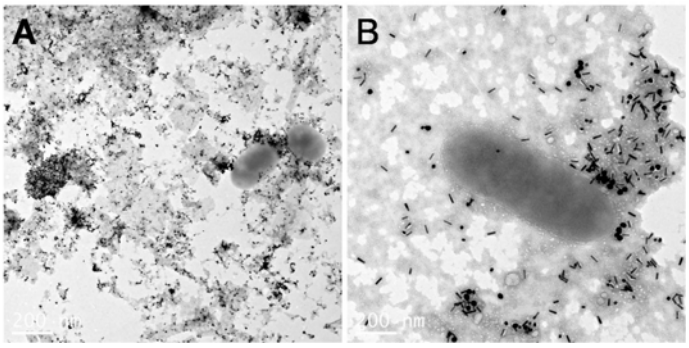

Fig. 4 TEM image of gold nano probe with anti-*E. coli* antibody incubated with (A) *E. coli* and (B) *Listeria*.

Although colloidal nanoparticles can be absorbed onto microbial cell wall from all directions due to electrostatic interaction, simply mixing bacterial cells with nanoparticles usually do not bring enough particles to the surface of bacterial cells to generate measurable SERS signals (Kahraman, Zamaleeva et al. 2009). With our specifically functionalized nanoprobes, however, as shown in the TEM image in Fig 4, more nano probes were attached to the surface of *E. coli* due to the anti-*E. coli* antibodies conjugated on the surface of GNRs.

We also use anti-*Listeria* antibody modified silver nano probe to test the binding effect between *Listeria* and anti-*Listeria* antibody. As demonstrated in Fig 5, *Listeria* antibody could recognize and bind with *Listeria* instead of *E. coli*. This result is consistence with what we obtained with gold nano probe: that is nanoprobes modified with anti-bacteria antibody, could bind to the surface of target bacteria, due to the specific recognition between antibody and antigen.

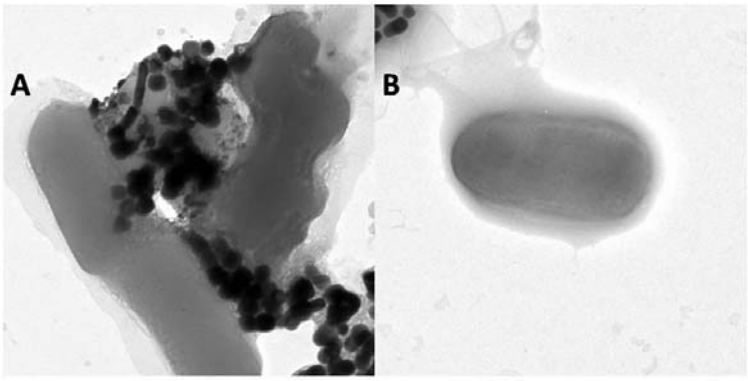

Fig. 5 TEM image of silver nano probe with anti-*Listeria* antibody incubated with (A) *Listeria* and (B) *E. coli*.

Figure 6 shows the SERS spectra of gold nanoprobes (specific towards *E. coli*) incubated with *E. coli* and *Listeria monocytogenes,* respectively. The illumination volume of the Raman microscope was not sufficient to cover a whole bacterial cell and adjacent nanoprobes, average spectra over 10 measurement was used instead to overcome this problem(Jarvis and Goodacre 2008). In fig. 4, peak at 723 cm^{-1} only appeared upon the binding of probes to their specific bacterial targets (*E. coli*). This peak is assigned as adenine from flavin, NAG and NAM (Kahraman, Zamaleeva et al. 2009).

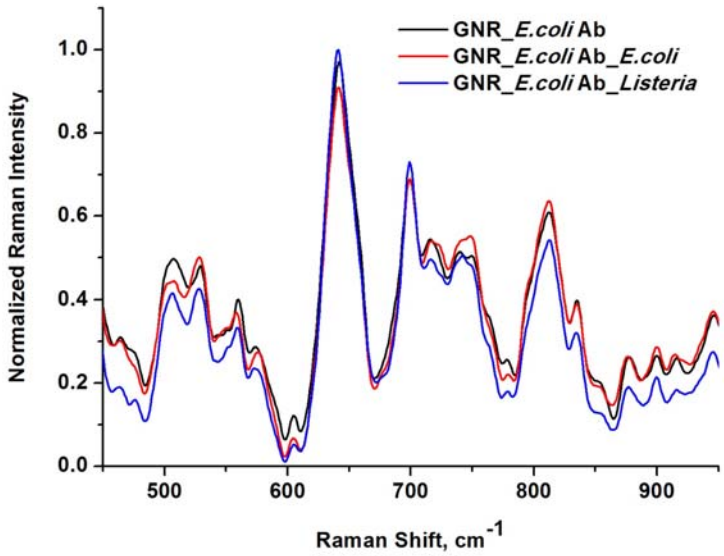

Fig. 6 SERS spectra of gold nano probe covered with anti-*E. coli* antibody (black), incubated with *E. coli* (red) and *Listeria* (blue).

Fig 7 shows the SERS spectra of silver nanoprobes (specific towards *E. coli*) incubated with *E. coli* and *Listeria monocytogenes,* respectively. The 1599 and 1554 cm^{-1} peaks only appeared upon the binding of probes to their specific bacterial targets (*E. coli*) (Kahraman, Zamaleeva et al. 2009). The appearance of these peaks indicated the existence of *E. coli* in the sample

solution. Moreover, these peaks were easier to be identified that the peaks introduced by gold nano probe. The main reason is that silver nanocubes are superior SERS enhancer than gold nanorods (Rycenga, Kim et al. 2009), they would be favored as choice of SERS nanoprobes.

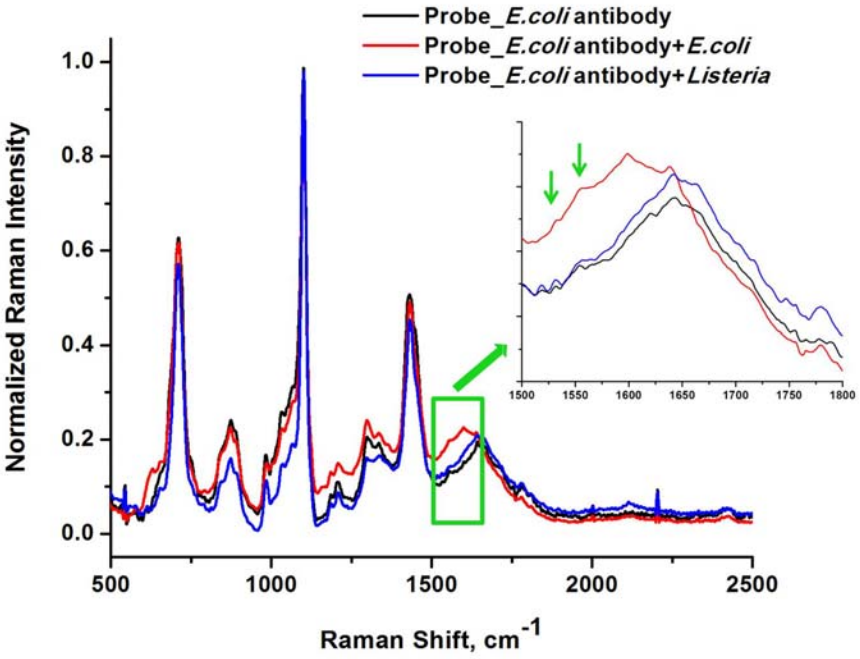

Fig. 7 SERS spectra of silver nano probe covered with anti-*E. coli* antibody (black), incubated with *E. coli* (red) and *Listeria* (blue).

Sensitivity and Selectivity of the Dual-recognition probing scheme

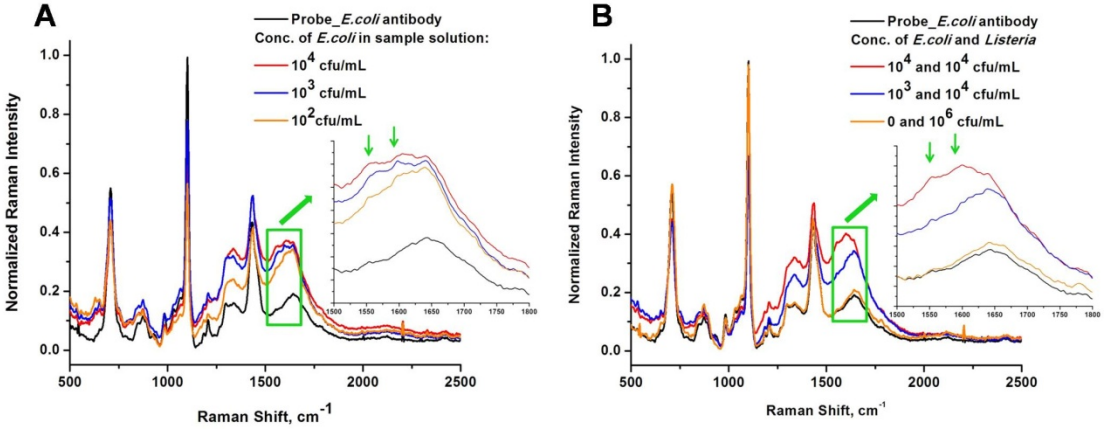

Fig 8. SERS spectra of silver nano probe covered with anti-*E. coli* antibody interacted with: (A) *E. coli* sample solution with different *E. coli* concentration; (B) *E. coli* and *Listeria* mixture sample solution.

The sensitivity and selectivity of the superior silver nanocube probes were investigated. As shown in fig 8A, the two bacterial peaks were identified at 10^2 cfu/mL *E. coli* concentration, comparable with that of high-end ELISA assay, without going through any washing steps.

Also, for a sample solution with concentration of 10-fold higher *Listeria monocytogenes* than target *E. coli*, the specific *E. coli* peaks could still be identified, as shown in Fig. 8B. Relatively

high level of interferences from other bacteria (*Listeria monocytogenes*) did not diminish the sensitivity and accuracy of the dual-recognition probing scheme, indicating that this scheme would be extremely attractive to in-field pathogen detection applications, where interference from other co-existed microorganism species will be omnipresent.

Conclusion

A dual recognition mechanism was successfully established for single-step detection of bacterial target in a lab-in-a-tube setting using SERS spectroscopic sensing. The detection time for this method is relatively short, taking about 30 minutes incubation of nanoprobes with a sample to achieve a definite spectral signal to determine whether or not the targets are present in the sample. The sensitivity of the dual-recognition probing scheme is high (100 cfu/ml), and it was demonstrated that interference from other sources was minimal even at high concentrations (10 times higher than the targets).

The binding coefficient between nanoprobes and target bacteria need to be further improved. So that stronger SERS enhancement of the finger printing peaks of the bacterial targets could be obtained. Also, multiplexing detection scheme will be developed with a multi-channel sensor design in the near future.

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