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Ambient electrospray deposition Raman spectroscopy (AESD RS) using soft landed preformed silver nanoparticles for rapid and sensitive analysis[†]

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We introduce a technique called ambient electrospray deposition Raman spectroscopy (AESD RS) for rapid and sensitive surface-enhanced Raman scattering (SERS) based detection of analytes using a miniature Raman spectrometer. Using electrospray, soft landing of preformed silver nanoparticles (AgNPs) was performed for 30–40 seconds for different concentrations of analytes deposited on conducting glass slides. Using AESD RS, SERS signals were collected within 4–6 minutes, including sample preparation. Transmission electron microscopy (TEM) and dark-field microscopy (DFM) were used to characterize the preformed AgNPs before and after electrospray. We achieved the nanomolar and micromolar detection of *p*-mercaptobenzoic acid (*p*-MBA) and 2,4-dinitrotoluene (2,4-DNT), respectively. In this work, 0.3 μ L of preformed AgNPs were used, which is ~33 times less in volume than the quantity needed for conventional SERS. Quantitation of unknown concentration of analytes was also possible. A similar amount of electrosprayed AgNPs was utilized to characterize *Escherichia coli* (*E. coli*) bacteria of different concentrations. Viability of bacteria was tested using fluorescence microscopic imaging. Besides reduced analysis time and improved reproducibility of the data in every analysis, which is generally difficult in SERS, the amount of AgNPs required is an order of magnitude lower in this method. This method could also be used to probe the real-time changes in molecular and biological species under ambient conditions.

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Introduction

Surface-enhanced Raman spectroscopy (SERS) is used widely as a powerful tool for sensitive detection of surface adsorbed species.^{1–5} It has also been used to probe molecular orientations over nanoparticle surfaces.^{6–8} Numerous analytical applications in different fields including electrochemistry, catalysis, biology, medicine, art conservation and materials science have been reported using SERS because of the rich

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vibrational spectroscopic information it provides.9-11 It has also extended applications in the field of sensors such as chemosensors, biosensors, etc.¹²⁻¹⁴ Silver nanoparticles (AgNPs) are well known SERS substrates used for analyzing chemical and biological systems. They are easy to synthesize, have a high surface area to volume ratio, and possess excellent plasmonic properties. Raman scattering using AgNPs is useful in chemical sensing and catalysis, as in the catalytic reduction of p-nitro thiophenol (p-NTP) to p-amino thiophenol (p-ATP).^{15,16} However, the toxicity of AgNPs has been a hindrance for their efficient use in biological studies. They affect bacterial cells by disrupting the cell membrane and consequently, the cellular functions by causing oxidative damage.^{17,18} Limiting the exposure time of AgNPs can reduce their toxic effects and minimizing their amount used for analysis is a small step toward lessening the impact of AgNPs on the environment.¹⁹ This is particularly important as AgNPs are one of the most marketed nanoparticles (NPs) worldwide.²⁰

Conventional SERS substrates are fabricated by three usual methods: (i) mixing plasmonic NPs with a dilute solution of analytes, (ii) spin/drop-casting of analyte solution on a solid/

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[†]Electronic supplementary information (ESI) available: Optical photograph of the actual AESD RS set-up, characterization of the as-synthesized AgNPs by UV-Vis and DFM, comparative plot between conventional SERS and AESD RS signals, tables showing error bar calculations of calibration curves of *p*-MBA and 2,4-DNT, band assignment tables of SERS features of *p*-MBA and *E. coli*, 'replica plating' of ITO slide dropcast with *E. coli*, viability test of bacteria using fluorescence microscopic imaging and enhancement factor calculations. See DOI: 10.1039/c9an01700c

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dried film of NPs, and (iii) incubating particles with a dilute solution of analytes for a period of time for better adsorption.^{21,22} In these approaches, the preferred adsorption of NPs at specific sites do not happen efficiently within a short period. However, there are other ways of preparing SERS substrates using templated nanostructures on surfaces which are stable, reproducible and convenient to handle.^{23,24} These substrates have mastered the technology of engineering hotspots that result in high SERS enhancement factors and extraordinary signal uniformities over large sampling areas.²⁵⁻²⁷ But their fabrication procedures have rendered them considerably more expensive and uncommon. An alternative approach for the preparation of SERS substrates is possible by ambient electrospray deposition (AESD).²⁸⁻³⁰ Ambient electrospray is a process of producing charged micro or nano-droplets by applying direct current (DC) voltages of the order of a few kilovolts (kV) on liquids confined in a micro-capillary, while soft landing is a process where polyatomic ions are deposited directly onto a specified location of the surface at near-zero kinetic energy.^{29,30} Previously reported results have shown the synthesis of plasmonic NPs using AESD.^{31–33} A combination of ambient electrospray and soft landing can produce a uniform distribution of localized NPs on conducting surfaces.30 Localization of NPs can limit the effective area for SERS examination, but better enhancement of signals can be achieved. Tremendous efforts have been made to obtain efficient SERS substrates by modifying the nanoparticle surfaces. This has enhanced their utility in various analytical applications.^{33,34} In this regard, AESD of preformed AgNPs can be used as a promising technique to prepare SERS substrates for the rapid detection of analytes with a good enhancement factor. In this approach, a stream of electrosprayed droplets composed of AgNPs was sprayed on the dropcast film of an analyte and SERS spectra were recorded from the opposite side of the support.

Here, we report the rapid SERS detection of *p*-mercaptobenzoic acid (p-MBA - a Raman tag), 2,4-dinitrotoluene (2,4-DNT an explosive organic compound) and Escherichia coli (E. coli a biological analyte) by AESD of preformed citrate-capped AgNPs. For this, we introduce the AESD RS technique where ambient electrospray deposition was coupled with a miniature Raman spectrometer. This technique helped us in reducing the exposure time and the amount of AgNPs used for SERS analysis. Nanomolar and micromolar concentrations of p-MBA and 2,4-DNT, respectively, were detected with a small volume of sprayed AgNPs (0.3 µL) in shorter durations (30-40 s). Complete SERS measurements were performed within 4-6 minutes, starting from the sample preparation to data acquisition. Calibration curves were plotted for p-MBA as well as for 2,4-DNT. A linear correlation between SERS signals versus the logarithm of concentration was observed which could be used for the quantitation of unknown analytes, within the uncertainties in intensity measurements. We have also shown the capability of the AESD RS technique to detect *E. coli* at its lowest concentration limit of 10^2 CFU mL⁻¹ and performed live dead imaging of bacteria to show their viability after electrospray deposition (ESD).

Experimental methods and materials

Materials

Trisodium citrate (>99%) was purchased from Merck Life Science Private Limited. Silver nitrate (99.9%) from RANKEM, India. *p*-MBA and 2,4-DNT were purchased from Sigma Aldrich. Luria Bertani (LB) broth and growth media were obtained from HiMedia. *E. coli* (MTCC 443) was obtained from Microbial Type Culture Collection and Gene Bank. A LIVE/ DEAD BacLightTM bacterial viability kit was purchased from Molecular Probes, Eugene, OR. Other reagents were of analytical grade and used without any further purification or treatment. Deionized water (DI) (~18.2 M Ω) obtained from Milli-Q® was used throughout the experiments.

Synthesis of AgNPs

Citrate-capped silver sols were prepared using the modified Turkevich method,³⁵ wherein 17 mg of $AgNO_3$ was dissolved in 100 mL of DI H₂O, and the solution was heated to 100 °C. A solution of 1% sodium citrate (4 mL) was added dropwise to the boiling solution. The solution was kept boiling for 10–15 min until it became pale yellow. This colored solution was allowed to cool to room temperature and was finally stored in the dark at 4 °C. The prepared sols were diluted with DI water (1:1) for electrospray SERS measurements.

Sample preparation and SERS measurements

SERS sample preparation was done by drop-casting analyte solution (40 µL) on the conductive surface of a clean indium tin oxide (ITO), in a controlled manner (2 µL solution spotted every 2 s, 20 times at the same spot) such that the analyte solution spreads equally in all directions. However, after drying, it forms a ring. Four spots near the periphery of the ring (Fig. S2[†]) were selected and used for AESD. Spots more than 4 were not used to prevent interference between AgNPs electrosprayed at these spots. Diluted concentrations of analyte were made from a stock concentration by dissolving 5 mg of a compound (p-MBA and 2,4-DNT) in 1 mL of ethanol. Signals were collected instantly after electrospraying preformed AgNPs for 30-40 s based on the analyte concentration. We have monitored 2 samples of each concentration, with 3 spectra each from 4 different locations of the sample. A total of 24 spectra (2 samples \times 4 locations \times 3 spectra) was averaged, plotted and used for statistical variance calculations. Note that no Raman signals were obtained from the analytes at such concentrations dropcast onto ITO glass slides, without AgNPs.

Preparation of bacterial samples

For this study, *E. coli* cells were grown overnight in LB broth at 37 °C and 220 rpm in an orbital shaker. The cells were then diluted to a concentration of 8×10^8 CFU mL⁻¹ in LB (OD₆₀₀ = 0.1). The cells were separated from the media by centrifugation at 3000 rpm for 5 min. The pellet was washed twice with DI water by centrifugation. Other dilutions ($10^{2}-10^{5}$ CFU mL⁻¹) of bacteria samples were prepared similarly to ~ 10^{8} CFU mL⁻¹ concentration. For fluorescence microscopic analysis, 40 µL of

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the bacterial sample was dropcast onto cleaned ITO glass slides and 20 μ L of propidium iodide-SYTO 9 mix (1:1) was loaded on top of the sample. The mixture was sealed with a 0.145 mm thick cleaned coverslip (Schott) and incubated in the dark for 5 min.

Instrumentation

UV-Visible spectroscopic measurements were performed using a PerkinElmer Lambda 25 spectrophotometer in the range of 200-1100 nm. Transmission electron microscopic (TEM) measurements were performed using a JEOL 3010, 300 kV instrument. As-synthesized AgNPs were spotted on a carboncoated copper grid by drop-casting followed by air drying. For an electrosprayed sample, the copper grid was placed near the silica capillary tip and preformed AgNPs were sprayed for 40 s on a grid followed by air drying. Confocal Raman imaging experiments were performed using WITec alpha300 S equipment. Frequency-doubled Nd:YAG laser (532 nm) with a maximum output power of 20 mW was used for the excitation of the sample. For dark-field imaging, an attachment was designed to use a CytoViva[™] high-resolution dark-field condenser (oil immersion) and 100X oil immersion objective (UPLFLN, Olympus) in the above-mentioned confocal Raman set-up. For white light illumination (400 to 1000 nm), an L1090-Halogen lamp from International Light Technologies Inc. was used. The laser was focused onto the sample using a

100X oil immersion objective (UPLFLN, Olympus). Signals after passing through a 532 nm bandpass filter were dispersed using a grating spectrometer (600 grooves per mm) onto a charge-coupled detector (CCD). Spectral images were scanned using the sample mounted on a piezo stage. Fluorescence staining experiments were performed using a CytoViva[™] microscopy system.

AESD RS set-up

A custom-built AESD RS set-up (schematic in Fig. 1A) incorporates a nanoESI emitter with a high voltage DC power supply of ~2.5-3 kV and a Raman spectrometer (Research India Co.) with a 532 nm excitation laser of 20 mW power on the sample. A grating of 1800 grooves per mm and an accumulation time of 0.3 s were used. The home-made electrospray source was made by continuously infusing a dilute solution of preformed AgNPs (1:1 AgNPs:water, 0.284 nM) through a fused silica capillary using a 500 µL Hamilton syringe and a syringe pump. The inner and outer diameters of the fused capillary were 150 and 300 μ m, respectively. The flow rate was set to 0.5 μ L min⁻¹ that generated a gentle electrospray plume. The positive polarity of a high voltage DC power supply was connected to the needle of the syringe to apply the required potential. A fused silica capillary was connected to the syringe through a finger tight union connector. 100-120 nA current was observed for the electrosprayed AgNPs at ~3 kV, using a picoammeter.



Fig. 1 (A) Schematic representation of the AESD RS set-up, (B) characterization of the soft landed AgNPs before and after electrospray, (i and ii) TEM images of the preformed AgNPs before and after electrospray, respectively, (iii) DFM image of the soft landed AgNPs, and (iv) plasmonic scattering spectra corresponding to each of the AgNPs encircled in (iii).

The tip of the capillary was placed in such a way that the soft landing of nanoparticles can be done over one side of the ITO plate containing the analyte of interest. The ITO plate was then connected to the ground to dissipate the charge of the droplets.

Data processing by the clustering algorithm

Cluster analysis for confocal Raman spectral image was performed using an in-built clustering algorithm of the WITec software.

Results and discussion

Ambient electrospray deposition Raman spectroscopy (AESD RS)

The set-up incorporates a nanoESI emitter (a fused silica capillary) with a high voltage module and a miniature Raman spectrometer with a green laser as the excitation source. Details of the set-up are discussed in the Experimental section. The charged AgNPs generated by the ion source were soft landed on the analyte film which was dropcast onto an ITO glass plate placed at a distance of 3–5 mm from the tip of the silica capillary. Instead of placing the analyte coated glass slide facing the Raman objective, it was placed in an inverted position such that it faces the tip of the silica capillary. A schematic illustration of the set-up is shown in Fig. 1A and an optical photograph of the actual set-up with a zoomed-in image of the sample region is shown in Fig. S1.[†]

Experiments involved the recording of SERS spectra from dropcast analyte on ITO after spraying AgNPs for 30-40 s until enhanced SERS signals appeared. Signals were recorded from four locations of the sample (see Fig. S2[†] for details). In this work, we have probed p-MBA, 2,4-DNT, and E. coli for analysis. Characterization of the as-synthesized AgNPs was performed by UV-Vis absorption spectroscopy and DFM, as shown in Fig. S3.† The absorption peak of AgNPs appeared at 420 nm in solution (see Fig. S3[†]), which confirmed the formation of plasmonic nanoparticles. Subsequent studies were performed on the electrosprayed AgNPs by TEM and DFM. TEM images of the preformed AgNPs before and after electrospray are shown in Fig. 1B(i and ii), showing that applying such high voltage brings about some changes in the morphology of the NPs, although this aspect has not been investigated here. These modified AgNPs served as better SERS substrates having more hotspots due to the increase in polydispersity and sharper edges of the particles. Increased polydispersity after electrospray is influenced by the parent sample, surfactants in the medium, and electrospray parameters. These need to be optimized, and we are currently pursuing this study to obtain ideal conditions. Increased polydispersity of AgNPs leads to SERS enhancement, but it causes spatial variations in intensity. Post-characterization of the sprayed AgNPs was performed by DFM and plasmonic scattering spectroscopy, as shown in Fig. 1B(iii & iv). Multiple colors of AgNPs in the corresponding DF image indicate the polydispersity of the sample, which has been supported by the TEM image (ii). The particle size distribution of the AgNPs before and after electrospray is shown in Fig. S4.[†] Size distribution calculations were performed using DF images and the ImageJ software. These plots suggested that before and after electrospray, the average size of AgNPs remained approximately the same. However, some aggregates were formed by electrospray as evidenced by the DFM image and the scattering spectra. With better understanding and characterization of the SERS substrates, the detection of analytes was pursued, and the corresponding data are presented in the next section.

Rapid and sensitive detection of *p*-MBA and 2,4-DNT using AESD RS

For SERS detection, 40 µL of an ethanolic solution of analytes were dropcast onto ITO glass slides and air-dried. We achieved detection in the range of 100 μ M to 1 nM (15 μ g mL⁻¹ to 0.15 ng mL⁻¹) for *p*-MBA and 3 to 0.5 μ M (0.45 μ g mL⁻¹ to 0.075 μ g mL^{-1}) for 2,4-DNT, respectively. The compound *p*-MBA, being a good Raman probe, has been used widely for SERS detection and also for pH sensing which is nowadays used for cellular imaging.³⁶ Hence, it was used as a model analyte for AESD RS but to prove the ability of our technique over a wide range of analytes, 2,4-DNT (an explosive surrogate) and E. coli (a biological species) were also tested. A waterfall plot of SERS spectra of p-MBA at different concentrations is shown in Fig. 2A. The spectra are dominated by two features at 1586 and 1080 cm⁻¹, which are assigned to ν_{8a} and ν_{12} aromatic ring vibrations, respectively.³⁷ A second sharp peak appears at 1377 cm⁻¹ due to the symmetric stretching of the carboxylate. Also, the bending mode of carboxylate appears at 844 $\rm cm^{-1}$, which was broad and weak at lower concentrations. In our experiments, the absence of 910 and 2580 cm⁻¹ peaks, which correspond to δ (CSH) and ν (SH) bonds, respectively, indicates that the analyte was bound with the electrosprayed AgNPs. Major peak assignments are shown in Fig. 2A and complete assignments of the vibrational bands are listed in Table S1.† A pictorial representation of p-MBA molecules bound to the Ag surface is shown in the inset of Fig. 2A. The signal intensity of the molecule increases gradually with an increase in the concentration from 1 nM to 100 µM. A calibration curve was plotted between SERS intensity (counts) of the 1377 cm⁻¹ peak versus the logarithm of concentration, as shown in Fig. 2B. Intensities used in the calibration plot were taken after averaging 24 spectra at each concentration. The linear correlation (R square value is 0.9895) observed could be helpful in the quantification of unknown analytes.³⁸ Mean and standard deviation values of the calibration curve calculated from the weighted intensities of the sample (p-MBA) are shown in Table S2.† Variance observed in the calibration plot is due to the morphological changes occurred in AgNPs as a result of ESD. Such morphological changes will alter the number and position of hotspots on the nanoparticles. As the variance is large, the quantitation of analytes using AESD RS is semiquantitative. We also calculated the enhancement factor^{30,39} (EF) for the SERS system probed by AEDS RS. An EF of 2×10^8 was observed for 1 µM concentration. EF calculations were per-



Fig. 2 (A) SERS spectra of *p*-MBA of various concentrations ranging from 100 μ M to 1 nM, the inset shows the pictorial representation of *p*-MBA molecules adsorbed on the AgNP surface and (B) calibration curve of SERS intensity of the 1377 cm⁻¹ peak *vs.* logarithm of the concentration of *p*-MBA. Data are fitted with a straight line.

formed using the weighted intensity counts of the 1586 cm⁻¹ peak, and the details are discussed in the ESI.†

Control experiments of blank ITO and citrate-capped AgNPs were also performed to show that p-MBA signals were significantly different from those of citrate-capped particles and ITO. The spectra of ITO and citrate are shown in Fig. S5.† ITO signals appeared as two broad bands in the region of 500-700 and 900–1200 cm⁻¹, respectively, which are in good agreement with the values reported in the literature.⁴⁰ Since citrate has very weak Raman scattering cross-section, sharp and wellresolved SERS features were not observed in the preformed AgNPs before and after spray. Symmetric and asymmetric COO⁻ stretching features of citrate in the 1370-1385 and 1580-1590 cm⁻¹ window were detectable with reduced intensity.41 In addition to the control experiments, a comparative study was performed between dropcast and electrosprayed SERS signals, as shown in Fig. S5.[†] We observed that in the case of dropcast colloidal AgNPs (10 µL) solution on dried p-MBA (40 µL), SERS was observed, but intensity and sharpness of the signals were much lower than that obtained from

the electrospraved AgNPs. Signal intensities for the dropcast AgNPs were in the range of 1500-2000 counts (for 1586 cm⁻¹), whereas, in the electrospray method, intensities enhanced to 10 000 counts which were \sim 5 times that obtained for the same analyte concentration (see Fig. S5[†]). In the AESD method, a colloidal solution of preformed AgNPs was sprayed for 40 s at a flow rate of 0.5 μ L min⁻¹ to obtain the signals of the analyte. It was observed that the utilized volume of the colloidal solution is ~33 times lesser than the volume used in the conventional dropcasting methods for SERS analysis. We also compared the EF between dropcast and electrosprayed SERS signals using 10 µM concentration of p-MBA with 0.3 µL of AgNPs. Calculations showed that the EF of electrosprayed SERS is 5.2 times higher than that of the dropcast SERS (shown in the ESI[†]). Similar concentration-based experiments were performed on 2,4-DNT, an organic compound used as a surrogate for trinitrotoluene (TNT). Its detection was performed sequentially from 3 µM to its toxicity threshold limit $(0.5 \ \mu M)$, and the corresponding data are listed in Fig. S6⁺ and Table S3.† A stacked plot of different concentrations of DNT is shown in Fig. S6A[†] and a calibration curve was also plotted (Fig. S6B[†]). Error calculations of the calibration curve are shown in Table S3.† With the help of this curve, spiked DNT concentrations can also be quantified approximately. Assignments of the vibrational bands are shown in Fig. S6A.⁺⁴² Hence, soft landing of preformed AgNPs using AESD RS serves as a robust method for making useful SERS substrates for the rapid detection and semi-quantitation of analytes.

Post-characterization of electrosprayed AgNPs by DFM and confocal Raman imaging

The SERS experiments presented in the previous section showed that electrospray facilitates the rapid and efficient detection of analytes. However, it is equally essential to ensure that SERS signals appeared only from the locations where the spray has happened and are absent otherwise. To do this, we performed confocal Raman imaging aided with dark-field microscopy on the sprayed samples separately, as our AESD RS system was not integrated with the imaging technique.

However, the intensity of SERS signals collected in AESD RS will be different from those measured in the confocal system as the sample was dried before performing dark-field assisted confocal Raman measurements. At first, the optical image stitching of the sprayed sample was performed, shown in the inset of Fig. 3A, then the boundary of the electrosprayed AgNPs was focused with a dark-field objective followed by confocal Raman imaging of an area of $20 \times 20 \ \mu\text{m}^2$. The observed boundary in the inset (Fig. 3A) is due to the size distribution of droplets in electrospray, which suggests that some droplets are deposited on the substrate in the wet form, while many nanoparticles are deposited directly. However, the fraction of charged droplets vs. NPs is not clear from the AESD RS data. As micro and nano-droplets travel more distance, the solvent keeps evaporating and thus increasing the distance would increase the soft landing of dry and charged NPs and concomitantly it will decrease the number of droplets from being de-

posited. To ensure that the formation of charged droplets is minimum during AESD, we have increased the tip to collector distance from ~4 mm (original distance) to ~8 mm and ~12 mm, respectively. At larger distances, SERS signals (Fig. S7[†]) were still observed (for 10 μ M *p*-MBA) but with the reduced intensity as compared to ~4 mm distance. Thus, we conclude that at a distance of ~4 mm, the relative contribution of charged droplets on SERS signals will be more compared to that of the dry NPs. It will be difficult to distinguish quantitatively the fraction of charged droplets vs. dry NPs on the observed signals when AESD and SERS are happening simultaneously. The optical image, DFM image of the boundary with sprayed AgNPs and the corresponding confocal Raman imaging of one of those regions are shown in Fig. 3. It is evident from the confocal Raman map that only at the electrosprayed AgNP locations, SERS signals were observed, while no signal was detected where AgNPs were absent. Raman imaging was performed for 10 μ M concentration of *p*-MBA.

Fig. 3 Image mapping of the electrosprayed AgNPs. (A) Dark-field

image of one of the portions of the boundary of electrosprayed AgNPs, and (B) confocal Raman imaging of the region encircled in (A) with a heat map shown next to it, where 0 and 5363 indicate the CCD counts

in the SERS spectra. An image in (B) corresponds to the total SERS inten-

sity of the spectrum. An optical image of the sprayed region shown in

the inset of (A), scale bar in the optical image is 500 μ m. The dotted

Confocal Raman map analysis by the clustering algorithm

A clustering algorithm is one of the simplest algorithms for spectral image analysis. It groups the spectra according to their similarity in peak positions, widths and signal intensities, forming clusters which represent the regions of the image with identical molecular properties.43 Confocal Raman image of the electrosprayed AgNPs shown in Fig. 3 has been subjected to cluster analysis to obtain the SERS spectra of p-MBA at different regions of the map. Cluster analysis gives the average spectra of various regions. The cluster analyzed spectra are shown in Fig. 4, which resulted in two sets of groups as (iiv) and (v). The first group (i-iv) corresponds to those regions of the map where SERS signals were observed due to the presence of sprayed AgNPs on the dropcast analyte. However, the other group (v) corresponds to that region of the map where no SERS was observed. Insignificant intensity for the whole region in this spectrum (component v) shows that *p*-MBA at

this concentration (10 μ M) could not be detected without AgNPs being present. Along with the cluster spectra (Fig. 3B), the corresponding Raman images (Fig. 3A) are also shown (i-v). The SERS spectra obtained for different regions of the Raman map were similar with spatial variations in the intensity which could be due to nanoparticle reshaping caused by laser irradiation as reported recently.³⁵ Thus, the clustering of the Raman map ensured that the SERS signals of *p*-MBA were observed only in the regions of spray and were absent otherwise.

AESD RS as a tool for biological applications

The bio-molecules of bacteria interacting with AgNPs show distinct SERS spectra.^{17,44–46} Contact between bacteria and AgNPs is necessary for obtaining reliable SERS signals. Although AgNPs perform well as SERS substrates, they are known to possess antimicrobial properties.⁴⁷ AgNPs induced apoptosis influences the spectra to a large extent.^{47,48} Overcoming this toxicity of AgNPs while utilizing them as SERS substrates have been a challenge.

We present a technique, wherein the volume of AgNPs sprayed on bacterial cells is similar to the amount used for the detection and quantification of previous analytes. In this case, the time for exposure to AgNPs was 40–50 s, which was slightly more as compared to *p*-MBA and 2,4-DNT. During this short time, the signals can be obtained without much effect on the bacterial cells. As the time of exposure to AgNPs is shortened, the uptake of AgNPs by the bacterial cells will be reduced. The cells are not suspended in colloidal AgNPs solutions,⁴⁹ as done in conventional techniques. Thus, we can use the AESD RS method without much sample preparation for analyzing bacteria in water, food, clinical, and environmental samples. DFM images of the untreated bacteria and the bacteria treated with sprayed AgNPs are shown in Fig. 5A and B, respectively. Expanded DFM images of the individual bacterium are shown



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(B)

Fig. 4 Cluster analysis of the electrosprayed region, (A) confocal Raman map of the region as shown in Fig. 3, (i)–(iv) indicate the respective regions of map A, where SERS signals of *p*-MBA were observed in the presence of AgNPs, and (v) shows the region of map A, where no SERS was observed in the absence of AgNP spray, and the spectra corresponding to regions (i–v) are shown in (B), scale bar in all images is 4 μ m.

region is expanded in (A).



Fig. 5 (A and B) DFM images of the bacteria before and after electrospray. An individual bacterium is expanded in the inset of (A) and (B), and (C) SERS spectra of *E. coli* (MTCC 443) at different concentrations $(10^5-10^2 \text{ CFU mL}^{-1})$ with their vibrational band assignments.

in the insets of Fig. 5A and B. Average spectra of different concentrations of E. coli are shown in Fig. 5C. The resultant SERS spectra are dominated by peaks at 1370-1420, 950-1000 and 560-640 cm⁻¹ which were attributed to symmetric COO⁻ stretching, membrane phospholipids and carbohydrates, respectively.¹⁷ Complete band assignments are listed in Table S4.† Although AESD RS can analyse bacteria, we wanted to know whether these organisms are indeed alive during analysis. Therefore, we have performed 'replica plating' of the ITO glass slide, dropcast with E. coli, on two types of agar-based growth media. Nutrient agar (general nutrient media for algae, bacteria, etc.) and MacConkey agar (media specific to Gramnegative bacteria) were used for the replica plating. The first replica was made with the glass slide dropcast with E. coli on the agar plate. Then, the same slide was removed from the agar plate and used for electrospray deposition. This AgNP deposited plate was used for the second replica on a fresh agar plate. These plates were incubated at 37 degrees for 24 hours. Results for these four plates are shown in Fig. S8.[†] The growth of the bacteria after the electrospray showed that they were not killed during deposition.

Our claim that E. coli have survived after electrospray deposition is to show that the conditions at which the electrospray was performed enable analysis on live bacteria as well. The exposure time of AgNPs to bacterial cells was just 60 s. The volume and concentration of AgNPs used for the sample are 0.5 µL and 0.284 nM, respectively. The effect of AgNPs at this condition cannot be ascertained quantitatively as the current AESD RS set-up is not integrated with an imaging system. Thus, we have shown the effect qualitatively. We have also performed fluorescence microscopic imaging (see Fig. S9[†]) to show the viability of bacteria and a statistical count of live and dead bacteria.⁵⁰ For *E. coli* after electrospray deposition, the death rate of bacteria was somewhat higher than that of E. coli before electrospray. However, it is evident from images (Ai, Aii, Bi, and Bii, Fig. S9[†]) that a large number of bacteria survived even after ESD. Statistical calculations were performed using ImageJ analysis and it was seen that 94.2% bacteria were alive after ESD, while 96.7% bacteria were alive prior to ESD. The location of nanoparticles can be ascertained using correlated optical DFM and fluorescence images (Ci, Cii, Di, and Dii, Fig. S9[†]), but the understanding of the effect of nanoparticles on bacteria cannot be quantified as the exposure time is very short and the number of NPs interacting with each bacteria is different. Inferences from replica plating and fluorescence imaging experiments support that AESD RS can help in the analysis of live bacterial cells.

Conclusions

In conclusion, the technique of AESD RS was introduced, which is capable of rapid and sensitive SERS detection of molecules in diverse systems. Nanomolar and micromolar concentrations of p-MBA and 2,4-DNT were detected within 4-6 minutes, including sample preparation. A linear plot of SERS signal intensity versus logarithm of concentration was observed, which can be used for the semi-quantitative analysis of spiked analytes. AESD RS provides an enhanced signal intensity of the order of 10^8 for 1 μ M concentration, which is 5.2 times higher than that of the drop-casting SERS method. As the AESD RS set-up was not integrated with an imaging technique, post-characterization of the electrosprayed AgNPs was performed using confocal Raman imaging aided with DFM. Post-characterization helped us to ensure that the SERS signals appeared only from the sprayed locations. Cluster analysis of the Raman image of p-MBA resulted in two groups, where one showed SERS signals in the sprayed region, and another group where no SERS was observed. Furthermore, our technique proved to be an important tool for detecting and characterizing *E. coli* at its lowest concentration of 10^2 CFU mL⁻¹. Qualitative experiments of replica plating showed that bacteria survived after ESD. Live and dead fluorescence imaging of E. coli provided a statistical count of live and dead bacteria after ESD. The AESD RS technique can also serve the purpose of single bacterium detection if an imaging system is coupled with it. We believe that the real-time changes in

molecular and biological species can also be performed efficiently using the presented technique to get better insights into the orientations and intermediates of the analyte in a similar time scale.

Conflicts of interest

There are no conflicts to declare.

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