Fano Resonance Enhanced Raman Spectroscopy for Ultrasensitive *Escherichia coli* Detection

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Abstract

Strategies for in-liquid micro-organism detection are crucial in clinical and pharmaceutical industries. While Raman spectroscopy is a promising label-free technique for micro-organism detection, reaching detection limits similar to those obtained using culture-based methods, while keeping a high accuracy, remains challenging due to weak bacterial Raman signals. Taking advantage of the unique electromagnetic properties of metamaterials, we demonstrate bacterial identification in liquid using an array of Fano-resonant metamolecules that act as an enhanced Raman scattering (FERS) platform. The metamaterial is designed to exhibit a Fano resonance close to the protein amide group fingerprint regions. Under the off-resonance condition, Raman signatures of *Escherichia coli* were recorded at several locations on the metamaterial. We observe that the intensity of the amide characteristic peaks increases during the bacteria exponential phase while it decreases at their stationary phase. This study provides a new set of opportunities for developing
ultrasensitive FERS platforms suitable for large-scale applications and will be particularly useful for diagnostics and environmental studies.

**Keywords:** Fano resonant, metamaterial, amide vibrations, micro-organism detection

1 Introduction

Microbial contamination testing is a key point in the quality control of pharmaceutical drug products [1] before their commercial release and the pharmaceutical industry continuously strives to develop new methods for this purpose [2, 3]. Standard procedures for the accurate detection of micro-organism contamination are limited in terms of sensitivity and specificity given their dependency on growing conditions [4]. A complete absence of micro-organisms is usually required for drug products to pass quality control tests. For this reason, novel biotechnological approaches for micro-organism detection must secure detection of their potentially present during each step of drug production [1, 5]. Determining the absence of micro-organisms using conventional growth-based microbiological methods [6, 7] presents a further challenge in the laboratory environment as the risk of false positive test results caused by laboratory contamination during sample handling [8] must be minimised.

In recent years, the fabrication of novel optical nanostructures [9–18] illustrates the potential for using optical techniques to detect micro-organism contamination with high accuracy and sensitivity. Among the various optical biosensor schemes [19], metamaterial-based plasmonic biosensors [9, 17] show great potential because of their exotic effects, such as negative refraction [20], perfect lenses [21], and even optical cloaking [22], enabling unprecedented control of light at the subwavelength scale. Fano-resonant asymmetric metamaterials [23, 24] have been utilised for both biomolecule detection [11] and precise nanoparticle trapping [25, 26]. Periodically arranged metamolecules with nano-aperture structures are characterised by strong localised electromagnetic fields [25], enabling detection of extremely small amounts of chemical and biological substances [27]. Each metamolecule in an array can be treated as a single-dipole resonator with a polarizability that depends on its material, size, and shape [28–30]. The diffractive scattering of photons by each metamolecule can excite localised surface plasmons of adjacent metamolecules instead of decaying into free space, leading to suppression of the radiative loss by the lattice resonances [28]. Such metallic nanostructures organised into ordered arrays can support surface lattice resonances, thereby enabling the manipulation of light at the nanoscale as well as permitting a plethora of applications [30]. In addition, metamaterials operating at THz frequencies [17] have micron-sized apertures and can serve as ideal platforms for fungi and bacteria [19] detection since the sizes of these micro-organisms are on the order of \( \lambda/100 \) (\( \lambda \) is the wavelength of laser), which is comparable with the micro-aperture size.
Bacteria and several micro-organisms are responsible for many human diseases, and the rapid and accurate identification of bacteria species is crucial for effective treatment and the prevention of further infections. Currently, culture-based diagnostic techniques, such as polymerase chain reaction (PCR) [31] and enzyme-linked immunosorbent assay (ELISA) [32] are the commonly used for bacterial identification. Although culture-based methods can provide accurate results for a wide range of infectious bacteria, they are time consuming, expensive, usually require specialised technicians, and lack the ability to distinguish between live and dead bacteria differences owing to their similar chemical compositions that may lead to inaccurate readings [33].

Surface-enhanced Raman spectroscopy (SERS) offers a potential solution for label-free cellular identification with minimal sample preparation and low cellular damage. Despite considerable advances in the characterisation of bacteria using SERS [19], many previous studies used dried samples and long spectral acquisition i.e. few minutes. However, clinical or pharmaceutical samples are generally in liquid and drying can remove or modify important biological information from the SERS spectra. Furthermore, the majority of SERS-based bacterial biosensing has been performed with plasmonic nanoparticles [34, 35], where nonuniform nanoparticle distribution can lead to spot-to-spot variations in SERS enhancement, making quantification difficult. The generation of reproducible SERS spectra remains a challenging task for some complex biospecimens, such as bacteria. Even though advances with SERS are promising, new approaches should improve accuracy, reproducibility and effectiveness to be suitable for pharmaceutical and clinical control. The use of nanostructure arrays [25, 26] with long-range ordered features that eliminate spot-to-spot spatial variation could lead to periodic enhanced Raman scattering hot-spot generation.

Here, we demonstrate a liquid bacterial Fano-Resonant Enhanced Raman Spectroscopy (FERS) platform with large-area FERS enhancement and sensitivity for the amide modes of proteins. We designed and fabricated an array of asymmetric split-rings (ASRs) consisting of two types of nano-apertures, C-type and l-type, that support a Fano-resonant mode, allowing for strong background suppression, increased sensitivity, and significant field enhancement. The Fano resonance properties of metamaterials are highly sensitive to modification of their surrounding medium [23] making them particularly attractive for use as biosensors. Fano resonant biosensors can provide accurate detection of low-weight biological specimens at fairly low concentration. Our metamaterial is designed to exhibit a Fano resonance peak at 1676 cm$^{-1}$, which is spectrally in the proximity of the amide vibrational modes. In the Raman measurements, *Escherichia coli* signature peaks were clearly observed. This metamaterial approach achieves sufficient sensitivity by using off-resonance matching conditions, paving the way for the realisation of ultrasensitive bacterial identification technology.
2 Methods

To determine the theoretical absorption spectral peak of the metamaterial used in this study, we applied the finite element method using the COMSOL Multiphysics software package [36]. The spatial metamolecule geometric characteristics from our simulations are noted in the caption of Figure 1. Periodic boundary conditions were imposed in the $x$- and $y$-directions to account for the periodic arrangement of the metamolecules, and the array was modeled using Floquet periodicity. Absorption peak spectra are used as indicators of the theoretical resonance position of the metamaterial device. We designed our metamaterial to operate in the THz regime because biological samples possess mid-infrared vibrational fingerprints that can be used for their identification, leading to improved biodetection specificity. In addition, the micro-sized gap of our metamaterial is compatible with the size of an $E.coli$ (radius: 0.5 $\mu$m), allowing for local bacterium immobilisation and thus an increase in sensitivity. Figure 1(a) shows the theoretical absorption peak at 1676 cm$^{-1}$, which is in close proximity to protein amide group vibrations [11, 35].

Our Raman spectroscopy system (3D Laser Raman Microspectrometer Nanofinder 30) consists of a Nd:YAG laser beam ($\lambda = 532$ nm with maximum incident power of 17 mW) focussed using a high numerical aperture (NA = 1.25) oil immersion objective lens (Plan-Neofluar 100×, Carl Zeiss) onto the metamaterial. A monochromator with a 1800 grooves/mm grating was used for spectra collection. Figure 1(b) shows a scanning electron microscope (SEM) image of the metamaterial device. It consists of an array of 17×17 ASRs and was fabricated using focussed ion beam milling on a 50 nm thick gold film. The zoomed in SEM image shows the metamolecule’s geometrical characteristics of periodicity, $p = 3.6 \pm 0.2$ $\mu$m, and gap size, 0.41 ± 0.3 $\mu$m (Fig. 1(b)). The device was sealed with a cover glass and an adhesive microscope spacer of
10 μm to form a microwell. One microliter of bacterial solution was pipetted into the well and the device was mounted on top of the piezoelectric translation stage, as shown in Figure 1(c). We used *E. coli* BL21 cells (a B-Strain derivative), without any plasmid construct for antibiotic resistance. The primary culture was set up in Luria-Bertani (LB) broth medium and cultured overnight in a shaking incubator set at 25°C and 180 rpm. The following day, the overnight culture was subcultured in LB broth without any antibiotics (0.5 to 1 % inoculation) and grown to mid-exponential phase (optical density-OD, 0.5 to 0.6) in a shaking incubator set at 37°C and 180 rpm. The *E. coli* cultures were diluted 1/10 - 1/100 before FERS observation. *E. coli* is a Gram-negative bacterium and interacts strongly with gold nanostructures via lipopolysaccharide carboxylate groups [37].

### 3 Results and Discussion

Figure 2(a) shows Raman spectra from live bacteria (OD 0.5 to 0.6) on the gold and glass substrate. We notice that spectra from samples without the metamaterial did not have the same peaks as spectra with the metamaterial (Fig. 2(c)) and under the same interrogation settings; we hypothesise that the detectable spectra peaks were attributable to enhancement from the Fano-resonant mode supported by the metamaterial. Figure 2(b) shows Raman spectra from live *E. coli* on the metamaterial recorded at different incident laser powers. At high laser powers, i.e. higher than 3.0 mW, two broad, strong intensity bands located at 1365 cm$^{-1}$ and 1567 cm$^{-1}$ are noted. The photoinduced degradation of biological samples [38] often results in the presence of these characteristic bands in recorded Raman spectra owing to the formation of amorphous carbon [37]. By using lower incident laser powers, this carbonisation effect was minimised, allowing for the Raman signature of *E. coli* to be recorded.

Figure 2(c) shows a Raman spectrum in which live (grey line) and dead (orange line) *E. coli* were recorded separately on the metamaterial, with a laser power of 2.0 mW before the objective lens. No Raman scattering response was recorded when all the bacteria had been killed (orange line in Fig. 2(c)), whereas the Raman signal was very strong in the sample without dead bacteria (grey line in Fig. 2(c)). Note that each spectrum was collected with 10 s acquisition time while the concentration of bacteria for each measurement was kept constant (OD 0.5 - 0.6). We determined the Raman peaks by fitting the experimental spectra using a Lorentzian function [39]. With laser excitation at 532 nm and a Fano-resonance at around 1676 cm$^{-1}$ (Fig. 1(a)), signature bacterial amide spectral peaks appeared near 1280 cm$^{-1}$ and 1624 cm$^{-1}$. The presence of these bands corresponded with amide III and amide I protein vibrations [35, 40]. Additionally, Raman peaks arising from components of the bacterial cell membrane, such as phospholipids, liposaccharides, and other polysaccharide moieties, have also been observed [37, 41, 42] (noted in Fig. 2(c)). Raman signals obtained with excitation wavelengths of 532 nm
contain high fluorescence backgrounds and cause photodamage to the samples [37]. However, in this study, the measurements conducted with excitation wavelengths of 532 nm and lower laser incident powers on the metamaterial gave the most satisfactory results, as the obtained FERS spectra showed many bands originating from bacterial components.

The diagnostic spectral signatures and the intensities of the FERS signals from the bacteria scale with the percentage of live bacteria in the population. Therefore, the FERS experiments were repeated several times for both the exponential and stationary phases of the growth cycle of the bacteria and at random positions on the metamaterial to obtain reproducible spectral fingerprints and the corresponding intensity. Since bacteria are living organisms and do not necessarily respond or grow identically over several days, even if the growth conditions are kept the same, we note that the bacterial optical density (OD 0.5 to 0.6) we use might be the optimum to provide the most reproducible spectra. These observations might have a diagnostics implication for patient care in several clinical settings.

A comparison between the non-FERS (green line in Fig. 2(a)) and FERS (grey line) spectra of live bacteria (see Fig. 2(c)) revealed that, besides the significant enhancement of the Raman signal, the FERS spectra are characterised...
(a) Microscope image of stationary phase bacteria population on the metamaterial where the distribution of *E. coli* is noted by the shaded areas. The yellow dashed rectangle shows the map area. (b) FERS mapping of the stationary phase bacteria population on the metamaterial in a liquid environment. The red and green positions show live bacteria whereas blue positions show where no organism could be detected. (c) FERS spectra after baseline correction and normalization for the red, green, and blue positions. The red positions provide a broad characteristic band at 1334 cm\(^{-1}\) that indicates CH deformation vibrations [40]. The applied concentration of *E. coli* bacteria was OD 1.2 diluted 1/100 before FERS observation, a 100x objective lens was used and the incident laser power was 3.0 mW.

by a larger number of peaks which is indicative of more chemical information. Although it is very difficult to estimate the enhancement factor for the spectra, from the intensities of the strongest bands and the acquisition conditions used, we assumed the FERS enhancement factor to be equivalent to the magnitude of the ratio between FERS and non-FERS intensities for the same probe and Raman peak. We obtained a FERS enhancement factor on the order of 10\(^4\) from a comparison between the intensity magnitude of FERS with the metamaterial and non-FERS values with the glass substrate, while a factor of 10 was noted between the metamaterial and gold substrate at the 1624 cm\(^{-1}\) spectral peak. Signal enhancement in SERS can appear when both surface and resonance effects are combined [43]. The resonance effect is high when the plasmon resonance is located between the excitation wavelength and the wavelength that is Raman scattered by the analyte [37, 43]. Typical values of SERS enhancement factors [43] are on the order of 10\(^4\)–10\(^8\). In our case, the resonance and surface effects were not in close proximity, resulting in a FERS enhancement factor at the lower end of this range. As discussed above, when using nanostructures with resonances matched to the excitation laser, both the laser and the Raman scattering light absorbed by the nanostructures themselves. The absorbed photon energy leads to the excitation of the electrons and the subsequent nonradiative decay of the excited electron converts the energy to heat. The thermodisturbance caused by plasmonic heating may affect the photochemical degradation of the bacterial cells showing low surface enhanced Raman scattering spectrum reproducibility. In this work, the resonance of the metamaterial is red-shifted from the laser excitation, thereby minimising the laser-induced heating that can contribute to photodamage of biological entities. Our work shows a relatively high reproducibility and significant enhancement profile.
Fig. 4  (a) FERS spectra of live bacteria with OD 0.5 - 0.6 over time on a metamaterial device. Inset shows a linear relationship of amide peak intensity magnitude versus time. Time-varying experiments of the solution containing live and dead *E. coli* on metamaterial (b) between 0 and 50 min and (c) between 60 and 255 min. Insets: The intensities of the amide characteristic peaks at 1280 cm$^{-1}$ and 1624 cm$^{-1}$ decrease until they approach the values measured on the glass substrate after 255 min. The dashed lines are a guide for the eye.

It is important to note that the Raman cross-section can only be enhanced for molecular components that are sufficiently close (within 10 nm) to the SERS active surface because the electromagnetic enhancement scales with the 12th power of the distance ($d$) between the analyte and SERS substrate \([44]\). Therefore, as the gap size of the metamaterial is comparable to the size of a single bacterium, the latter may be captured into the micro-aperture and can interact strongly with the electromagnetic field leading to an increase in device sensitivity and selectivity to the spectral bacterial fingerprints.

To validate the sensitivity of the metamaterial and collect information on the distribution of bacteria, FERS mapping was performed with a solution containing live and dead bacteria at the stationary phase (OD 1.2 diluted 1/100 before FERS experiments). A drop of 1 µL of the stationary phase bacteria solution was added on to the metamaterial and FERS mapping was performed with a 4 µm step size, covering an area of 60 $\times$ 40 µm$^2$ as shown in Figure 3(b). Note that the stationary phase is when bacteria growth ceases, but they remain metabolically active \([45]\). During this phase, several physical and molecular changes can take place \([45]\). In addition, the characteristic proteins synthesised in this stage are indispensable as they confer viability to the bacteria. Figure 3(a) shows a microscope image in which the distribution of stationary phase, *i.e.* live-dead, bacteria on the surface of the metamaterial can
be observed (shaded areas). To analyse the distribution of various substances in the stationary phase bacterial solution, it was important to choose characteristic frequency regions or marker bands for them. Therefore, the SERS band at 1280 cm$^{-1}$, was used to image the contribution of the amide modes. If no Raman signals were detected, we inferred that the area had only dead bacteria. Conversely, if strong Raman signals were obtained, we inferred that the area contained live bacteria at the time of signal collection. In Figure 3(b) and (c), each red and green position represents the detection of a FERS signal for live bacteria, whereas the blue positions indicate no bacterial signals were obtained. Moreover, spectral differences between the red and green positions may be explained by the chemical enhancement effect, induced by direct interactions between the analyte and metamaterial. This resulted in a significantly strong vibration near 1334 cm$^{-1}$ (red spectrum in Fig. 3(c)) which is often assigned to the deformation of CH mode [40] and results in the peak splitting into two: one at 1280 cm$^{-1}$ that is characteristic of amide III vibrations [40] and the other at 1380 cm$^{-1}$ that is predominantly due to the symmetric carboxylate stretching mode of polyanionic polysaccharides [40]. Note that the peak at 1334 cm$^{-1}$ is often used as a marker of adenine and DNA in SERS analyses of biological specimens [46].

Furthermore, we investigated the FERS signals at different time points (Fig. 4) and found that the FERS spectra remained largely unchanged throughout the experiment. Figure 4(a) shows the time-dependent Raman spectra of *E.coli* at mid-exponential growth (OD 0.5 - 0.6) over a 30 min period. We observe that the intensity magnitude of both characteristic peaks, *i.e.*, 1280 cm$^{-1}$ and 1624 cm$^{-1}$, increases with time between 0 and 30 min, demonstrating production and accumulation of bacteria during the mid-exponential phase. However, a trend of lower signal intensities toward the end of a long measurement series was observed in Figure 4 (b) and (c) for bacteria with an OD of 1.2. This observation can be explained by an increasing number of dead bacteria over time; as bacteria die and lose integrity of their cell walls and membranes, which are the main contributors to a recorded FERS spectrum, the recorded signal decreases. Therefore, the experimental data are consistent with the fact that the bacteria of OD of 0.5 - 0.6 are dividing and are metabolically active during the first 30 min, while their metabolic activity is likely slowed at an OD of 1.2. This may lead to the observed differences in intensity of the amine peaks. Hence, the time-depended FERS signals may be directly related to bacteria metabolism.

### 4 Conclusion

Rapid and reliable identification of pathogenic bacteria is vital in many fields such as health care, food and environmental sciences. Here, we have demonstrated a FERS platform that can be employed to produce valuable and repeatable bacterial spectral information in a liquid environment. A Fano-resonant ASRs metamaterial was fabricated on a thin 50 nm gold film to
identify signature peaks of *E. coli*. By suitably engineering the Fano line-shape, we produced an efficient FERS-active substrate with spatially localised hotspots and capable of significant FERS enhancement. This enhancement stems from the remarkable sensitivity of metamaterials to minor variations in the dielectric permittivity of the surrounding medium. The power of the irradiating laser and the repeatability of the Raman signals were investigated to determine the spectral resolution and specificity of the device. Compared to other analytical methods or SERS schemes, our approach is especially promising for precise and rapid identification of bacteria in their natural environment due to the unique properties of the metamaterial. We envision that the proposed Fano-resonant nanostructure possesses strong potential to be employed for practical and modern on-chip devices, enabling high specificity detection of biological substances in various environments.

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**Data Availability.** The data that support the findings of this study are available from the authors upon reasonable request.

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**Author contributions.** DGK and VGT conceived the idea, executed it in discussion with RRS, and revised the experimental plan. DGK performed the experiments, simulations, analyzed the data, and drafted the initial manuscript. RRS prepared the solution of bacteria in accordance with the experiment. SNC supervised all the stages of the project. All authors discussed the results and commented on the manuscript.

**Highlights**

- An array of Fano-resonant asymmetric metamolecules is used as a diagnostic/clinical platform for ultrasensitive detection of *E. coli*.

- For off-resonant matching conditions, where the photodamage caused by the plasmonic heating is minimum, the metamaterial provides sufficient sensitivity and FERS enhancement factor.

- We observe that the time-dependent FERS signals reflect the bacteria growth curve. Hence, the FERS platform not only provides an alternative micro-organism sensing method, but also suggests a feasible pathway to monitoring the bacterial growth.
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