Raman spectroscopy: lighting up the future of microbial identification

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Over the last decade Raman spectroscopy has become established as a physicochemical technique for the rapid identification of microbes. This powerful analytical method generates a spectroscopic fingerprint from the microbial sample, which provides quantitative and qualitative information that can be used to characterize, discriminate and identify microorganisms, in both bacteria slurry and at the single-cell level. Recent developments in Raman spectroscopy have dramatically increased in recent years due to the enhancement of the signal by techniques including tip-enhanced Raman spectroscopy and coherent anti-Stokes Raman spectroscopy and due to the availability of user-friendly instrumentation and software. The result of this has been reduced cost and rapid collection time, and it has allowed the nonspecialist access to this physical sciences approach for biological applications. In this article, we will briefly explain the technique of Raman spectroscopy and discuss enhancement techniques, including the recent application of tip-enhanced Raman spectroscopy to microbiology, as well as the move towards rapid microbial identification with Raman spectroscopy. Furthermore, recent studies have combined Raman spectroscopy with microfluidic devices, giving greater control of sample conditions, which will no doubt have an important impact in the future development of Raman spectroscopy for microbial identification.

The potential of Raman spectroscopy as a noninvasive and rapid identification technique for microorganisms has been well established [1–3]. In biomolecular studies Raman spectroscopy has the advantage of providing information on both chemical composition and the structure of biological molecules ranging from proteins to nucleic acids, as well as lipids and carbohydrates [3,4]. In microbial studies this approach is often referred to in the literature as a 'whole-organism' fingerprint as different microorganisms each produce a unique spectrum. The specificity of the analytical technique in combination with appropriate chemometric processing enables the characterization, discrimination and identification of bacteria, fungi and yeasts at both species and subspecies levels [1,5]. Moreover, this detailed spectral information may be used to monitor phenotypic changes that can result from environmental stress [3,6], as well as cell heterogeneity due to cell processes such as cell aging, cell cycle and metabolic rhythms [7], thus providing an extremely versatile and sensitive technique. Advancements in the analysis of Raman spectra have been greatly aided by the use of a range of chemometric methods and algorithms. The appropriate application of these methods and mathematical background behind them has been extensively reported elsewhere [8,9] and consequently not discussed in detail in this article. Despite the established advantages of Raman spectroscopy for microbial studies, the migration of Raman spectroscopy from the research scientific laboratory to a routinely used tool in a clinical setting has been held back by the traditional challenges of Raman scattering. These include an inherently weak signal that can necessitate long acquisition times and the use of large amounts of biomass, combined with backgrounds signals arising from fluorescence, sample medium and instrumental differences. In addition, initial instrument costs and automated data processing methods have hampered the adoption of Raman spectroscopy in the clinical laboratory. In more recent years rapid developments in the technique have started to change this, and here we discuss how these advancements are taking Raman spectroscopy a step nearer to being accepted as a noninvasive, reagent free, rapid, reliable, user-friendly, and low-cost alternative for microbial identification.

Special Report
Future Microbiology

Keywords

■ bacterial identification ■ characterization ■ mapping ■ microfluidic devices ■ Raman spectroscopy ■ surface-enhanced ■ tip-enhanced ■ UV resonance Raman

future medicine part of fsg

Raman spectroscopy utilizes the interaction of light with molecules to measure functional group vibrations. When incident light, or photons (usually supplied from a highly focused laser), interact with a molecule, they can induce a transition in energy states that leaves the molecule in an excited vibrational state with a corresponding loss of energy in the photon [10]; this loss of energy results in the incident light changing color due to a frequency shift, and this is what is measured. While most of the scattered photons from the molecule have the same energy as the incident light (referred to as Rayleigh [elastic] scattering), a small fraction of the photons are scattered at different frequencies, referred to as Raman (inelastic) scattering, eponymously named after Sir Chandrasekhara Venkata Raman who was awarded the Nobel prize for physics in 1930 for this discovery. This difference in scattered frequencies, or Raman shift, is measured in units of cm^{-1} (wavenumbers; which is related to the Raman shift from the excitation laser). The frequency of the Raman-scattered photon can be either less than the incident frequency (Stokes scattering) or greater than the incident frequency (anti-Stokes scattering). In general it is the Stokes scattering that is measured, as this is the more common phenomenon resulting in higher Raman intensities.

The application of Raman spectroscopy for bacterial identification initially escalated after combining this method with optical microscopy [3,11]. Raman microscopy enabled high lateral and axial resolution sensitive enough to allow identification from small sample volumes (e.g., ~1 μm^3) including the measurement of single cells. Furthermore, this coupling of an excitation laser to a confocal microscope system improved spatial resolution (and the possibility of 3D measurements) so that detailed chemical maps could be generated for the discrimination of specific elements. Figure 1 gives an example of Raman mapping of a mixed sample of *Bacillus subtilis* (blue), *Saccharomyces cerevisiae* (pink) and *Staphylococcus epidermidus* (red). As can be observed in Figure 1, after appropriate preprocessing (WiRE 3, Renishaw plc, UK) each species can easily be identified by their spectral variation (for further details see the Figure 1 legend) without complex sample preparation or the addition of any identifying markers. Work by Hermelink *et al.* [7] demonstrates the specificity of confocal Raman microscopy to measure heterogeneity at the single-cell level in genetically uniform microbial populations, without the addition of any dyes or contrasting agents. By investigating three model systems, *Bacillus thuringiensis*, *Legionella bozemanii* and *Bacillus cereus*, these authors showed pronounced cellular heterogeneity, as a result of principal control processes that regulate cell function, even in colonies cultivated under the same laboratory conditions [7]. Although this sensitivity at single-cell level has vast potential for understanding chemical and structural cell-to-cell variation, and with improving instrumentation recording single-cell spectra is becoming easier and faster, Raman scattering still has the disadvantage that the actual interpretation of the spectra is complex. The vast amount of data generated requires robust data analysis and a very extensive database for reliable and consistent interpretation. Despite the fact that heterogeneity between cells is masked in bulk sample Raman studies, identification of microbial species and subspecies can still be achieved from alternative Raman techniques without having to acquire and process such large amounts of data.

As previously stated, Raman spectroscopy has been traditionally held back by the fact that it results in an inherently weak signal with approximately only 1 in every 10^6–10^8 photons being inelastically scattered. As a consequence, spectral collection can have the disadvantage of requiring long acquisition times and, for microbial studies, require relatively large amounts of biomass. However, the continual development of enhancement techniques, including surface-enhanced Raman scattering (SERS) and UV resonance Raman (UVRR) spectroscopy, have made a vast impact in overcoming some of these challenges. SERS has been extensively applied to the investigation of bacteria, using a range of excitation wavelengths and colloidal metal preparations (usually of gold or silver), utilizing the fact that Raman scattering is amplified when molecules are absorbed onto, or microscopically close to, a suitable roughened metal surface [12]. SERS has the advantage of not only reducing collection times but also requiring far smaller concentration of analytes compared with conventional Raman spectroscopy [1]. In more recent years the importance of the optimization of all experimental conditions for SERS measurements has been highlighted [13–15]. One challenge in SERS investigations has been reproducibility, with fluctuations of spectral features being induced by variations in colloid batches [12], colloid concentration dependence [16], and aggregation of the nanoparticles [17], resulting in inconsistent enhancement. However, as illustrated by the studies mentioned previously, with careful

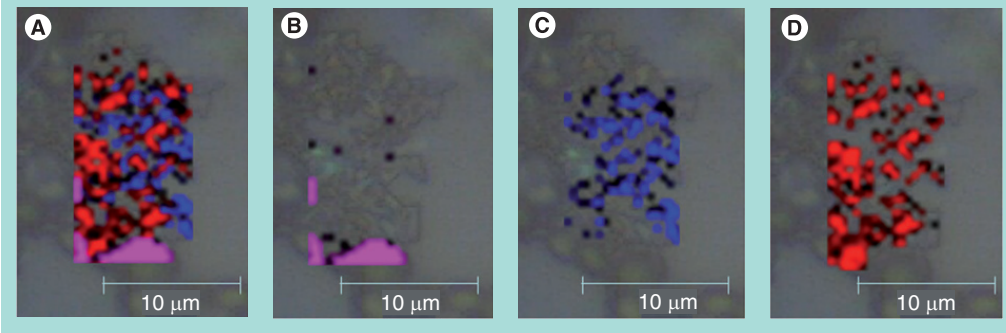

Figure 1. Raman images overlaid on white light images of three different cultures: two bacteria strains *Bacillus subtilis* (blue) and *Staphylococcus epidermidis* (red) and a yeast *Saccharomyces cerevisiae* (pink). The microorganisms were mixed and then deposited on a calcium fluoride window and air dried. Raman mapping was performed using a confocal Raman system (inVia, Renishaw plc, Wotton-Under-Edge, UK) coupled to a 532 nm wavelength laser. StreamLineHR mapping (which uses a laser-spot focus) was carried out using a 100× objective, in high confocal mode, at a step size of 0.5 μm. A power intensity of ~10 mW on the sample and exposure time of 10 s were applied. By applying direct classical least squares component analysis (WiRE 3, Renishaw plc, UK), using the reference spectra obtained from each individual strain, all spectra in the map were identified and assigned a color. These false-color images were then used to further locate the microorganisms within the chemical maps displayed: **(A)** is the composite image of **(B–D)** showing the location of all three microorganisms.

optimization of all conditions for each specific analyte, more reproducible SERS data are being acquired. More recently, alternative SERS methodologies have been reported, including; SERS detection in the interior of a microorganism from colloidal particles precipitated within the cell [2,18], combining SERS with microfluidic devices [19,20], and *in situ* detection of microorganisms on microarray chips using SERS [21].

Another important enhancement technique in Raman spectroscopy, particularly for bacterial studies, is UVRR. Resonant Raman spectroscopy occurs if the energy of the incident laser is within the molecular absorption bands of chromophores in the molecules [10]. Using an excitation laser in the deep UV (180–260 nm) the Raman signal can be enhanced by a factor of 10^3–10^5 over conventional Raman scattering through this resonant Raman effect. The resulting spectrum predominantly contains aromatic chromophores, such as nucleic acids and aromatic amino acids [22]. Moreover, UVRR spectroscopy has the further advantage of reducing fluorescence. The emission of fluorescence, which is particularly strong from biological samples, has also challenged the development of Raman spectroscopy as it can often mask the Raman signal [10]. The amount of fluorescence is strongly correlated to the laser excitation wavelength, decreasing in intensity with increasing wavelength; for example, less fluorescence is observed with an excitation wavelength of 785 nm compared with 633 and 532 nm for the same sample. However, due to the inverse fourth power dependence of Raman scattering, efficiency of wavelength scattering power also decreases, with increasing wavelength resulting in a trade-off between fluorescence reduction and scattering efficiency. At excitation wavelengths below 250 nm no fluorescence interference exists, but extreme care needs to be taken to avoid photo-degrading samples due to the highly efficient lasers required. Sample degradation can be avoided by reducing laser power to the minimum required for good quality spectra and by continually moving the sample under the laser beam, either on a turntable for dried samples or in flow for solution samples.

In the characterization of *Staphylococcus epidermidis*, Neugebauer *et al.* [23] successfully applied UVRR spectroscopy to identify nucleic acid components of the cells. They demonstrated that GC content, the percentage of guanine (G) and cytosine (C) in the DNA of the cell, could be determined for *S. epidermidis* from the ratio of the area under the curve of two bands: one assigned to cytosine (~1521 cm^{-1}) and the other to guanine (~1475 cm^{-1}). As shown in the UVRR spectra of suspended cells of *E. coli*, *B. subtilis* and *S. cerevisiae* in physiological saline (Figure 2), the main variation between species is the relative intensity of bands. Three spectral bands can be observed arising from tyrosine (tyr; ~1614 cm^{-1}), guanine (G; ~1580 cm^{-1}), adenine and cytosine (A & C; ~1495 and ~1338 cm^{-1}) and thymine (T; ~1256 cm^{-1}). Although all five bands can be observed in each spectrum, the relative ratios of one band compared with

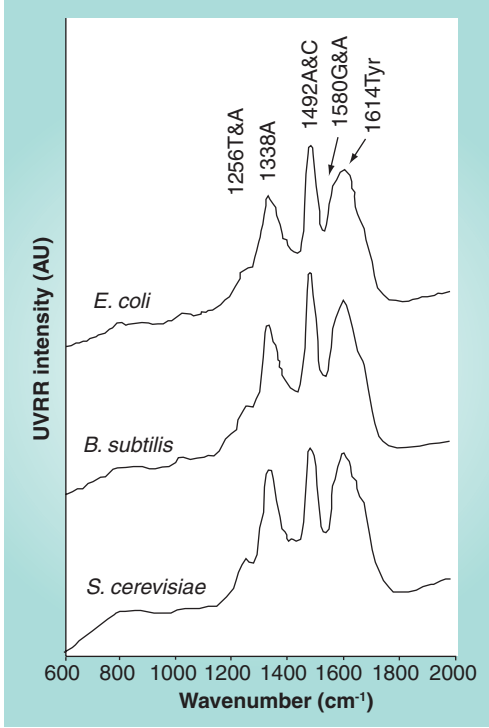

Figure 2. UVRR spectra of suspensions of *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* in physiological saline. 30 µl of each sample was pipetted onto a small well on the lid of a polystyrene microplate. The laser was then focused into the suspension with care being taken not to focus on the lid itself. The well was continually rotated during laser interrogation to reduce photodegradation. All spectra were collected for 120 s, with an excitation wavelength of 244 nm with a power on the sample of ~1 mW. UVRR: UV resonance Raman.

another clearly vary between the microorganisms. Knowledge of bands assigned to chemical structures within the cell offers the potential to monitor metabolic changes in bacteria caused by growth and aging, as well as abiotic stress such as the addition of antibiotics to growing cells [24].

In Neugebauer and colleagues' [23,25] applied study of *S. epidermidis* they also employed the rapidly developing technique of tip-enhanced Raman spectroscopy (TERS). This technique combines the nanoparticles employed in SERS with atomic force microscopy (AFM) by reducing the SERS active metal site onto an AFM tip with apex sites of less than 50 nm in diameter. As the tip is moved across the sample a strong local enhanced electromagnetic field is generated close to the tip, allowing enhanced Raman spectra to be measured. Consequently, TERS can be used to obtain simultaneously detailed chemical and topographical information with nanometer resolution [6]. The combination of SERS and AFM has the advantage of enabling high lateral resolution and chemical specificity, demonstrated in the mapping of the surface of single *S. epidermidis* cells (Figure 3) [23]. TERS provided valuable chemical information about the bacterial surface, and has the potential to monitor fluctuations in the cell wall components of the outer membrane over time. Further biological applications of TERS have included the investigation of bacterial biofilms where the combination of AFM images, providing structural details, along with Raman measurements of chemical composition to provide insight into the complex heterogeneous system [25,26]. However, as with SERS, there can be difficulties with reproducibility and production of the metal-coated AFM tip, making collection of TERS data a specialized skill. Although enhancement techniques such as UVRR, SERS and TERS are making vast advancements, all require expertise for robust spectral collection; needing either reliable sample preparation (SERS and TERS) or complex instrumental set-up (TERS and UVRR), which prevents routine introduction into the clinical setting.

Further developments in instrumentation are also aiding the application of Raman spectroscopy to biomolecules. Improvements in lasers, optics, and charged-coupled device cameras along with advances in computer processing, have increased the availability of Raman spectrometers in a range of sizes, sensitivities and costs. These improvements have been particularly significant in the development of a nonlinear type of Raman scattering, known as coherent anti-Stokes Raman scattering (CARS). CARS utilizes two incident lasers to induce coherent molecular vibrations, which can scatter light more efficiently than in conventional, spontaneous Raman scattering [27]. Technological advancements have increased the stability and sensitivity of CARS, resulting in the application of this methodology to microspectroscopy, including the detection of single bacterial endospores [27].

Advancements in instrumentation are also enabling the production of affordable, portable Raman spectrometers with options for fiber-optic probes, specific types of sample compartments and programming for precise tasks. Willemse-Erix *et al.* [28,29] have demonstrated the feasibility of applying conventional Raman spectroscopy to the continuous, automated, and real-time epidemiological monitoring of bacterial infections with a custom built Raman system. This approach was successfully applied in the discrimination of isolates of methicillin-resistant *Staphylococcus aureus* [28] and coagulase-negative staphylococci [29] as

well as *Mycobacterium tuberculosis* [30] in a short timescale (typically a few minutes to a few hours) compared with typical and time-consuming genotyping methods. A key element of their bacterial strain identification methods is the use of dedicated data-preprocessing software to eliminate contributions from water and media in the spectra, combined with the application of chemometric-based classification techniques. This combination of sensitive instrumentation, dedicated hardware and software development demonstrates the potential of providing easy-to-use, rapid, and automated analysis of multiple samples with negligible reagent costs, facilitating the introduction of specialist Raman equipment into clinical microbiological laboratories in the near future. However, the success of this type of equipment greatly depends on a reliable database. Correct identification of species requires an extensive database containing the spectra of all bacteria of interest grown in all relevant incubation conditions. Without this, no phenotypic typing-based technique can be feasibly applied to the clinical setting.

Another significant development is the combination of microfluidic devices and Raman spectroscopy in the characterization of microorganisms. Wilson *et al.* [20] constructed a microfluidic system from a glass capillary tube that could also be used as a SERS chamber to collect SERS with silver colloids. The detection of scytonemin, the sheath pigment unique to cyanobacteria, at nM concentrations was measured using a specifically designed small, light and low-cost system. In another example Walter and workers [19] demonstrated the advantages of combining microfluidic devices and SERS from silver colloids in the classification of nine strains of *E. coli*. SERS spectra were recorded from a lab-on-a-chip device mounted on the microscope table for Raman measurements. The micro-fluidic device was an all-glass chip with six injection ports measuring 16 × 25 mm and allowed the fast, reproducible and reliable discrimination between small sample volumes of different bacteria strains. The use of microfluidic devices has the potential of allowing the integration of a number of sample preparation steps and the isolation and concentration of bacteria from clinical fluids. Cheng *et al.* [31] recorded *in situ* SERS of bacteria in a microfluidic chip capable of continuously sorting and concentrating bacteria via 3D dielectrophoresis (DEP). The device was designed with DEP microelectrodes between glass slides and a roughened metal surface for SERS measurements rather than using colloids. From the SERS data they were able to distinguish between *S. aureus* and *Pseudomonas aeruginosa* and, via DEP, *S. aureus* was continuously separated and concentrated out of a sample of blood cells. The integration of manipulation processes and well-defined detection chambers in microfluidic chambers opens up a wealth of experimental conditions not only for bacterial identification but also for monitoring complex biological processes. Although not yet combined with microfluidics, Xie and colleagues [32] as well as other workers [33], have demonstrated that it is possible to trap bacteria cells optically. After using these so-called 'laser tweezers', the trapped bacteria can then be interrogated by the same laser, or one of a different frequency, to yield a unique Raman spectrum for the single bacterium. This exciting advance has also been used by others in the analysis of environmentally important microbes to assign function at the single-cell level [34]. Finally, it has tantalizingly been demonstrated that Raman spectroscopy can even be coupled with flow cytometry [35], although this is yet to be realized with bacteria.

The combination of these numerous advances in Raman spectroscopy are moving the technique nearer to providing routine microbial identification as well as characterization-based measurements as the organism adapts to a new environment. Improvements in enhancement methods, including UVRR, SERS, and in particular TERS, are enabling rapid and detailed spectral collection from minute amounts of sample. Developments in instrumentation

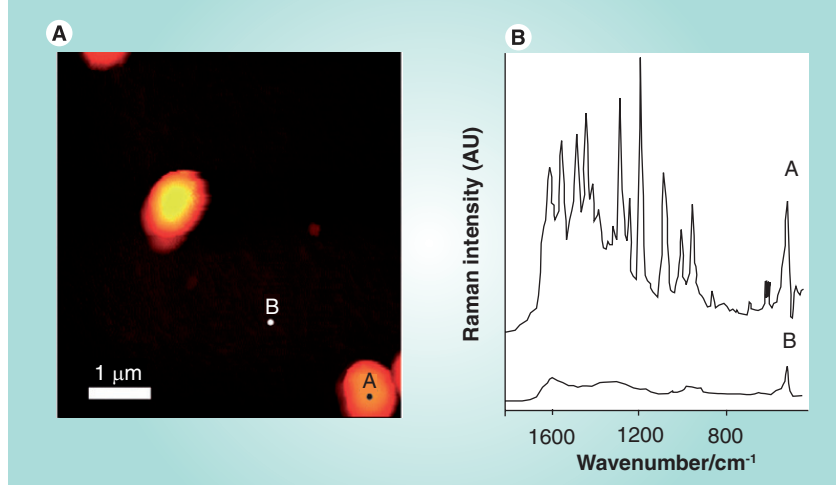

Figure 3. An example of tip-enhanced Raman spectroscopy of bacterial cells. Topographic image of *Staphylococcus epidermidis* cells **(A)** with the positions marked from where Raman spectra **(B)** were measured. Position A corresponds to a tip-enhanced Raman spectroscopy spectrum on the cell surface, and position B corresponds to a reference tip-enhanced Raman spectroscopy experiment on the glass surface.
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are reducing the size and cost of Raman spectrometers while improving sensitivity, thus facilitating specialized instrumental design to meet specific clinical needs. Finally, the use of microfluidic devices with Raman spectroscopy is providing a platform for integrating SERS, instrumental design and control of sample preparation, ultimately facilitating single-cell measurements with the concomitant advantage of reducing the lengthy times usually needed to culture the pathogen from the host prior to analysis.

Future perspective

Raman spectroscopy's advantage of providing detailed and vast amounts of phenotypic information on the bacteria under investigation can also be its disadvantage. For future developments in microbial identification, new instrumental designs need to utilize recent advancements while exclusively focusing on specific clinical needs. Furthermore, by building up extensive and reliable databases with probabilistic identification algorithms, Raman spectroscopy has real potential as a noninvasive, easy-to-use, fast and reliable microbial characterization technique, ultimately giving unequivocal identification at the single-cell level.

Acknowledgements

The authors would like to acknowledge the support of Renishaw plc (Old Town, Wotton-Under-Edge, Gloucestershire, GL12 7DW, UK) and the Bioprocessing Research Industry Club, UK.

Financial & competing interests disclosure

R Goodacre and CL Winder would like to thank the UK BBSRC and EPSRC (BB/C008219/1) for financial support of the Manchester Centre for Integrative Systems Biology (MCISB). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

- Raman microscopy has enabled high lateral and axial resolution, ideal for investigations at the single-cell level, and has thus provided detailed chemical maps for discrimination.
- Improvements in instrumentation, including better lasers, optics, charge-coupled device cameras and computer processing, has reduced the size and cost while improving signal sensitivity.
- Enhancement techniques including UV resonance Raman, coherent anti-Stokes Raman scattering, surface-enhanced Raman scattering and tip-enhanced Raman spectroscopy overcome the traditional challenge of weak Raman signals, and enable rapid analysis times.
- Combining microfluidic technology with Raman spectroscopy provides a platform for integrating surface-enhanced Raman scattering, instrumental design and robust control of sample preparation.
- With continuing developments, Raman spectroscopy has real potential to provide a noninvasive, rapid and reliable alternative for microbial identification.

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