MEDDOCS Open Access Publisher

ISSN: 2637-4900

Journal of Community Medicine

Open Access | Review Article

Raman Imaging Microscopy: A Mini - Overview on Its Role in Curtailing the Menace of Drug - Resistant Bacterial Pathogens

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Received: Nov 11, 2021

Accepted: Nov 28, 2022

Published Online: Nov 30, 2022

Journal: Journal of Community Medicine

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: Raman imaging microscopy; Antibiotic resistance; Diagnosis; Infectious disease.

Abstract

Raman microscopy is an emerging group of tools for molecular imaging of cells. It is currently a phenotypic method that can identify and test microbial susceptibility to antibiotics. Raman Spectroscopy (RS) a useful instrument in biological systems. Owing to its low-cost, label-free, and non-destructive characteristics, RS has remained broadly examined for its impending use in medical education as it has high-throughput and real-time applications in clinical diagnostics. Furthermore, it is a growing technique for identifying bacterial infections since it can act as a rapid and efficient tool to identify bacterial cells and antibiotic resistance. The sample preparation phases are easy, and the spectroscopic processes can be accomplished within minutes or seconds, which sorts it as a favorable technique for identifying bacterial infection. There are, however, still a lot of gaps moving from simple research to practical use, which restrict RS from being a routinely used laboratory technique. Here, we briefly described the application of Raman imaging in curtailing the menace of infectious diseases.

Introduction

Antimicrobial Resistance (AMR) has resulted in major threats to human health due to out-of-control and inappropriate use of antibiotics, particularly broad-spectrum antibiotics. According to reports, antimicrobial resistance causes one million deaths worldwide each year, and it is predicted that by 2050, antimicrobial resistance will cause ten million fatalities **(Figure 2)** and $100 trillion in manufacturing costs if no action is taken [1]. While AMR's development is a natural occurrence that cannot be totally avoided, there are numerous things we can do to slow down the rate at which AMR emerges and spreads over the world.

For example, we need inexpensive and quick diagnostics to detect all sorts of infections in various contexts and then modify or amend treatment to provide best clinical practices to avoid antibiotic overuse [1]. As a result, rapid identification of bacteria pathogens and antibiotic resistance profiling could substantially aid in developing an effective infectious disease treatment approach [2]. Raman imaging microscopy, also known as Raman Technology or Raman scattering imaging, is a contemporary quick phenotypic technology that may identify and quickly produce AST results in femtoseconds, or 10-15 seconds [3]. In addition, several studies have shown that Surface-Enhanced Raman Scattering (SERS) can quickly identify and discriminate biological material, including medically important microorganisms [4].

Cite this article: Nkiruka O, Anekpo CC, Adanma E. Raman Imaging Microscopy: A Mini - Overview on Its Role in Curtailing the Menace of Drug - Resistant Bacterial Pathogens. J Community Med. 2022; 5(2): 1046.

The gold standard for AST remains a culture-based phenotypic approach, such as disk diffusion or broth dilution. However, this strategy is too slow and not robust enough to suggest a direct treatment decision for different drug-resistant infectious diseases. There are also genotypic techniques, which can detect identified resistance genes and provide faster findings. Still, they are not always correct for various bacteria and do not provide a mechanism of resistance or results for therapy decisions [5]. Here, we briefly described the role of Raman imaging in identifying and curtailing the menace of infectious diseases through prompt and accurate diagnosis of drug-resistant pathogens.

An overview of Raman technology-based method

Raman spectroscopy is a rapidly evolving technique for detecting bacterial infections because it can identify bacterial cells and antibiotic resistance quickly, efficiently, and non-harmful manner. It also has the potential for high-throughput and real-time applications in clinical diagnostics [2]. The Raman Effect is based on the idea that once the smallest unit of light passes through any medium, the light scattered by other molecules impacts the frequency change. This implies that the Raman Effect is influenced by molecular vibrations and may thus be explained by energy levels [6]. According to quantum physics, the Raman Effect is the inelastic impact that happens when photons collide with molecules. The molecule will be excited to a high energy level or virtual state after the exciting light interacts with it, and then the molecules and electrons in the virtual state will transition to the excited state, creating dispersed light. The agitated photon can transport energy to the molecule in this mechanism, but the photon loses energy in the process **(Figure 1)**.

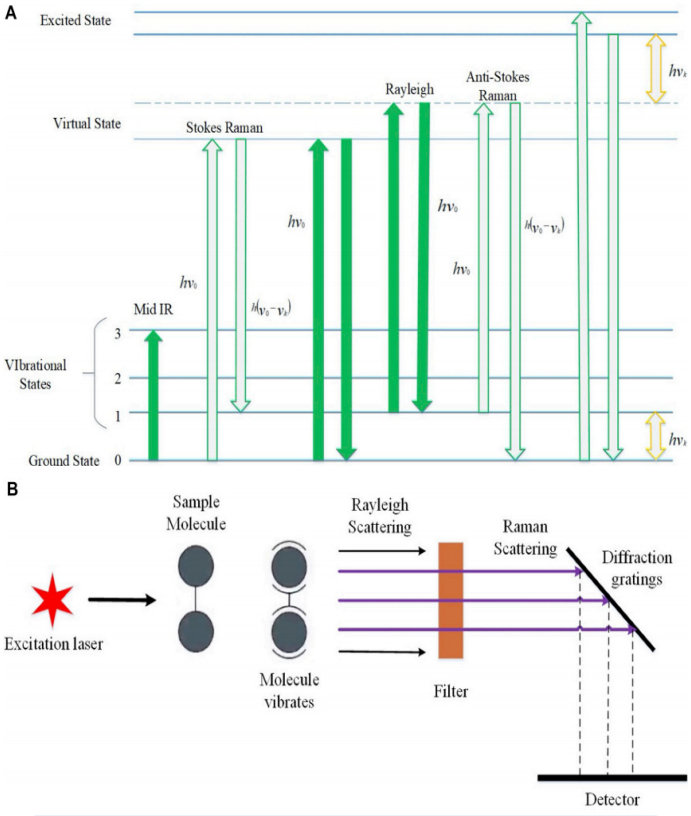

Figure 1: Representation design of the basic principles of Raman effects and the Raman spectroscopy's brief structural design **(A)** The change in course of infrared light irradiation, Stokes rays, anti-Stokes rays, Rayleigh scattering, and Raman scattering are all shown on a map of Raman spectrum energy levels. E0, ground state; E1, vibration excited state; E0+hv0 and E1+hv0, excited virtual states; hvk, initial irradiation energy; E0, ground state; E1, vibration excited state; E0+hv0 and E1+hv0, excited virtual states. (B) Raman spectroscopy representation map. Later, the incident light is irradiated, causing the molecules to become excited. Raman scattering is a changing frequency of light that occurs throughout the scattering process and is seen on the grating and caught by the detector [2].

Subsequently, the molecule that changes to the excited state obtains energy. There are some areas where the incident light frequency is low. In this case, the identified dispersed light is called Stokes Line. Conversely, it is called the anti-Stokes Line in the opposite case [6]. When photons collide with molecules, the energy between them does not change, but the path is altered, which is known as Rayleigh scattering [7]. In general, RS creates a strong fluorescence background that can disturb the original spectrum, resulting in poor bacterial identification quality. It can be removed using a method such as polynomial baseline fitting [8]. When taking measurements of a specific sample, RS generates a sequence of spectral indicator lines. The Raman shift is the frequency difference between Raman scattered light and the previously mentioned Rayleigh scattered light [9]. Selected compounds in biological samples will have different peaks, and the amount of a specific molecule in the sample will influence the molecule's concentration **(Figure 1)**.

Furthermore, Raman spectroscopy provides information on both chemical compositions and biomolecular structures, such as DNA, RNA, proteins, lipids, and carbohydrates, in bacterial samples and is sometimes referred to as a whole-organism fingerprint [2,10].

The potential of the Raman spectroscopy-based method in helping to curtail the AMR crisis

Raman spectroscopy has been useful in bacterial research [2]. When comparing gram-positive and gram-negative bacteria, it was discovered that selected peaks at 540cm-1 and 1,380 cm-1 had significant differences for gram-positive bacteria when compared to gram-negative bacteria. This difference was attributed to glycosidic bonds in n-acetyl glucosamine and n-acetyl muramic acid of peptidoglycan [11]. Because of the low concentration of bacteria in medical samples, research using RAMAN spectroscopy on clinical bacterial pathogens has previously required growth in agar plates [12,11]. However, culture-based RS can produce sufficient biomass throughout testing, resulting in a higher signal-to-noise ratio, and it is time-consuming.

There are similar RAMAN spectroscopic efforts on tissues with in situ infectious disease diagnostics. For example, the ascitic fluid was examined straight for pathogen identification using RAMAN spectroscopy and chemometric evaluation in a study by [2]. It was demonstrated that 97.7% of the spectra from gram-positive bacteria were correctly allotted on the genus level and 83.6 percent on the species level. In another investigation, Raman spectra were utilized to quickly identify bacterial and fungal pathogens recovered from 115 blood cultures following a 6- to 8-hour culture in an automated blood culture method [2]. This study shows the potential of RAMAN spectroscopy to detect bacterial illnesses in medical samples directly.

The discovery of changes in characteristic spectral variations of vulnerable and resistant bacteria with or without antibiotic treatment and the quantification of Carbon Deuterium (CD) peak strength changes after deuterium labeling of bacteria and antibiotic treatment are two key methods for rapid AST using Raman spectroscopy. The AST methods based on detecting

characteristic Raman spectral changes, such as specific Raman peak changes after antibiotic treatment or Raman spectra-based differentiation of susceptible or resistant bacteria, must be evaluated case by case for different bacteria species or antibiotics and thus are not universal methods. Several types of research that coupled Raman spectroscopy with stable isotope tagging to detect bacterial metabolism and determine antimicrobial susceptibility present an alternative method for universal AST. For example, by measuring the metabolic action of heavy water (D_2O)-labeled bacterial cells, Raman spectroscopy was used to investigate bacterial reactions to various medicines [13]. Another previous investigation by [14] reported similar findings. Raman spectroscopy using the Raman shift at the C-D band from 2040 to 2300 cm-1 can be used to determine the degree of C-D bond formation [15]. The D_2O-Raman can be used to detect and evaluate the metabolic activity of cells because the use of H_2O or D_2O is a basic attribute of living cells [16].

Stimulated Raman Scattering (SRS) Imaging

The advancement of Stimulated Raman Scattering microscopy (SRS) in biology and medicine has shown notable signals of improvement. SRS has been used for metabolic imaging in cells, tissues, and model organisms. SRS is also suitable for bacteria examination at specific bacterial cell concentrations due to the advantages of high-speed imaging and sub-micron resolution. D2O was used to grow bacteria in clinic-relevant circumstances with SRS, revealing a quick phenotypic AST approach. In addition, SRS can be used to identify The study by and [17] have helped demonstrate this technology's clinical potential.

Surface-enhanced Raman spectrometry (SERS) for sensing trace levels of bacteria

Surface-Enhanced Raman Spectroscopy (SERS) is a common biochemical fingerprinting technique because it can precisely show macromolecular profiles as well as changes in bacterial cells as a result of antibiotic treatment [18]. SERS has been used to investigate bacterial resistance or susceptibility to antibiotics, as well as to test the working mechanism of antibiotics based on the spectral fingerprint of the entire cell. SERS can detect resistant bacteria quickly, accurately, and ultra-sensitively with minimal sample preparation and handling [19]. **Figure 2** shows a schematic diagram of the Raman spectroscopy and Surface-Enhanced Raman Spectroscopy (SERS) workflow.

Clinical samples may include just trace numbers of bacterial organisms in some cases. Surface-Enhanced Raman Spectroscopy (SERS) can be used to boost weak Raman signals in clinical samples such as blood and urine when bacterial amounts are low, thereby facilitating the development of culture-free bacterial pathogen detection. SERS is a type of enhanced RS that involves sample molecules interacting with surface plasmons of nanoscale structured metal surfaces, which typically employs spherical silver or gold nanoparticles with diameters ranging from 20 to 100 nm [2]. [20], for example, investigated bacterial pathogens in 108 urine samples sourced from urinary tract infection patients; according to the study, 93 samples were detected with a single bacterial species via SERS, while 97 samples were confirmed pathogen positive through medium culture, resulting in detection accuracy of 95.87 percent.

Despite SERS being a highly promising analytical approach, it has yet to be employed as a standard diagnostic procedure in the clinical laboratory, and a number of issues are inhibiting its widespread adoption. Although much effort has been put into this area, one of the primary difficulties is the manufacture of suitable substrates with distinctive properties in SERS-related detections [21]. As a result, developing new low-cost, reproducible substrates for SERS would greatly increase its sensitivity and accuracy, allowing it to be employed in more places. SERS nanoparticle fabrication procedures have demonstrated tremendous promise in the reliable detection of parasites and viruses [22,23,24].

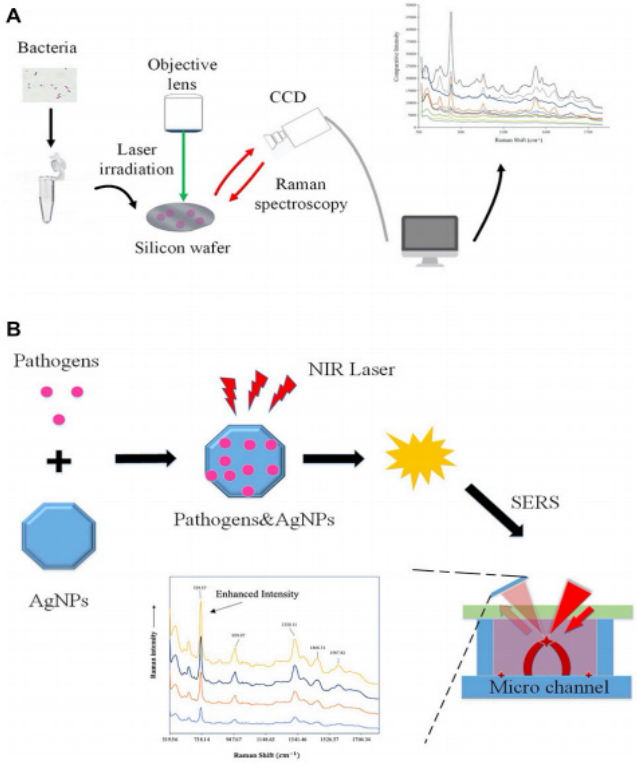

Figure 2: The process of Raman spectroscopy and surface-enhanced Raman spectroscopy is depicted schematically in (SERS). **(A)** Raman spectroscopy workflow for bacterial categorization and antibiotic resistance profiling using molecular vibrations and rotations to gather information on molecular structure. **(B)** Surface-enhanced Raman spectroscopy procedures. To improve signal intensity, samples were measured while adsorbed on the surface of colloidal metal nanoparticles such as silver (AgNPs), gold, or copper [2].

Raman imaging microscopy in antibiotic resistance profiling

AST is an important step in the clinical diagnosis of significant bacterial infection, and precise and successful identification of bacterial antibiotic resistance is critical for bacterial infection treatment [18]. Although the standard technique for fastidious bacteria often takes 3-4 days or longer to produce final AST results [25], with MALDI-TOF MS-based approaches, such as for positive blood culture bottles, a result can be provided in as little as 24 hours, and in some cases the same day. RS, particularly SERS, has been used to assess antibiotic resistance phenotypes in a variety of bacterial species, including *Escherichia coli* [26], *Staphylococcus aureus* [27], and *Pseudomonas aeruginosa* [28].

In terms of bacterial antibiotic resistance and susceptibility, several signatures have been discovered that could be used to quickly identify antibiotic resistance at sub-lethal dosages [29,25]. Furthermore, a single study found that a portable Raman spectrometer with a paper-based SERS may be utilized to screen for tetracycline residues in milk with peak intensity ratios of 455 cm-1/1,280 cm-1 and 874 cm-1/1,397 cm-1. Thus, RS could function as a potential tool for on-site monitoring of antibiotics [30].

Despite that SERS was investigated to detect antibiotic-resistant phenotypes in some studies, current datasets are small, limited, and often involve environmental settings. Furthermore, the ability of RS to detect resistance phenotypes differs from antibiotic resistance testing, which relies on the finding of minimum inhibitory concentrations rather than the existence of resistance markers [29]. As a result, the generalizability of these Raman signatures, biomarkers, or metabolites in predicting antibiotic resistance profiles should be investigated further before they are used in clinical settings.

Furthermore, SERS can detect the effectiveness of antibiotic therapy and the antibiotic resistance patterns of bacterial cells since it can record the macromolecular fingerprints of the bacterial cell membrane and cell wall [29]. Although traditional Raman spectroscopy has been widely used to characterize the phenotypic response of bacteria to antibiotic treatment, the collection of Raman signal requires a high concentration of bacterial culture [31]. Furthermore, antibiotic-sensitive bacteria may be distinguished from antibiotic-resistant bacteria within 1 hour of antibiotic exposure by looking at the SERS spectral profile alterations. SERS has the capacity to identify and characterize antibiotic resistance in real-world samples rather than in pure bacterial culture, as demonstrated by this technique. Liu and colleagues demonstrated that SERS could monitor the reduction of specific bacterial biomarkers along with the treatment of antibiotics within two hours [32].

Nanochip technology can also be incorporated with SERS to test antibiotic susceptibility. In this approach, the Nano Chip technology is used in SERS to detect molecules using nanoparticles as Raman substrates (reference). Several studies are available that show the potential of technologies using Raman imaging approaches are available [33, 34,16,4, 3,2,36].

Challenges, prospects, recommendations, and conclusion

Stimulated Raman scattering (SRS) microscopy has been used to image cell metabolic processes and to detect the minimal inhibitory concentration in single cells precisely. Additionally, the Stimulated enhanced Raman Scattering (SERS) approach could lead to the creation of the biosensor, which would enable quick and convenient point of care testing for the clinical diagnostics of body fluids, pathogens, cells, and tissues. It can be labeled or unlabeled, which means biomarkers can be used or not.

Furthermore, since the global outbreak of coronavirus 2019 (COVID-19), several studies have focused on the rapid identification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) utilizing RS. For example, [37] developed a Raman-based saliva analysis approach that can distinguish healthy persons from infected patients with greater than 95% accuracy, precision, sensitivity, and specificity. SERS was also used to examine 177 serum samples using RS (63 confirmed COVID19 patients, 59 probable cases, and 55 healthy individuals) and 20 independent individuals for external validation. According to the study, the COVID-19 and healthy controls had an accuracy of 0.90, indicating that RS has the potential to be a safe and effective tool for COVID-19 screening [38]. This potential use in COVID-19 screening can be translated to bacterial pathogens.

A more detailed method is required when using Raman spectroscopy to study antibiotic resistance. Furthermore, deep learning is necessary to compute the Raman spectra and compare them to the old method of antimicrobial resistance detection. Also, a comprehensive Raman library must be produced, as well as Principal Component Analysis (PCA) plots from combinations of two distinct bacteria. In addition, Raman spectroscopy results can be compared to traditional clinical procedures and interpreted in accordance with the Clinical And Laboratory Standards Institute's Guidelines (CLSI).

Patients with Bloodstream Infections (BSI) and those in Intensive Care Units (ICU) require a simple and direct separation of bacteria from positive blood culture bottles for identification and AST. However, this process takes time, wastes people, and may result in excessive pollution; however, if automated ways can be created, these issues will be resolved. Shortening the time to determine antimicrobial susceptibility of bacteria from a Positive Blood Culture Bottle (PBCB) using a direct and rapid AST approach based on Stimulated Raman Scattering (SRS) imaging of D2O metabolism (almost 5- 6 hours) could be helpful. This approach has a lot of clinical potential because it uses simple isolation to identify the bacteria and AST. However, the current scheme continues to use a manual method, which cannot avoid issues such as sample contamination and labor costs. This approach will be an effective method procedure to solve rapid and direct AST by rapidly automating the separation of bacteria from samples and using SRS imaging. SERS can also detect UTI germs without the need for time-consuming sample preparation. The evolution of a bacteria-specific Raman signal peak can then be used to determine antibiotic susceptibility.

Therefore, Raman Spectroscopy (RS) helps illustrate biological systems because molecular vibrations create spectral characteristics in the [2]. Because of its low cost, lack of labels, and non-destructive nature, RS has been widely studied for its potential use in medical education. It's simple to add to the sample preparation phases, and the spectroscopic processes can be completed in minutes or seconds, making it a good technique for detecting bacterial infection [39]. However, there is still a significant gap in its practical application, preventing RS from becoming a standard laboratory procedure. For example, the Raman Effect is weak, resulting in long measurement periods; sample fluorescence also results in long measurement times [40]. Furthermore, the addition of intense laser energy can heat up the sample, resulting in sample damage and a disordered Raman spectrum. As a result, biological samples must be investigated in aqueous solutions or using a low-energy near-infrared wavelength for excitation, such as 785 or 830 nm [41].

Until now, SERS has not yet been approved for use in clinical practice. There are insufficient standard-reference Raman spectra from known bacteria for recognition and software to correctly identify patients' urine samples 100 percent of the time. More studies and efforts are needed to position Raman imaging as an indispensable tool for curtailing the menace of infectious diseases through prompt and accurate diagnosis.

Conflict of interest: The authors report no conflict of interest

Funding: The authors received no funding for this study.

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