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REVIEW

Progress in the Applications of Raman Spectroscopy in Microbial Identification

ADIL EMIN

School of Physical Science & Technology, Lanzhou University, Lanzhou 730000, P.R. China

Corresponding author: E-mail: adl19@lzu.edu.cn

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After more than 40 years of development, surface-enhanced Raman spectroscopy (SERS) has become a powerful and mature analytical tool. It has been widely used in surface science, materials science, biomedicine, drug analysis, food safety, environmental testing, etc. SERS technology has molecular-level detection accuracy, which can effectively amplify signals and has obvious advantages in realizing trace substance detection. In present article, a comprehensive review of the SERS technology and related applications in microbial identification is carried out, and its future research hotspots and development directions are discussed.

Keywords: Surface enhanced Raman spectroscopy, Microorganism, High-accuracy detection.

INTRODUCTION

Although the current analysis and testing methods are still very good, but can not meet the development of modern life sciences and technology, so we need a more sensitive and better analytical methods [1-6]. Raman spectroscopy is characterized by rapid, sensitive, non-destructive, real-time monitoring and is widely used in the field of microbiology [1-7]. Microbiology is a discipline that studies the morphology, physiology, biochemistry, and the evolution, classification and ecology of microorganisms under certain conditions and their applications [6-11]. The analysis and detection of trace substances in complex systems is an important part of life sciences and technology. Surface-enhanced Raman spectroscopy has many advantages in this respect [1,2,7-9]. Present goal is to develop high sensitivity and universality in continuous research. Wide range of enhanced Raman spectroscopy methods and techniques. The Raman spectroscopy technique uses a laser with a good monochromaticity as a light source. According to the analysis of the vibration spectrum obtained by the experiment, the molecular fingerprint information of the sample in various states of solid, liquid and gas can be obtained, which it is widely used in the analysis and identification of various samples [10-15]. Raman scattering is a kind of light scattering phenomenon [16]. When a photon of a monochromatic beam interacts with a molecule, elastic collision and inelastic collision occur [16,17]. Raman scattering is generated by inelastic collision of photons. The scattering process in which elastic collision occurs is also called Rayleigh scattering. During the collision, there is no exchange of energy between the molecules and the photons. After the collision, the molecules only change the direction of motion without changing the frequency of motion. Unlike elastic collisions, energy exchange occurs between photons and molecules during this inelastic collision, *i.e.* photons transfer a part of the energy to the molecules, or the vibration and rotational energy of the molecules are transmitted to the photons, so the photons change direction of motion. It also changed the frequency of the movement. In 1928, Raman and Krishman first discovered Raman scatter in liquid scattered light [1,8,16-19]. The detection limit for CCl_4 is generally only 10^{-3} g/L, and when the wavelength of the excitation light falls within the electron transition absorption wavelength range of the molecule. When the Raman scattering intensity is increased by 10^3 to 10^4 times [1,17,19-22]. With the development of science and technology, a nanostructure-based surface enhanced Raman spectroscopy (SERS) was invented, which improves the signal-to-noise ratio of the spectrum by using the interaction between the metal particles and the measured molecules. So that the experimental results are more significant. Surface-enhanced Raman spectroscopy (SERS) has the ability to analyze the composition of

nanoscale mixtures for environmental analysis, pharmacy, materials science, art and archaeological research, forensics, drug and explosives testing. In addition, in the field of microbiology research, the application of microbial identification in food has been initially explored and little research has been done on pathogenic microorganism, and detection of single algae cells. SERS combined with plasma sensing can be used for highly sensitive quantitative detection of biomolecular interactions [17,19]. In this article, a comprehensive review of the application of SERS technology and related technology in microbial identification is carried out, and its future research hotspots and development directions are discussed.

Surface enhanced Raman spectroscopy (SERS)

Definition, principle and characteristics of surface enhanced Raman spectroscopy: When some molecules are adsorbed to the surface of some rough metal (gold, silver, *etc*.), the Raman signal can be greatly enhanced by the interaction between the two molecules [9,17,20,23]. Thus, a high signal-to-noise ratio spectrum, which produces a surface-enhanced Raman scattering effect, is a special surface optical phenomenon. In 1928, Indian physicist C.V. Raman discovered the Raman scattering effect [16,17]. This technique experienced a long and bumpy development due to various experimental conditions and was almost untouched during the first 40 years [7,12,24,25]. Until the 1960s, the development of a series of technologies such as lasers, beam splitters, detectors and computers effectively led to the study of Raman spectroscopy, making Raman spectroscopy stand out in the field of molecular spectroscopy [17,19,26,27]. However, the number of molecules on the surface interface is small and the Raman scattering signal intensity is weak, which makes the application of Raman spectroscopy in the research of surface interface still difficult. In 1974, a high-quality Raman spectrum of pyridine adsorbed on the surface of a rough silver electrode was obtained by electrochemical roughening method, Fleischmann [18] (Fig. 1a). Subsequently, Van Duyne *et al*. [28] and Creighton *et al*. [29] after rigorous experiments and calculations, concluded that the signal enhancement of pyridine on the roughened silver electrode is derived from a certain enhancement effect rather than a simple increase in surface area, which is later called surface enhanced Raman spectroscopy (SERS) [18,30].

SERS's study of Raman spectroscopy can be said to be a historic breakthrough, with a profound impact on surface interface science and spectroscopy. It makes the surface interface

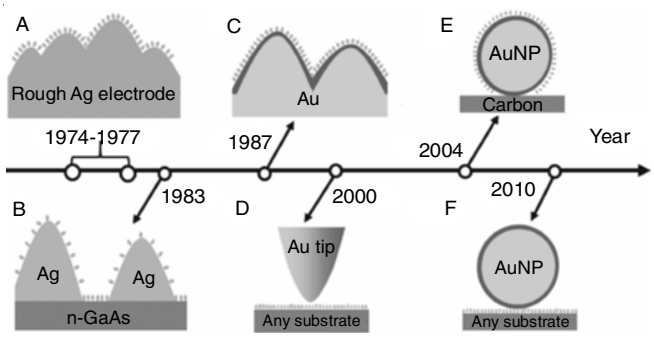

Fig. 1. Development of the "borrowing SERS activity" strategy

Raman spectroscopy no longer subject to the inherent shortcomings of low detection sensitivity, enabling a wider range of applications in many fields such as electrochemistry, biomedicine, catalysis, environmental science, and materials science. According to the most generally accepted view, the enhancement of SERS is mainly due to the localized surface plasmon resonance (LSPR) effect, which is also called the electromagnetic field enhancement mechanism of SERS [1,2,31]. The mechanism believes that if the wavelength of the excitation light satisfies the resonance frequency of the conduction band electrons in the metal, the surface plasmon resonance can be excited on the metal surface with a certain nanostructure and the resonance around the metal surface is stronger due to the resonance interaction [28,29]. The localized optical field, which in turn enhances the Raman signal of the molecules in the local optical field. Although the emergence of SERS has greatly expanded the field of application of Raman spectroscopy, researchers have gradually found two universal problems in SERS. First, the universality of the base material, only in the gold, silver, copper and some of the commonly used alkali metal surface can get a strong SERS effect, in addition to the metal system has not been able to detect high SERS effect in the experiment. The second is the universality of the surface morphology [1,8,14,18,32,33]. Only the rough or nanostructured metal surface can obtain high SERS activity. The smooth surface and even the single crystal surface commonly used in surface interface research cannot be used for SERS study, which makes SERS technology has not been recognized by surface scientists for a long time [8,9,34].

The SERS method is based on the principle of surface-enhanced Raman spectroscopy combined with advanced confocal laser Raman spectroscopy to quickly detect and image the sample to be tested. The application of this technology can provide more reference for researchers, the choice of experimental programs and design is inspiring.

Due to its fast and convenient detection, low sample size and no damage to samples and high sensitivity, SERS technology has been widely used in food, chemistry, medicine, industry and other fields in recent years.

Applications in food discipline: In our daily intake of food, there may be a variety of pesticides and pesticide residues, and the rapid detection of these residues can be achieved by SERS technology. Müller *et al*. [35] used a portable Raman spectrometer to detect the presence of thiabendazole (insect repellent) in bananas and citrus fruits. The results showed that the thiabendazole was safely used in bananas in the market. Within the range, the content in citrus fruit is too severe. Toman *et al*. [36] and Kneipp *et al*. [37] studied the modification of organochlorine pesticides and parathion pesticides based on modified nanosilver particles and silica-coated nanogold particles as base materials, confirming that modification and control of particle arrangement are beneficial. The detection sensitivity is improved, as shown in Figs. 2 and 3.

In addition, Wijaya *et al*. [38] used untreated apple skin and apple juice as research objects to rapidly detect SERS in a food residue of a new alkali insecticide, acetamiprid. The samples were not pretreated and it was found that at least 3 μg/mL of

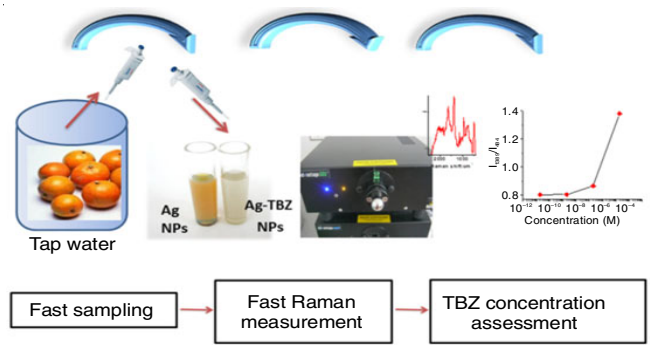

Fig. 2. Graphical sketch as pre-standardized three steps protocol for SERS detection of TBZ in fruits

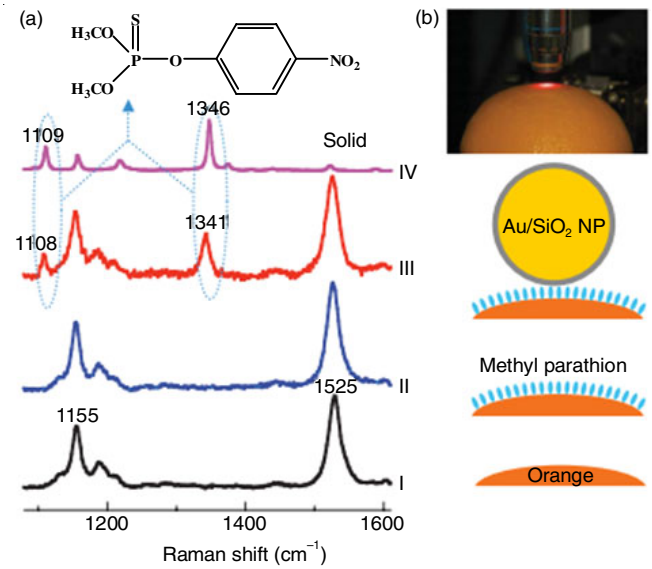

Fig. 3. *in situ* Inspection of pesticide residues on food/fruit (a) Normal Raman spectra on fresh citrus fruits. Curve I, with clean pericarps; curve II, contaminated by parathion. Curve III, SHINERS spectrum of contaminated orange modified by Au/SiO_2 nanoparticles. Curve IV, Raman spectrum of solid methyl parathion. Laser power on the sample was 0.5 mW and the collected times were 30 s (b) Schematic of the SHINERS experiment

acetamiprid present in apple juice, and the concentration of acetamiprid in the apple peel was 2.5 µg/kg, far below the maximum concentration of 1000 µg/kg.

Generally offenders may incorporate a variety of prohibited ingredients in the production and processing of food. It is important to quickly identify whether the food contains prohibited additives, which is important for food safety. The use of SERS technology meets the needs of this test. For example, Roy *et al*. [39] used gold nanoparticles as 20-30 nm as a reinforcing agent to detect melamine content in milk. In addition to the use of gold nanoparticles, the study found that the synergy of graphite and silver nanoparticles can further optimize the effect of SERS [6,40,41]. In this way, the researchers performed SERS on several pigments that were banned in food, which proved that this technology can quickly distinguish a variety of mixed pigment components through their respective characteristic peaks. The work provides strong technical support. Li *et al*. [4,42] prepared PS/Ag nanoparticles as a dynamic SERS detection active substrate, and detected low concentrations of organophosphorus insecticides such as paraoxon and pinch pine. The detection concentration was as low as 10 nmol/L detection effect.

Applications in chemistry: SERS was applied to the chemical field earlier. With the development of nano-preparation and characterization techniques, it was found that several metal ions can significantly improve the experimental results of Raman spectroscopy. Dasary *et al*. [43] used SERS technology to determine the high selectivity and sensitivity of iodide ions present in water and salt. The results show that the surface of gold nanoparticles attracts many charged Rh_6G molecules and is in the form of polymers. Significantly induced an increase in the number of hot spots, and the use of hydrogen peroxide (H_2O_2) to oxidize I^- to I_2 was successfully used to screen for bromide ions (Br^-) that would cause severe interference at high concentrations (Fig. 4). In addition to gold nanoparticles, silver sol can also be used as a surface enhancer in experiments.

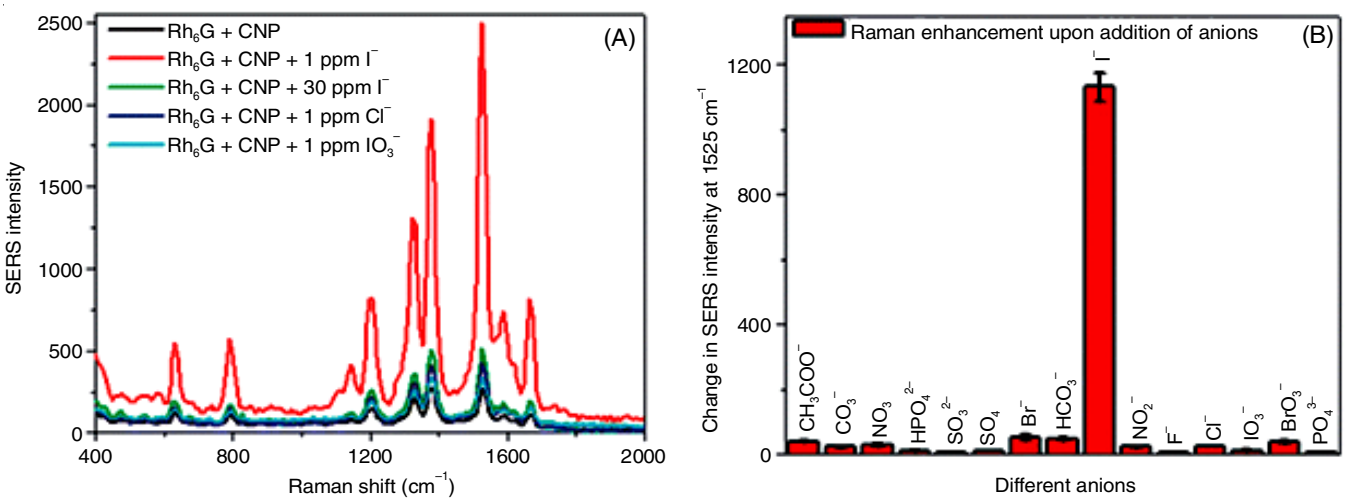

Fig. 4. (A) Change in SERS intensity induced by different anions, (B) a plot demonstrating the selectivity of our SERS assay for I^- ions over other common anions. All the anions have been used at a concentration of 5 ppb. Our results clearly demonstrate that the GNP-based assay is highly selective for I^- ions and at a concentration of ppb our assay shows a negligible contribution from Br^- ions towards the change in SERS intensity

Temiz *et al*. [44] used four new synthetic compounds that were chemically modified with 1,8-naphthalimide at the edge of macromolecules. Poly(acrylamide) dendrimers act as ligands for SERS detection of heavy metal ions (Fig. 5). The Raman spectra of low-concentration heavy metal ions can be detected by the combination of these macromolecules and silver sol and the obtained data are consistent with the results of principal component analysis.

Applications in medicine and industry: SERS technology also plays an important role in the pharmaceutical and industrial fields. Researchers can quickly identify drugs that contain prohibited ingredients and can also screen drugs to ensure the safety of drugs in the market. Zhang *et al*. [45] used silver sol-based SERS technology to analyze the illegal ingredients in traditional chinese patent medicines and found illegal drugs such as rosiglitazone hydrochloride, phenformin hydrochloride, metformin hydrochloride, pioglitazone hydrochloride and sibutramine hydrochloride. These ingredients can improve the patient's condition in a short period of time, but can cause unpredictable side effects. In addition, SERS technology is also used for diseases such as urinary tract diseases, cancer diagnosis and detection of cancer cells *in vitro*. MUC4 is a serum marker for early pancreatic cancer [46], which can be quantitatively detected by surface Raman enhanced spectroscopy, but for multiple organisms. The detection of markers makes people more accurate in the diagnosis of lung cancer. In addition, by establishing DNA-gold nanoparticle detection technology, CD44+/CD24- cells were found in three different breast cancer cell lines, indicating that SERS technology can identify CD44+ in breast cancer stem cells by detecting cell surface marker proteins. The content of CD24- [47]. In the field of industrial science, *Bacillus* has attracted much attention due to its strong ability to produce extracellular enzymes, so it is particularly important to identify it quickly. Deng *et al*. [48] used SERS to detect 14 strains of *Bacillus* isolated from Tibet, China. The multivariate analysis clearly classified these strains into three categories (Fig. 6), which is related to the phylogenetic analysis of their 16S rRNA. It is exactly the same.

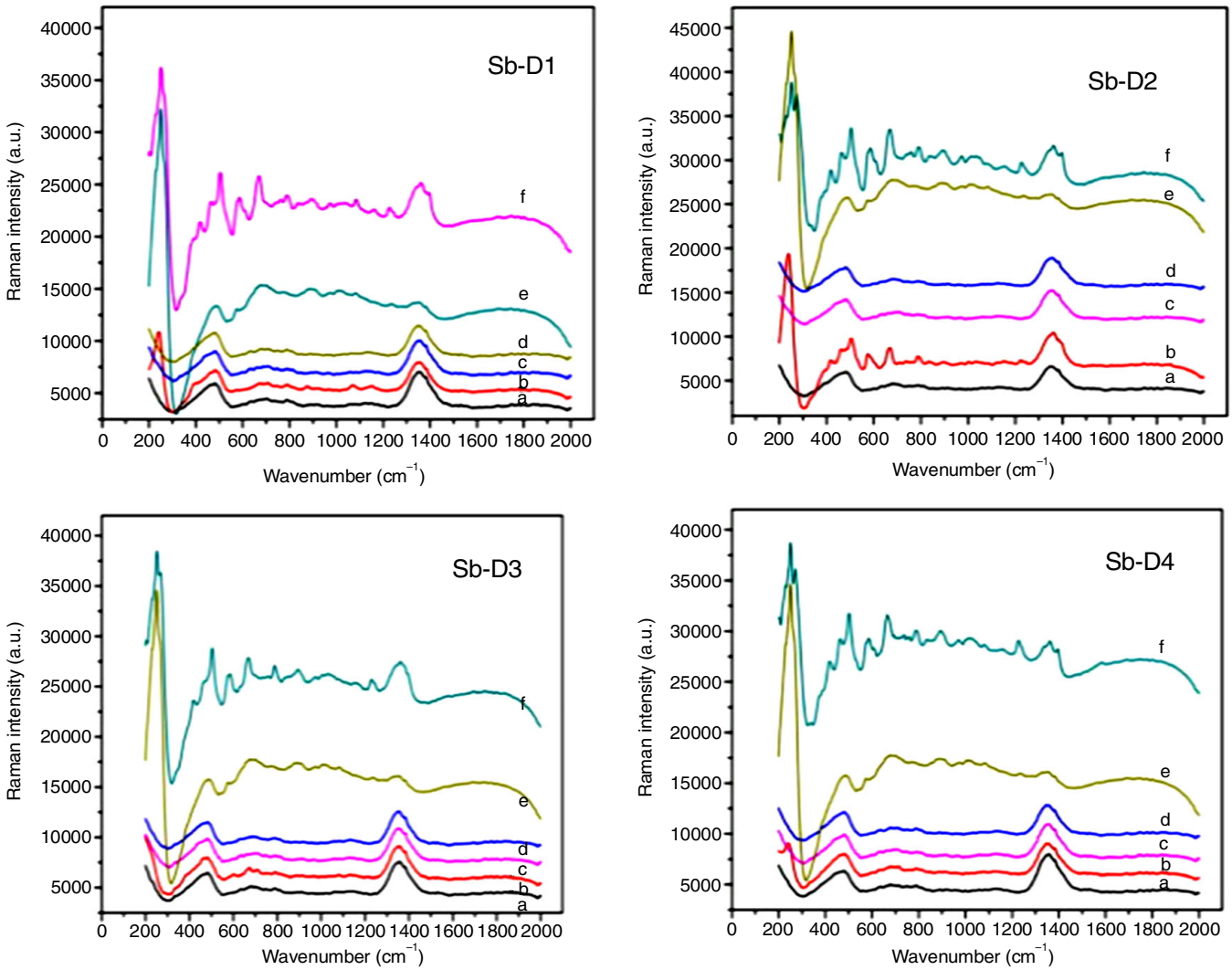

Fig. 5. SERS spectra of Sb^{2+} ions (5×10^{-4} M) interacted with D1-D4 (1×10^{-6} M) in the presence of Ag colloids. Each graphic contains following spectra; (a) dendrimer molecule, (b) dendrimer molecule with Ag colloids, (c) Sb^{2+} ions, (d) Sb^{2+} ions with dendrimer molecule, (e) Sb^{2+} ions with Ag colloids and (f) Sb^{2+} ions with dendrimer molecule and Ag colloids

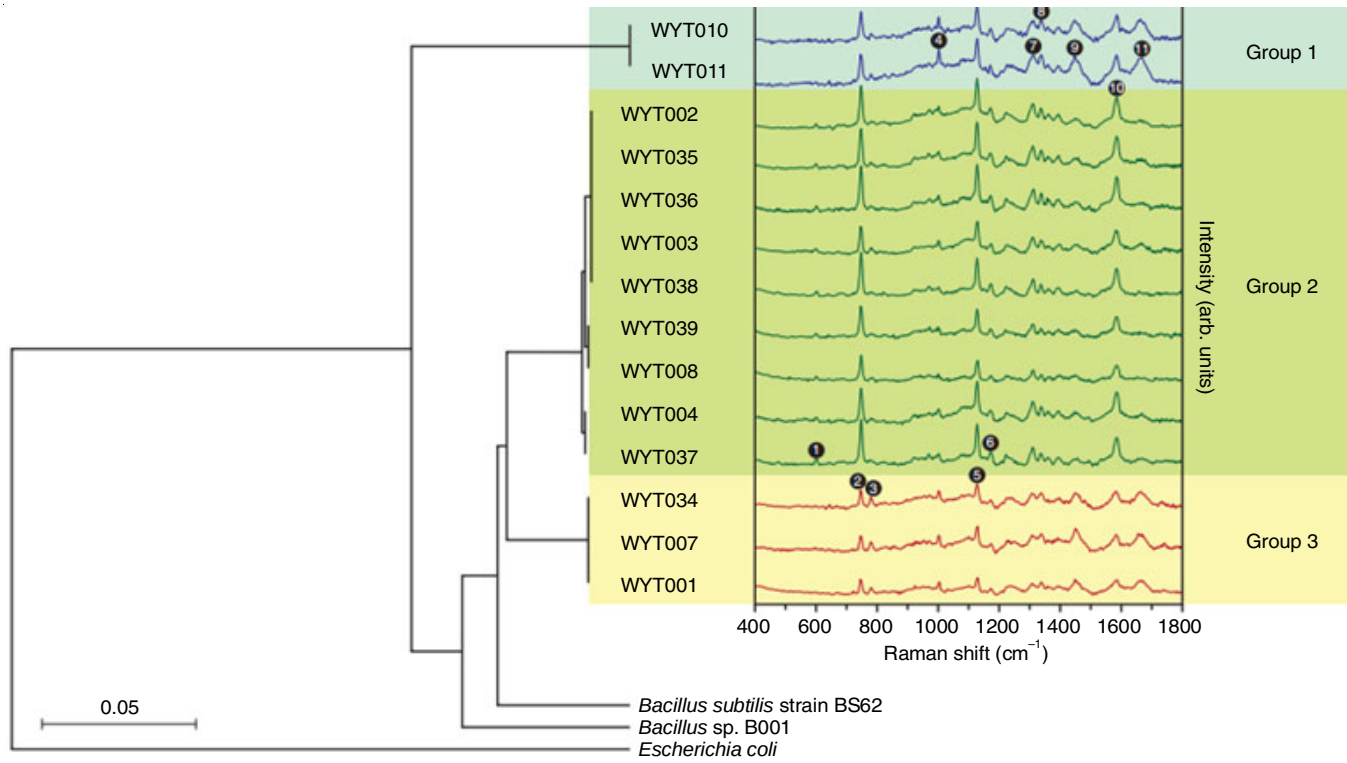

Fig. 6. Isolation and identification of 14 Bacillales strains by SERS in industrial microbiology

SERS technology in pathogenic bacteriology

Identification of mixed strains using SERS: An important application of SERS technology in pathogenic microorganisms is the rapid detection and identification of pathogens isolated directly from the environment or in the patient's body without relying on the medium, thereby increasing efficiency and saving costs. The method can not only identify a single species, but also accurately screen the mixed species. Xu *et al.* [49] found that because of the large amount of biochemical components on the surface of bacterial cell membranes, they can be used as markers for rapid identification and identification of strains. Using SERS, seven strains of *Vibrio parahaemolyticus*, four genotypes isolated from clinical patients and the environment in Washington, USA, were identified. The results showed that seven strains of bacteria had characteristic peaks distinguishable from other strains. They also mixed the two strains numbered 551 and 3652 in a volume ratio of 2:1, 1:1, 1:2, respectively, and found that the two bacteria were clearly distinguished from the respective characteristic peak maps. The characteristic peaks of *Vibrio parahaemolyticus* No. 551 appeared at 1002, 1177 and 1532 cm^{-1}, while the characteristic peaks of *Vibrio parahaemolyticus* No. 3652 appeared at 525, 738, 1319 and 1639 cm^{-1} indicating that the method can obtain good results in single sample and multi-strain mixture samples. Zhou *et al.* [50] mixed *Escherichia coli* and *Shigella* with nanosilver particles and detected them with SERS. It was found that these two bacteria produced completely different characteristic maps, and the Raman vibration of Escherichia coli the peaks appeared at 658, 728, 960 cm^{-1}, and the vibration peaks of Shigella appeared at 670, 820 and 1330 cm^{-1}. The experiment was repeated three times, and the test results were all reproducible. The researchers found that based on the use of stoichiometry, SERS can be used to distinguish between bacterial species and serotypes. They use silver nanoparticles as a substrate for six foods such as *Salmonella*, *Stanley salmonella* and *Staphylococcus epidermidis* in mung bean sprouts. The pathogenic bacteria were identified and differentiated by SERS [51]. Meisel *et al.* [52] performed SERS on 19 major food-borne pathogens and the data obtained were graded. The first grade was to divide the test bacteria into Gram-negative bacteria and Gram-positive bacteria, and then distinguish the species of pathogenic bacteria by two other important nodes. The experimental results show that the accuracy of each level is within the range of 90.6-99.5%.

Rapid detection of common pathogens using SERS: Lu *et al.* [53] used microfluidic chips combined with SERS technology to rapidly identify methicillin-resistant *S. aureus*. Qiu *et al.* [54] also used gold nanoparticles. Mixing with the sample for rapid detection of SERS in *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *etc.* in the food, the study shows that the whole detection process takes a very short time, the detection process only takes a dozen seconds, and the tradition polymerase chain reaction, colony isolation, serological identification experiments and other methods yield the same accurate results, which can be used as a rapid diagnostic method in food hygiene supervision.

Urinary tract infection is a common condition and the current gold standard for the detection of infectious bacteria is the traditional culture method [38], but this method takes a long time. Kloß *et al.* [55] performed a combination of SERS and chemometrics on patients with urinary tract infections. The results showed that SERS technology can accurately detect

urine samples in patients without medium. And the main flora of infection can be determined, the accuracy can reach more than 92%.

The rapid identification and differentiation of common pathogens such as *E. coli*, *Staphylococcus* and *Salmonella* have also been implemented using SERS technology. The SERS after the combination of the cells and the silver nanoparticles can reach the level of single cell detection. Fan *et al*. [56] used silver nanoparticles to identify SERS for common foodborne pathogens such as *E. coli* O157:H7, *Staphylococcus epidermidis*, *Listeria monocytogenes* and *Enterococcus*, and found that at 500 cm^{-1} the difference in peak profile of the different strains in the 800 cm^{-1} interval is most pronounced. Wang *et al*. [57] used a reverse microemulsion method (cetyltrimethylammonium bromide) as a surfactant and tetraethyl orthosilicate as a silica precursor) to make a silica-coated magnetic probe with a diameter of 100 nm. In order to verify its sensitivity, the researchers mixed *Staphylococcus aureus* in PBS buffer and found that the probe can detect a minimum concentration of 10^2 CFU/mL, demonstrating that this SERS-based probe is highly sensitivity. Sundaram *et al*. [58] used silver nanoparticles to detect SERS in typhoid fever, *Escherichia coli*, *Staphylococcus aureus* and *Listeria*, and found that Gram-positive and negative bacteria were fine. There is no significant difference between the cell wall and the cell membrane structure, but the main difference is nucleotides and amino acids, and the characteristic peaks of each are mainly concentrated between 1700-1200 and 700-400 cm^{-1}. At present, the rapid detection of pathogens by SERS is mainly concentrated on intestinal pathogens [59-61], but there are few studies on the rapid identification of respiratory pathogens, and there is also a lack of a complete reference data system.

Applications in virology and other disciplines in pathogenic microorganisms: In terms of optimization of SERS technology, in addition to working with metal ions, virus detection can also be achieved using SERS technology. Cao *et al*. [62] used nucleic acid hybridization technology to simultaneously detect six pathogenic microorganisms such as A/B hepatitis virus, HIV, Ebola virus, Variola virus and *Bacillus anthracis* antigen. Simple spherical gold nanoparticles can be used for quantitative antigen detection, while dimerized gold nanoparticles can reduce the minimum concentration of detection. Lee *et al*. [63] used a bifunctional viral SERS probe at one end as an antibody to bind to gold nanoparticles for SERS detection. Kneipp *et al*. [37] used silver nanoparticles as a substrate to identify SERS for three different strains of influenza virus A/HKx31, A/WSN/33 and A/PR/8/34. The collection was completed within 1 min of the Raman spectrometer with a virus sample volume of less than 5 μL and different strains could be clearly identified based on the difference between the peak maps (Fig. 2 gray area). The results show that SERS can be used to identify different strains of the same pathogen.

In addition to common pathogenic bacteria and viruses, some microorganisms such as mycoplasma and chlamydia have also caused serious harm to the development of agriculture. At present, mycoplasma testing in poultry in the United States mainly relies on skilled workers and does not guarantee the accuracy of the test results. Hennigan *et al*. [64] used NA-SERS to rapidly detect *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, cholestyramine, tylosin, *etc*. in poultry. The results showed that this method requires lower detection conditions. It also works better than standard PCR and real-time PCR.

SERS can also play an important role in judging normal colon tissue and malignant tumors. Lopes *et al*. [65] measured 11 human colon samples and obtained 55 maps, and observed peak maps of proteins, lipids and amino acids. Strips with large differences in strip strength differences may be key differences. A linear analysis of the band strength and intensity ratios then shows that the accuracy of this method is 81% [36,66-68]. At present, colon cancer mainly tends to use some small molecules for targeted therapy. The detection of targeting molecules and their metabolites generally requires the carrying of fluorescent labels, which may carry toxins and cause harm to the patient. Researchers have used SERS technology to study the spatial distribution of intracellular drugs and found that drugs accumulate on the cell membrane epidermal growth factor receptor and induce receptor internalization. This study illustrates the drug at the atomic level in the cell. The molecular targeting mechanism [4,38,39,42,50,69-71].

Miscellaneous Applications

Development kit for microbial detection and identification: SERS technology due to the unique nature of the spectrum, there are some differences in the SERS of different microorganisms [71,72]. Just like human fingerprint identification, different microorganisms can be distinguished by using the difference of "spectral fingerprint" of different microorganisms. The first feature of this method is that there is no need to mark the sample, the detection process is fast, usually a Raman spectrum can be completed within 30 s; the second feature is that the detection cost is lower, the nano-matrix is lower and can be used repeatedly, except for the Raman spectrometer, there is basically no need for other consumables, so the whole set of equipment is relatively low in price and has market promotion. The third feature is that the Raman spectrometer can be miniaturized and portable, and can realize on-site, field testing. At present, there is no application of this technology to clinical identification at home and abroad, especially for the identification and detection of pathogenic microorganisms, and its timely and accurate prediction and early warning plays an important role in disease prevention and control. SERS technology is used to test clinical products, and further explore the conditions and indicators such as sensitivity, reproducibility, specificity, cost analysis, *etc*., and carry out research and development and promotion of kits or test strips for microbial detection and identification.

Establish SERS technology network database platform: The current research status shows that SERS technology only detects and identifies certain microorganisms and does not widely collect and summarize microscopic SERS technology images and establish a searchable and operable database and a shared network platform. The establishment of a multi-microbial Raman spectral fingerprint database and network sharing platform will enable the sharing of resources across

multiple research institutions across time and regional boundaries. Analyze the Raman spectrum to determine the characteristic peaks. By obtaining the Raman spectral fingerprints of known microorganisms and matching with the microbial information identified by the traditional methods, a specific spectral fingerprint database is established, which is the cutting result of our next work point. In summary, the application of SERS technology can realize the high-precision and rapid identification of microorganisms, and gradually complete the research and development of related products and the transformation of results. At the same time, the establishment of a variety of microorganism "Raman spectral fingerprint" library can be completed and relevant technical reserves can be completed. It has developed into a world-leading rapid identification network sharing platform to meet the rapid and onsite needs of clinical samples in the microbiology field.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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