In situ food-borne pathogen sensors in a nanoconfined space by surface enhanced Raman scattering

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Abstract
The incidence of disease arising from food-borne pathogens is increasing continuously and has become a global public health problem. Rapid and accurate identification of food-borne pathogens is essential for adopting disease intervention strategies and controlling the spread of epidemics. Surface-enhanced Raman spectroscopy (SERS) has attracted increasing interest due to the attractive features including simplicity, rapid measurement, and high sensitivity. It can be used for rapid in situ sensing of single and multicomponent samples within the nanostructure-based confined space by providing molecular fingerprint information and has been demonstrated to be an effective detection strategy for pathogens. This article aims to review the application of SERS to the rapid sensing of food-borne pathogens in food matrices. The mechanisms and advantages of SERS, and detection strategies are briefly discussed. The latest progress on the use of SERS for rapid detection of food-borne bacteria and viruses is considered, including both the labeled and label-free detection strategies. In closing, according to the current situation regarding detection of food-borne pathogens, the review highlights the challenges faced by SERS and the prospects for new applications in food safety.

Keywords Bacterium · Virus detection · Food pathogens · Food analysis · SERS · Nanoconfined space · Nanostructures

Introduction
The outbreak of novel coronavirus (COVID-19) pneumonia associated with high morbidity and mortality has caused ever-increasing attention and public panic throughout the world [1, 2]. COVID-19 has been detected in various frozen foods, particularly in their packaging and the storage environments [3–5], which makes food safety an important issue of global concern nowadays. In fact, the presence of food-borne pathogenic microorganisms during the processes of food production and supply would cause food contamination, spoilage, and deterioration, thus resulting in nausea, vomiting, stomach pain etc., and in serious cases, organ damage [6, 7]. For example, after Shigella enters into human intestine, it can destroy the intestinal mucosa due to the dual effects of endotoxins and exotoxins and cause human intestinal disease [8, 9]. Therefore, it is vital to access a sensitive and selective technique for rapid monitoring of food pathogens, including bacteria and viruses, to protect people against potential health risks.

Conventional methods for detection of pathogens mainly include culture analysis and metabolic tests [10, 11], which are known as the gold standards for bacterial identification. However, the detection process may take several days to a week that could hardly be adaptable in rapid emergency events. Current methods for pathogen detection generally employ the nucleic acid-based polymerase chain reaction (PCR) and enzyme-linked immunoassay, showing the advantages of high specificity, high sensitivity, and ease of observation [12–14]. Although these methods can produce results within a few hours, they require complex steps, time-consuming sample pretreatments, and skilled personnel.

The demand for rapid and cost-effective pathogen diagnostic methods has prompted researchers to focus on the development of real-time biosensing platforms [15–17]. Among the
various biosensor technologies, surface-enhanced Raman spectroscopy (SERS) as a vibrational spectroscopic technique has attracted increasing attention due to its prominent characteristics such as high sensitivity, low water interference, short detection time, and the rapid, in situ, and noninvasive capabilities [18–21]. The SERS effect mainly derives from the local electromagnetic field enhancement generated on or near the surface of coinage-metal nanostructures, and the resulting chemical interaction between the nanostructure surface and the adsorbed molecules [22, 23]. This interaction-based construction of SERS-active nanomaterials is one of the most important prerequisites for expanding SERS applications in biological analysis. It has been shown that only substrates at the nano-scale can generate high SERS enhancements that are also known as the enhancement of nanoconfined space provided by the diversified nanostructures. The nanoconfined space refers to nanoscale geometry of the structure surface or the gaps between, where the plasmon resonance or the electron oscillate occurs. The shape, size, composition, and particle spacing of metallic nanostructures are the key factors that affect surface plasmon resonance (SPR) and the electromagnetic fields [24–27], which need to be carefully designed to ensure SERS enhancement and excellent signal reproducibility. The commonly used substrates for biological applications mainly include colloidal nanoparticles, surfaces modified with nanostructures, and colloidal SERS tags [28–31]. Colloids and surface substrates are generally designed to measure the inherent SERS signal of the analytes, while SERS nanoparticle tags are usually combined with targeting ligands such as aptamers and antibodies for detection.

The research on SERS detection and differentiation of pathogens can be dated to 1998, when Efrima et al. [32] obtained the SERS spectra of Escherichia coli with colloidal Ag nanoparticles deposited on the outer membrane of Escherichia coli. Since then, the application of SERS technique in pathogen identification and classification has blossomed rapidly [24, 33–36]. The combination of handheld portable Raman spectrometers with nanostructured substrates provides strategies for rapid identification of pathogens in a nanoconfined space, though there are still some challenges such as batch-to-batch consistency and multiple analysis [37–39]. Nowadays, SERS can be used for the detection of single cell bacteria or a single spore [40, 41]. In this review paper, we focus on the latest progress in rapid detection of food-borne pathogenic microorganisms by SERS. The mechanisms and sensing strategies for SERS detection of pathogens are firstly discussed, followed by the details on the SERS nanostructures. Finally, we discuss the challenges and opportunities in developing next-generation SERS nanostructures for pathogens sensing in real food samples.

SERS mechanisms and advantages

Since the discovery of SERS, the enhancement mechanism has become a hot research topic. Two primary mechanistic models have been proposed, namely, electromagnetic (EM) field enhancement and chemical enhancement (CE) [22, 23, 42]. EM enhancement makes a major contribution to the SERS phenomenon. With a certain frequency EM radiation incident on noble metal nanostructures, the conduction electrons oscillate collectively under the action of the radiation electric field, which is termed surface plasmon resonance (SPR) [43]. The surface of the nanostructure will generate a redistribution of the local field and a great increase in the EM field. The Raman mode of molecules confined in these EM fields will be greatly enhanced. Therefore, the EM has a long-range effect because molecules on or near the metal surface experience the enormous field enhancement. In CE, the metal and adsorbed molecules generate charge transfer states, which increases the possibility of Raman transitions by providing resonance excitation [44]. CE is position-specific and analyte-dependent and provides one or two orders of magnitude increase in Raman signal intensity. The analyte molecule should be directly adsorbed to the metal surface to allow a charge transfer state. Due to the complex charge transfer process, many factors can influence charge transfer, such as the nature of the analyte molecules, metal nanostructures, excitation wavelengths, and molecular vibration modes. It can be concluded that the EM enhancement emphasizes the role of the nano-substrate to provide a long-distance EM field, which depends on the inherent characteristics of the nano-substrate. However, CE can be achieved by changing the scattering cross section of the analyte attached to the metal surface. Thus, the degree of enhancement depends on the chemical characteristics of the analytes.

One of the biggest challenges in SERS is related to the activity of the molecular system being studied. Theoretically, all molecules with changeable polarizability could produce SERS, but not all molecules are good SERS probes [45, 46]. SERS activity mainly depends on three factors: the electromagnetic field generated by the plasmonic nanostructure used as an optical intensifier, the intrinsic Raman properties of the molecule, and the affinity between molecule and plasmonic surface. The first factor, the electromagnetic field or the local surface plasmon resonance (LSPR), is the basis of SERS, and the other two dominate the sensitivity in different systems. Therefore, they are all related to the bacteria detection.

Compared with other pathogen detection methods, SERS detection platform has clear advantages. For instance, compared with fluorescence, the instantaneous features of Raman Scattering enable the excitation energy relax at a faster rate [47, 48], inducing a more stable SERS signals which is resistant to photodegradation or photobleaching. The peak width
of the Raman signal is about 1–2 nm, which is 10–100 times narrower than the fluorescence emission of traditional organic dyes or quantum dots [49], hence the Raman signal is more conducive to multi-component analysis. In addition, unlike infrared spectroscopy, water exhibits negligible Raman scattering [50] which is beneficial for biological sensing.

**SERS detection strategies for pathogens**

There are two SERS configurations for bacteria and virus detection, namely unlabeled or labeled SERS, as shown in Scheme 1. In label-free detection, bacterial cells and viruses or their metabolites can be directly introduced onto the nanostructures surface. The intrinsic Raman spectra of the biomolecules in the nanoconfined space can be directly measured to identify the samples. While in labeled detection, Raman reporter molecules are introduced to generate SERS signals. To facilitate target capture and enable the specific detection, ligands such as antibodies, aptamers, or related molecules are typically immobilized on the nanostructures surface. The differences in Raman spectral before and after capture of the targets can be used to identify the targets. For example, Au nanoparticles (AuNPs) immobilized with Raman reporter can be used as SERS active substrate. A core-shell nanostructure can then be formed by covering this structure with another layer of medium such as silica or polymer, where the shell may be decorated with capture molecules. Therefore, the target bacteria can be captured and detected by this elaborate design of sandwich structure.

**Detection of food-borne bacteria**

SERS-based technology has long been applied for the detection of food-borne bacteria, which serves as a rapid alternative for evaluation of food safety. These methods mainly include the confined nanostructures-based detection of bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*, which are summarized in Table 1.

**Label-free detection of bacteria**

Bacteria are generally in large volumes, contain complex chemical components including proteins, nucleic acids, enzymes, and various inorganic and organic substances [50, 68]. Although SERS spectrum owns the fingerprint characteristics, the complex spectral characteristics of bacteria make it difficult to identify specific targets in some cases. Consequently, chemometrics is employed to assist in the qualitative and quantitative analysis of SERS spectrochemical measurements. Multivariate data analysis including principal component analysis (PCA) and partial least squares (PLS) regression are the commonly used statistical methods [34, 69]. PCA is a primary mathematical method to reduce the data dimensions by identifying correlations among a set of variables and then projecting the original set of variables into a new set of uncorrelated variables called principal components. PLS uses a linear combination of the predictor variables to project the original data to a new set of coordinates to generate a positive/negative prediction. Thus, PCA analysis is generally employed for the classification of bacteria with given features, like Gram stain results.

Label-free SERS detection is generally employed to directly obtain SERS spectra of bacterial cells without the need for synthesis of Raman dyes and SERS tags. This method has the advantages of simplicity and convenience.

**Detection of bacterial cells**

There are typically three strategies for label-free detection of bacterial cells in SERS: mixing the bacteria with colloidal nanoparticles to induce aggregation, placing the entire bacteria cell directly on the solid SERS substrate, and assembling colloidal nanoparticles onto the bacteria surface directly. Silver
nanoparticles (AgNPs) were initially employed as a SERS substrate to rapidly distinguish *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Escherichia coli O157:H7*, and *Listeria monocytogenes* in water and food [51]. Subsequently, a series of nanostructures such as Au@Ag nanoparticle-coated mussel shell (Fig. 1a, b) [52], HfTe₂-Au nanocomposites [53], and black phosphorus-Au filter paper [54] were fabricated for the rapid detection of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*. To achieve higher sensitivity and improved reproducibility, AgNPs were in situ synthesized on the bacterial cell surface for label-free SERS detection. For example, living bacteria in drinking water could be detected by in situ coating bacterial cell wall with AgNPs [35]. The enhancement of the specific Raman signal via this method was nearly 30-fold greater than that for a simple mixed colloid-bacterial suspension.

Compared with nanostructures (typical dimensions 10–100 nm), most large bacteria are typically with diameter of 0.5–1 μm and length of 1–2 μm. This mismatch in size hampers contact between bacteria and nanostructures [70, 71]. The measurement principles of SERS distance dependence indicate that the intensity increases with the reducing surface-to-target distance R. Considering the field enhancement decays with $R^{-3}$, the whole distance dependence should scale with $R^{-12}$ followed with $E^4$ approximation. Experimentally, the distance dependence should be $R^{-10}$ by including a rectified $R^2$ due to the increased surface area scaling, that is the shell of

| Table 1 | Food-borne bacteria detected by SERS |
|---------|-------------------------------------|
| **Escherichia coli** | **Detection strategy** | **SERS Substrates** | **LOD** | **Ref.** |
| | Label-free | AgNPs | Single cells | [51] |
| | | Au@AgNP-coated mussel shell | – | [52] |
| | | HfTe₂-Au nanocomposites | 10 CFU mL⁻¹ | [53] |
| | | Black phosphorus-Au-based filter paper | 10⁷ CFU mL⁻¹ | [54] |
| | | In situ synthesis of AgNPs on cell | 2.5 × 10⁵ cells mL⁻¹ | [35] |
| | | Vancomycin functionalized AgNR | 10⁶ CFU mL⁻¹ | [55] |
| | | Nanoplate-bacteria-nanorod supercrystals | 10⁷ CFU mL⁻¹ | [56] |
| | Labeled | AuNPs and Fe₃O₄ | 10 CFU mL⁻¹ | [57] |
| | | AuNPs and Fe₃O₄ | 10 CFU mL⁻¹ | [58] |
| | | AuNPs and Fe₃O₄ | 10 CFU mL⁻¹ | [59] |
| | | Gold nanobones & Fe₃O₄ | 10 CFU mL⁻¹ | [60] |
| **Staphylococcus aureus** | **Detection strategy** | **SERS Substrates** | **LOD** | **Ref.** |
| | Label-free | HfTe₂-Au nanocomposites | 10 CFU mL⁻¹ | [53] |
| | | Black phosphorus-Au-based filter paper | 10⁷ CFU/mL | [54] |
| | Labeled | AuNPs and Fe₃O₄ | 25 CFU mL⁻¹ | [58] |
| | | AuNPs | 10⁴ CFU mL⁻¹ | [61] |
| | | AuNPs and Fe₃O₄ magnetic AuNPs | 35 CFU mL⁻¹ | [62] |
| | | Ag-coated magnetic nanoparticles and AuNPs/PDMS & Au-Ag core-shell nanoflowers | 13 CFU mL⁻¹ | [64] |
| **Salmonella typhimurium** | **Detection strategy** | **SERS Substrates** | **LOD** | **Ref.** |
| | Label-free | HfTe₂-Au nanocomposites | 10 CFU mL⁻¹ | [53] |
| | | Vancomycin functionalized AgNR | 10⁵ CFU mL⁻¹ | [55] |
| | Labeled | AuNPs | 10⁵ CFU mL⁻¹ | [62] |
| | | AuNPs and Fe₃O₄ magnetic AuNPs | 15 CFU mL⁻¹ | [65] |
| | | AuNPs and Fe₃O₄ | 8 CFU mL⁻¹ | [59] |
| | | AuNPs/PDMS | 27 CFU mL⁻¹ | [66] |
| **Staphylococcus epidermidis** | **Detection strategy** | **SERS Substrates** | **LOD** | **Ref.** |
| | Label-free | Vancomycin functionalized AgNR | 10⁵ CFU mL⁻¹ | [55] |
| **Listeria monocytogenes** | **Detection strategy** | **SERS Substrates** | **LOD** | **Ref.** |
| | Label-free | HfTe₂-AuNPs | 10 CFU mL⁻¹ | [53] |
| | | Black phosphorus-Au-based filter paper | 10⁷ CFU mL⁻¹ | [54] |
| **Staphylococcus xylosus** | **Detection strategy** | **SERS Substrates** | **LOD** | **Ref.** |
| | Label-free | Nanoplate-bacteria-nanorod supercrystals | 10⁷ CFU mL⁻¹ | [56] |
| **Bacillus** | **Detection strategy** | **SERS Substrates** | **LOD** | **Ref.** |
| | Label-free | Silver film over nanosphere | 2.6 × 10⁴ spores | [67] |
| | | Silver colloids | 29.9 nM | [37] |
| | | Colloid meso-droplets on superhydrophobic wires | 18 spores | |
| **Vibrio parahaemolyticus** | Labeled | AuNPs/PDMS | 18 CFU mL⁻¹ | [66] |
targets at the increased distance from substrate surface [72]. Hence, reducing the distance between the bacterium and the nanostructure would greatly improve the detection sensitivity of the SERS-active substrates. Both chemical bonding and manipulation through physical forces can be the right solutions [23, 73]. Given that the nuclei of lipopolysaccharide molecules of bacteria are phosphate- and carboxylate-rich, the surface of the bacterial membrane is negatively charged. The synthesized positively charged Au nanorods can be adsorbed onto the bacteria surface via electrostatic adsorption. Using such an approach and with the aid of multivariate analysis, four types of *Pseudomonas* isolated from rotten chicken were classified [74]. Vancomycin, a well-known glycopeptide antibiotic capable of strongly interacting with the bacterial cell wall via hydrogen bonds [55], was used to modify SERS-active substrates and subsequently capture the bacteria. An atomic force microscopy (AFM) image of the bacteria on vancomycin modified substrates indicated the deformation of the bacterial cell, which suggested that the bacteria were bonded more closely to the SERS-active substrates. The corresponding results demonstrated that the SERS intensity of the bacteria increased at least 1000 times as a result of decreasing the distance [75]. Similarly, a 3D bio-inorganic scaffold-based confined nanostructure was constructed for the detection of Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus xylosus* [56]. This method allowed the detection of *Escherichia coli* and *Staphylococcus xylosus* with LODs of $10^7$ CFU/mL and $10^6$ CFU/mL, respectively (Fig. 1c, d). PCA analysis of the single and mix bacteria samples was further implemented for the discrimination of different kinds of bacteria. The detection of bacteria in soft drinks demonstrated the feasibility of this method in quality control analysis of foods and drinks. Another significant method to improve the detection sensitivity is to combine SERS with sample pre-concentration whereby a maximus number of bacteria are captured onto a large fixed surface area such as magnetic nanoparticles (MNPs). For example, magnetic-plasmonic nanoparticles were fabricated and used for the enrichment of bacteria by applying an external magnetic field [76].
The above studies have demonstrated that SERS can be used for the identification and classification of bacteria. However, none of the label-free bacteria detection methods have considered the real-world scenarios whereby large numbers of bacterial species often exist together in a biological sample, along with bacteria from different serotypes and strains. Although chemometric analysis of SERS signals is applied to distinguish bacteria present in species, strains, and serotypes, it has limitations in the presence of mixtures of different bacteria [77–79]. When two or more kinds of bacteria are combined, the spectrum of the mixture sometimes shows spectral characteristics of the individual bacteria, because the SERS process relates to the vibrational modes of the chemical bonds.

Detection of bacterial metabolites

As mentioned above, the Raman signals of bacteria originate from cell membranes with similar chemical structures and composition for most bacterial species. Thus, it is still challenging to distinguish closely related species, especially in bacterial mixtures [80, 81]. In addition, cells should be washed repeatedly and resuspended in pure water to avoid the potential interference of the residual matrix or buffer. The interruption of osmotic balance would result in bacterial cell disruption, which may affect the repeatability of analysis due to inconsistent sample composition. For these reasons, the detection of whole bacterial cells via Raman method is still challenging. The indirect sensing of bacteria thus becomes an alternative method, which is mainly performed by identifying specific markers released by organisms in the matrix fluid. Calcium dipicolinate (CaDPA), an important biomarker for bacillus spores, was detected via its SERS spectra on Ag film over nanosphere (AgFON) substrates. The capability of this approach for bacillus spores sensing was demonstrated with LOD of 2.6 × 10^3, which was lower than the infectious dose level of 10^4 spores for anthrax (Fig. 2a) [67]. In addition, the SERS spectra of dipicolinic acid (DPA) extracted from spores of Bacillus was also obtained using nanoparticles. Cowcher et al. demonstrated that colloidal nanoparticles as SERS substrates could be used for rapid detection and quantification of the DPA at the 5 ppb (29.9 nM) level [37]. Cheung et al. performed SERS measurements of <1 μL of analyte/colloid meso-droplets on superhydrophobic wires with hydrophilic tips, which enabled the detection of DPA at 10−6 mol dm−3. This sensing performance is approximately to 18 spores, a level which is greatly below the infectious dose and nearly two orders of magnitude more sensitive than previous methods [83]. Based on the loading of Au nanostars on a flat filter, Guo et al. developed user-friendly SERS devices for measurement of gaseous metabolites of bacteria (Fig. 2b, c). The impressive performance of such devices for the detection of gaseous metabolites of common foodborne bacteria such as Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa from inoculated samples achieved extremely high sensitivity. The approach further enabled the detection of spoiled food at an early stage and tracking of the degree of food spoilage in real-time [82].

Detection of bacterial DNA

Instead of detection and differentiation of the bacterial whole cell, the bacterial gene (DNA or RNA) is an attractive alternative given that the genes could reflect the unique genetic information of bacteria species, strains, and serotypes [84–86]. The specificity of the SERS-based gene probe relies on its hybridization with complementary target bacterial gene sequence, where SERS is used to characterize the change before and after such hybridization [87–90]. The facile synthesis of genetic sequences and their relatively small size confer unique advantages compared to whole cell detection [91–95].

The SERS-based DNA gene probe was first reported by Vo-Dinh et al. [96] in 1994 and was applied for the detection of DNA biotargets via hybridization to complementary DNA sequences. During the last decades, numbers of SERS-based DNA sensing assays have been developed to detect bacterial genomes. Typically, MNPs have been fabricated for separation of the DNA strands. Following the binding of target DNA, a short synthetic ssDNA with specific dye modification was hybridized, which served as label for SERS sensing. The PCR product of the bacterium Mycoplasma mycoides subspecies mycoides small colony type (MmmSC) was amplified and then detected by SERS. The multiplexing capability of the SERS method has been further verified by simultaneous detection of three independent PCR products with different dyes modification [97]. Van Lierop et al. [98] reported a separation-free SERS method by employing a well-designed SERS primer, which showed a distinct enhancement in the SERS intensity in the presence of target DNA. The presence of specific bacterial DNA from Staphylococcus epidermidis was amplified by PCR and then detected by SERS. Multiplexing bacterial DNA detection was achieved by Kang et al. [99] via an Au particle-on-wire platform. Au nanowire immobilized with probe DNA and Au nanoparticle linked with reporter DNA was employed in such platform. Both the probe and reporter DNA were complementary to the target DNA, forming a sandwich hybridization of reporter-target-probe DNAs (Fig. 3). The nanoconfined space for SERS enhancement could be created with the coupling between Au nanowire and Au nanoparticle. Thus, only after the formation of sandwich hybridization, the Raman dye on the reported DNA could be within this confined space, whereafter SERS detection was performed. Using this SERS platform, PCR products of the 4 different bacteria from 7 clinical isolates (Enterococcus faecium, 2 isolates; Microchim Acta (2021) 188: 201
Staphylococcus aureus, 2 isolates; Stenotrophomonas maltophilia, 2 isolates; Vibrio vulnificus, 1 isolate) were simultaneously identified, demonstrating the potential for multiplex bacterial detection. The successful detection of bacterial DNA discussed above was realized based on using artificially synthesized DNA or PCR products [100]. Unfortunately, nearly few of the aforementioned studies realized the detection of bacteria in real samples without the use of PCR, which inevitably increases the time required for PCR reaction to the total detection time.

Fig. 2 A SERS detection of CaDPA from B. subtilis spores. Reprinted with permission from ref. 67. B Schematic for monitoring of gas metabolites from contaminated samples. C Photos of a SERS “nose” and pork inoculated with Au nanostars-coated flat filter and bacteria. b Photo showing the point-of-care sensing with a user-friendly SERS device. c–f Typical SERS spectra of (i), E. coli (ii), S. aureus (iii), and P. aeruginosa (iv) acquired from (c, d) medium and (e, f) inoculated pork samples. Reprinted with permission from ref. 82

Fig. 3 a Scheme of the DNA detection with the Au particle-on-wire SERS platform. b Representation of the SERS detection of patterned multiplex pathogen DNA. Reprinted with permission from ref. 99
Labeled detection of bacteria

The differentiation of bacteria based on their SERS signals poses many challenges, especially for a mixture of different bacteria. Hence, a secondary confirmation is often required, which mainly base on assessing the immunological properties of target bacteria by using immune molecules such as antibodies and aptamers [61, 101, 102]. The core of the immune-SERS platform is the immune-conjugated nanoparticles, where the conjugated immune molecules are specific to the target bacteria and the nanoparticles serve as the SERS-active substrates. These immune molecules are often labeled with Raman reporter molecules, which are small molecules with strong and distinguishable Raman signals. The detection and differentiation of the bacteria can thus be achieved through the different labeled Raman reporter molecules.

Combining SERS with classical immunoassay method offers the possibility to selectively capture and detect target bacteria with high sensitivity. A sandwich type SERS sensing platform was established by using 4-mercaptobenzoic acid (MBA)-coated Au nanoparticles conjugated with polyclonal antibodies and MNPs (Fe3O4) modified with monoclonal antibodies. Escherichia coli O157:H7 (10 CFU/mL) from both pure cultures and ground beef samples were detected within 1 h and 3 h, respectively [57]. Dual antibodies including Escherichia coli and Staphylococcus aureus were used for the functionalization of Au nanoparticles with magnetic beads were employed to construct a simultaneous SERS detection system. The Au nanoparticles with SERS probes of triple bonds at 2105 and 2227 cm⁻¹ could prevent the interferences from the coexisting constituents in the fingerprint area. This method showed the LODs for Escherichia coli and Staphylococcus aureus were 10 and 25 CFU mL⁻¹, respectively. Moreover, it enabled simultaneous detection of these two bacteria both in bottled water and milk samples, demonstrating the application to real samples [58].

Antibodies were used in the abovementioned studies as sensitive and selective recognition agents. However, it has been shown that there are still limitations in the use of antibodies for recognition in biosensing and bioanalysis [103–105]. For example, antibody production is expensive and time-consuming, and the specific binding greatly depend on the environmental conditions, which may be detrimental to the analysis of environmental or food samples. Therefore, more and more attentions have been focused on the development of alternative recognition molecules. Aptamers are rapidly emerging with high specificity and environmental robustness [61, 106, 107]. They are single-stranded DNA or RNA sequences that can potentially recognized any targets selectively. Compared with antibodies, aptamers have the advantages of small size, readily amenable to chemical synthesis, cost-effective, high stability, reproducibility between batches, and unlimited target size or immune response [108–111]. In line with the potential advantages of aptamers being used as excellent capture biomolecules, they have been explored as one of the most promising candidates for manufacturing next-generation biosensors. Diaz-Amaya et al. described the effective sensing of Escherichia coli O157:H7 by using multi functionalized gold nanoparticles with specific aptamer and Raman reporter. This SERS-based method exhibited high sensitivity and specificity for Escherichia coli and could be used both for both pure culture and ground beef samples with LOD of ~10 CFU mL⁻¹ and ~100 CFU mL⁻¹, respectively [61]. A aptamers-based SERS screening method was developed to detect and filtrate Salmonella enterica serovar Enteritis in water. A DNA aptamer was used for targeting Salmonella Enteritidis and labeling with specificity, which was attached to an adenine and fluorescein (FAM) chain to determine the presence or absence of Salmonella Enteritidis. Target bacterial cells were identified and further concentrated by combining with a vacuum filtration system. Through an extra filtration of Au NPs, the aptamer signals were obtained and employed for SERS mapping, indicating whether the existence of specific bacterial strains. The specificity of this approach was verified and the sensitivity was reported as 10³ CFU/mL within the detection time of 3 h [62]. Simultaneous quantification of Salmonella typhimurium and Staphylococcus aureus was also demonstrated by employing aptamers and nanostructures. A SERS-based platform was developed with the well-designed signal probe and capture probe, among which the former referred to gold nanoparticles (AuNPs) functionalized with both Raman reporter and aptamers, and the latter referred to aptamer-coated AuNPs. The detection limit for Staphylococcus aureus and Salmonella typhimurium were 35 CFU mL⁻¹ and 15 CFU mL⁻¹, respectively [65]. To further improve the sensitivity, a magnetically assisted biosensor consisting of both SERS substrate (magnetic NPs coated with Ag) and SERS tag (Au-DTNB@Ag-DTNB core-shell plasmonic NPs) was designed for detection of Staphylococcus aureus. The positions of double-layer DTNB and LSPR matched well with the laser excitation wavelength, which enabled 10 times of enhancement in the Raman signals compared with the commonly used SERS tag AuNR-DTNB. This nanostructure-based SERS method achieved the LOD of 10 cells/mL for Staphylococcus aureus [63].

In further development work, various morphology-based SERS tags were developed for the simultaneous and sensitive detection of Escherichia coli O157:H7 and Salmonella typhimurium, which was based on co-functionalized Au NRs with aptamer and Raman reporter. The one-pot synthesized tags provided characteristic SERS signals with high stability, as well as specificity for biorecognition. In the Au NR-mediated regeneration process, SERS tags with octahedral cracks and small protrusions were prepared and coated by specific aptamers and two kinds of Raman reporter. With the aid of antibody-conjugated bulk-modified MNPs, Salmonella
typhimurium and Escherichia coli in the range of 10–10⁶ CFU mL⁻¹ were detected simultaneously with LODs of 8 and 5 CFU mL⁻¹, respectively (Fig. 4a) [59]. Similarly, the same group also reported SERS detection of E. coli O157:H7 with a one-pot using self-prepared multifunctional Au nanobones, showing a LOD of 3 CFU mL⁻¹ [60]. Moreover, a uniform approach for Staphylococcus aureus enterotoxin B (SEB) sensing was established by applying core-shell Fe-Au MNPs and Raman reporter-tagged Au nanorods as SERS probes. Magnetic Au nanorods modified with aptamers were used for targeting SEB. After SEB separation from matrix, the SERS signals were obtained with Au nanorods serving as SERS probes. It showed that SERS signals were linearly related with the concentration of SEB in the range 2.5 fM to 3.2 nM, with a LOD for homogeneous analysis of 224 aM. This specificity and real application of this method was evaluated by SEB measurement in artificially contaminated milk, blood, and urine [112].

However, the colloid-based methods usually face limitations in reproducibility due to the small changes both in the cluster size and shape caused by the colloidal solution [113, 114]. To solve this problem, several solid phase substrates have been proposed for the labeled detection of bacteria. It has been reported that bacterial cells could be identified by the aptamer and further concentrated on a membrane with an incorporated vacuum filtration system [115–117]. A polydimethylsiloxane (PDMS) film modified with Au nanoparticle has been used as an active substrate. Aptamer-functionalized AuNP film combined with specific Raman reporters (4-mercaptobenzoic acid/Nile-blue A) was further prepared as a pathogen-specific SERS probe. After successful capturing by Apt-Au-PDMS membrane, the pathogen was then combined to the SERS probe, forming a sandwich type analysis for multiple pathogens. Using Vibrio hemolyticus and Salmonella typhimurium as model targets, respective concentrations of 18 and 27 CFU mL⁻¹ were detected with high selectivity. This platform can be successfully applied for the detection of pathogenic bacteria in marine food samples, showing the recovery rate between 82.9% and 95.1% [66]. In addition, the aptamer-functionalized AuNP-PDMS enabled the detection of Staphylococcus aureus in fish samples with high sensitivity and selectively. It exhibited a linear detection range of 4.3 × 10⁻⁸–4.3 × 10⁷ CFU mL⁻¹, with a detection limit of 13 CFU mL⁻¹ (Fig. 4b) [64].

**Detection of food-borne viruses**

Food-borne viruses that include norovirus, hepatitis A and E viruses, swine fever virus, rotavirus, and coronavirus are mainly transmitted through the fecal-oral route as well as from the intake of contaminated water and food such as fruits, uncooked vegetables, and shellfish [118–120]. These viruses have low infectious doses and survive well in the host environments. Although many colonized individuals are asymptomatic or develop gastroenteritis within a few days, the excretion of the virus may last for several days or weeks [121–123]. Therefore, it is challenging to construct an effective disease surveillance system and expand the scope of virus surveillance through real-time virus identification. The SERS technique paves the way for a simplified, robust, and low-cost detection strategy for food-borne viruses. A summary of these typical SERS-based methods for the detection of food-borne virus is shown in Table 2.

**Label-free detection of viruses**

Viruses could be quickly and selectively distinguished with a direct SERS configuration in a confined nanostructured space. For example, Au substrates coupled with SERS were demonstrated for the detection of food- and water-borne viruses including norovirus, adenovirus, parvovirus, rotavirus, coronavirus, the Sendai virus, and herpes virus [124]. An Ag nanorod array substrate-based SERS method was devised to detect rotavirus quickly and sensitively. The SERS spectra of 8 rotaviruses were qualitatively detected for identification and classification of rotaviruses based on the G and P genotypes and strains. The accuracy was >96% and the LOD was about 1 × 10⁴ ffu/mL [126]. A similar direct method was reported to obtain the Raman spectra of respiratory syncytial virus (RSV) strains A/Long, B₁, and A₂ [128]. The collected SERS spectrum for each virus exhibited high repeatability. Based on the SERS spectra, 4 virus strains were classified by combining PCA and hierarchical cluster analysis. To help understand the origin of viruses and control disease outbreaks, it is usually necessary to isolate viruses from the infected sample for characterization. For this purpose, a portable microfluidic platform with carbon nanotube arrays featuring differential filtration porosity was constructed for rapid enrichment of the viruses and subsequent SERS identification. Rhinoviruses, influenza viruses, and parainfluenza viruses were successfully enriched such that a stoichiometric proportion of the viruses was maintained and when the samples contained multiple types of viruses, thus simulating co-infection. It took only a few minutes to capture and detect the viruses with an enrichment factor of 70-fold. Highly sensitive detection could be achieved with 10² EID₅₀/mL (50% egg infective dose/μL) and the virus specificity of 90% [129]. This enrichment method combined with SERS identification constitutes an innovative system that can be used for rapid tracking and monitoring of virus outbreaks in real time. Therefore, the uniqueness of SERS provides molecular fingerprints for specific detection of viruses, laying the foundation for the direct application in biosensing.
Fig. 4  a Schemes of various SERS tags and the application in bacteria detection. Reprinted with permission from ref. 59. b Schematic representation of the solid phase substrate for SERS detection. Reprinted with permission from ref. 64
Labeled detection of viruses

SERS substrate with antibodies modification could be employed to improve the detection sensitivity for viruses by comparing the Raman responses before and after viruses capture. A plasmonic Au-Ag-coated GaN substrate with unique fuchsin-labeled immuno-Au nanoflowers was prepared for the detection of HBsAg antigen in the blood plasma at 0.01 IU/mL [130]. Magnetic Fe$_3$O$_4$ nanoparticles anchored with Au/Ag nanostructures have drawn widespread attention due to their superior versatility and stability, especially for SERS measurement of complex samples. Combining the SERS capability of Au/Ag NPs and the enrichment advantages of Fe$_3$O$_4$, these magnetic SERS substrates enabled not only great enhancement in the Raman signals but also quick separation process under magnetic field. A series of immunosensors have been constructed for sensing of various viruses based on the magnetic SERS substrates. An antibody-attached 3D plasmonic-magnetic architecture was employed for SERS detection and complete removal of rotavirus from water samples at a concentration of about 6 × 10$^5$ PFU/mL (Fig. 5a) [127]. A magnetic SERS immunosensor was constructed to detect intact but inactivated influenza virus H$_3$N$_2$ via sandwich type structure consisting of 4-mercaptobenzoic acid-labeled Au nanoparticles, target influenza viruses and Fe$_3$O$_4$/Au NPs. With a portable Raman spectrometer, H$_3$N$_2$ down to 10$^2$ TCID$_{50}$/mL could be detected and showed a linear detection range of 10$^2$ to 5 × 10$^3$ TCID$_{50}$/mL [131]. These methods which exploit magnetic nanocomposites in SERS detection of viruses are limited in the ability to act as a magnetic separation tool with the SERS substrates. To expand the biochemical application, a SERS-based lateral flow immunoassay strip was designed for the detection of influenza A H$_1$N$_1$ virus and human adenovirus (HAdV) simultaneously. Fe$_3$O$_4$@Ag NPs was prepared and employed as SERS nanotags, which were dual modified with Raman reporters and specific antibodies (Fig. 5b). It exhibits the several advanced functional features: identification of specific virus and accumulation on the magnetic NPs surface, direct SERS analysis of these virus on the lateral strip. H$_1$N$_1$ and HAdV with concentrations of 50 and 10 PFU/mL could be detected, showing three orders of magnitude higher sensitivity compared with the standard gold strip method [132].

SERS nanotags may suffer from unstable signals due to the aggregation or desorption of Raman reporters from the nanostructure surface [133, 134], that would weaken the analytical performance of the SERS method, including sensitivity, reproducibility, and overall reliability. To address these problems in norovirus detection, a dual SERS immunoassay was developed based on plasmonic/magnetic molybdenum trioxide nanocubes anchored on single-layer graphene oxide (Fig. 5c, d). Detaielly, the immune magnetic nanoparticles and graphene oxide were employed as SERS substrate/capture platform with molybdenum trioxide nanocubes using as SERS tag. They served as the signal reporter and were able to accommodate an additional Raman molecule as a co-reporter. Attributing to the applied magnetic-based separation process, norovirus was concentrated and identified in clinical samples with the favorable detection range of 10$^2$ to 10$^6$ RNA copies/mL [125].

In conclusion of the presenting label-free and labeled methods, label-free methods own the advantages of simplicity and convenience and enable the collection of SERS spectra directly, but have difficulties in real sample analysis. Generally, pretreatment of samples or separation techniques are included to perform the SERS analysis, with further chemometrics employed for better understanding the results, while the labeled methods generally employ Raman reporter

| Table 2 | Food-borne viruses detected by SERS |
|---------|-----------------------------------|
| Food-borne viruses | Detection strategy | Substrates | LOD | Ref. |
| norovirus | Label-free | AuNPs | 10$^2$ Pfu ml$^{-1}$ | [124] |
|           | Labeled | Molybdenum trioxide nanocubes aligned on a graphene oxide substrate | 5.2 fg/mL; 60 RNA copies/mL | [125] |
| rotavirus | Label-free | Au NPs | 10$^2$ Pfu ml$^{-1}$ | [124] |
|           | Label-free | Silver nanorod array | 1 × 10$^4$ Pfu ml$^{-1}$ | [126] |
|           | Label-free | 3D Au/Fe$_3$O$_4$-graphene oxide architecture | ——— | [127] |
| respiratory syncytial virus (RSV) strains A/Long, B1, and A2 | Label-free | Silver nanorod array | 10$^3$ Pfu ml$^{-1}$ | [128] |
| rhinovirus, influenza virus, parainfluenza viruses | Label-free | Portable microfluidic platform containing carbon nanotube arrays | 10$^2$ EID$_{50}$/mL | [129] |
| hepatitis B virus antigen | Labeled | Au-Ag-coated GaN and Au nanoflowers | 0.01 IU/mL | [130] |
| Avian influenza virus | Labeled | AuNPs and Fe$_3$O$_4$/Au | 10$^3$ TCID$_{50}$/mL | [131] |
| H1N1 virus human adenovirus | Labeled | Fe$_3$O$_4$/AgNPs | 50 and 10 Pfu/mL | [132] |
molecules for SERS sensing. Taking advantage of the recognition agents such as antibodies or aptamers, these methods show a great increase in the specificity, which are more adaptable for real sample analysis.

Conclusions and perspectives

In this review, the advances on the SERS detection of pathogens over the past decades have been reviewed, focusing on the improvements in sensitivity, reproducibility, specificity, and the performance of in complex analytical scenarios. The progress in research demonstrates that SERS-based methods enable rapid detection of food-borne bacteria and viruses with high sensitivity as well as high specificity. Pathogen species can be successfully differentiated either through chemometric analysis or via secondary confirmation. Although these exciting research studies have resulted in dramatic advances in the field, the application of SERS in pathogens analysis still faces a number of challenges: (1) Most studies have not focused on real samples, hence there are clear differences in SERS detection capabilities between standard culture medium and real samples. The real sample components will interfere with the SERS response and result in certain difficulties to practical applications. (2) The SERS performance of different methods is quite different, mainly reflecting differences in the design and structure of the nano-enhanced substrates. Therefore, more in-depth research is needed to further optimize the conditions to improve practical application of SERS. (3) The relatively high cost of SERS-active substrates and the complications associated with complex samples may be the biggest hurdle towards development of widely recognized and robust SERS platforms for large batch sample analysis. Thus, deepen understanding of the relationship between nanostructure substrate and the SERS enhancement is significant for construction and design biological sensors with high sensitivity and reproducibility [135]. In recent years, combination of SERS with other analytical methods offers a potential solution for these challenges [136]. An electrochemical SERS (EC-SERS) platform was proposed to improve the sensitivity and selectivity, as well as stimulate more biological relevant electric field environment [137]. It has been proposed for the detection and identification of food-borne pathogens by using carbon screen printed electrodes with nanoparticles coating on the working electrode [138]. Gram-positive *B. megaterium* and Gram-negative *E. coli K-12* were detected and differentiated.
with improved SERS peak intensities and spectral richness, showing a promise for bacterial screening. Further coupled the EC-SERS method with filter system, control studies were performed to ascertain the EC-SERS signals for bacterial sample [139]. It was reported that the SERS spectra collected for bacterial cultures were due to the released small molecules metabolites in response to environmental stressors. Other analytical methods, such as separation and microfluidic techniques have also been reported for bacteria concentration, which would further improve the sensing ability [136]. In time, we are confident these problems can be solved through fast developments in nanotechnology and integration with biology. Thus, we anticipate more research efforts will be devoted to applying SERS in clinical diagnostics and safety evaluation, where dynamic and ultra-fast detection is a crucial requirement.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Conflict of interest The authors declare that they have no competing of interests.

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