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REVIEW

Optical Biosensing of Bacteria and Bacterial Communities

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Abstract Bacterial sensing is important for understanding the numerous roles bacteria play in nature and in technology, understanding and managing bacterial populations, detecting pathogenic bacterial infections, and preventing the outbreak of illness. Current analytical challenges in bacterial sensing center on the dilemma of rapidly acquiring quantitative information about bacteria with high detection efficiency, sensitivity, and specificity, while operating within a reasonable budget and optimizing the use of ancillary tools, such as multivariate statistics. This review starts from a general description of bacterial sensing methods and challenges, and then focuses on bacterial characterization using optical methods including Raman spectroscopy and imaging, infrared spectroscopy, fluorescence spectroscopy and imaging, and plasmonics, including both extended and localized surface plasmon resonance spectroscopy. The advantages and drawbacks of each method in relation to the others are discussed, as are their applications. A particularly promising direction in bacterial sensing lies in combining multiple approaches to achieve multiplex analysis, and examples where this has been achieved are highlighted.

Keywords Bacterial sensing · Biosensors · Microscopy · Raman · Fluorescence · Plasmonics

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Rationale for Bacteria Sensing

Bacteria are the progenitor organisms from which all complex life ensues. They have a huge impact on natural systems—agriculture, the environment and ecosystems—as well as health and wellness of humans and livestock. Most bacteria are benign, or even essential, in converting basic components to required substances for life. For example, *Azotobacter* and *Rhizobium* convert inert atmospheric N_2 gas to ammonia, and subsequently to other organic nitro-compounds, which function broadly as nutrients for plants [1]. Similarly, *Cyanobacteria* direct nitrogen fixation for marine life [2]. As part of the human microbiome, bacteria in our gut not only extract nutrients for us by breaking down our food, but also are critical in maintaining the robustness of our immune system [3]. Thus, the detection of these bacteria plays a central role in maintaining their populations, understanding their roles, and ensuring the health of ecosystems.

Not all bacterial life is beneficial, however. Many bacteria are, or can become, pathogenic to humans as well as animals, and can cause a variety of diseases or even death. At a coarse level of classification, pathogenic bacteria can be foodborne, airborne, and bloodborne. Currently, multidrug resistant (MDR) pathogens are garnering the most interest, because the wide spread of antimicrobial resistance exponentially increases the difficulty of developing effective treatments. According to the Center for Disease Control (CDC), in the United States alone more than 2 million people are infected with antibiotic resistant bacteria each year, leading to ~23,000 direct deaths [4]. MDR bacteria are not only a domestic issue. The World Health Organization reports worldwide MDR in common bacterial infections and the failure of treatments of last resort in several countries [5]. Because the indiscriminate use of

broad-spectrum antibiotics plays a crucial role in the development of MDR pathogens, new diagnostic tools, capable of selective and sensitive identification of bacteria, are critical for the development of targeted antimicrobials.

While bacterial sensing techniques are needed across a wide spectrum of technologies, the principal targets are: (1) the food industry to ensure food quality and prevent the outbreak of food-borne illnesses [6–10]; (2) clinical settings to identify the source of, and thus prevent, nosocomial infections; and (3) national security applications to detect biological weapons and prevent bioterrorism. Across the spectrum of food, clinical and bioterrorism applications new bacterial sensing platforms are needed which allow us to accurately and rapidly detect pathogenic bacteria at pre-infectious levels.

Analytical Challenges

The current challenge in the field of bacterial sensing centers on the dilemma of rapidly acquiring quantitative information about bacteria with high detection efficiency, sensitivity, and specificity, while operating within a reasonable budget and making optimal use of ancillary tools, such as multivariate statistics. From an analytical standpoint, obtaining accurate and sensitive readouts is a function of both signal amplification and background reduction, which in turn depends on the approach used to identify and quantitate bacteria. An ideal biosensor would be fast, accurate, sensitive, selective, cost effective, portable, robust, need minimum sample preparation and post-measurement data processing, as well as have a large dynamic range. Additionally, it would be accessible to operation by non-specialized personnel and suitable for point-of-care and field operation.

Currently, microbiological culturing methods are the gold standard in bacterial detection due to their extraordinary sensitivity and specificity. These methods have the capacity to detect a single bacterial cell in a sample; however, they are not rapid—often taking up to several days to identify bacteria—which may delay the start of targeted therapy. To solve this issue, a large variety of biosensors have been developed in the past twenty years. The interest in the field of biosensor and bacterial detection has blossomed and is still growing (Fig. 1). Broadly stated, the bacterial biosensor development seeks to simultaneously optimize all of the figures-of-merit mentioned above. However, this is quite challenging. Some biosensors are fast, requiring limited sample processing, but they suffer from relatively low sensitivity. Some biosensors have high sensitivity, but they are slow due to the requirement for cumbersome sample preparation protocols. Some biosensors have both fast readout and high sensitivity, but require expensive instrumentation and require technically skilled operators. The goal of achieving mutual optimization of all figures-of-merit in a single measurement platform has yet to be achieved.

Aside from bacterial identification, it is also challenging to obtain quantitative information. Sometimes a simple yes or no answer, obtainable from a threshold test, is just not enough, and information about the quantity of bacteria is critical. For example, quantitative information would have a great impact in diagnosing the onset of sepsis based upon clinically relevant cutoff points [11]. Unfortunately, in contrast to molecular sensors, bacterial biosensor signals do not always correlate with bacterial populations.

Additionally, it is challenging to achieve selectivity and specificity in bacterial sensing. There is a tremendous value in accurately revealing identities of bacteria down to strain level within a complex matrix. Rapidly identifying methicillin-resistant *Staphylococcus aureus* (MRSA) from methicillin-sensitive *S. aureus* (MSSA) in a clinically relevant timeline (less than 3 h) would save precious treatment time and could potentially save lives. Unfortunately, biosensors with the required detection speed, selectivity and specificity are not yet widely available.

Another current issue is the proper integration of statistical analysis methods to enhance the selectivity and specificity of sensing modalities. Sophisticated multivariate statistical methods such as principal component analysis (PCA), hierarchical cluster analysis (HCA), discriminant function analysis (DFA), partial least-squares (PLS), kernel PLS (KPLS), and canonical correlation analysis (CCA) can be used to determine small differences among samples, which would be extraordinarily difficult to identify from the sample spectra or biosensor response alone [12–15]. Multivariate statistical analysis methods can be key to averting false positive and false negative results. Consequently, sensing techniques which can optimize figures-of-merit and make optimal use of statistical methods are highly desired.

General Bacterial Sensing Methods

Bacterial sensing methods can be classified according to type of analytes, recognition elements, and detection methods. Depending on the situation, the target of the analysis could be bacterial metabolites or small molecules [16–18], bacterial lysates, e.g., proteins [19, 20] and nucleic acids [21, 22], bacterial spores [23, 24], whole bacteria [13, 25–32], and even biofilms [33, 34]. Selectivity is most frequently achieved through use of recognition elements, such as antibodies, enzymes and affinity reagents. Due to their inherent simplicity, affinity reagents, including antibodies [27, 31, 35–37], aptamers [30, 38–42],

Fig. 1 Comparison of publications in PubMed by searching "Biosensor" + "Bacteria" versus "Optical Detection" + "Bacteria"
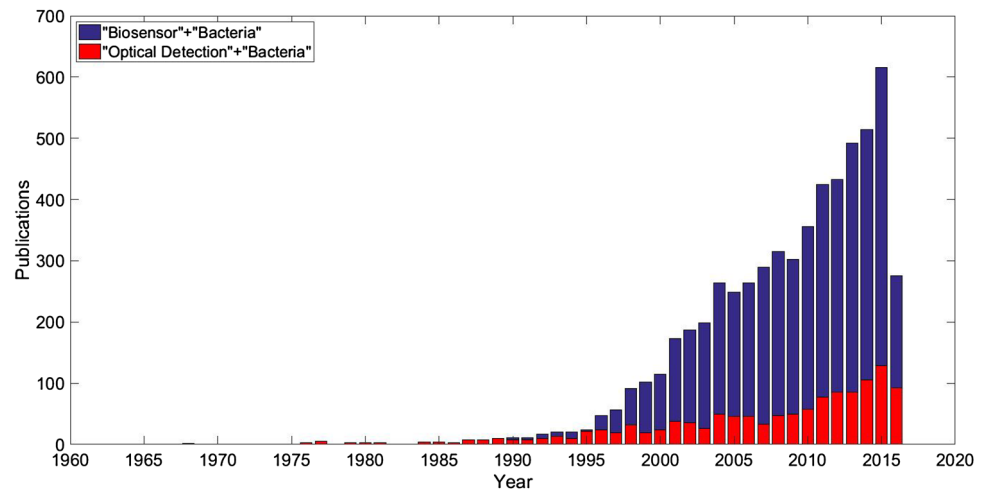
siderophores [29, 43], bacteriophages [32, 44, 45], antimicrobials [46, 47], and lectins [48], are commonly used. Among recognition agents, antibody–antigen recognition is most frequently used because of its high affinity (attractive K_d values) and specificity. However, commercial antibody production suffers from batch to batch variation, temperature and pH instabilities, buffer incompatibility, cost, concerns over killing animals to harvest the antibodies, and potential loss of affinity to specific bacteria due to mutations of bacterial receptors.

To circumvent the technical challenges of antibody–antigen recognition, "immutable ligands" such as aptamers and siderophores have been developed. Aptamers, single strand oligonucleotides, can be specifically selected against bacteria with high affinity through systematic evolution of ligands by exponential enrichment (SELEX) [49]. Once recognition-competent aptamer sequences are determined, aptamers can be easily synthesized in large quantity at relatively low cost and are stable under a wide range of conditions. However, it is tedious to select bacterial specific aptamer via SELEX, and it is still possible for the aptamer to lose its affinity to the target bacteria over time. In this context, siderophores, a group of small molecules released by bacteria to acquire nutrients, e.g., Fe(III), from the environment, are of particular interest, because they exhibit enormous binding affinities for their targets. Since siderophores are part of the bacterial nutrient uptake system, inactivating mutations, which would cause loss of affinity, are less likely [29]. Taking advantage of their high affinity, immutability, and stability, siderophores are promising new affinity agents in bacterial sensing. Finally, another alternative is offered through bacteriophages, which are the primary regulators of bacteria growth and prevalence. Aside from being cost effective, bacteriophages are highly selective and specific for live cells [50].

Detection of bacteria may be transduced by a variety of mechanisms, including electrochemical, mechanical, and optical. Within each category, preferences are based on the specific bacterial identification/measurement task. Due to complexity of the topic, it is not possible to cover all electrochemical and mechanical biosensors, but the interested reader is referred to excellent reviews [51, 52]. This review will focus on bacterial detection using optical methods—an area that has seen an explosion of recent activity.

Vibrational Spectroscopy

A great deal of effort has been invested in bacterial detection with vibrational spectroscopies, such as conventional Raman spectroscopy, which has the advantages of being label-free, non-invasive and which presents vibrational "fingerprints" of analytes that are uniquely determined by chemical composition and structure. The major drawback of conventional Raman spectroscopy is that the Raman scattering is inherently weak [53], for example as a rough guide, ca. 1 in 10^8 incident photons is inelastically scattered [54]. Also, the sample matrix can interfere with the acquisition of Raman signal by producing an overwhelming fluorescence background. To address these issues, a variety of Raman techniques have been developed in the past twenty years. These derivative approaches include surface enhanced Raman spectroscopy (SERS), tip-enhanced Raman spectroscopy (TERS), coherent anti-Stokes Raman spectroscopy (CARS), resonance Raman (RR) spectroscopy, and Raman imaging, all of which are designed to enhance the signal relative to the background, thereby improving the signal-to-noise ratio in general, thus facilitating bacterial detection.

Recent instrumental advances in fast data acquisition and processing have greatly extended the capabilities of Raman scattering, enabling the collection of thousands of spectra within moderate acquisition times (~ 1 h). However, the information content of Raman spectra and images alone is often insufficient to differentiate among bacterial populations. To fully exploit the spectral information, to the point that minor differences from acquired Raman spectra or images can be used to assign bacterial species with confidence, sophisticated statistical analyses are usually required. With the help of these chemometric tools, Raman spectroscopy and its derivatives have the capacity to be both selective and specific, as evidenced by the fact that bacteria have been differentiated at the strain level from pure culture [14, 55], tap water samples [29], and clinical samples [13, 25, 54] using Raman techniques.

Surface-Enhanced Raman Spectroscopy (SERS)

SERS enhancement was first reported by Van Duyne and coworkers in 1977, in the form of a $\sim 10^6$ increase in the Raman signal compared to the expected value [56]. Since then, various methods to enhance Raman scattering have been developed. Raman signal enhancement is facilitated by SERS substrates, such as aggregated Ag or Au nanoparticles, or SERS tags [57] to reach a maximum SERS enhancement of 10^{10}–10^{11} [58, 59]. In the SERS experiment, biomolecules with low abundance can be detected at relatively low laser power. In contemporary practice, the principal challenge is reproducibility arising from variations in SERS substrate properties and uncontrolled aggregation of metallic nanoparticles.
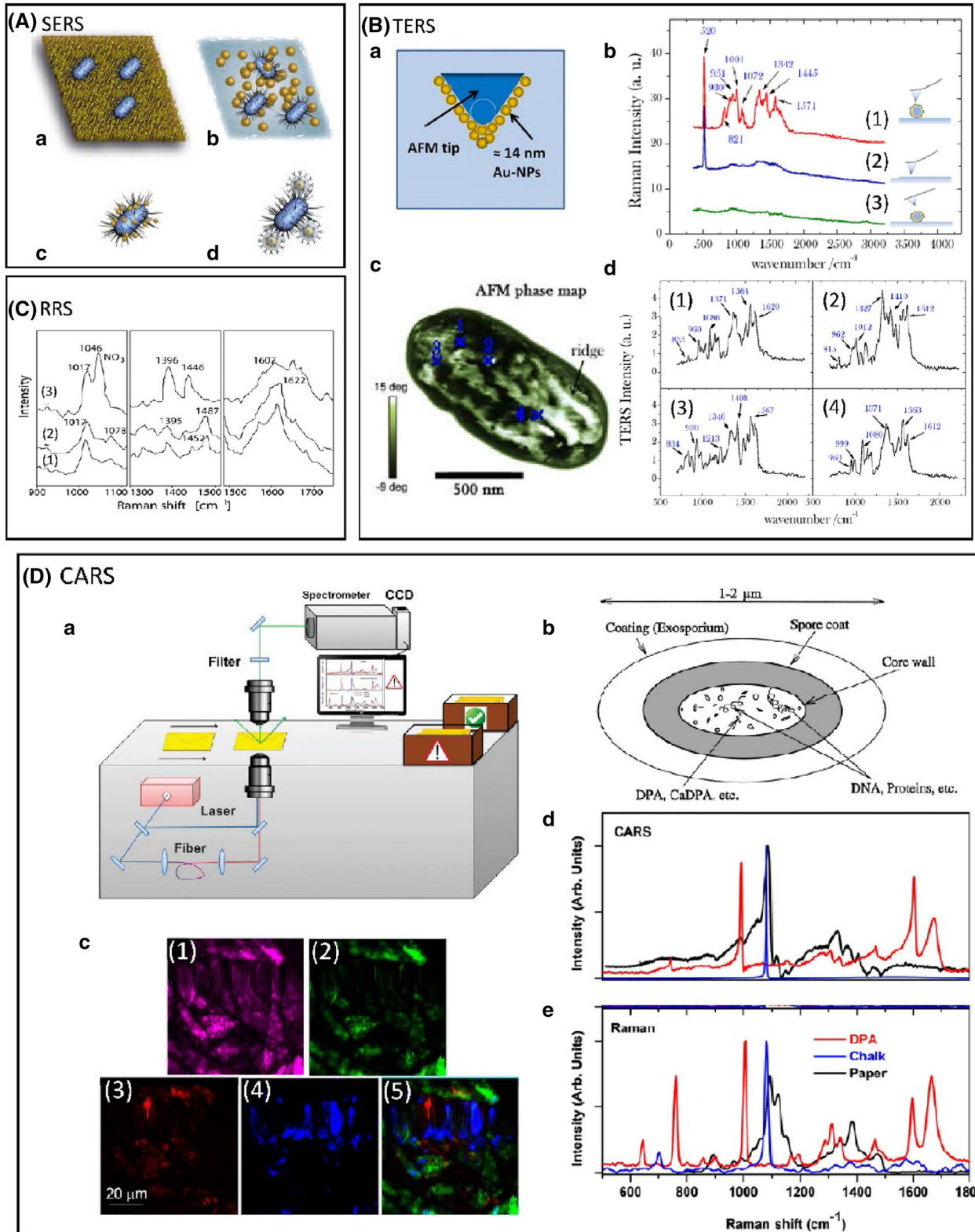
SERS has been widely applied to detect a large number of different types of bacterial samples, both in solution [60, 61] and on surfaces [17, 25, 57], viz. Fig. 2A. The most striking characteristic of SERS in the context of bacterial biosensing is its versatility. For example, *Bacillus* spores have been detected using dipicolinic acid as the biomarker on a SERS substrate [17] as well as in solution [62], with both studies generating a limit of detection (LOD) of $\sim 10^3$ spores. Furthermore, SERS detection schemes can be quite simple. For example, just mixing bacterial samples with Au or Ag nanoparticles prior to detection can lead to LODs of 10^3 CFU mL^{-1} [61], although the signal has to be carefully calibrated with respect to nanoparticle concentration. The specificity and selectivity of SERS can be improved by conjugating metal nanoparticles with affinity agents. For example, with two sets of antibodies, SERS has been utilized to detect bacteria with LODs as low as 5 or 8 CFU mL^{-1} [63, 64]. Moreover, SERS assays have been shown to be robust and capable of analyzing environmental water samples [63, 64].

Fig. 2 Raman spectroscopy for bacterial sensing. **A** SERS enhancement methods: *a* bacteria on a metallic substrate; *b* bacteria with metallic nanoparticles on a substrate; *c* bacteria with metallic nanoparticles in solution; *d* bacteria with metallic nanoparticles labeled antibodies in solution. Figure from Ref. [57]. **B** TERS bacterial detection: *a* Illustration of a TERS tip consisting of a 14 nm gold nanoparticle coated commercial AFM tip; *b* TERS spectrum of (*1*) *B. subtilis* spore with the AFM tip in contact, (*2*) a clean glass coverslip, and (*3*) spore with the retracted AFM tip; *c* AFM phase map of a *B. subtilis* spore; *d* TERS spectra in four selected positions on the spore in AFM phase map. Figure modified from Ref. [66]. **C** RR spectra of (*1*) *B. megaterium* spores, (*2*) *B. cereus* spores, and (*3*) calcium dipicolinate. Figure from Ref. [71]. **D** CARS detection of anthrax in the mail: *a* illustration of CARS microscopic imaging scheme; *b* schematic drawing of a bacterial endospore; *c* CARS image of (*1*) control, (*2*) paper, (*3*) DPA powder, and (*4*) calcium carbonate powder in a mailing envelop. (*5*) An overlaid image of paper (*green*), DPA (*red*), and calcium carbonate (*blue*); *d, e* are CARS and Raman spectra respectively of DPA, chalk (calcium carbonate), and paper. Figure modified from Ref. [23, 71]. All adapted figures reproduced with permission

Additionally, SERS has great possibilities with regard to achieving multiplex detection. For example, SERS has been coupled with electrokinetic transport for rapid human blood pathogen identification with an LOD of 10^3 CFU mL^{-1} [25]. With a nanostructured SERS substrate, e.g., quasi-3D gold, Gram-positive bacteria can be selectively identified in the presence of three different species of Gram-negative bacteria. In the same study, these three different species of Gram-negative bacteria were differentiated from each other with the help of principle component analysis (PCA) [15]. More recently seven different strains of *Vibrio parahaemolyticus* were differentiated with a LOD of 10^5 CFU mL^{-1} [14]. Moreover, SERS has been coupled with microfluidics and confocal microscopy to rapidly differentiate between methicillin-resistant *S. aureus* and methicillin-sensitive *S. aureus* from clinical isolates [13]. Despite the challenges of associating relatively large bacterial cells with nanoscopic SERS-enhancing hot spots, SERS is already well entrenched as an established bacterial detection and identification approach.

Tip-Enhanced Raman Spectroscopy (TERS)

TERS was proposed in 1980s to optimize the use of hot spots and allow acquisition of Raman spectra from smooth or single crystal surfaces [58]. In 2000 Zenobi and coworkers reported the first TERS experiment [65]. TERS utilizes a scanning probe with a tip (<50 nm in diameter) that plays a vital role in mediating the TERS enhancement by creating hot spots to enhance the near-field plasmon which further enhances Raman scattering of molecules in the junction, viz. Fig. 2B [65, 66]. TERS exhibits an approximate 10^6 enhancement factor, which, given the small areas addressed, enables single molecule detection

(A) SERS

a   b   c   d

(B) TERS

a

AFM tip   ≈ 14 nm Au-NPs

b

(1) (2) (3)

520 951 1001 1072 1342 1445 1371 821 939

wavenumber /cm⁻¹

c

AFM phase map

15 deg

-9 deg

500 nm

ridge

d

(1) (2) (3) (4)

wavenumber /cm⁻¹

(C) RRS

(3) (2) (1)

1046 NO₃ 1017 1396 1446 1012 1078 1395 1452 1487 1607 1622

Raman shift [cm⁻¹]

(D) CARS

a

Spectrometer   CCD   Filter   Laser   Fiber

b

1-2 µm   Coating (Exosporium)   Spore coat   Core wall   DPA, CaDPA, etc.   DNA, Proteins, etc.

c

(1) (2) (3) (4) (5)   20 µm

d

CARS

e

Raman   DPA   Chalk   Paper

Raman shift (cm⁻¹)

[58]. In addition, TERS collects chemical information and also provides topographic information at nanometer spatial resolution [66].

However, given that it has been developed relatively recently, there are only limited applications of TERS to bacterial sensing. The first example of bacterial biosensing with TERS was reported by Neugebauer et al. [67], who simultaneously obtained topographic AFM images and TERS spectra of a single *Staphylococcus epidermis* bacterium with an estimated enhancement factor of 10^4–10^5. One drawback of TERS is that it produces spectra in which the intensity and position of some Raman bands vary depending on location on the bacterial surface, a feature that is exacerbated by the heterogeneity of the bacterial cell surface at the nanoscale [68]. Nevertheless, with its superior spatial resolutions and excellent mass sensitivity, TERS has great potential to contribute to understanding bacterial surface composition, distribution of surface components, mechanism of bacterial cell adhesion to surfaces, and biofilm formation, thus adding great values to the suite of Raman-based approaches to bacterial sensing.

Coherent Anti-stokes Raman Spectroscopy (CARS)

CARS is an intriguing addition to the collection of vibrational spectroscopies for bacterial assays, because it is relatively insensitive to fluorescence interference due to the scattered photons appearing at a higher frequency than the incident photons. CARS has been applied to identify *Bacillus subtilis* endospores in a single laser shot with an approximately LOD of 10^4 spores [24]. Despite its fast detection capabilities, the LOD of CARS is limited. To achieve single bacterium detection, CARS can be combined with microscopy. CARS imaging has the potential to collect Raman images of biological samples in real time, and has been applied to visualize carotenoid chemical fingerprints from cyanobacteria [69]. This label-free technique permits non-invasive mapping of carotenoid distributions at the subcellular level with high spatial resolution.

Hyperspectral imaging can further extend these powerful vibrational spectroscopies. For example, a hyperspectral Raman imaging instrument can record a stack of images in which each pixel in the image contains a full Raman (or SERS, TERS, CARS) spectrum. Specifically, hyperspectral CARS imaging has been utilized to identify anthrax spores in postal materials, which has obvious impact in combating threats from bioterrorism, viz. Fig. 2D [23]. Recently, a fiber laser-based hyperspectral CARS imaging approach was used to detect single live *Escherichia coli* cells in situ within 2 min [12]; the approach combining phase retrieval and multivariate curve resolution to detect and identify single *E. coli* cells in milk and urine samples with minimal sample preparation. CARS microscopy has great potential for clinical detection due to its inherent advantages as well as the portability and cost effectiveness of the instrument.

Resonance Raman (RR) Spectroscopy

Resonance Raman spectroscopy utilizes excitation wavelengths in resonance with electronic states, typically in the ultraviolet region, to excite Raman scattering. RR features nearly complete elimination of fluorescence background at excitation wavelengths below 260 nm [70], as well as selective excitation of scattering from aromatic amino acids, i.e., tyrosine, tryptophan, phenylalanine, and nucleic acids in bacterial cell metabolites and whole cell bacteria, viz. Fig. 2C [71]. Compared to conventional Raman spectroscopy, the enhancement factor of RR spectroscopy can be as high as 10^6 [72]. However, biological samples may suffer photo-degradation as a result of exposure to high energy UV photons. Furthermore, RR spectroscopy cannot identify or differentiate bacterial species and strains on its own. Typically, multivariate statistical methods have to be applied to tease out bacterial identification information. For example, RR spectroscopy has been used to analyze 20 clinical bacterial isolates (whole cell) from urinary tract infection patients and differentiate four bacterial species with the help of chemometric cluster analysis [73]. In combination with various chemometric methods, RR spectroscopy has also been used to classify different strains of lactic acid bacteria from yogurt [70].

Raman Microspectroscopy and Raman Imaging

Another challenge in using Raman signals to identify bacteria originates from the heterogeneity of bacterial samples on a microscopic, i.e., μm, lengthscale [53]. To solve this problem, confocal Raman microscopy (CRM), a hyperspectral imaging approach combining Raman spectroscopy and microscopy, can be applied to complex bacterial samples, such as bacterial communities, producing information about bacterial morphology, quantity, spatial distribution, and the detailed mechanisms of chemical communication bacteria use to organize multicellular communities.

Instead of recording a single image or discrete images, CRM acquires hyperspectral Raman scattering in either point or line mapping mode. In the mapping mode, Raman microspectra are collected utilizing a tightly focused excitation laser (<1 μm diam.), providing spatial resolution [74] that is commensurate to the size of typical bacteria (1–2 μm, typ.), thus allowing individual planktonic bacteria to be distinguished and even probed individually [34]. These characteristics have been exploited to detect bacterial meningitis and discriminate various bacterial species

with the assistance of hierarchical cluster analysis [28]. In addition to studies that seek to identify a single bacterial species (or strain), CRM has been used to study the interactions between bacteria and other species. For example, the bacterium *Pantoea* sp. YR343, an important component of the rhizosphere, was imaged by CRM and correlated with electron microscope images in co-culture with *Arabidopsis thaliana* [75].

Raman microscopy is also well suited to being combined with other techniques, such as mass spectrometry (e.g., secondary ion mass spectrometry (SIMS)), to acquire multiplex information that is simultaneously multi-modal and hyperspectral, i.e., each pixel represents two different complete spectra—e.g., a Raman spectrum and a SIMS mass spectrum. This leads to an incredibly rich array of possible spectral correlations that can be exploited in probing cooperative microbial behavior and biofilm formation. For example, intercellular communication is based on quorum sensing (QS), in which individual cells differentially produce an array of molecular messengers which, upon achieving a threshold concentration produces either up- or down-regulation of target genes [76, 77]. To illustrate the approach, CRM was combined with SIMS imaging to study how the spatial and temporal organization of quinolones and quinolines in *Pseudomonas aeruginosa* microbial communities correlate with the growth in free-floating planktonic cells, colonies on agar plates, and static biofilms, viz. Fig. 3 [33].

CRM can also be productively combined with other Raman strategies, such as SERS, to build in situ spatiotemporal maps of chemical messengers. For example, CRM-SERS was combined with PCA to obtain detailed spatial distributions of the virulence factor pyocyanin in communities of *P. aeruginosa*. CRM-SERS was able to circumvent interference from sample autofluorescence and to counter the inherent problems with reproducibility that attend some SERS experiments. The production of pyocyanin was found to depend both on the growth carbon source and on the specific strain of *P. aeruginosa* studied [78].

Infrared (IR) Spectroscopy

As a complement to Raman spectroscopy, IR spectroscopy also has the capacity to identify bacteria based on patterns of molecular vibrations. Although Fourier transform infrared spectroscopy (FT-IR) has several advantages, including rapid spectral acquisition, sensitivity that can be improved by averaging spectra over multiple scans, and non-invasive detection, it has the major drawback that water gives rise to strong background absorption. Thus, biological samples, including those containing bacteria need to be dried prior to IR measurements. Nevertheless, IR spectroscopy has been productively used in surface and material characterization, bacterial spore detection, and dried biological sample analysis. For example, with the
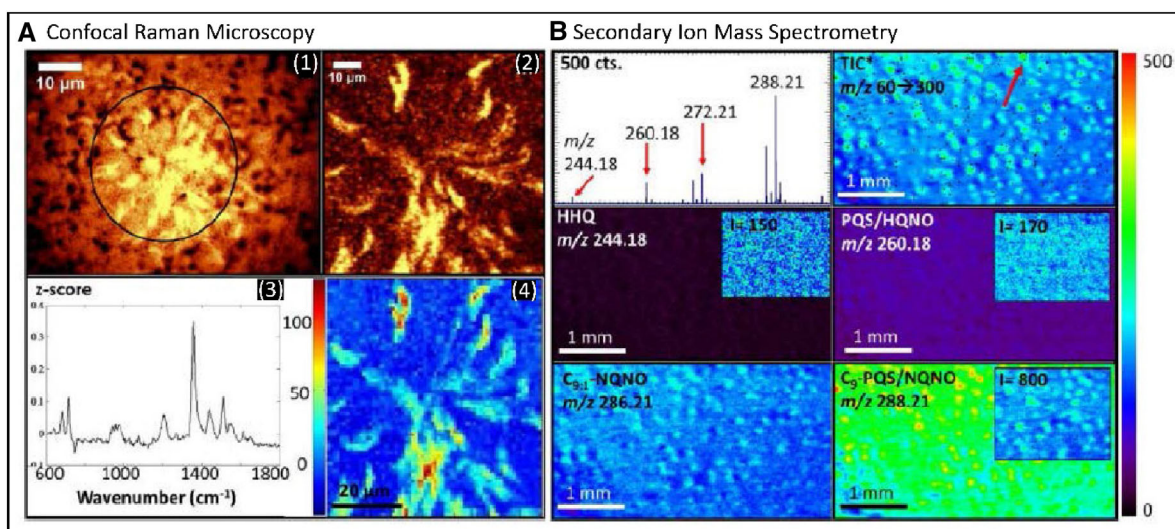

Fig. 3 A Characterization of *P. aeruginosa* metabolites and signaling molecules using confocal Raman microscopy from a 7-h *P. aeruginosa* biofilm: (*1*) bright field microscope image of a quinoline rich region in biofilm, (*2*) Raman image acquired from the circled region in (*1*) and integrated over 1338–1376 cm^{-1}, (*3*) principle component 3 from the Raman image, and (*4*) its heat map showing the spatial distribution and magnitude. **B** As a complementary characterization method, secondary ion mass spectrum and images acquired from the high analyte abundance region and *color maps* showing the spatial distribution and magnitude of analyte. Figures modified from Ref. [33] and used with permission
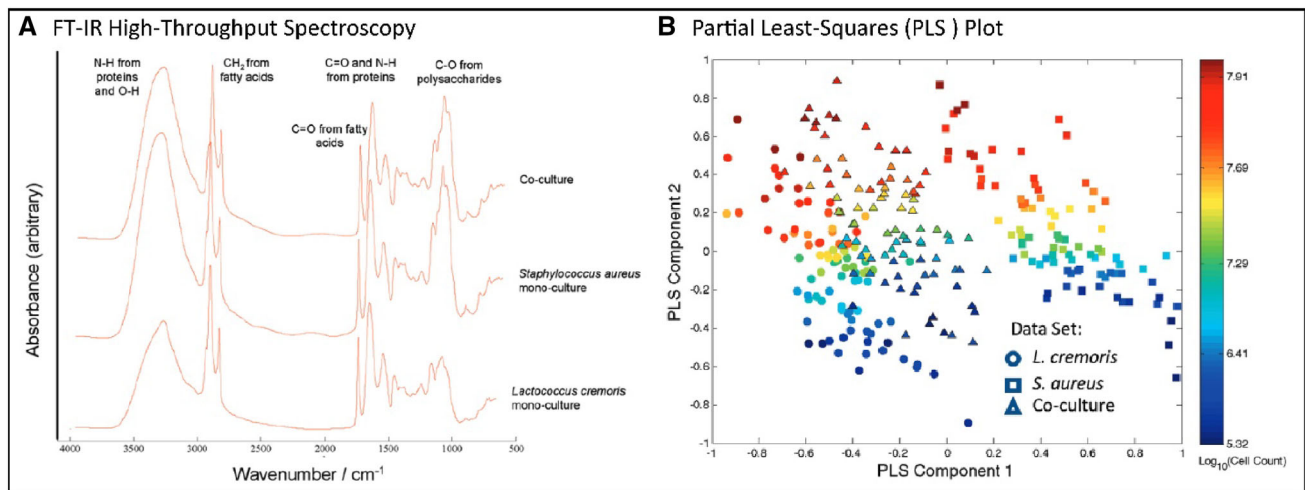
Fig. 4 A Characterization of *S. aureus*, *L. cremoris*, and their co-culture in ultraheated milk samples at 6 h using FT-IR high-throughput spectroscopy. **B** PLS projection analysis illustrating the similarities of metabolic fingerprints in mono-cultures as well as co-culture. *Symbols* and *colors* represent different cultures, and log(cell counts), respectively. Figures modified from Ref. [79] and used with permission

assistance of multivariate statistical analysis FT-IR spectroscopy was used to enumerate *S. aureus* and *Lactococcus cremoris* in milk within a few minutes, viz. Fig. 4 [79]. FT-IR was also used to identify six of the most clinically relevant Gram-negative non-fermenting bacilli isolates from cystic fibrosis (CF) patients' sputum samples as well as to discriminate four of the common *Burkholderia cepacia* complex bacteria among CF patients [80].

Fluorescence Spectroscopy

Despite the ubiquitous nature of biological fluorescence (autofluorescence), a number of approaches have been developed for bacterial assays based on characteristic luminescence properties of bacteria. Endogenous fluorescence typically arises from: (1) biological structures, e.g., the extracellular matrix, (2) small biological molecules or metabolites including nicotinamide adenine dinucleotide phosphate (NADPH) [81], flavins [82], and quinolones [83], or (3) proteins containing aromatic amino acids [84], and natively fluorescent proteins, e.g., green fluorescence protein (GFP) [85, 86] and the related family of inherently fluorescent proteins, e.g. YFP [85, 87], mCherry [88–90], etc. GFP not only has high quantum yield ranging from 0.72 to 0.85 [86], but the GFP gene can be inserted into various biological samples via transgenic techniques to produce GFP either alone or as chimeric fusion proteins. With the exception of GFP, the expression of which can be controlled at the genetic level, endogenous fluorescence is typically only useful for non-specific detection. For aforementioned reasons, GFP is the only endogenous

fluorescence which has been widely used for targeted bioimaging.

Exogenous fluorescence involves chemically tagging appropriate sites with synthetic fluorophores. These fluorophores can be organic dyes, quantum dots, or upconversion nanoparticles. Synthetic fluorophores have been engineered to have tunable excitation and emission, high quantum efficiency, good photostability, and excellent specificity when conjugated to a recognition element. With recent advances in the engineering of fluorophores, a wide range of biomolecular phenomena can be studied with all of the inherent advantages of fluorescence, especially the sensitivity and background discrimination that make it possible to visualize spatial structure [91] and to detect single molecules and/or bacteria [26, 47, 92–97].

Counterbalancing these powerful advantages, there are some caveats to the effective use of fluorescence for bacterial biosensing. Users have to be cautious to avoid photobleaching by choosing proper excitation power or in some cases by the use of additives that suppress photo-oxidation. Quantum dots are robust and can be used to counter this issue [32, 98, 99]. Moreover, to achieve specific detection, fluorophores are usually modified with a recognition agent, e.g. antibodies [100–102] and aptamers [40]. However, conjugation with fluorophores can in some cases interfere with protein function, causing the antibody to lose affinity. Nevertheless with proper attention to experimental design, fluorescence is a powerful and widely utilized method of bacterial sensing.

Fluorescence spectroscopy has been applied to selectively detect *Listeria monocytogenes* in pure culture as well as artificially contaminated food with a LOD of

10^3 CFU mL^{-1} using an antibody as capture molecule and a fluorophore-conjugated aptamer as reporter molecule [100]. Fluorescence obviously also offers the possibility of spectral multiplexing. Employing two different antibodies, three pathogens were simultaneously detected from artificially contaminated food with a LOD of 10^3 CFU mL^{-1} [101]. More recently, a droplet based fluorescence immunoassay was developed to selectively detect *E. coli* and *Salmonella typhimurium* in a mixture with a LOD of 10^2 CFU mL^{-1} for both bacteria [102]. Furthermore, the detection was accomplished in ∼12 min, and could be carried out using smartphones with proper filters. In some cases, sample preparation can be time-consuming. To shorten sample preparation and minimize the use of statistical analysis, Mouffouk et al. [26] used pH-sensitive nanopolymeric micelles containing fluorescent dyes to achieve a LOD of 15 *E. coli* mL^{-1}, viz. Fig. 5A. These examples illustrate the principal advantage of fluorescence approaches, namely the excellent sensitivity that can be achieved with proper care in experimental design.

Fluorescence in vivo imaging, another powerful diagnostic tool, has great potential to detect bacterial infections, given that it can be highly species/strain-specific and non-invasive while providing real-time imaging at high resolution and at relatively low cost [92]. However, potential cytotoxicity of the imaged fluorophores remains a concern that needs further testing. Typical LOD values for fluorescence in vivo imaging are consistent with the clinical infection levels (∼10^7 CFU mL^{-1}). Various groups have reported using (1) fluorescently labeled vancomycin to selectively detect Gram-positive bacterial infections in a mouse myositis model, viz. Fig. 5B [92], (2) fluorescently labeled maltodextrin to detect metabolically active bacteria [93], (3) fluorescently labeled prothrombin to detect *S. aureus* induced endocarditis in mice [94], and (4) a fluorescently labeled β-lactam moiety with a fluorescence quencher to detect *Mycobacterium tuberculosis* in live mice [103]. Thus, the application of fluorescence in vivo imaging is a promising tool that is likely to grow in applications going forward.

Plasmonics

Nanometer-sized metallic objects exhibit extraordinary optical properties not observed in bulk materials. When an incident photon strikes a metal nanoparticle, the resulting collective oscillation of electrons, or plasmons, produces an extinction peak at a characteristic wavelength. The extraordinary optical properties of metal nanoparticles can be explained using Mie theory to express the extinction $E(\lambda)$ in terms of the optical response functions of the media involved [104, 105],

$$E(\lambda) = \frac{24\pi N_A a^3 \varepsilon_m^{3/2}}{\lambda \times \text{In}(10)} \times \left[\frac{\varepsilon_i}{(\varepsilon_r + \chi\varepsilon_m)^2 + \varepsilon_i^2} \right],$$

where λ is the wavelength of the absorbing radiation, $E(\lambda)$ is the extinction, N_A is the areal density of metallic nanoparticles, a is the radius of a spherical metal nanoparticle, χ is the polarization factor corresponding to the aspect ratio of metal nanoparticle ($\chi = 2$ for a sphere), ε_m is the dielectric constant of the surrounding medium, and ε_i and ε_r are imaginary and real parts of the dielectric response function of the metal nanoparticle, respectively. According to Mie theory, plasmons arising in metal nanoparticles can be systematically tuned by changing the shape and material of the nanoparticles, excitation wavelength, and—most importantly for plasmon-based sensing—the dielectric constant of the surroundings.

Propagating surface plasmon resonances (SPR) are most frequently studied in planar thin metallic films. As predicted by Mie theory, the spectral position of the surface plasmon resonance can be tuned by changing the material and thickness of the metallic thin film as well as the refractive index of its local environment. In the context of SPR, the local environment can be understood as the volume over which the plasmon field decays in the dielectric medium [106]. An especially useful variant of SPR, localized surface plasmon resonance (LSPR) spectroscopy, utilizes surface confined plasmons from nanoparticles. The position of LSPR peaks are sensitive to material, size—both in-plane width and out-of-plane height—shape, e.g., sphere, triangle, rod, and interparticle spacing, as well as the refractive index of the surroundings [105].

Both SPR and LSPR spectroscopy are refractive index based sensing techniques, viz. Fig. 6A, B[6, 105, 107]. The refractive index of local surroundings can be altered by changing the solvent and by molecular adsorption. Because the magnitude of the wavelength shift upon molecular (or bacterial) adsorption can be correlated to analyte concentration and to the rate of association and dissociation of target molecules, plasmon sensing can be used to extract both analytical (concentration) as well as kinetic information. Both SPR and LSPR spectroscopy are rapid and label-free sensing techniques, but neither of them is inherently selective. The selectivity of both sensing platforms comes from surface confined biorecognition elements. Moreover, the sensitivity of both techniques is affected by the size, concentration, and surface confined binding constant ($K_{a,surf}$) of analytes, as well as the distance of the analyte to the sensor surface [104]. Typically, larger molecules, higher analyte concentrations, larger $K_{a,surf}$ and shorter capture agents—which bind the analyte closer to the surface—give rise to larger plasmon shifts [104, 107].
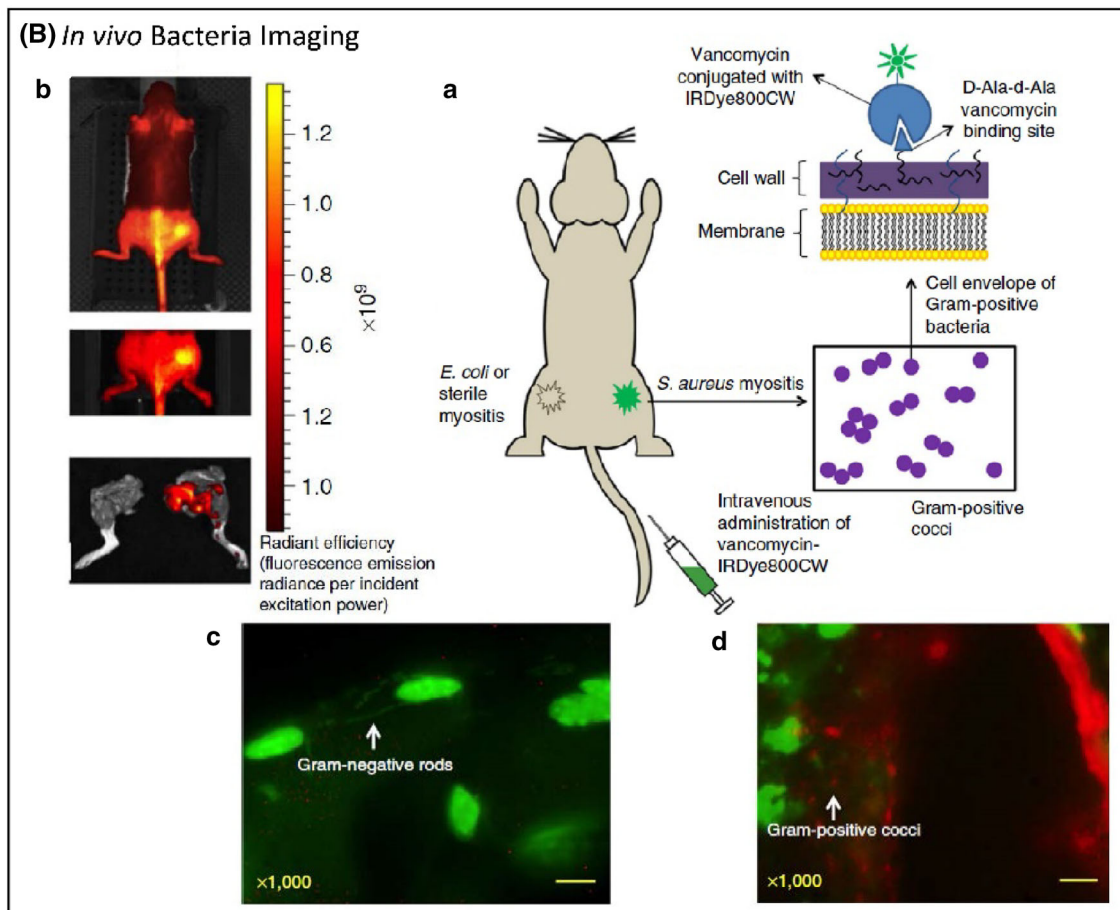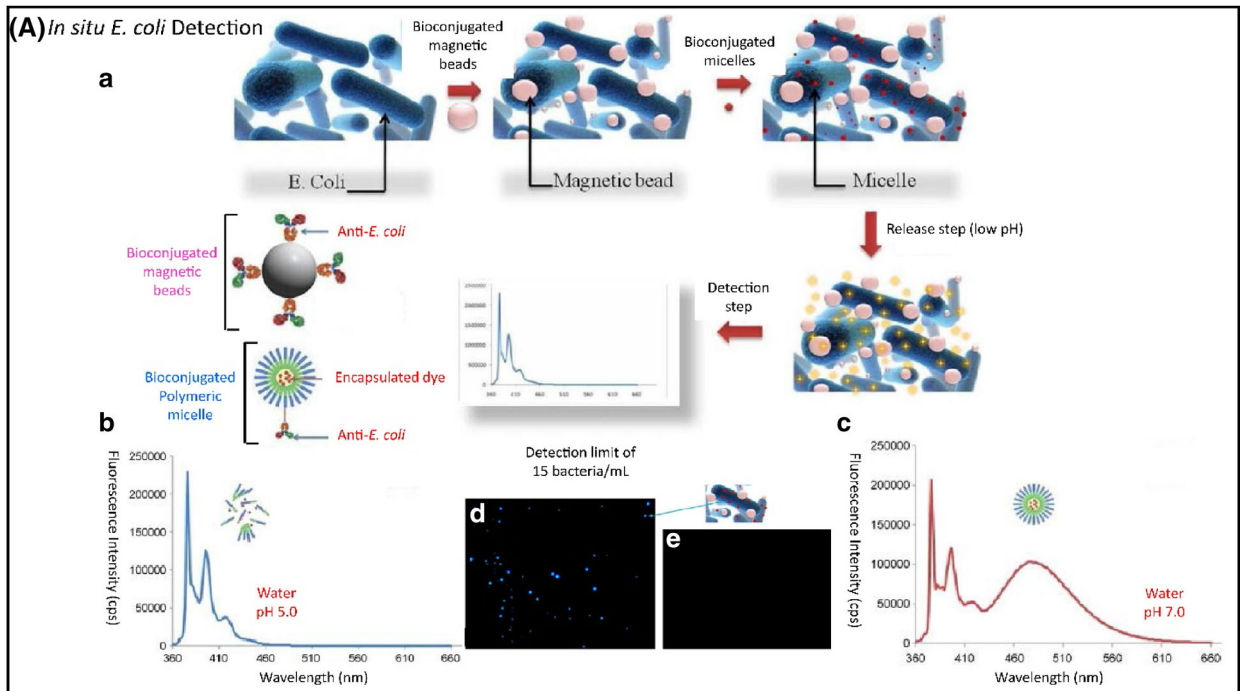
(A) *In situ E. coli Detection*

a

Bioconjugated magnetic beads

Bioconjugated micelles

E. Coli

Magnetic bead

Micelle

Release step (low pH)

Detection step

Bioconjugated magnetic beads
- Anti-*E. coli*

Bioconjugated Polymeric micelle
- Encapsulated dye
- Anti-*E. coli*

b

Water pH 5.0

Detection limit of 15 bacteria/mL

d

e

c

Water pH 7.0

(B) *In vivo* Bacteria Imaging

b

Radiant efficiency (fluorescence emission radiance per incident excitation power)

a

Vancomycin conjugated with IRDye800CW

D-Ala-d-Ala vancomycin binding site

Cell wall

Membrane

Cell envelope of Gram-positive bacteria

E. coli or sterile myositis

S. aureus myositis

Gram-positive cocci

Intravenous administration of vancomycin-IRDye800CW

c

Gram-negative rods

×1,000

d

Gram-positive cocci

×1,000

◄**Fig. 5 A** Fluorescence spectroscopy and microscopy for in situ *E. coli* detection: *a* a general work flow of using bioconjugated dye loaded micelles and bioconjugated magnetic beads to sense *E. coli*; *b*, *c* are fluorescence spectra of dye loaded polymeric micelle at pH 5.0 and 7.0, respectively; *d* fluorescence image of bioconjugated dye loaded micelles bound to *E. coli*; *e* fluorescence image of dye loaded micelles. Figures modified from Ref. [26] and used with permission. **B** Fluorescence in vivo bacterial imaging: *a* schematic drawing of a general work flow of using fluorophore labeled vancomycin to selectively detect Gram-positive bacteria; *b* fluorescence images of fluorophore labeled vancomycin in mouse with bacterial induced myositis; *c*, *d* are fluorescence images of *E. coli* and *S. aureus* in infected muscle tissue. Figures modified from Ref. [92] and used with permission

The challenges of using both SPR and LSPR spectroscopy in bacterial sensing are several. The similarity of refractive index among target analytes, e.g., biomolecules, bacteria, and surface recognition motifs inherently limits sensitivity, even for efficient analyte capture. This limitation can be addressed by the addition of an amplification agent, such as gold nanoparticle-labeled DNA [108, 109]. The typical use of diffusion to drive mass transport to the surface limits the speed of analysis. This can be addressed by using active transport, for example using dielectrophoresis at interdigitated electrode arrays [110]. In common with all surface adsorption-based approaches the possibility of non-specific adsorption giving false positive signals is a pervasive issue that is commonly addressed by decorating the non-active parts of the surface with a non-specific adsorption-resistant coating, such as poly(ethylene glycol) [111]. Finally, a special challenge in the use of SPR and LSPR bacterial sensing arises from the relative magnitude of the sensing depth and the size of bacteria for probing whole bacteria [27].

Surface Plasmon Resonance (SPR) Spectroscopy

Despite the challenges facing the use of SPR, it has been widely utilized for bacterial detection. With anti-*E. coli* as an affinity reagent, SPR spectroscopy was able to detect *E.coli* in pure culture and from spinach leaves with LODs of 10^3 and 10^4 CFU mL^{-1}, respectively [35, 36]. With immobilized T4 bacteriophage as a recognition element, flowing *E. coli* past a metal-decorated optical fiber produced a LOD of 10^3 CFU mL^{-1} [45]. Tawil et al. [44] reported detection of *E. coli* O157:H7 and methicillin-
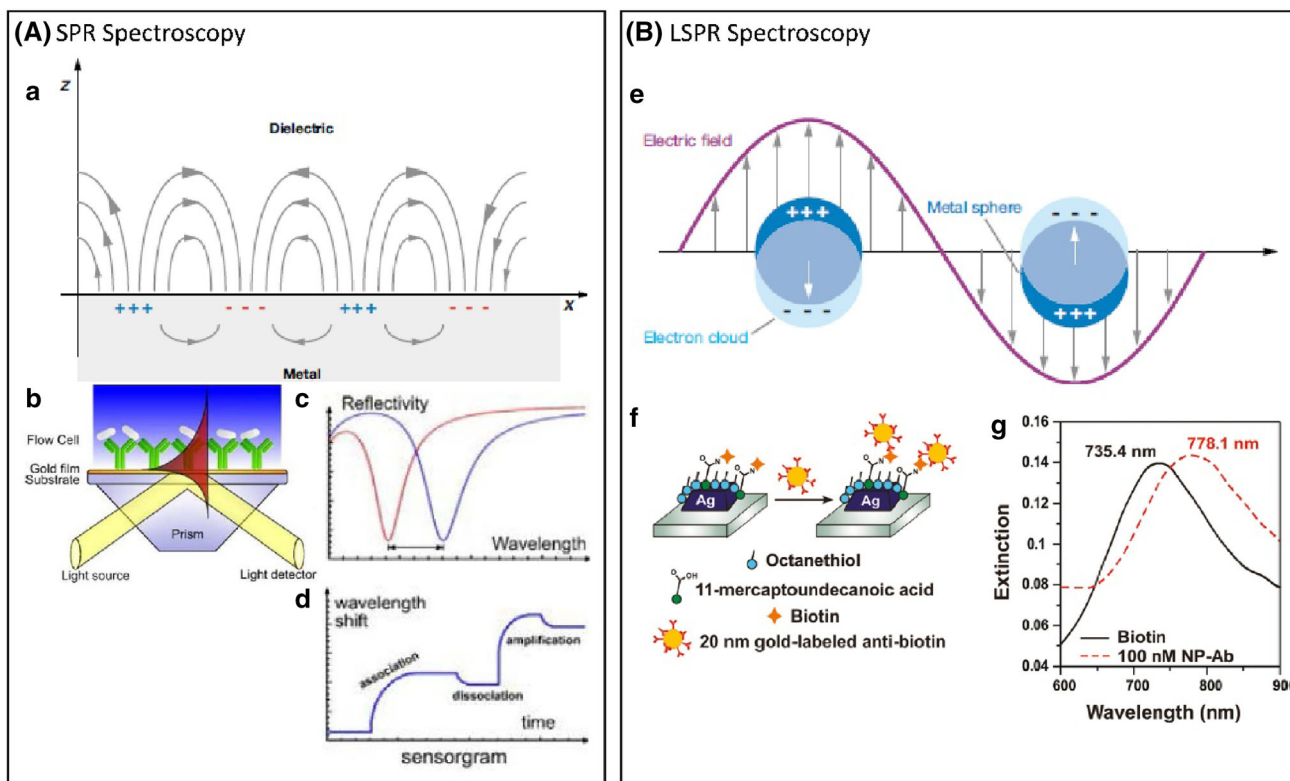

Fig. 6 SPR (**A**) and LSPR (**B**) spectroscopy and sensing modalities. *a*, *e* are illustrations of surface propagating plasmon and localized surface plasmon, respectively; *b* schematic drawing of SPR sensing platform based on Kretschman configuration and wave modulation (*red*); *c* SPR peak shifts in wavelength; *d* SPR sensorgram measuring wavelength shift as a function of analyte binding and dissociation; *f* illustration of LSPR sensor used to detect anti-biotin labeled gold nanoparticles binding to surface confined biotin; *g* Extinction spectra of subsequent addition of biotin (*black*) and anti-biotin labeled gold nanoparticles (*red*) onto LSPR sensor. Figures modified from Refs. [6, 105, 107] and used with permission
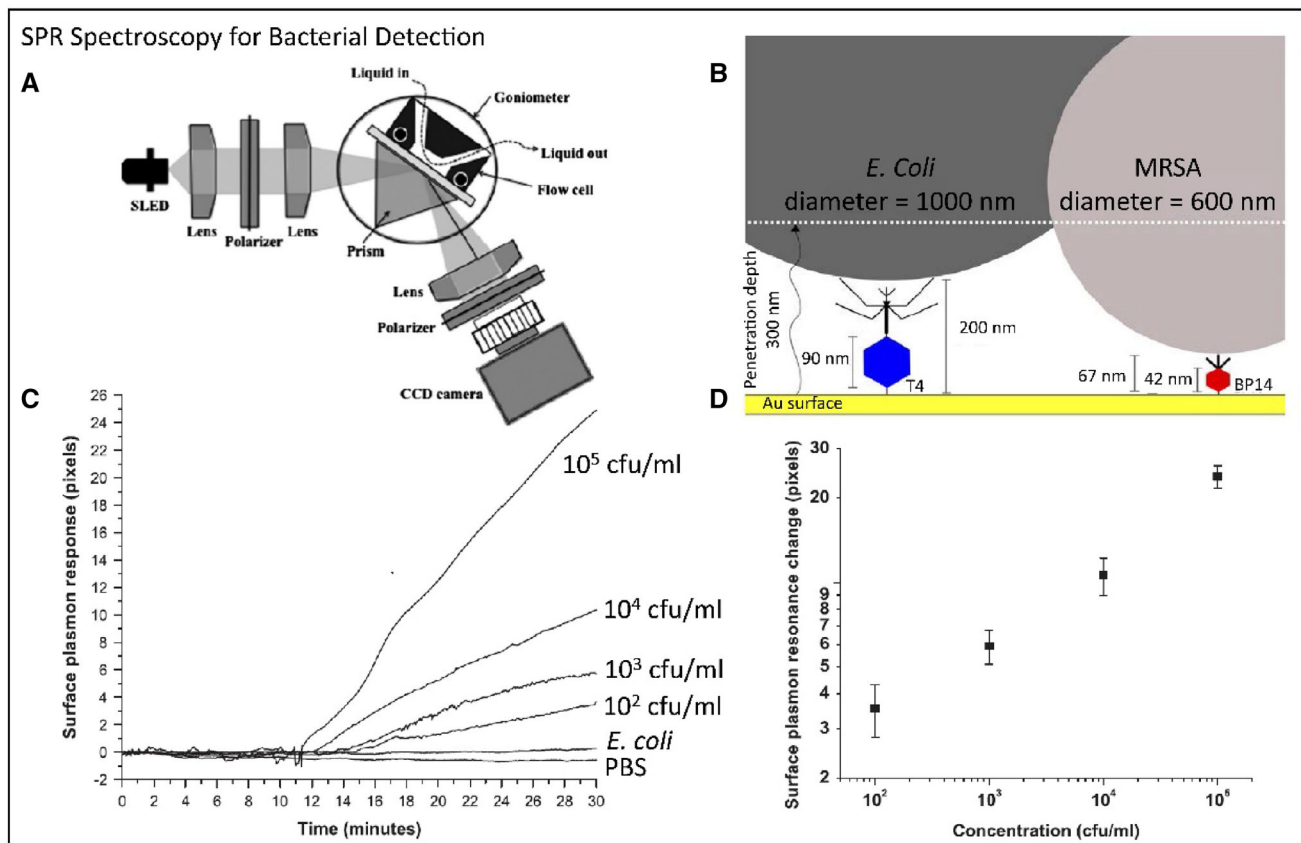
Fig. 7 Detection of *E. coli* and MRSA using SPR spectroscopy: **A** schematic drawing of experimental set-up; **B** cartoon illustration of sensing bacteria with bacteriophages on sensor surface; **C** SPR responses from various concentrations of *E. coli* as a function of time; **D** SPR changes as a function of *E. coli* concentration. Figures modified from Ref. [44] and used with permission

resistant *S. aureus* using surface immobilized T4 and BP14 bacteriophage, respectively, with a LOD of 10^3 CFU mL^{-1} in both cases, viz. Fig. 7.

Additionally, parallel bacterial sensing with multichannel SPR sensing has also been demonstrated. With this sensing platform, four food-borne bacterial pathogens were simultaneously detected using immobilized antibodies as affinity agent. Sensing was demonstrated in both buffer and apple juice with LODs ranging from 3.4×10^3 to 1.2×10^5 CFU mL^{-1} [6]. A SPR biosensor based on grating-coupled long range surface plasmons and coupled with antibody labeled magnetic nanoparticles was developed to detect *E. coli* O157:H7 with an extraordinary LOD of 50 CFU mL^{-1} [27].

Localized Surface Plasmon Resonance (LSPR) Spectroscopy

The LSPR characteristics of metal nanoparticles have been appreciated for several decades. However, initially the dispersion in size, and thus resonant wavelength, prevented their widespread use in biosensing. In 1995, Van Duyne and coworkers developed nanosphere lithography (NSL) [112], which can generate highly monodisperse metallic nanoparticle

arrays on surfaces. The resulting metallic nanoparticle arrays exhibit strong and highly wavelength-specific LSPR peaks, and thus serve as nearly ideal LSPR substrates. Utilizing these NSL-derived substrates, Van Duyne and coworkers published the first demonstration of LSPR detection in 2000 [113] and have systematically investigated LSPR sensing since then. The introduction of NSL-fabricated arrays of monodisperse nanoparticles to LSPR sensing was a seminal advance, because it dramatically reduced the sample-to-sample variation, resulting in structures that are typically limited by surface defects and possible impurity adsorption on surface.

Biomolecular systems, such as biotin and streptavidin [114], biotin and anti-biotin [6, 37, 105, 107], as well as other antibody–antigen [27, 31, 35, 36], and aptamer-based recognition systems [41, 115, 116] have been extensively explored using LSPR. In addition, LSPR sensors can be reusable in immunoassays with relatively weak binding, e.g., biotin/anti-biotin, where the recognition surface can be regenerated by mild changes in solution conditions [37]. In general, LSPR sensors have been applied to detect biomolecular interactions, but to-date they have only rarely been applied for bacterial sensing. Fu et al. demonstrated the detection of whole cell *Salmonella* via anti-*Salmonella* antibody on surface confined
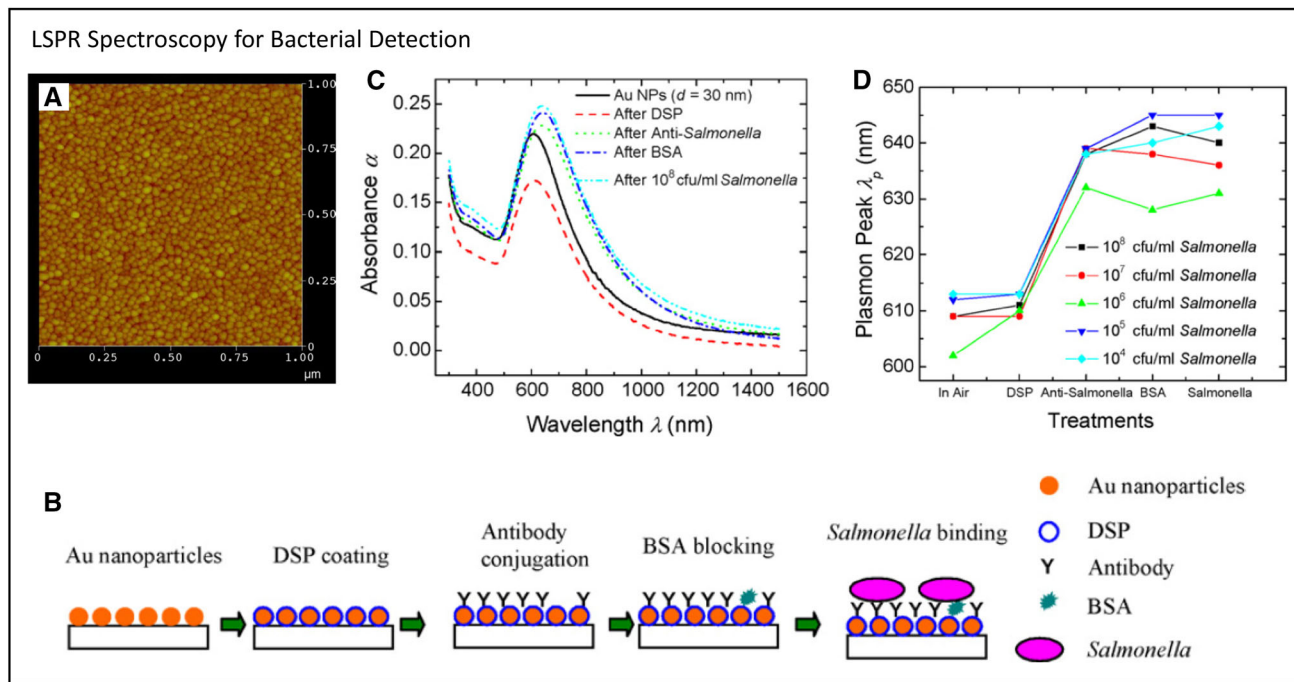
Fig. 8 LSPR spectroscopy for detection of *Salmonella* via anti-*Salmonella* antibody on Au nanoparticle substrates: **A** a typical atomic force microscope image of Au nanoparticle substrate; **B** schematic drawing of bacterial sensing strategy; **C** absorption spectra showing a corresponding wavelength shift after each step of surface modification and bacterial binding; **D** LSPR peak shifts as a function of surface modification and sensing various concentrations of *Salmonella*. Figures modified from Ref. [31] and used with permission

Au nanoparticles. A plasmonic peak shift was observed when bacteria were bound to the surface, but there was no correlation in terms of bacterial concentration, viz. Fig. 8 [31]. Nevertheless, the prospects for LSPR bacterial biosensing are promising. The chief hurdles to overcome are the relative sizes of bacteria and LSPR motifs as well as the existence of many different capture targets on the surface of bacteria. Bacteria are many times larger than the typical NSL-derived nanoparticle elements, so in contrast to molecular sensing with LSPR, where many molecules are resident on a single nanoparticle, even relatively small bacteria are likely to span many nanoparticles. In addition, the vast majority of bacteria are non-spherical; so the point of attachment can determine the orientation of the bacterial cell on the nanoparticle thus affecting its dielectric response function in the near-particle volume. However, these problems are certainly no worse than the sensitivity and reproducibility issues already solved in molecular LSPR biosensing; so in the future we expect to see LSPR added to the armamentarium of optical biosensing strategies for bacteria.

Summary and Future Prospects

In this review, we addressed the importance of sensing bacteria and the challenges and successes associated with each of three broad classes of bacterial biosensors based on Raman scattering, fluorescence, and plasmonics. Each class exploits a fundamentally distinct feature of bacteria. Raman scattering takes advantage of characteristic vibrational signatures that are enhanced—either by way of molecular abundance or signal generation mechanisms—to the exclusion of other signals. In typical samples of planktonic bacteria, for example, the bacteria signals are dominated by nucleotide scattering, whereas adding SERS-enhancing nanoparticles accentuates cell membrane components. Fluorescence bacterial biosensing exploits the inherent sensitivity associated with the detection of an exogenous fluorescence label. As such, it is largely limited by background and by the specificity of the recognition motif used to convey the label to the bacteria. Recently SPR and LSPR approaches have grown in their use for bacterial sensing. Here the presence of the bacteria is signaled by the change in the dielectric response function of the medium in the near-field of plasmonic nanoparticles. Because bacteria are large compared to the sensing structures, they can, in principle, be detected quite sensitively.

Conventional Raman spectroscopy and its derivatives are powerful optical techniques in the field of bacterial sensing. Raman spectroscopy provides characteristic molecular fingerprints; is insensitive to water, which enables detection of biological samples in their native environment; and is non-invasive. However, conventional

Raman spectroscopy is inherently weak; so various Raman derivatives have developed to address the sensitivity issue. SERS exhibits great versatility, good sensitivity, and specificity, and it is the most widely utilized Raman technique for bacterial detection. TERS not only records chemical information of analytes, but also topographic information with nanometer-scale spatial resolution, which is extremely useful in studying bacterial surface compositions, distribution of surface components, cellular structures, and even biofilm structure. Currently, TERS has only infrequently been used for bacterial sensing applications, but this should change as commercial TERS instruments become more readily available. CARS, especially recent broadband variants, combines rapid detection with moderate sensitivity. Essentially every type of Raman spectroscopy can be coupled with microscopy to achieve bacterial imaging, and the resulting hyperspectral data sets open up a rich set of analysis possibilities, including combinations with other imaging modalities, such as mass spectrometry imaging, to enable multiplex sensing and imaging. Furthermore, although the spectra of bacteria arise from a common set of organismal components, i.e., nucleotides, proteins, lipids, etc., judicious use of chemometrics and multivariate statistical approaches renders Raman techniques capable of differentiating bacterial species, even down to the strain level.

Fluorescence spectroscopy has also been extensively used for bacterial sensing because of its high sensitivity and the specificity imbued by fluorophore-conjugated affinity agents. Exogenous fluorescence, as it is used in bacterial biosensing, is ultimately limited by the background, however, under the right conditions single molecules and single bacteria can be detected. Furthermore, the use of the spectral dimension makes it possible to achieve spectral multiplexing and thereby simultaneously determine multiple bacterial targets. Recently the combination of bacterial recognition and fluorescence readout has been combined to achieve in vivo fluorescence imaging, wherein bacteria can be detected directly in living organisms and/or biopsy tissues, making it a promising diagnostic tool for the clinic.

The most recent addition to optical bacterial biosensing, SPR and LSPR detection, has been made possible by advances in nanotechnology. These sensing modalities which detect changes in the local dielectric response function are extraordinarily sensitive to the material, size, shape, thickness, and interparticle spacing of the sensor surface, as well as the refractive index of the surroundings. SPR and LSPR approaches are both sensitive to the size and concentration of analytes, and they have been employed extensively for chemical and biochemical sensing. In contrast bacterial sensing, to the extent that plasmonic approaches have been used, has far more frequently used SPR, although the characteristics of LSPR would seem to make it a promising approach for the future.

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