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Raman microspectroscopy, surface-enhanced Raman scattering microspectroscopy, and stable-isotope Raman microspectroscopy for biofilm characterization

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Abstract Biofilms represent the predominant form of microbial life on our planet. These aggregates of microorganisms, which are embedded in a matrix formed by extracellular polymeric substances, may colonize nearly all interfaces. Detailed knowledge of microorganisms enclosed in biofilms as well as of the chemical composition, structure, and functions of the complex biofilm matrix and their changes at different stages of the biofilm formation and under various physical and chemical conditions is relevant in different fields. Important research topics include the development and improvement of antibiotics and medical devices and the optimization of biocides, antifouling strategies, and biological wastewater treatment. Raman microspectroscopy is a capable and nondestructive tool that can provide detailed two-dimensional and three-dimensional chemical information about biofilm constituents with the spatial resolution of an optical microscope and without interference from water. However, the sensitivity of Raman microspectroscopy is rather limited, which hampers the applicability of Raman microspectroscopy especially at low biomass concentrations. Fortunately, the resonance Raman effect as well as surface-enhanced Raman scattering can help to overcome this drawback. Furthermore, the combination of Raman microspectroscopy with other microscopic techniques, mass spectrometry techniques, or particularly with stable-isotope techniques can provide comprehensive information on monospecies and multispecies biofilms. Here, an overview of different Raman microspectroscopic techniques, including resonance Raman microspectroscopy and surface-enhanced Raman scattering microspectroscopy, for in situ detection, visualization, identification, and chemical characterization of biofilms is given, and the main feasibilities and limitations of these techniques in biofilm research are presented. Future possibilities of and challenges for Raman microspectroscopy alone and in combination with other analytical techniques for characterization of complex biofilm matrices are discussed in a critical review.

Introduction

More than 99% of the microorganisms on Earth are organized within biofilms. Biofilms are solid–liquid, liquid–liquid, liquid–air, and solid–air interface associated communities of microorganisms (e.g., Gram negative and Gram positive bacteria as well as protozoa, fungi, and algae) embedded in a matrix of extracellular polymeric substances (EPS). In microorganisms from biofilms, large suites of genes are differentially regulated, leading to phenotypic and physiologic differences between biofilm cells and planktonic cells of the same organism, which can float or swim in an aquatic environment as single cells [1–4]. EPS are biopolymers of microbial origin such as polysaccharides, proteins, glycoproteins, nucleic acids, and lipids. In addition, EPS may include humic-like substances as well as materials from the surrounding environment (e.g., minerals, colloids, and soil particles). Depending on the enclosed microorganisms, the environmental conditions, and the biofilm

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edge, EPS can have various compositions and may account for up to 90% of the particulate fraction of the biofilm. The major component in the biofilm matrix is water, which can make up to 98% of the biofilm mass. The three-dimensional EPS framework in biofilms acts as a water reservoir and protects the microorganisms against environmental stress such as from detergents and antibiotics (e.g., antibiotic resistance of bacteria in biofilms can be increased 100 or even 1000 times [5]). Besides physicochemical advantages, microbial cells living in biofilms benefit from intracellular and intercellular signaling quorum sensing (QS), multispecies synergisms (allowing proliferation of microorganisms that cannot form a monospecies biofilm) and gene transfer within the same species and between different species [1, 5, 6].

The identification and characterization of microorganisms enclosed in biofilms as well as detailed information on the chemical composition of the EPS matrix are important in various fields (e.g., medicine, biology, and technological processes). This knowledge will allow accurate disease diagnostics, the development of new antibiotics, biocides, and detergent agents, optimization of antifouling strategies, and optimization of biological wastewater treatment. However, the actual chemical composition and structure of biofilm matrices differ strongly, depending on several factors: the microbial cells present, their metabolic activity, the available nutrients, the prevailing physicochemical conditions (temperature, shear, pH, etc.), and the biofilm development stage (which includes initial and then irreversible attachment of cells, microcolony formation, biofilm development, maturation, and detachment as well as formation of planktonic cells; Fig. 1) [53]. Therefore, the development of nondestructive methods for in situ characterization of complex biofilm matrices with high spatial resolution is desired but remains a very challenging issue.

In the last few decades various techniques have been applied for the identification and characterization of biofilms, including their molecular structures and associated functions (for a comprehensive review, see [54]). There are conventional chemical and biochemical methods, such as nucleic acid assays, genetic assays, fatty acid profiling, and proteomics in general. Usually these methods are very time-consuming and labor-intensive, require expensive instrumentation and highly trained personnel, and have only limited reproducibility and low specificity [54, 55]. Another commonly used method is fluorescence in situ hybridization (FISH). It allows the detection of different microorganisms in a multispecies biofilm by applying ribosomal-RNA-targeted nucleic acid probes [56, 57]. For biofilm analysis, FISH is often combined with fluorescence-induced confocal laser scanning microscopy (CLSM). This approach provides nondestructive, three-dimensional information about the biofilm structure [58, 59]. However, the preparation procedure is quite extensive and the staining of the total EPS for CLSM analysis is rather

expensive and complicated, since EPS are complex mixtures of different biopolymers with a large number of potential binding sites. Simultaneous analysis of at most two biofilm components is usually possible with CLSM [54]. Furthermore, the spatial resolution is limited to several micrometers. In contrast, scanning electron microscopy (SEM) offers higher resolution for visualization of biofilms (down to the nanometer range). However, for conventional electron microscopy, sample preparation involves the fixing and/or drying of the samples before examination, which may lead to changes of the morphology of the biofilm and hence may negatively affect the reproducibility and authenticity of the images [60]. To prevent a collapse of the EPS matrix during the sample preparation, cryo-SEM or environmental SEM (ESEM) can be applied for the analysis of unfixed biofilms [54]. Another visualization technique is optical coherence tomography. The spatial resolution of this technique is significantly lower than that of SEM, but it allows in situ two-dimensional and three-dimensional imaging of biofilm structures and monitoring of biofilm distribution and detachment without staining of the sample [61, 62]. In addition to these techniques, analyses of biofilm topography as well as mechanical and physical properties of microbial systems in the nanometer range can be achieved by atomic force microscopy (AFM) [63]. When a depth-resolved analysis of physical properties, such as biofilm density or thickness, is important, the photoacoustic technique, which allows an analysis to depths of more than 1 cm, can be applied. This method allows, for example, the monitoring of growth and removal of a biofilm with high temporal resolution [64]. Also solid-state nuclear magnetic resonance spectroscopy methods can be used for biofilm characterization; however, a spatially resolved analysis is not possible with this technique [65]. For biofilm identification, imaging, and characterization of matrix components many different mass spectrometry (MS) methods are available, including secondary ion MS (SIMS), matrix-assisted laser desorption ionization MS, and desorption electrospray ionization MS. However, MS imaging techniques require a complicated sample preparation and are destructive, and the measurement is very expensive and rather time-consuming. Also, the quantitation of the imaged molecular species is a major challenge for MS imaging techniques [54].

Two complementary vibrational techniques—namely, Fourier transform IR (FTIR) spectroscopy and Raman spectroscopy—provide intrinsic chemical information on biofilms [44, 45]. Although these techniques both analyze vibrations of molecules or groups of atoms, their physical mechanisms are different. In IR spectroscopy, vibrations that change the permanent dipole moment of the molecule are detected, whereas in Raman spectroscopy, vibrations that change the polarizability are visible in the spectrum. The molecular vibrations involved are excited via absorption of light or the inelastic scattering of light respectively. In Raman spectroscopy the
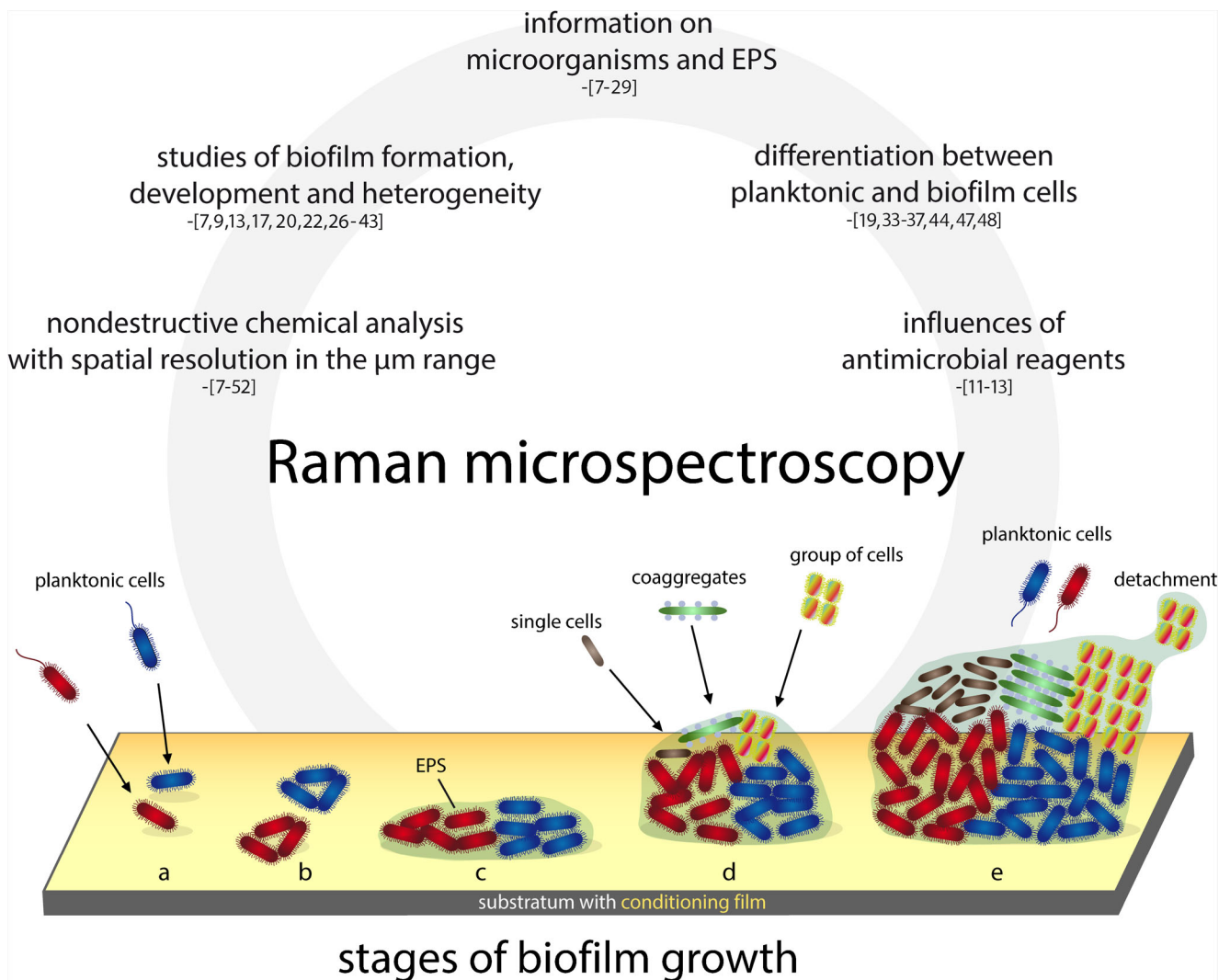
Fig. 1 Biofilm development and various corresponding applications of Raman microspectroscopy: *a* initial adhesion or attachment of cells (i.e., primary colonization of a substratum covered with "conditioning film" composed of polysaccharides and proteins by planktonic cells); *b* irreversible cell attachment; *c* microcolony formation (i.e. cell growth, division, and production of extracellular polymeric substances, *EPS*); *d* development of a multispecies biofilm (e.g., coadhesion of single cells, coaggregated cells, and groups of cells); *e* maturation of biofilm, detachment, and formation and dispersion of planktonic cells. For more information on stages of biofilm growth see [1, 53, 54]

scattered photons transfer a certain amount of energy that corresponds to specific molecule vibrations. When the frequency of the scattered light is lower than the frequency of the incident light, the scattering is referred to as Stokes scattering; when the frequency of the scattered light is higher than the frequency of the incident light, the scattering is denoted as anti-Stokes scattering. The different physical principles of both methods lead to different intensities of the same vibrational bonds. For example, the OH stretching vibration is highly pronounced in IR spectra but only weakly visible in Raman spectra. Therefore, Raman spectroscopy is better suited for the analysis of water containing samples such as biofilms. On the other hand, the (high) fluorescence background in spectra is a major problem for Raman spectroscopy. Raman microspectroscopy (a combination of Raman spectroscopy and optical microscopy) offers confocality, which allows the analysis of samples below the surface area and even through cover plates such as those made of glass or quartz. Furthermore, excitation with visible light can be applied, which allows the use of standard optics. Therefore, the spatial resolution of Raman microspectroscopy is higher (down to 1 μm) than that of IR techniques (e.g. down to around 10 μm for micro-FTIR spectroscopy). The higher spatial resolution of Raman microspectroscopy makes possible analysis of the heterogeneity of the sample in greater detail. In combination with its insensitivity to water, which is often a major component in natural samples, Raman microspectroscopy is the better of these two vibrational spectroscopic techniques for the analysis of biofilms. It allows nondestructive characterization and imaging of biofilms and

provides vibrational fingerprint spectra of cellular and extra-cellular components (such as proteins, carbohydrates, nucleic acids, and lipids) and hence biochemical information on the biofilm matrix, without the necessity to dry or stain the samples before analysis (see Fig. 1). Fingerprint spectra can be used to identify and discriminate microorganisms on the strain and species level and to analyze the EPS composition of biofilms [66, 67].

The significant disadvantage of Raman microspectroscopy is the low quantum efficiency of the Raman effect (typically 10^{-8}–10^{-6}), which leads to a limited sensitivity. Hence, the analysis times for microbiological samples, which often possess only a little biomass per measurement spot, are usually quite long, even when powerful laser equipment and sensitive detectors are used. Therefore, methods that enhance the Raman effect are often required. Raman signals can be enhanced either by attachment of nanometer-sized metallic structures (Ag or Au) or by use of the resonance Raman effect. This first enhancement effect is known as surface-enhanced Raman scattering (SERS). It uses electromagnetic and chemical enhancement effects to achieve higher sensitivity. The latter occurs when the incident photon energy is equal to or is close to the energy of an electronic transition of a molecule. Therefore, when a biological sample possesses resonance Raman-active substances (i.e. chromophores), a rapid Raman analysis is possible if an appropriate laser wavelength is chosen [68]. Furthermore, the sensitivity of spontaneous Raman scattering can be increased by many orders of magnitude by use of, for example, stimulated Raman scattering (SRS) and coherent anti-Stokes Raman scattering (CARS) [69].

Raman microspectroscopy can also be combined with a stable-isotope labeling technique, which allows Raman microspectroscopy to be used to investigate ecophysiology and metabolic functions in situ of microorganisms and biofilms. When atoms in a molecule are (partially) replaced by stable isotopes of the same element, there are no or only negligible changes in the chemical structure and in the intensity of corresponding vibration bands. However, the vibrational frequency of the bond(s) involved is affected significantly (Raman shift of 3–5 cm^{-1} or more). For heavier isotopes the corresponding Raman band shows a characteristic shift toward lower frequency (so called redshift) in the spectrum. Therefore, Raman microspectroscopy has great potential for analysis of stable-isotope tracer incorporation into biomass.

In this article Raman-based techniques for the analysis of microorganisms and biofilms are presented and discussed in a critical view. An overview of the Raman-based techniques covered can be found in Fig. 2. The first part of this review focuses on Raman microspectroscopy for analysis of biofilms. Since biofilms consist of microorganisms, information about Raman microspectroscopy as a tool for single-cell analysis is given first. Achieving an understanding of biofilms in their

entirety, which requires identification, characterization, and quantitation, is a very challenging task for one analytical method alone, and approaches with multiple analytical methods in combination are also presented here. This is followed by discussion of resonance Raman microspectroscopy for biofilm characterization. The next section is devoted to SERS-based approaches for biofilm analysis. The main challenges and solutions for SERS methods for analysis of microbiological samples are also discussed in that section. Then the applicability of the nonlinear Raman techniques for biofilm analysis is addressed. In the following section the combination of Raman microspectroscopy with stable-isotope labeling is presented. This approach is quite new and until now studies have focused on the characterization of individual microbial cells. Additionally, the challenges in the analysis of Raman data are addressed. Finally a critical summary and outlook is given and future possibilities of and challenges for Raman microspectroscopy alone and in combination with other analytical techniques for the characterization of complex biofilm matrices are discussed.

Raman microspectroscopic analysis of biofilms

Since biofilms consist of many microbial cells close to each other, Raman microspectroscopy was established initially for single-cell analysis of microorganism cultures. The first Raman microspectroscopic characterization of single cells was reported by Schuster et al. [70] in 2000. They presented an approach to use Raman microspectroscopy to investigate the chemical composition and the heterogeneity of a *Clostridium acetobutylicum* colony. Many groups then applied Raman microspectroscopy as a tool for identification and characterization of single microbial cells. For example, the Popp group [71–74] analyzed and classified single bacterial cells of different species by means of Raman microspectroscopy in combination with various multivariate techniques—for example, principal component analysis (PCA), cluster analysis, and support vector machine analysis. Especially the rapid identification of microbial pathogens in clinical microbiology is an important topic. Maquelin et al. [75, 76] demonstrated species and even strain identification and discrimination of different microbial pathogens. More information about isolation and identification of bacteria by means of Raman microspectroscopy can be found in a recent review by Pahlow et al. [77].

The early investigations of biofilms by means of Raman microspectroscopy focused mainly on diffusion processes. Suci et al. [46] analyzed the spatial and temporal distribution of chlorhexidine in *Candida albicans* biofilms with Raman microspectroscopy and attenuated total reflection FTIR spectroscopy. Marcotte et al. [30] characterized the diffusion of polyethylene glycol in *Streptococcus mutans* biofilms. For

Technique	Schematic setup	Physical principles	Advantages	Limitations	Applications
Raman micro-spectroscopy (RM)			• Molecular/chemical fingerprintig • Water insensitivity • Spatial resolution in the µm-range • No staining required	• Fluorescence interference • Long analysis time • Low sensitivity	• Biochemical imaging • Identification and characterization of single microbial cells and EPS • Analysis of biofilm development and heterogenity
Resonance RM			• High sensitivity and selectivity • Rapid analysis	• Chromophore needed • Photobleaching	• Biochemical imaging • Rapid identification and characterization of microorganisms • Cell sorting
Surface-enhanced Raman spectroscopy (SERS)			• High enhancement • Fluorescence quenching • Rapid analysis	• Difficult to obtain reproducible spectra • Depends on the analyte and the adsorption site	• Biochemical imaging • Rapid identification and characterization of microorganisms and EPS • Analysis of quorum sensing (QS)
Tip-enhanced Raman spectroscopy (TERS)			• Lateral resolution below diffraction limit • Correlation of structural and topographic information	• Production of reproducible tips • Contamiantion of the tip • Analysis in aeriferous environment	• Biochemical imaging • Characterization of single microorganisms • Analysis of dynamics on cell surface
Nonlinear Raman spectroscopy (e.g. coherent anti-Stokes Raman scattering)			• Insensitive to fluorescence • High signal-to-noise ratio • Rapid analysis	• Nonresonant background noise • Usually only narrow band implementation • Instrument complexity	• Biochemical imaging (CH/OH-stretch, lipids) • Non-invasive cell tracking

Fig. 2 Various Raman-based techniques for the analysis of microbiological samples. *AFM* atomic force microscope

the analysis of the biomass distribution, the CH stretching region at around 2900 cm^{-1} was used. They found a heterogeneous penetrability of the biofilm for polyethylene glycol, which is modulated by the biomass content.

The following studies described in this section illustrate the applicability of Raman microspectroscopy for the analysis of (1) the biofilm formation and development, (2) the influence of different stresses and metabolic histories on the chemical composition of a biofilm, (3) chemical differences between planktonic and biofilm cells, (4) the overall heterogeneity in a biofilm in combination with the physiologic states of the microorganisms, and (5) the impact of antimicrobial reagents on biofilms.

The development of microcolonies and heterogeneity of microorganism growth was analyzed by Choo-Smith et al. [31]. They found a colony heterogeneity in samples cultured

for 12 and 24 h. Hierarchical cluster analysis of spectra from various positions and depths revealed the presence of different layers in the colonies. Furthermore, it was shown that the surface of the colonies exhibited higher levels of glycogen, whereas higher RNA levels were typical for the deeper layers. Sandt et al. [7] applied Raman microspectroscopy as a tool to study the in situ chemical heterogeneities, composition, structure, and development of a fully hydrated biofilm grown on glass in a flow cell. They used the Raman signature of cells and EPS to characterize the biofilm composition (see Fig. 3). They treated some samples with ethylenediaminetetraacetic acid to remove bound EPS due to calcium complexation. Spectra with sharp but mostly unidentified bands, which were obtained by the subtraction of spectra of biofilm cells treated with ethylenediaminetetraacetic acid from those of the untreated biofilm cells, were used as marker for EPS.
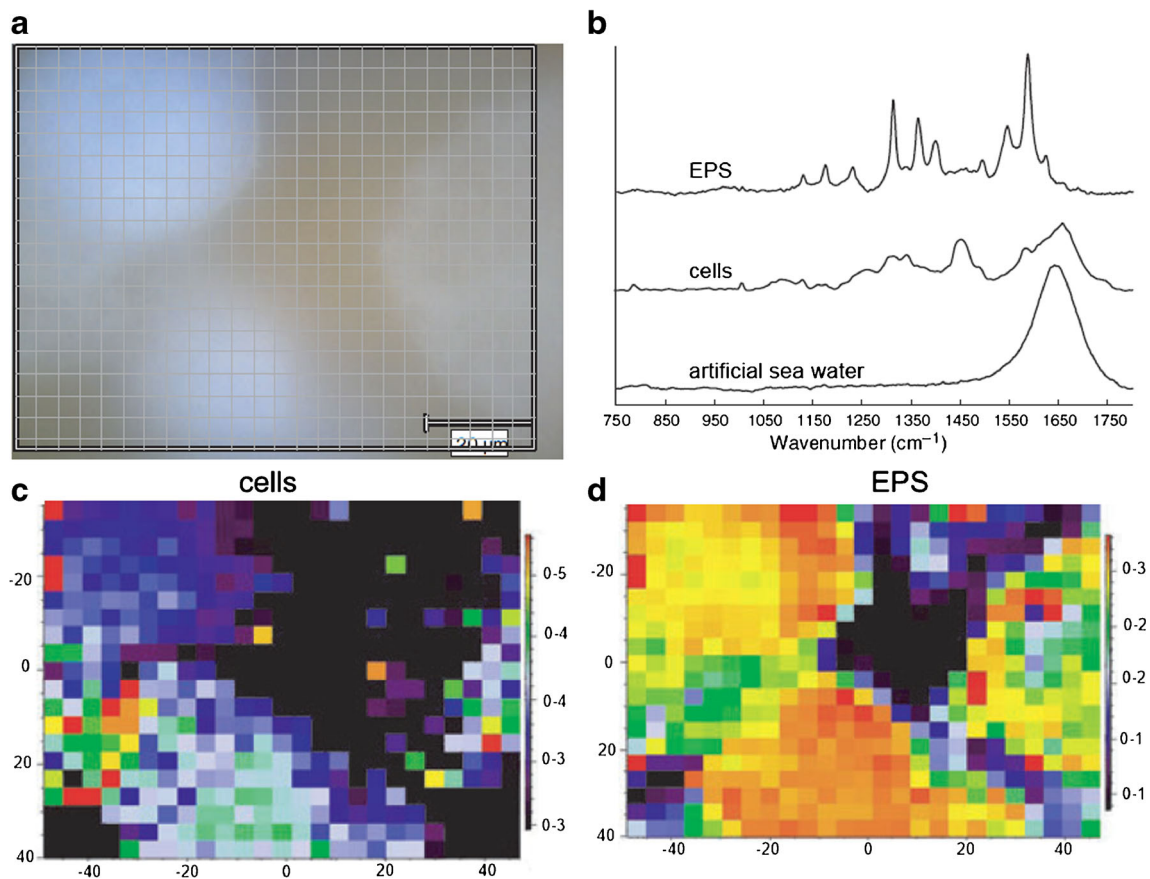
Fig. 3 Development of microcolonies and heterogeneity of microorganism growth. Lateral heterogeneity in a 19-day-old biofilm, 20 μm below the coverslip. **a** Bright field image showing a water channel between colonies with a 4×4 μm^2 grid superimposed. **b** Spectra of EPS, cells, and artificial seawater used in the principal component analysis (PCA); the component maps were generated from the contributions of the component spectra to the experimental spectra. The colors range from blue to red as this contribution increases from 0 to 1. Maps showing the contribution of **c** the cell-rich biofilm and **d** the EPS. (Adapted from [7], copyright 2007 Wiley)

Later the same group [32] demonstrated that Raman microspectroscopy can be used as a method to obtain information about the species- and strain-related variation of water content and biomass density in *Pseudomonas aeruginosa* and *Pseudoalteromonas* sp. NCIMB 2021 biofilms. They used the ratio of the area of the O–H stretching band at 3405 cm^{-1} (water) to that of the C–H stretching bands at 2950 cm^{-1} (biomass) to estimate the relative biomass water content. Samek et al. [33] demonstrated that it is possible to distinguish between two *Staphylococcus epidermidis* strains that show a very close clonal relation. One was a biofilm-positive strain and the other a biofilm-negative strain. Because the biofilm-positive strain produces a polysaccharide intercellular adhesin, the main differences in the spectra were found for polysaccharide bands. But only the use of PCA allowed clear discrimination of the two strains. Beier et al. [34] detected two different oral bacteria (*Streptococcus sanguinis* and *S. mutans*) in mixed biofilms, and the spatial maps of biofilms in the hydrated environment were created by means of Raman microspectroscopy. Huang et al. [35] demonstrated the

potential of Raman microspectroscopy to differentiate between *Pseudomonas fluorescens* cells that were cultivated in different environments with different stresses and metabolic histories. The whole-cell in vitro chemical composition varied significantly when cells were grown on different carbon sources and after transition into starvation. In another study with the same microorganism, Huang et al. [36] compared planktonic cells and cells recovered from a biofilm and reported that the Raman spectra could be differentiated by multivariate analysis.

In this context the chemical differences of planktonic and biofilm cells of *Cronobacter sakazakii* were investigated by Garima and Alka [44]. Especially the intensity of bands that can be assigned to proteins was increased in cells from biofilms. Kusić et al. [37] used Raman microspectroscopy to investigate differences in the chemical composition of both planktonic cells and sessile cells of monospecies biofilms of *P. aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and six strains of *Legionella*. For the *Legionella* species biofilms, the main difference was found in an increase of the synthesis

of lipids that may be associated with the potential of *Legionella* to form biofilms. For *E. coli*, *K. pneumoniae*, and *P. aeruginosa* biofilms an increase in the production of polysaccharides was detected. A follow-up study [47] evaluated immunomagnetic separation for its compatibility with Raman microspectroscopy to isolate *Legionella* spp. from biofilms grown in tap water and their subsequent identification on a single-cell level by application of multivariate analysis on the Raman spectra of single bacterial cells without any cultivation step. Although the polyclonal antibodies used cross-reacted with other bacteria, the Raman spectra of isolated biofilm cells of *Legionella* spp. were separated from spectra of isolated nontargeted bacteria and correctly identified via multivariate analysis. An overall accuracy of 86.3% was obtained for the identification of legionellae via a support vector machine model. It is important to note that in this proof-of-principle study only monospecies biofilms from diverse bacterial species were analyzed. Therefore, further studies are essential to prove the applicability of this approach for multispecies biofilms grown under real environmental conditions. Liu et al. [48] analyzed the chemical composition of *Enterococcus faecalis* cells in biofilms. They reported that, mainly because of changes of nucleic acids and protein signatures in the spectra, they could differentiate the biofilm of starved *E. faecalis* from planktonic starved cells or biofilm cells from other physiologic states (i.e., exponential and stationary phase).

Ivleva et al. [8] studied a whole multispecies biofilm, including microbial constituents and EPS matrix. A wide range of reference samples (biofilm-specific polysaccharides, proteins, microorganisms, and encapsulated bacteria) were analyzed to allow the identification of characteristic frequency regions and marker bands specific for different biofilm components. In this context, Andrews et al. [9] reported Raman microspectroscopy as a method to identify the diversity of macromolecules that play a role in cell attachment and biofilm growth. They showed that for different species, various extracellular macromolecules influenced the initial cell attachment to surfaces and even the growth behavior. Chen et al. [10] extracted EPS, including soluble EPS, loosely bound EPS, and tightly bound EPS, in biofilms growing on different carriers and investigated them to obtain their different biochemical compositions and functional group characteristics.

Biofilms can be a serious problem in health care and other areas where unwanted biofilm development (e.g., on medical devices) can easily lead to contamination and ultimately to diseases. Therefore, the influence of antimicrobial reagents on certain biofilms of interest is an important topic that can be investigated by means of Raman microspectroscopy. Lu et al. [11] analyzed the antimicrobial effect of diallyl sulfide on *Campylobacter jejuni* biofilms. Raman microspectroscopy was used to study the survival of sessile cells within biofilms. Raman maps were generated, and the chemical compositions

of cells and EPS with and without diallyl sulfide were determined and compared. Another study of antibiotic effects on biofilms using Raman microspectroscopy and multivariate analysis was performed by Jung et al. [12]. They used *P. aeruginosa* biofilms, and reported the changes in Raman spectra as response to three different antimicrobial reagents. The antibiofilm activity of functionalized polycaprolactam on *E. coli* biofilms and the growth of the biofilm was investigated by Prabhawathi et al. [13]. A large reduction in the amounts of colony-forming units, proteins, and carbohydrates was found.

Raman microspectroscopy in combination with other techniques, especially a multidisciplinary approach with microscopic and spectroscopic techniques, can yield complementary information and a comprehensive view of biofilms. Often used techniques include CLSM, FISH, SEM, AFM, SIMS, and classic MS methods.

The first combination of Raman microspectroscopy with FISH for in situ identification was reported by Huang et al. [57]. They described the development, calibration, and application of Raman microspectroscopy and FISH with pure cultures and illustrated its potential with a proof-of-principle experiment in naphthalene-degrading groundwater samples. An optimized version of this technique was reported recently by Kniggendorf et al. [14]. By use of separated equipment and settings for Raman and fluorescence analysis, it was possible to overcome several technical limitations. Without the need for fluorescent dyes used to label the targeted microbes, a high fluorescence background and photobleaching steps could be avoided by Raman microspectroscopy measurements. They showed a direct cell-on-cell comparison of confocal Raman microspectroscopy and FISH analysis performed on the same sample with precise cell localization over different measurements and instruments. This allowed each microbial cell to be connected with its Raman spectrum and its FISH identification.

Schwartz et al. [15] studied natural biofilms grown on filter materials at waterworks by means of Raman microspectroscopy in combination with molecular biology taxonomy and ESEM imaging. Raman microspectroscopy was used to analyze the EPS (nucleic acids, carbohydrates, and fatty acids) as well as other substances inside the biofilm (inorganic compounds and humic substances). Molecular biology polymerase chain reaction combined with denaturing gradient gel electrophoresis provided a deeper insight into the bacterial biofilm diversities. Additionally, ESEM (which has the ability to keep a sample hydrated and hence allows imaging of biological samples without a complex preparation procedure) revealed the differences in surface colonization between sand filter materials and carbon particles. Thus, by combination of these techniques, detailed information on the spatial distribution of biofilms, bacterial composition, and matrix composition could be obtained.

CLSM and Raman microspectroscopy were combined to further investigate the EPS matrix and the chemical changes during biofilm development in situ. Since Raman microspectroscopy requires no staining and provides biochemical information in a nondestructive way, it is an excellent complement to CLSM, which provides information only on the distribution of stainable EPS and nucleic acids [16]. The in situ characterization of *P. aeruginosa* biofilms cultivated in the Raman-spectroscopy-based microfluidic lab-on-a-chip platform in combination with CLSM analysis was performed by Feng et al. [17]. They monitored the increase in the intensity of certain Raman bands (e.g., those of proteins, lipids, and carbohydrates) during biofilm development, quantified the biofilm thickness by CLSM, and could therefore discriminate biofilms at different developmental stages (early, mid, and late stages). These observations were additionally verified by use of PCA. Masyuko et al. [18] analyzed the chemical composition in samples of *P. aeruginosa* (biofilms and planktonic cells) by Raman microspectroscopy in combination with matrix-assisted laser desorption ionization MS. Planktonic cells were mainly dominated by DNA/RNA spectral features, which were significantly weaker in spectra from cells situated in the biofilm. Instead, spectra that were dominated by lipids were found in the biofilm. In a second study by the same group, Raman microspectroscopy (which was shown to provide information on proteins, carbohydrates, and for the first time quinolone signaling molecules) was combined with SIMS to visualize molecular distributions of metabolites (multiple quinolone species) in *P. aeruginosa* biofilms and determine the specific molecular identities via in situ tandem MS (see Fig. 4) [19]. The filamentation-mediated *Xylella fastidiosa* adhesion, biofilm formation, and the distribution and chemical composition of different EPS at each stage of the entire bacterial life cycle were investigated ex vivo by Janissen et al. [20] using a multidisciplinary approach with

different microspectroscopic techniques. They combined Raman microspectroscopy, wide-field epifluorescence microscopy, spinning disk confocal fluorescence microscopy, CLSM, SEM, and scanning probe microscopy to gain comprehensive knowledge of *X. fastidiosa* biofilm formation, starting at single adhesion and progressing to biofilm maturation.

Thus, Raman microspectroscopy seems to be a suitable and very promising technique that makes possible nondestructive in situ characterization and chemical imaging of complex biofilm matrices including microorganisms, EPS, and other substances inside biofilms (inorganic compounds and humic substances). The combination of Raman microspectroscopy with other techniques can help to provide comprehensive information on biofilm composition, structure, and functions. However, Raman microspectroscopy studies of (environmental) biofilm samples are often hampered by (strong) interference from fluorescence, and hence optimization of measurement parameters (excitation laser wavelength, photobleaching, magnification of objective lens, confocal mode, etc.) is required. Since most bacteria and EPS compounds (polysaccharides, proteins, nucleic acids, etc.) show relatively weak Raman signals at biofilm-related concentrations, long acquisition times have to be considered. Especially by the analysis of the initial stage of biofilm development, Raman microspectroscopy often provides only limited sensitivity. This problem can be solved by application of resonance Raman microspectroscopy and SERS microspectroscopy, as discussed in the following sections.

Resonance Raman microspectroscopy of biofilms

One major problem with Raman microspectroscopy is its limited sensitivity caused by the low quantum efficiency of the

Fig. 4 Combination of Raman microspectroscopy and secondary ion mass spectrometry (*SIMS*) to visualize the distribution of metabolites. **a** With the help of a microdroplet array, regions of interest can be analyzed by Raman microspectroscopy and SIMS. Molecular distribution images of a quinolone signaling molecule in the selected regions of interest for **b** Raman microspectroscopy and **c** SIMS. *CRM* confocal Raman microspectroscopy. (Adapted from [19], copyright 2015 American Chemical Society)
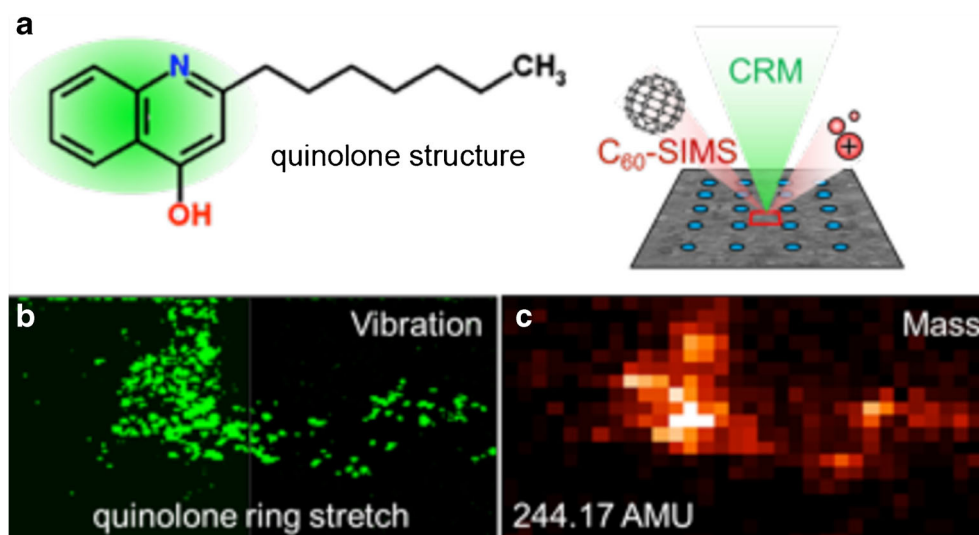
Raman effect (typically 10^{-8}–10^{-6}). This usually leads to long acquisition times, especially for the analysis of single cells with very little biomass. Fortunately, there are strategies to amplify the Raman signal and shorten the acquisition time. One of them is resonance Raman scattering. In resonance Raman spectroscopy, the wavelength of the excitation laser is selected in such a way that the incident photon energy is equal to or is close to the energy of an electronic transition of an analyte. This leads to an increase of Raman scattering intensity by a factor of 10^2–10^6 and hence to a significant decrease of detection limits and acquisition times. The sample must possess substances that are resonance Raman active (e.g., a chromophore containing molecules such as heme [78], vitamin B_{12} [79], chlorophyll [80], cytochrome c [49], carotenoids [81], rhodopsin [82], or flavin nucleotides [83]). If such substances are present, a rapid resonance Raman analysis is possible if an appropriate laser wavelength (mostly green) is chosen [68]. If none of these chromophores are present, a UV laser can be chosen so that aromatic amino acids and nucleic acids are in resonance. Since amino acids and nucleic acids exist in every microorganism, the resulting spectra of different bacteria will be very similar, which makes the identification rather problematic.

The first report on resonance Raman analysis of biofilms was by Pätzold et al. [21]. They demonstrated the detection and spatial distribution of wastewater-related microorganisms directly in their natural environment by means of resonance Raman spectroscopy. They used, among others, the resonance

Raman bands of cytochrome c and also the Raman bands of crystalline calcium phosphate and lipids to visualize the lateral distribution of the biofilm samples. The Raman spectra of different microorganisms with resonance Raman bands of cytochrome c and a map of the biofilm colony, where the resonance Raman band of cytochrome c at 748 cm^{-1} was used, are shown in Fig. 5. By application of a hierarchical cluster analysis, the averaged spectrum from the colony was assigned to *Brocadia anammoxidans*. A follow-up study reported the in situ mapping of nitrifiers and anammox bacteria in aggregates by means of resonance Raman microspectroscopy. The required measurement time was reduced by a factor of 100 and allowed a discrimination down to strain level within microbial aggregates by use of the resonance Raman effect of cytochrome c present in the microorganisms [49].

Kniggendorf and Meinhardt-Wollweber [22] used resonance Raman microspectroscopy as a rapid tool for the in situ identification of microorganisms expressing cytochrome c in biofilms and coexisting microcolonies of *Nitrosomonas* embedded in the outer layers of anammox granules. They investigated granules from two sequencing batch reactors for the anammox process. The chromophores in spectra from the outer layer of granules, which originated from a reactor without inoculation, were identified as carotenoids. The main chromophore found in the granules from the reactor inoculated with seeding sludge was cytochrome c. Here two types of resonance Raman fingerprints were obtained. With use of hierarchical cluster analysis, the first bacterial fingerprint was
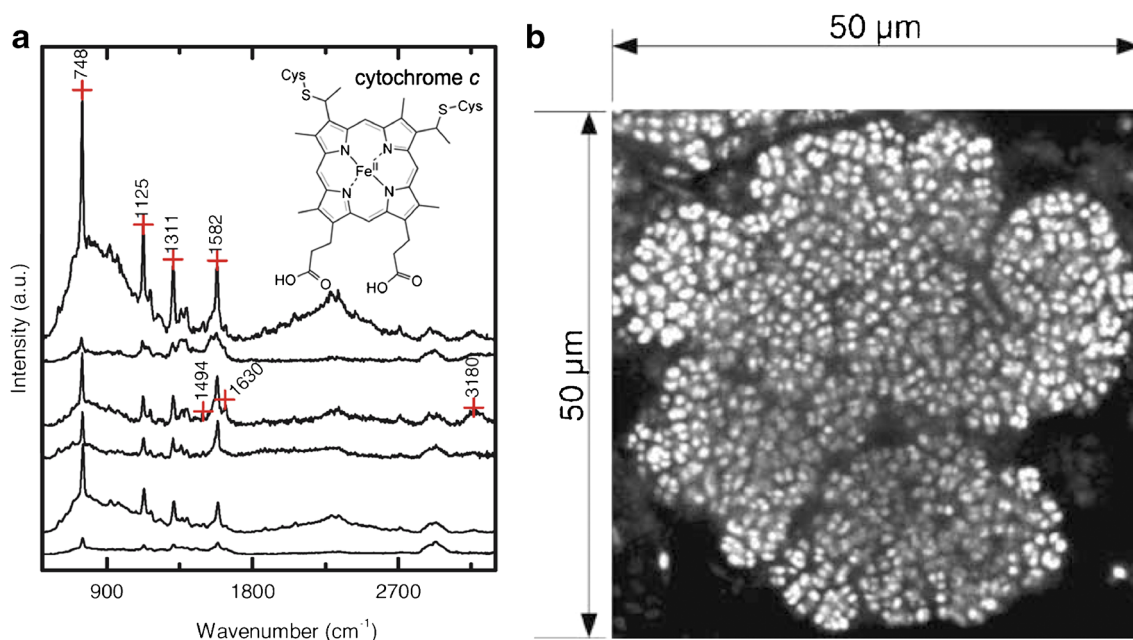

Fig. 5 Resonance Raman spectra of different microorganisms and map of biofilm colony. **a** Spectra of different microorganisms with resonance Raman bands of cytochrome c at 748, 1125, 1311, and 1582 cm^{-1} (*from top to bottom*, two averaged spectra from different colonies in a biofilm; enriched culture of *Candidatus Brocadia anammoxidans*, *Nitrobacter hamburgensis* DSM 10229, *Nitrosomonas eutropha* Nm 57 and *Nitrosomonas europaea* Nm 50). **b** Lateral distribution of the resonance Raman band from cytochrome c at 748 cm^{-1} at a depth of 10 μm below the biofilm surface. (Adapted from [21], copyright 2004 Springer)

identified with 94% certainty as *Nitrosomonas communis* Nm 02. A best match to *Nitrosomonas europaea* references (Nm 50, Nm 53) with a spectral similarity of approximately 80% was found for the much rarer second type of bacteria. Additionally, mineral titanium dioxide microparticles (rutile and anatase) were found embedded in the outer layer of the granules. Recently, Smith-Palmer et al. [38] used optical microscopy and the relative amount of cytochrome *c* compared with phenylalanine, as determined from Raman spectra, to analyze the development of *Pseudoalteromonas* sp. NCIMB 2021 biofilms on various surfaces (glass with different coatings) in a flow cell. As each flow cell was assembled with use of a glass coverslip, biofilm growth or inhibition, under the influence of fluid flow, could be compared among four different surface treatments. They reported that bacteria were able to attach to, and multiply on, all surfaces tested, but biofilm development proceeded at different rates and with differing structures and chemical compositions.

A high density of cytochrome *c* is often found in electroactive bacteria as the cytochrome plays an important role in, for example, the production of electricity or electron transfer processes. Hence, resonance Raman microspectroscopy is a suitable method for the characterization of biofilms containing such microorganisms. Virdis et al. [39] studied electrochemically active microbial biofilms to monitor biofilm development at different growth stages. Furthermore, they demonstrated a noninvasive investigation of the spatial redox electrochemistry of the biofilm by exploiting the resonance Raman effect of cytochrome *c*. They observed that the resonance Raman spectrum of cytochrome *c* depends on the redox state of the heme protein, which suggests that cytochrome *c* can be used as a metabolic indicator of redox variations within the electroactive aggregates. Another study on redox states of *c*-type cytochromes in electroactive biofilms [40] reported that cytochromes in their biofilm were mainly in the reduced redox state. This showed that only aged mixed-culture biofilms accumulate electrons during anode respiration, which suggests different bottlenecks in current production for mixed-culture and *Geobacter sulfurreducens* biofilms. Lebedev et al. [41] observed an intrabiofilm cytochrome redox gradient in *G. sulfurreducens* biofilms. Their results also indicate that most *c*-type cytochromes detected in the biofilm have oxidation potentials too negative to be directly involved in biofilm electron transport between cells and the anode surface.

Sandt et al. [50] analyzed the distribution of a carotene-containing yeast in *P. aeruginosa* biofilms by means of resonance Raman microspectroscopy. The resonance Raman carotene signal was used as a marker for the yeast, whereas certain EPS pigment signals (in the range of 1570–1605 cm^{-1}) were used as a marker for *P. aeruginosa* biofilms. A complete mapping of the whole biomass distribution was done with the C–H stretching band.

Thus, resonance Raman microspectroscopy is a very powerful tool to analyze microbial samples with short measurement times, but it can be applied only for microorganisms that possess chromophores, which limits its use. Furthermore, only if the microbial cells demonstrate clear differences in the type and/or concentration of the chromophore(s) can a reliable identification be performed. Additionally, it should be underlined that the chromophores can be photobleached easily, and interfering autofluorescence can also occur, which further complicates the analysis. If the application of resonance Raman microspectroscopy on a specific sample is not possible, SERS is another method to enhance the Raman scattering and shorten the analysis time.

Surface-enhanced Raman scattering microspectroscopy of biofilms

Raman signals of analytes can be enhanced if they are located close to or are attached to nanometer-sized metallic structures (silver or gold). This effect is known as surface-enhanced Raman scattering (SERS). Enhancement factors of the Raman signal in the range of 10^3–10^{11} can be achieved because of electromagnetic ("localized surface plasmon resonance") and chemical ("charge transfer") enhancement effects [84, 85]. The electromagnetic enhancement is the dominant effect of the two and is associated with electromagnetic interaction of light with nanoscale metals that generate collective oscillations of conduction electrons [86]. This effect leads to an enhancement of the incident light and Raman scattering signal of molecules on or close to the surface of the metallic substrate. The chemical enhancement is based on the modification of the polarizability of the adsorbed analyte, which leads to a higher Raman cross section of the vibrational bonds involved and may also allow new vibrational transitions. By an increase in the Raman sensitivity with SERS, rapid analysis of different organic and (micro)biological samples becomes possible. However, the choice of the SERS substrate is an important and difficult task, since the enhancement factor depends on the metal (silver versus gold), on the nanoparticle or nanostructure size and shape, and on the excitation wavelength and the Raman scattered wavelength. Furthermore, the affinity of different components (e.g., on the cell surface) for silver or gold surfaces and hence the associated enhancement is different, which results in the selectivity of SERS analysis. Because of different optical properties, different excitation wavelengths are optimal for diverse metal nanoparticles or nanostructures; for example, gold plasmons are red-shifted by about 100 nm compared with silver plasmons and therefore show a stronger excitation in the red and near IR ($\lambda > 600$ nm) [87]. Silver, however, is plasmonically more active and its SERS enhancement outperforms that of gold. Therefore, silver nanoparticles allow ultrasensitive analysis

and are used more often than gold. On the other hand, in applications where greater (chemical) stability and better biocompatibility are more important than maximum enhancement, gold can be used [88, 89].

The SERS substrates can be divided mainly into solution-phase suspensions of nanoparticles (with a size of 10–100 nm) and nanotextured solid substrates [86, 90]. The distance (d) between the analyte and the SERS-active surface is essential, since the SERS intensity (I) decreases dramatically with increasing distance ($I \sim d^{-12}$) in the case of electromagnetic enhancement. Hence, almost no enhancement can be achieved with distances larger than 10 nm. The chemical enhancement requires direct contact between the SERS-active surface and the analyte. Furthermore, so-called hot spots can provide extra field amplification, resulting in enhancement factors of up to 10^9–10^{11}, allowing single-molecule detection, not only for dyes, where enhancement factors of 10^7–10^8 are sufficient, but also for molecules with a normal Raman cross section [88]. But such high amplifications can be expected only with a low probability and in very restricted areas and are therefore hardly reproducible [91].

The basic feasibility of the use of SERS for the investigation of microorganisms was first shown by Picorel et al. [92] in 1988, where the cell membrane of photosynthetic bacteria adsorbed on a silver electrode was investigated. The first study showing SERS of whole bacterial cells was reported 10 years later by Efrima and Bronk [93]. From that time on, different SERS substrates and media were tested and verified for microbial research [94–96].

Since classic Raman microspectroscopy is an established and powerful tool for identification, classification, and discrimination of bacteria, in recent decades SERS was also applied to examine the possibilities for the analysis of microbial structures and bacteria. Pearman et al. [97] targeted specifically QS molecules of bacteria to determine the viability of SERS with silver colloids and an excitation wavelength of 514.5 nm to identify, characterize, and quantify these important biological molecules in situ. They used *N*-acyl homoserine lactones as reference compounds and illustrated that these compounds can be detected at concentrations down to 10^{-6} M, which are relevant for biofilms. The first measurements of QS substances directly in biofilms were performed recently by Bodelon et al. [98]. By use surface-enhanced resonance Raman spectroscopy with nanostructured gold substrates, it was possible to detect in situ QS in *P. aeruginosa* biofilm communities. The work focused on pyocyanin, an intercellular signaling molecule in the QS network of *P. aeruginosa*. It was demonstrated with mesostructured Au@TiO$_2$ substrate (submonolayer of gold nanospheres coated with a mesoporous TiO$_2$ film) and micropatterned Au@SiO$_2$ array (gold nanorods organized in micrometer-sized pedestal-like structures coated with mesoporous silica) platforms that *P. aeruginosa* engages in QS not only in densely populated biofilms but also

in the early stages of biofilm development. Pyocyanin detection down to 10^{-14} M was possible.

SERS spectra of whole bacteria originate mostly from the cell surface, with possible contributions from metabolic activity or molecular species detached from the bacterial surface during sample preparation [99]. It was demonstrated that SERS enhancement in spectra can also be particularly sensitive to one specific component on the cell surface: the resonance SERS signal of flavin adenine dinucleotide measured at an excitation wavelength of 514.5 nm [100]. Recently, Kubryk et al. [101] combined SERS and a stable-isotope approach (see "Raman microspectroscopy and stable-isotope labeling") to elucidate the origin of the strong band in the SERS spectra of bacteria around 730 cm^{-1}. The analysis performed with a 633-nm laser and in situ produced silver colloids allowed this band to be assigned to adenine-related compounds. Recently, Premasiri et al. [102] comprehensively studied the biochemical origins of the surface-enhanced Raman spectra of bacteria. They used a 785-nm laser and an aggregated-gold-nanoparticle-covered SiO$_2$ SERS substrate grown in situ. They focused on the dominant molecular species (purines) contributing to the SERS spectra of bacteria that possess known differences in the metabolism pathway. The observed SERS spectra of these species can be rationalized in terms of these active purine metabolic pathway reactions and the resulting relative purine degradation products. The authors confirmed their findings by MS analysis. The evidence presented establishes that the SERS spectra of bacterial cells excited at 785 nm are dominated by the reproducible SERS contributions of the free purine nucleobases adenine, hypoxanthine, xanthine, and guanine, uric acid, and AMP. Under certain conditions metal silver colloids can form not only on but also within bacteria [93]. For example, in a study by Jarvis et al. [103], SERS analysis of the interior of a bacterial cell was possible with use of colloidal gold substrates produced by the enzymatic reduction of gold salts by the bacteria themselves.

However, the reproducibility of SERS analyses remains challenging, because it directly hinders proper discrimination of different bacteria. Usually only averaged SERS spectra would be representative of a microbial sample and would allow comparison between different samples. Kahraman et al. [104] demonstrated that the experimental conditions for SERS must be carefully controlled to acquire reproducible spectra from bacterial samples. Proper reproducibility can be achieved by use of low-magnification lenses (as well as defocusing or distribution of the laser beam through a distinct area with special techniques) for cell suspensions or dried cells to analyze many cells at once and generate average spectra. When single cells are the target, the task is to use a reproducibly synthesized and stable SERS substrate that allows reproducible spectra of the cells with good enhancement to be obtained. For example, Knauer et al. [105] compared different

preparation procedures for SERS substrates and optimized a SERS colloid synthesis for the characterization of microorganisms. It was furthermore reported by Kahraman et al. [104] that by an increase of the concentration of the colloidal silver solution, the reproducibility of SERS spectra from single cells can be improved. Other studies also showed that layer-by-layer coating [106], convective assembly methods [107], in situ synthesis of nanoparticles directly on the cell surface (achieved by stepwise addition of the chemicals) [108], and core–shell SERS tags functionalized with antibodies [109] can be successfully used for highly sensitive, selective, and reproducible SERS analysis of microorganisms. Three exemplary techniques to achieve reproducibility are shown are shown in Fig. 6: (1) the averaging of the SERS signals from many cells of the sample and the use of a low-magnification lens, (2) spectra from single cells due to concentration of the SERS colloids on the cells, and (3) in situ production of the SERS colloids on the cells and the use of a high-magnification lens.

The first study achieving bacterial characterization, identification, and discrimination using silver nanoparticles and multivariate statistical techniques was by Jarvis and Goodacre [110] in 2004. An excitation wavelength of 785 nm was used, and silver colloids produced by reduction of silver nitrate with trisodium citrate were applied as the SERS medium. They showed that the use of cluster analyses in combination with spectra averaging allowed discrimination between microorganisms down to the strain level. However, a problem remains when one is comparing different SERS studies of bacteria. Namely, the vibrational signatures are strongly dependent on the morphology and nature of the substrate and excitation wavelength used, and therefore certain spectral information can differ from study to study [111].

The main challenge in the use of SERS for analysis of bacteria is still to define standard operating procedures (especially SERS substrates) that could be used on a widespread basis [94]. The requirements for successful SERS analyses include reproducible, easy, and cost-efficient preparation, chemical stability of SERS substrates or media, high enhancement factors, reproducible spectral features, and biological compatibility with the analyte. Moreover, the preparation of microbial samples with SERS substrates and the measurement conditions have to be optimized to achieve a reliable SERS analysis. Furthermore to be able to fully discriminate between bacteria, a database consisting of SERS measurements obtained under several different conditions seems to be necessary [77, 95].

Soon not only microbial structures or bacteria but also whole biofilms were analyzed in SERS studies. The first investigations on this topic were performed by Ivleva et al. [23]. In that study a complex multispecies biofilm matrix was analyzed in situ. Colloidal silver nanoparticles produced by reduction of silver nitrate with hydroxylamine hydrochloride were used as the SERS medium. Because of good reproducibility and an enhancement factor of up to two orders of magnitude, it was possible to sensitively characterize different components of the biofilm matrix. Follow-up studies [24, 25] reported the application of SERS imaging for microbial biofilm analysis, including the detection of different constituents and their spatial distribution in a biofilm in the initial phase of growth and also in the mature matrix (see Fig. 7). Especially at low biomass concentrations, SERS imaging, in contrast to classic Raman microspectroscopy, can help to reveal more detailed chemical information on the biofilm matrix. Apart from that, systems similar to biofilms can be also analyzed by SERS imaging. Szeghalmi et al. [112] investigated a fungal hypha grown on a gold-coated nanostructured substrate with high sensitivity by use of a laser wavelength of 633 nm. They observed remarkable time fluctuations in the SERS spectra. Some bands were present only for a few seconds, whereas most bands stayed unchanged. This effect was attributed to thermal diffusion of fungal constituents into the
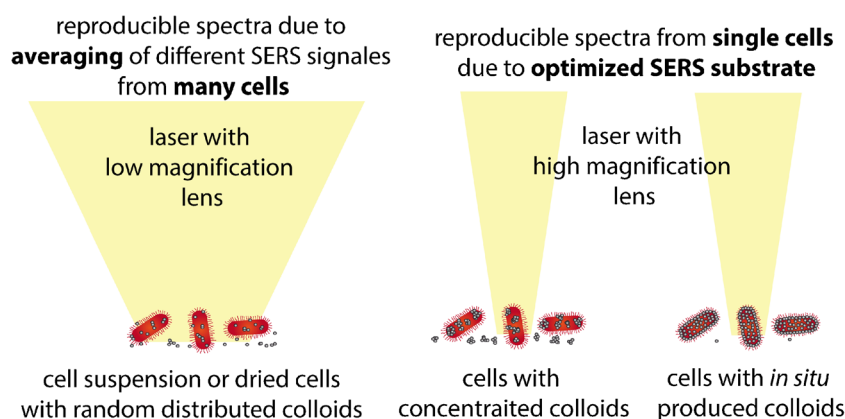

Fig. 6 Different methods to achieve reproducibility in the analysis of bacterial cells by means of surface-enhanced Raman scattering (*SERS*). Three exemplary techniques are (1) the averaging of the SERS signals from many cells of the sample and use of a low-magnification lens, (2) spectra from single cells due to concentration of the SERS colloids on the cells, and (3) in situ production of the SERS colloids on the cells and use of a high-magnification lens
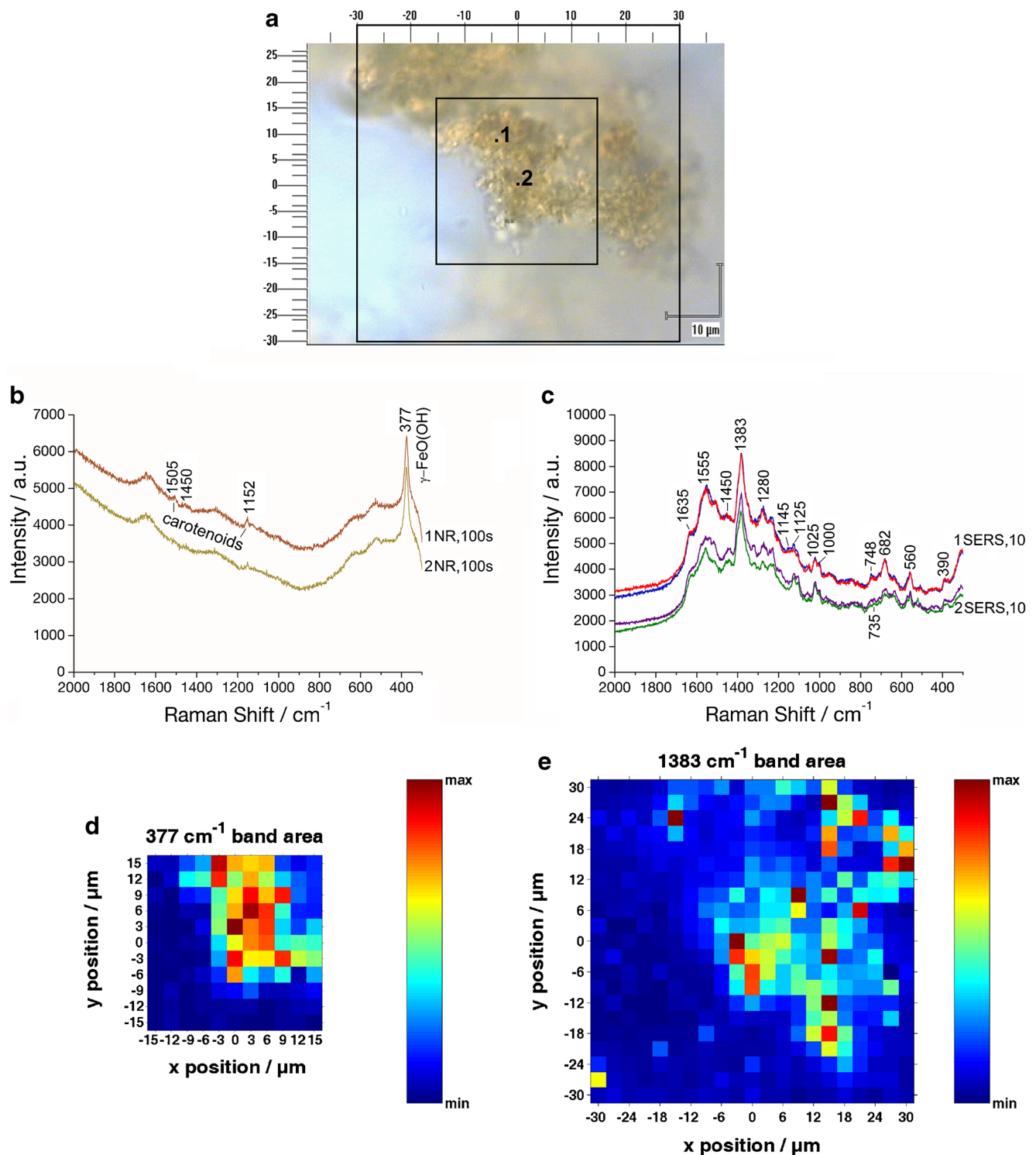
Fig. 7 Raman microspectroscopy and SERS imaging of biofilm. **a** Optical image of a biofilm (82 days old) with the marked mapping areas (*small area*, Raman microspectroscopy, *large area*, SERS). Corresponding Raman microspectroscopy (**b** and SERS (**c**) spectra, as well as Raman microspectroscopy (**d**) and SERS (**e**) maps for 377- and 1383-cm^{-1} band areas respectively. Spectra for maps were collected for 10 s (Raman microspectroscopy) or 1 s (SERS) from a 30×30 μm^2 or 60×60 μm^2 biofilm area respectively scanned with a 3-μm step. *NR* normal Raman. (Adapted from [24], copyright 2010 American Chemical Society)

hot spot in combination with high SERS enhancement for a short time.

Ramya et al. [26] performed Raman microscopic experiments on algae and *P. aeruginosa* biofilms grown on titanium

surfaces to analyze the chemical composition of the complex EPS in the biofilm. Additional SERS experiments were performed with monometallic and bimetallic (Ag and Cu) colloids and a laser excitation wavelength of 633 nm. They concluded that Raman microscopy coupled with the SERS technique was an effective tool for identification, differentiation, and quantification of algae and bacterial biofilms grown on titanium surfaces.

Chao and Zhang [27] reported the chemical variations in the matrix of biofilms at different growth phases of Gram-negative and Gram-positive bacteria (*E. coli*, *Pseudomonas putida*, and *Bacillus subtilis*) by means of the SERS technique with silver colloids prepared by reduction of silver nitrate with hydroxylamine and a laser excitation wavelength of 633 nm (see Fig. 8). Significant chemical variations for different phases of biofilm growth were found. The lipid, nucleic acid, and protein content in the biofilm increased significantly during cultivation. The peaks of the SERS spectra were sorted into different macromolecular classes (including carbohydrates, proteins, nucleic acid, and lipid clusters) and appearance probabilities for the different phases were calculated.

This approach seems more robust than studies where only one specific peak was considered. This is especially true for SERS analysis, since the acquisition of reproducible spectra remains a main challenge. Additionally, AFM images showed the even adhesion of the cells on the substratum at 0 h, the formation of larger colonies after 8 h, and a layer of mature biofilm with a large amount of EPS after 72 h of growth.

Another approach to monitor molecular changes during biofilm formation was reported by Efeoglu and Culha [28, 29], who used silver nanoparticles coated with chitosan. The silver substrates were placed directly on the biofilm during formation, and this allowed in situ characterization and discrimination of *E. coli* and *Staphylococcus cohnii*.

In the water industry, membranes are often used to improve water quality. Since biofilm adhesion on membranes and therefore biofouling is a major problem in the operation of such membrane systems, the development of biofilms and the development of membrane cleaning processes have to be investigated. Chen et al. [42] used SERS to monitor the development of a dual-species biofilm formed by *Brevundimonas diminuta* and *Staphylococcus aureus*. Gold
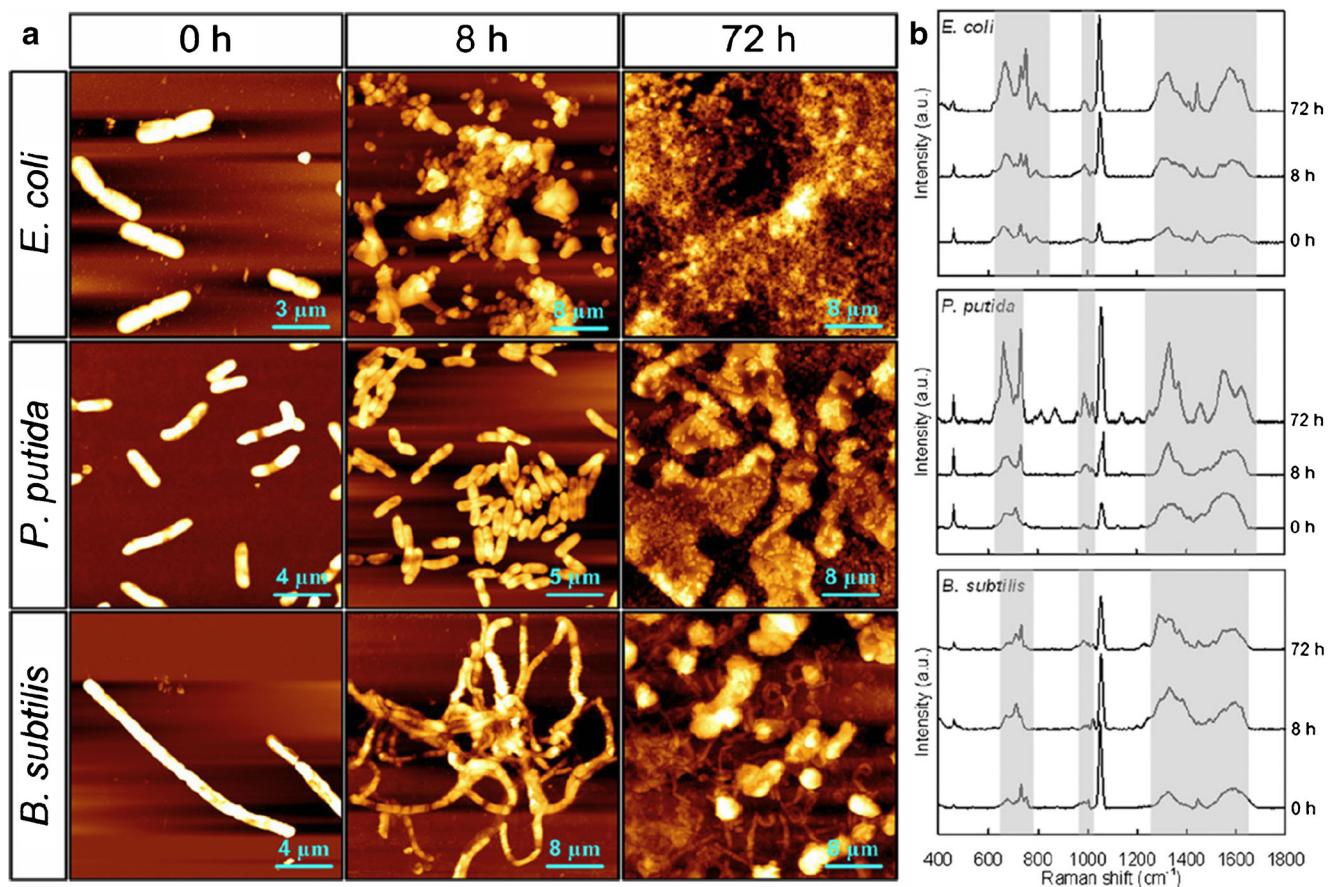

Fig. 8 Chemical variations in the biofilm matrix at different growth phases in a combined atomic force microscopy and SERS approach. **a** Atomic force microscopy height images showing the morphology of *Escherichia coli*, *Pseudomonas putida*, and *Bacillus subtilis* biofilms after 0 , 8 h, and 72 h cultivation. **b** Average SERS spectra of these biofilms ($\mathbf{n} = 40$–50). The sharp peak at around 1055 cm^{-1} might originate from amine bond vibrations of the silver nanoparticles used [27]. *Shadow regions* indicate the variation of peaks in different phases during biofilm formation. (Adapted from [27], copyright Springer 2012)

nanoparticles produced by reduction of chloroauric acid with trisodium citrate and a laser wavelength of 633 nm were used. Especially the ability of SERS spectroscopy to monitor the dynamic evolution of the dominant species in dual-species biofilms and the biomass changes was demonstrated. Also the different behaviors of bacteria in monocultured and dual-cultured biofilms were investigated and compared. Another study by the same group [43] focused on the chemical variations during both biofouling and cleaning processes by using layer-by-layer SERS. This technique makes it possible to obtain information on the surface composition of biofilms directly exposed to cleaning reagents. Detailed biochemical information about the composition of the biofilm, specifically the increase or decrease of certain Raman bands (e.g., proteins, phenylalanine, and polysaccharides) during biofilm formation and cleaning processes was given. Kögler et al. [51] described a novel concept for a compact real-time SERS online sensing approach for detection of biofouling in drinking water membrane filtration. They specially developed a gold nanoparticle SERS-sensing area on filter membranes to detect low concentrations of surface foulants in real time immediately after inoculation of an ultrapure water reservoir in conditions mimicking cross-flow membrane filtration. The SERS-sensing area developed was shown to be suitable as a SERS sensor in an online process with high water flux and pressure, although the fluorescence background and the influence of the SERS-sensing area on the membrane filtration efficiency have to be investigated further.

Because of the combination of SERS and resonance effects, the intensity of analytes (e.g., cytochromes) can be significantly enhanced. By exploiting the resonant effect of outer membrane cytochromes by using a laser wavelength of 413 nm, Millo et al. [52] studied a catalytically active *G. sulfurreducens* biofilm grown on silver electrodes by surface-enhanced resonance Raman spectroscopy. They demonstrated that coordinated heme cytochrome redox couples are involved in the direct electron transfer between the bacteria and the electrode.

Furthermore, when Raman analysis with a spatial resolution down to 20 nm is desirable, tip-enhanced Raman spectroscopy (TERS) can be applied. TERS is derived from SERS spectroscopy and uses a sharp metal or metal-coated tip positioned in the optical near field of the sample instead of classic SERS substrates. TERS is a noninvasive technique that does not require any labeling and provides detailed chemical and spatial information with a resolution below the diffraction limit. It was shown that TERS can give insight into the surface structures and help to follow the dynamics of the polysaccharide and peptide components on bacterial cells [113–115]. Schmid et al. [116] explored the feasibility of TERS for the study of complex biological matrices using a laser wavelength of 532 nm. Alginate was chosen as a sample because this biological macromolecule is a good model for the architecture of an EPS matrix at the

nanometer scale. Although the interpretation of the spectra can be complicated, especially because of contaminants on the tip and numerous interaction sites between the analyte and the tip, it should be possible in the future to analyze the distribution of different biopolymers inside a biofilm matrix. Until now all biofilm-related TERS studies were performed in an aeriferous environment. Use of the aqueous environment in future studies of biofilms is highly desired but can be very challenging because of potential contamination of the tip during measurements [115].

Thus, SERS microspectroscopy is a powerful and established method to overcome sensitivity limitations in Raman analysis and reveals new and more detailed chemical information on biofilms than classic Raman microspectroscopy. Only the rather complex operating procedures, with the difficulty in choosing the appropriate SERS substrates and optimizing measurement parameters to achieve reproducible data, have hindered the application of this approach for routine analyses.

Nonlinear Raman scattering microspectroscopy of biofilms

For the most part, the growing number of nonlinear Raman techniques remain in the realm of physical chemists or chemical physicists [117]. However, some methods (i.e., CARS and SRS microspectroscopy) are making progress as analytical tools for biological targets such as cells and living organisms [118]. In CARS microspectroscopy, two incident laser beams, the pump and the Stokes beam, which interact with the sample via a nonlinear, four-wave mixing processes, are used. When the beat frequency between these two beams is resonant with a Raman-active molecular vibration, the mode is coherently driven by the incident beam and the anti-Stokes signal is observed. In SRS microspectroscopy, like in CARS microspectroscopy, two incident beams are used. When the difference frequency matches a molecular vibration, a stimulated excitation process occurs, which results in intensity changes of the incident beams [67].

Mostly, nonlinear Raman scattering techniques are used for a wide variety of biomedical applications, such as chemically sensitive bioimaging [119, 120], imaging of nucleic acids [121], imaging of newly synthesized proteins [122], and detection of single bacterial endospores [123]. Recently, Hong et al. [69] demonstrated the applicability of CARS imaging for the in situ detection of single bacterial cells in a complex environment (milk and urine). However, the application for biofilm analysis is only at an experimental stage and has not yet been studied in detail. Also, major drawbacks exist. In CARS the nonresonant background limits the detection sensitivity and complicates image interpretation. Furthermore, the nonlinear scaling of the signal intensity interferes with robust

quantitative analysis. In SRS the narrowband implementation limits the specificity. This makes the differentiation of biochemical species with similar Raman spectra difficult. Although various methods to mitigate the influence of these effects exist, they typically require expensive and complicated experimental setups and complicated data analysis. Therefore, both CARS and SRS microspectroscopy have high potential, but their applicability is mostly limited by the practical implementation [67].

Raman microspectroscopy and stable-isotope labeling

In environmental and medical microbiology it is often not enough to identify and characterize microorganisms, and their ecophysiology and metabolic functions must be investigated in situ at the single-cell level [124]. Although MS methods, which provide very sensitive analysis of bulk samples, and techniques with a spatial resolution down to 50 nm (nanoscale SIMS) are available [125], these methods require complicated and time-consuming sample preparation and are destructive. Therefore, in the last decade Raman microspectroscopy was combined with use of stable isotopes to extend the capabilities of this method [126]. The pioneering study, which showed the Raman band shifts in isotope-labeled bacterial cells, was reported by Huang et al. [127] in 2004. They used *P. fluorescens*, which was grown in media containing different ratios of ^{12}C-glucose and ^{13}C-glucose as the sole carbon source. Clear redshifts of many different peaks, assigned to proteins, phenylalanine, and nucleic acids, were found in the spectra of the bacteria. Furthermore, with increasing incorporation of ^{13}C into the cells, the intensity of the redshifted bands (e.g., the phenylalanine band shifted from 1003 cm^{-1} in ^{12}C-cultivated bacteria to 966 cm^{-1} in bacteria grown in 100% ^{13}C-glucose) was more pronounced. However, the redshift for the bands of proteins, nucleic acids, lipids, etc., causes changes in the fingerprint spectra of the labeled cells, which makes the in situ identification of the bacterial cells rather complicated, especially in approaches with multiple microorganisms (which can be unlabeled, partially labeled, or completely labeled). Therefore, the combination of Raman microspectroscopy with in situ identification methods was the next logical step. Huang et al. [57] performed the first study that described such a system. They combined Raman microspectroscopy with FISH to simultaneously identify cells and determine the ^{13}C incorporation into the biomass. The labeling dyes used for FISH measurements showed no significant interference with the Raman signals and required only a short bleaching step to get rid of the remaining dye signals. An almost linear correlation between the known ^{13}C content of the cultivated microorganism and the phenylalanine peak ratio was described. With the help of this correlation, the detected

shift of phenylalanine could be used to determine the total ^{13}C incorporation into a single cell quite reliably. Only a minimum labeling of 10% ^{13}C content was required to discriminate between labeled and unlabeled cells. Instead of a direct identification with FISH, also molecular- or culture-based techniques can be used if the cells of interest are isolated before identification. Huang et al. [128] demonstrated this approach by combining optical trapping, using laser tweezers, with stable-isotope Raman microspectroscopy. An artificial mixture of ^{13}C-labeled and unlabeled bacteria was produced to explore the capability of isolating the different cells. While cells were trapped by the laser, a Raman spectrum was acquired. Selected cells were then moved to a capillary tube to separate them. These isolated cells were identified by incubation or genome amplification. However, only a small part of the sorted cells could be identified successfully, mainly due to cell misfunction, cell death, or the physical misplacement of the individual cells for the identification process. The approach of isolation and identification was further investigated by Wang et al. [129] using laser-induced forward transfer combined with Raman microspectroscopy at the subsecond level. This system allowed the identification, differentiation, location, and isolation of single ^{13}C- and ^{15}N-labeled cells in a complex microbial community. By combining Raman microspectroscopy and FTIR spectroscopy, Muhamadali et al. [130] demonstrated that it is possible to quantitatively analyze and differentiate *E. coli* cells that are simultaneously labeled with different ratios of both ^{13}C and ^{15}N at the community level. Data were analyzed by PCA and principal component–discriminant function analysis, which resulted in unique clustering patterns from specific spectral shifts on the incorporation of different isotopes for cells collected at the stationary phase. Partial least-squares regression was used for quantitative prediction of the ratio of ^{13}C and ^{15}N in cells. Furthermore, the single-cell Raman results allowed the separation of *E. coli* cells labeled with different isotopes and multiple isotope levels of carbon and nitrogen.

Raman microspectroscopy was applied to test whether *Protochlamydia amoebophila* takes up phenylalanine from its amoeba host during intracellular growth. Since *P. amoebophila* is unable to synthesize phenylalanine by itself, ^{13}C-labeled phenylalanine could be used directly as a tracer substrate [131]. Noothalapati and Shigeto [132] demonstrated the ability of mixed-stable-isotope-labeled Raman microspectroscopy in combination with multivariate curve resolution analysis of a marker band to study the carbon flow during ergosterol biosynthesis in yeast in vivo. Their multivariate spectral data analysis approach revealed the intrinsic spectra and relative abundances of all permissible isotopologues of ergosterol whose carbon atoms in the 5,7-diene moiety of the sterol skeleton are either partly or fully replaced by ^{13}C.

Usually a reduced analysis time is very favorable and often even vital for characterization of natural microbial communities. Therefore, rapid resonance Raman microspectroscopy in combination with use of stable isotopes was applied to investigate the carbon dioxide fixation by single cells by Li et al. [81]. This study demonstrated that by use of carotenoids as internal biomarkers, CO_2-fixing cells could be discriminated and imaged rapidly. It was also possible to quantify the CO_2 fixation of the photosynthetic microorganisms down to 10% ^{13}C content. By using this information, Li et al. demonstrated in another study the Raman-activated cell counting of photosynthetic microorganisms containing carotenoid on a microfluidic chip. A known mixture of ^{12}C- and ^{13}C-cultivated microorganisms could be determined correctly by this method [133].

Kubryk et al. [134] recently explored the potential of stable-isotope Raman microspectroscopy with resonance Raman spectroscopy and especially SERS for the rapid analysis of different microorganisms. For resonance Raman measurements *Geobacter metallireducen*s cells, containing cytochrome *c*, were cultivated with ^{13}C-labeled or ^2H-labeled substrate and compared with cells containing the natural abundance of those isotopes, revealing that a differentiation between those cells is clearly possible. *E. coli* cultivated with ^{12}C- glucose or ^{13}C-glucose was used as a model organism for SERS analysis with in situ synthesized silver nanoparticles as the SERS medium and a laser wavelength of 633 nm. A reproducible redshift of an adenine-related marker band in the SERS spectra for ^{13}C-labeled cells was observed. This study showed a successful combination of stable isotopes and SERS for

characterization of single microbial cells (see Fig. 9). The latest research on stable-isotope labeling [101] was focused on the origins of the SERS spectra of microorganisms. Usually whole bacterial cells exhibit fairly consistent SERS spectra; however the interpretation of the vibrational features of bacteria at the molecular level is difficult, since various vibrational bands of different organic compounds are anticipated to contribute to the SERS spectra. Especially ^{15}N isotopic vibrational frequency shifts were exploited here to identify purine bases as the major origin of SERS spectra when a laser wavelength of 633 nm was used. Recently Premasiri et al. [102], who studied several microorganisms with known differences in the metabolism pathway of purine, confirmed this finding.

Single-cell analysis of environmental samples is always a challenging task. For example, the combined approach of stable-isotope labeling followed by single-cell analysis has had limited application presumably because of the dispersal of microbial cells in a large background of particles. Eichorst et al. [135] developed a workflow for the efficient application of nanoscale SIMS and Raman microspectroscopy to soil microorganisms to assess the potential and limitations of stable-isotope incubation experiments of soils in combination with these methods. In proof-of-concept soil microcosm experiments, it was possible to detect ^{13}C and ^{15}N incorporation into the microorganisms by nanoscale SIMS and the incorporation of ^{13}C and deuterium into the microorganisms by Raman microspectroscopy.

Berry et al. [136] cultivated bacterial cells with portions of heavy water (D_2O) to identify and sort metabolically active
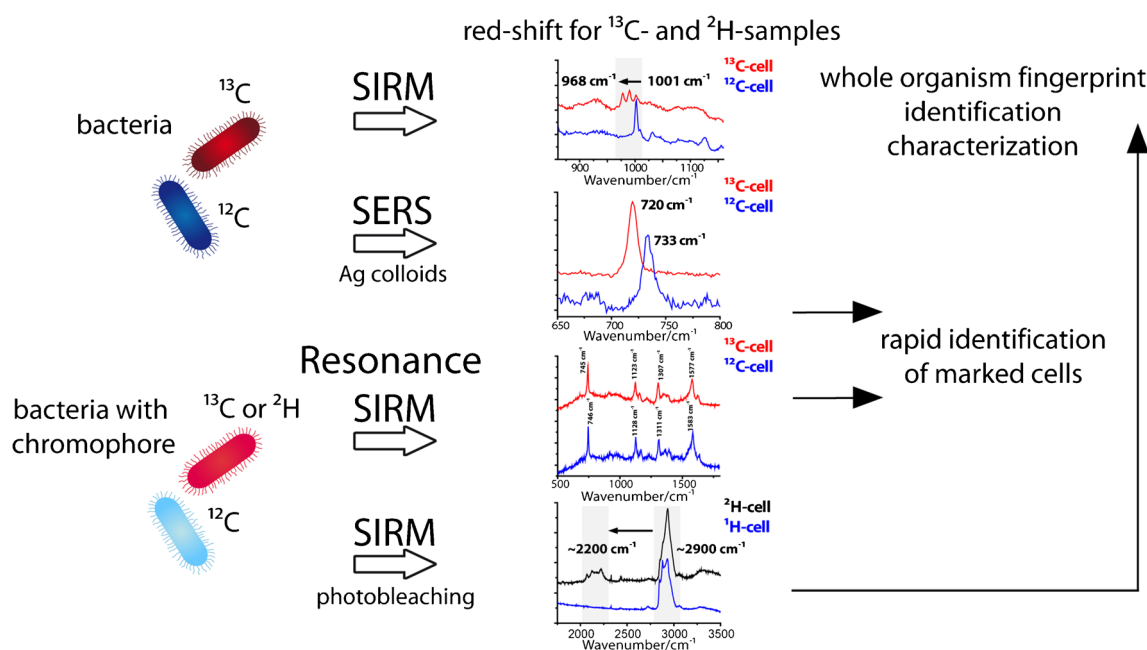

Fig. 9 Different methods for nondestructive quantitative and spatially resolved analysis of incorporation of ^{13}C and ^2H isotope-labeled compounds into microbial biomass. *SIRM* stable-isotope Raman microspectroscopy. (Reprinted from reference [134], copyright 2015 American Chemical Society)

cells without further sample preparation. *E. coli*, *B. subtilis* and *Bacillus thuringiensis* were analyzed. Cells exhibiting a strong C–D band were separated from the others and identified via genome amplification. This study shows that the incorporation of deuterium into the biomass of complex microbial communities allows the identification of individual metabolically active bacterial cells by Raman microspectroscopy.

Wang et al. [137] applied stable-isotope Raman microspectroscopy with ^{13}C-reverse and D_2O co-labeling to monitor substrate metabolism, cell activity, and their interactions of a two-species community consisting of *Acinetobacter baylyi* and *E. coli*. Stable-isotope Raman microspectroscopy with ^{13}C-reverse labeling revealed the carbon flow, and D_2O labeling was used to unravel the energy flow and general metabolic activity. This reverse labeling approach is especially useful to study the metabolic activity when isotopically labeled substrates are unavailable or expensive. Their results show that *E. coli* alone in minimal medium with citrate as the sole carbon source had no metabolic activity but became metabolically active in the presence of *A. baylyi*.

At the moment there are only a limited number of studies exploring mainly the possibilities of Raman microspectroscopy in combination with stable-isotope labeling (see Fig. 10). In the future, stable-isotope Raman microspectroscopy may be a promising method, especially in environmental and medical microbiology, that could complement other molecular biology techniques already available for the investigation of metabolic processes in bacteria and

biofilms in situ and in vivo at the single-cell level. Furthermore, it may be a powerful tool to nondestructively detect which microorganisms "eat what, where and when" [138] and hence to analyze and visualize (two-dimensional and three-dimensional) accumulation and degradation processes and to identify the communities responsible. Because of already existing methods to enhance the Raman signal—namely, resonance Raman scattering and SERS, even rapid and fast analyses are possible.

Data analysis

Raman spectra of microbiological samples contain Raman signals from the (macro)molecules within the laser focus and are therefore usually quite complex. Since Raman spectroscopy is based on the molecular vibrations of chemical bonds of proteins, carbohydrates, nucleic acids, lipids, etc., present in all microbiological samples, the spectra obtained are often quite similar. This makes the data interpretation difficult [139]. Therefore, besides the development of suitable sample preparation and Raman techniques, appropriate data processing methods are often necessary to get a better understanding of the chemical composition of biofilm samples [140]. In this context the importance of statistical/chemometric approaches for the reliable identification of microorganisms has to be underlined [77, 141]. The proper application of these techniques is restricted, however, to the case where all side effects

Fig. 10 Overview of present (with corresponding references) and future research topics for Raman microspectroscopy, resonance Raman microspectroscopy, and SERS in combination with stable-isotope labeling for cells and biofilms
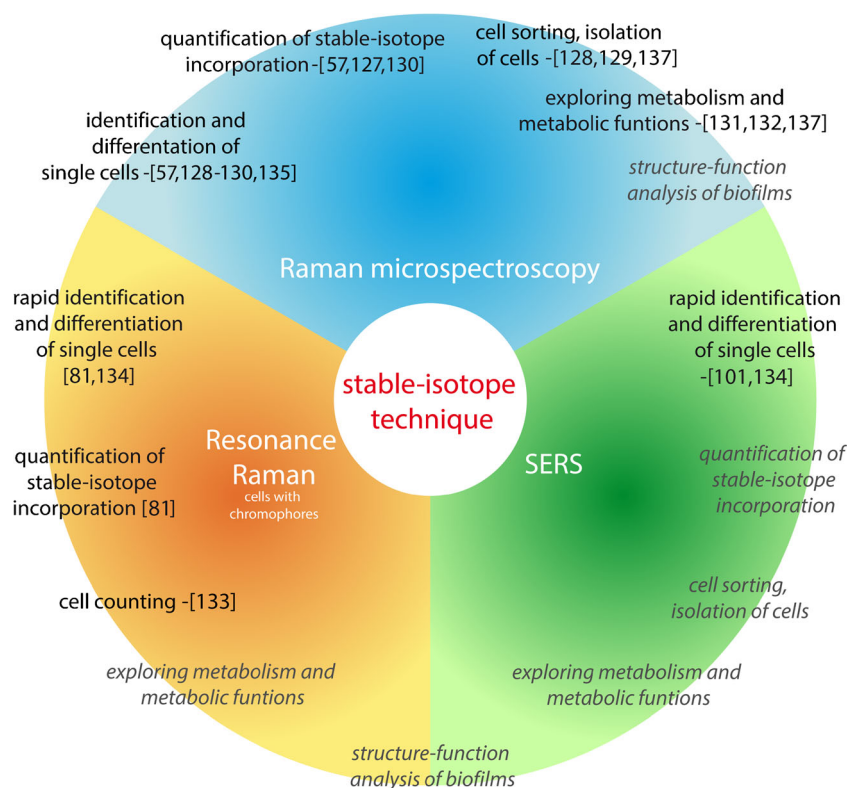
(e.g., fluorescence, background noise) that might influence the Raman spectra are separated from the Raman signals from the samples. Therefore, to exploit the unique potential of Raman spectroscopy for the characterization of microbiological samples, a method for choosing the best preprocessing together with chemometrics for studying microbiological samples has to be used [142].

In microbiological analysis two basic chemometric approaches are usually applied. The first is based on unsupervised classification methods, where no a priori knowledge of the sample is necessary. Examples include PCA [143] and hierarchical cluster analysis [144]. Diagnostic accuracies of around 97% are possible under optimal conditions. However, with these unsupervised methods it can be difficult or even impossible to solve classification problems for complex microbiological systems, such as biofilms, especially when only very subtle changes in the Raman spectra account for the effect studied. To solve this problem a second group of chemometric techniques (i.e., supervised multivariate methods) can be used. These, however, require a priori knowledge and therefore model training of the sample studied. With a set of well-characterized samples, a model can be trained so that it can properly predict the identity of unknown microbial samples [140]. Examples of supervised methods include support vector machines [71] and linear discriminant analysis [145]. An overall accuracy of more than 90% is possible with these methods under controlled conditions [77, 141].

Conclusions and outlook

In recent years the development and application of different techniques for biofilm analysis have led to improved understanding of these complex microbial aggregates. Yet much work remains, since biofilms are a very challenging microbiological system. Biofilms show a high (chemical) complexity and spatial heterogeneity. Significant variations in the biofilm composition and structure depend on (1) the microbial cells present and their metabolic activity, (2) the available nutrients and the environmental conditions, and (3) the biofilm development stage. For reliable analysis, these parameters have to be taken into account. The characterization of multispecies biofilms is especially challenging since various microbial populations coexist. Therefore, it is important to find techniques that allow a detailed analysis of biofilm composition (microorganisms and EPS) and even the two-dimensional and three-dimensional structure. With this knowledge a direct link to biofilm functions can be achieved, which leads to better understanding of these very complex and challenging samples.

Extensive efforts have been made with Raman microspectroscopy, resonance Raman microspectroscopy, SERS microspectroscopy, and techniques based on Raman microspectroscopy in combination with stable-isotope labeling. With the help of multivariate data analysis, more information from even very complex Raman spectra can be extracted. To strengthen these approaches in the future some requirements and challenges have to be considered: (1) increased understanding of the data obtained; (2) improvement of the reproducibility of the analysis (especially for SERS microspectroscopy); (3) construction, improvement, and extension of databases for spectral signatures including microbial and EPS constituents of biofilms; (4) increased sensitivity due to technical development (e.g. more sensitive CCD cameras), application of resonance Raman and SERS microspectroscopy; (5) optimization of the analysis for increased throughput; and (6) reduction of analysis time and costs.

It can be concluded that Raman microspectroscopy has a high potential for biofilm characterization in different fields (e.g., medical and environmental microbiology). In particular, the increase in the Raman microspectroscopy sensitivity through the resonance effect, SERS, and the combination of Raman microspectroscopy with stable-isotope labeling will allow improved analysis of complex biofilm matrices and improved understanding of a high variety of biofilm properties. Furthermore, the application of the stable-isotope technique to directly detect, qualify, and quantify isotopic labeling (for one or several isotopes) at the level of single cells and hence the analysis of the metabolic activity and metabolic functions of microorganisms in situ and in vivo in biofilms is an emerging topic. Finally, the combination with other methods for visualization and physicochemical characterization will lead to a comprehensive understanding of complex and heterogeneous biofilm matrices with high spatial and temporal resolution.

Compliance with ethical standards

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

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