Emerging Spectral Microscopy Techniques and Applications to Biofilm Detection

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This chapter reviews three spectral microscopy techniques based on Raman scattering, infrared absorption, and magnetic resonance spectroscopies. We include a brief review of the technical and scientific concepts related to general spectroscopy and imaging, followed by a more detailed look at the three spectral modes and their ability to operate as imaging technologies. We then demonstrate the use of spectral microscopy to study biofilms due to their current scientific relevance and their complexity, which makes them challenging to study by traditional microscopy. Specifically, we review the use of spectral microscopy for chemical and structural evaluation of the biofilm extracellular polymeric substance (EPS); the distribution, metabolites and biochemical composition of bacteria within the EPS; as well as physiochemical properties, such as diffusivity and uptake of foreign molecules.

Keywords: Spectral microscopy; imaging; infrared; Raman; magnetic resonance; biofilms

1. Introduction

Traditional microscopy has been essential in the advancement of a wide range of science and technology. However, the demand for high information density imaging is growing in order to accelerate discoveries. This need is particularly acute in materials sciences and microbiology, where development and understanding of structure-function relationships requires both morphological and chemical characterization at the same time. Thanks to the range of spectroscopic modes available, spectral microscopy can do this, and more. Spectroscopy is a general analytical approach that involves analyzing electromagnetic (EM) fields after their interaction with samples to provide profound insights into analyte chemical and physical properties without the need for sample preparation or the addition of foreign probes materials. Moreover, spectroscopy is generally passive, enabling consecutive, real-time measurements of systems as they evolve in time, without disturbance. Spectral microscopy expands the utility of standard spectroscopic tools to enable collection of spatially-resolved spectra, thereby filling the information gap that exists in pure microscopy. Techniques include Raman, infrared and magnetic resonance spectral imaging [1, 2] have been used as tools for studying biomedical systems [3-5], microfluidic environments [6], chemical reaction mapping and others. The purpose of this chapter is to review these three different techniques and demonstrate their potential for biofilm detection and characterization.

This chapter starts with a general review of concepts in spectroscopy such as absorption, scattering and electromagnetic (EM) fields, as well as a word about imaging and measurement dimensionality. This is followed by a review of Raman, infrared and magnetic resonance spectral modes and their use as imaging tools. The chapter concludes with a comprehensive review of spectral microscopy applied to biofilms. The main goal of this work is to provide current research results to benefit research on biofilms and other biological systems. Our goal is to achieving high impact by providing educational concepts, which should benefit members of the imaging community who are unfamiliar with spectral microscopy as well as students in materials sciences, microbiology and analytical chemistry.

2. Technical and scientific concepts

2.1 Spectroscopy: Absorption vs. scattering

Spectroscopy involves analyzing electromagnetic (EM) fields after their interaction with the sample. We briefly discuss fundamental concepts in spectroscopy: absorption and scattering.

2.1.1 Absorption

Absorption is the mechanism whereby energy from EM fields is transformed into internal energy within an absorbing material via direct excitation of an energy level transition of particular, molecular vibrations, rotations, electronic states or nuclear states. As we will briefly discuss, usually EM energy is supplied by radiating photons, but not always. An absorption spectrum shows attenuation of light intensity at certain wavelengths, which correspond to specific energy level transitions that are available within the absorber. Since each analyte has its own unique absorption spectrum, spectral microscopy can be used to identify different absorbers at the molecular and atomic level and visualize their distribution in space. Moreover, the amount of the material in the detection volume can be quantified based on the Beer-
Lambert law, which correlates absorbance with concentration, the path length of the light through the material and the materials’ inherent absorptivity as a function of wavelength:

\[ A = \alpha(\lambda) \cdot c \cdot l \]  
\[ A = -\log(I_T/I_0) \]  

Where \( A \) is the unitless absorptivity, \( \alpha(\lambda) \) (L·mol\(^{-1}\)·cm\(^{-1}\)) is the wavelength dependent molar absorption coefficient and \( c \) (mol·L\(^{-1}\)) is the concentration of the absorber which exists within the absorption path length \( l \) (m). In (2), \( I_0 \) and \( I_T \) are the intensities of light before and after interaction with the sample, respectively (Figure 1a). Therefore, in practice one determines the absorption by measuring \( I_0 \) and \( I_T \), which, for a known \( l \) and \( \alpha \), enables measurement of concentration of an analyte. If \( \alpha \) is not known, then a calibration curve is usually made that relates \( A \) to a series of known concentrations in an absorption cell with known \( l \) via (1).

\[ \alpha = \frac{-\log(I_T/I_0)}{lc} \]  

2.1.2 Scattering

Light scattering is the temporary absorption of a photon, followed by its near immediate re-radiation, causing deflection from its initial direction. Scattering can be elastic (Rayleigh) or inelastic (Raman). Scattering can contribute to light attenuation if light is scattered away from the detector (Figure 1b). Therefore, it is important to account for this process when measuring absorbance to ensure that scattered light is not misinterpreted as absorbed light. In Raman spectroscopy, inelastic scattered light is analyzed because changes to photon energy (and wavelength) reveals information about energy exchanged with vibrational modes in the analyte. Since the elastically (Rayleigh) scattered photons contains no spectral information and they are orders of magnitude stronger than Raman scattering, these photons must be filtered out. The remaining (Raman) photons are then collected and analyzed with a sensitive detector.

2.2 EM fields: Far-field vs. near-field

The EM fields are either in the form of radiating photons (far-field) or non-radiating (near-field). Both can be used for spectral microscopy.

2.2.1 Far-field

The majority of spectral microscopy techniques use radiating photons. In this case, the spatial resolution is determined by the diffraction limit:

\[ d = \frac{\lambda}{2\pi n \sin \theta} \]  

where \( \lambda \) is the photon wavelength, \( n \) is the index of refraction of the medium in which the collector lens is working, and \( \theta \) is the half angle of the cone of light that can enter the light collector. The term \( n \sin \theta \) is known as the numerical aperture, which quantifies the resolving power of an objective lens. Clearly, \( \lambda \)'s in the infrared will have lower spatial resolution (higher \( d \)) compared to those in the UV-vis. For microscope systems, the objective lens is often immersed in an optical oil with high \( n \) to reduce \( d \). Otherwise a diameter large lens can be used in order to increase \( \theta \). Another consideration when imaging in the far-field is the possibility of complete signal attenuation due to absorption and/or scattering. This places limits on solvents and support apparatus to those which minimally interact with the probe photons, in comparison to the analytes themselves. As well, the often complicated instrument optical paths can require costly optics to minimize internal light loss.
2.2.2 Near-field

In near-field modes, sensing occurs within an EM field that is not caused by radiating photons. Three types of near-field techniques include surface plasmon enhanced spectral microscopy via (i) surface enhanced Raman spectroscopy (SERS) and (ii) tip enhanced Raman spectroscopy (TERS). Another technique we consider here is (iii) evanescent fields in attenuated total reflection (ATR) infrared spectroscopy. In all cases, the diffraction limit is avoided, however, as we will discuss in Section 3, spatial resolution for (i) and (iii) are limited by other factors.

2.3 Imaging and measurement and dimensionality

We distinguish between the measurement dimensionality, which is the inherent dimensionality of the spectroscopic tool being used, and image dimensionality, which is the final dimensionality of the image after repeated measurements or data processing. In either case, we have: zero-dimensional (point measurements/imaging), one-dimensional (linear measurements/imaging), two-dimensional (area measurements/imaging) or three-dimensional (volume measurements/imaging). Figure 3 shows idealized spectral images of a bacterium imaged with different dimensionality.

Higher dimensionality spectral images are often constructed from lower order measurements by stitching multiple measurements together. An example is the creation of 1- and 2-, and 3-dimensional Raman images. In this case, images are routinely acquired by so-called “point-by-point imaging”, or “rastering”. That is, images are created by stitching together many individual zero-dimensional measurements into lines, planes or volumes, respectively. In Section 3, we discuss three spectral microscopy techniques, Raman, infrared and magnetic resonance. This discussion includes basic principles regarding the underlying spectroscopic mode and various variations, their inherent measurement dimensionality, and other practical matters related to their implementation. Section 3 is followed by Section 4, where we see these spectral microscopy tools in action for measurements of biofilms.

3. Three spectral microscopy techniques

The imaging dimensionality depends on the type of spectroscopy used. As we will discuss, they range from images comprised of many zero-dimensional measurements such as in Raman, to inherently three-dimensional imaging via magnetic resonance imaging, which uses structured magnetic fields in three dimensions to generate volumes images. Understanding a spectral technique, informs us about its measurement dimensionality and analytical niche.

3.1 Raman spectroscopy

As discussed in Section 2.1.2 Raman scattering is inelastic. The frequency of the scattered light is shifted down or up into Stokes and anti-Stokes bands, respectively. Raman spectral microscopy is a vibrational technique because the energy exchange occurs between the EM field and vibrational modes of the analyte. The Stokes and anti-Stokes bands are shifted by the same frequency (in different directions), but the Stokes shifts are more intense at ambient temperatures. Therefore, the Stokes bands are usually analyzed. In addition, low energy vibrations result in stronger scattering than higher energy ones. The result is that it is easier to image vibrations such as those involving single bonds in comparison to double or triple order bonds; weak bonds involving electrophilic atoms (i.e. metals); those bonds featuring heavy atoms; etc. Raman scattering is approximately $10^7$ times less likely to occur than Rayleigh scattering, therefore optical filters must be used to eliminate the unwanted photons from entering the detector. This also means that Raman sensitivity is low, and methods for increasing its intensity are important. Raman scattering intensity scales with the exciting laser frequency to the 4th power. For example, the Raman signal with a blue laser (ca. 490 nm) vs. a red laser (ca. 630 nm) are calculated to be approximately 3 times greater. As discussed below, there are other techniques that can improve the sensitivity of Raman, including SERS, TERS, and resonance Raman.

3.1.2 Raman microscopy

Raman spectroscopy acquires data from a fixed volume at a location defined by the positioning of the laser excitation beam. Therefore, it is a zero-dimensional measurement technique. Despite this low measurement dimensionality, Raman can be used for imaging by a point-by-point approach, where the probing volume can move by displacing the
excitation laser relative to the sample. This is usually achieved by moving the sample under a stationary laser beam usually with the use of an automated x,y mechanical stage. Drawbacks to this approach include motion-induced disturbances to the sample and the limited accuracy in positioning and repositioning. In some cases Raman spectrometers have confocal capabilities, enabling measurements along the z-direction.

3.1.3 Surface enhanced Raman spectroscopy

Surface enhanced Raman spectroscopy (SERS) relies on enhanced field strength a few nanometers near the surface of a metal nanoparticle and can result in increases to scattering efficiency by orders of magnitude higher. The enhancement is the effect of surface plasmons that develop on the nanoparticle upon absorbing photons with appropriate frequency. The plasmonic frequency of metal nanoparticles can be precisely tuned by manipulating their size. Microscopy using SERS leads to faster data acquisition times, which is important because Raman microscopy is already relatively slow due to long integration times and the necessity of multipoint measurements to obtain an image. It is important to note that while the interaction between the analyte and the EM-field occurs in the near-field, the diffraction limitation still determines the accuracy with which the laser spot can be focused on the sample.

3.1.4 Tip-enhanced Raman spectroscopy

Tip-enhanced Raman spectroscopy (TERS) is an imaging approach that combines the benefits of SERS sensitivity with spatial accuracy of AFM. The technique uses a sharp metal or metal-coated AFM needle which supports plasmonic resonance under laser irradiation. The AFM system can be accurately positioned, enabling point-by-point measurements with spatial resolution at the nanometer-scale [7]. The added benefit of this imaging system is that AFM images can be collected at the same time to obtain topographical maps. One drawback includes long data acquisition times, particularly for large images with high spatial resolution. Another is the need to have the sample accessible to the moving AFM needle. This eliminates closed systems, such as flow cells often used for cell cultivation.

3.2.1 Infrared spectroscopy

The infrared spectrum consists of three different regions including near-infrared (NIR) region (13000 to 4000 cm⁻¹); mid-infrared (MIR) region (4000 to 400 cm⁻¹) and far-infrared region (400 to 33 cm⁻¹). The mid-infrared is rich with well-resolved spectral bands pertaining to different molecular vibrational. It is therefore, the most used for spectral microscopy. Fourier transform IR (FTIR) is the most popular method because of a range of benefits, such as ability of simultaneous collection of information from all wavelengths, mechanical simplicity, high spectral resolution, fewer interferences and more sensitive detectors.

3.2.2 Infrared transmission and ATR microscopy

Infrared microscopy is usually conducted by an IR microscope in transmission mode or by ATR (Figures 6a and 6b, respectively). Transfection is another mode sometimes used. In each case, a glow bar and a focal plane array are generally the most popular choices for source and detector, respectively. In some cases, the infrared light produced from a synchrotron is used as the source light, which brings highly focusability, enabling the spot size to approach the diffraction limit (1-10 µm), and high light intensity. Synchrotron radiation is often used for hydrated biofilms, which would completely attenuate weaker sources. If the sample is not strongly absorbing, a defocused synchrotron beam can irradiate a relatively large portion of the sample, while maintaining enough signal intensity at each element in a focal plane array detector to generate good image without rastering. Microscopy via ATR mode occurs within a standing EM field (called an evanescent field), which is trapped between the surface of a high index of refraction (n) internal reflection element (IRE) and the (low n) sensing environment. The intensity of these non-radiating evanescent fields for
IR wavelengths is largely found within several microns from the IRE surface when the sensing environment is aqueous. This enables sapling in water and other highly absorbing environments. Typically, the IRE is made from crystalline material such as diamond or germanium with dimensions ranging from centimeters to a fraction of a millimeter. A single, small IRE can be used to sequentially make a series of zero dimensional measurements by displacing it relative to the sample, but the spatial resolution and placement accuracy are low. More often, imaging is achieved by coupling the light emerging from the IRE with a one- or two-dimensional focal plane array detector. Depth profiling can also be conducted by changing the angle of incidence of the photons relative to the sample/IRE interface, thereby enabling point-by-point measurements in the z-direction as well. It is important to note that spatial resolution using evanescent fields for imaging is not bound by the far-field diffraction limit. However, practically, it is hard to take advantage of this fact, because other components in the imaging system, such as the detector elements in a focal plane array detector, are usually the bottleneck for spatial resolution. Drawbacks include spectral interference from the IRE material itself. Table 1 shows IRE specifications for different materials.

Table 1  IRE specifications by material

<table>
<thead>
<tr>
<th>Crystal Plate</th>
<th>Spectral Cut-off (cm⁻¹)</th>
<th>Refractive Index *</th>
<th>Depth of Penetration **</th>
<th>pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diamond/ZnSe</td>
<td>&lt; 525</td>
<td>2.4</td>
<td>2.00</td>
<td>1–14</td>
</tr>
<tr>
<td>Ge</td>
<td>&lt; 575</td>
<td>4.0</td>
<td>0.66</td>
<td>1–14</td>
</tr>
<tr>
<td>Si</td>
<td>8900–1500, 475–40</td>
<td>3.4</td>
<td>0.85</td>
<td>1-12</td>
</tr>
<tr>
<td>ZnSe</td>
<td>&lt; 520</td>
<td>2.4</td>
<td>2.00</td>
<td>5–9</td>
</tr>
</tbody>
</table>

* at 1000cm⁻¹, ** for incident angle of 45 degrees

3.3 Magnetic resonance spectroscopy

Magnetic resonance (MR) is the basis of nuclear magnetic resonance (NMR) spectroscopy and its imaging counterparts, magnetic resonance imaging (MRI) and chemical shift imaging (CSI). NMR relies on the net magnetization of a sample in an external magnetic field (B₀) and the manipulation of the sample’s net magnetic vector in a second field, B₁. The B₁ field is applied by an EM coil transmitting in the radio frequency (RF), which selects certain nuclei based on their precessional frequency about B₀. This frequency is known as the Larmor frequency, given by ω₀ = γB₀, where γ is the gyromagnetic ratio, which is unique for each nucleus. Following the application of B₁, the RF coil is switched to receiver mode and the precession of the sample’s rotating net magnetization for the selected nuclei induces an AC voltage inside the coil by Faraday induction. Relaxation measurements monitor the return to equilibrium after application of B₁ to study molecular dynamics, liquid viscosity, surface wettability and material porosity. Chemical shift measurements monitor small shifts inω₀ due variances in the local magnetic field strength, due to shielding from nearby electron spins. MR techniques are highly versatile. First, EM fields are highly penetrating, meaning that MR is not limited to optically transparent samples or sample holders. Second, MR can be applied to a range of nuclei including ¹H, ¹³C, ¹⁹F, ²³Na, ³¹P, ³⁵Cl, ¹²⁹Xe. ¹H nuclei, and to a lesser degree ¹³C, are most widely due to their natural abundance and good sensitivity. More exotic nuclei can be used to track liquid flow and differentiate from background H₂O.

3.3.1 Microscopy with MRI and CSI

Magnetic resonance imaging (MRI) and chemical shift imaging (CSI) give physical and chemical images, respectively. Usually they are conducted with the same instrumentation. They are typically found in clinical diagnostic settings, serving as safer and more information intensive techniques than X-ray imaging, but non-clinical applications are increasingly appearing [8]. In MRI the external magnetic field has a gradient in space rather than the uniform B₀ field used in NMR. This defines a position-dependent magnetic field, B₀(x,y,z), and the resulting in spatial encoding via different Larmor frequencies ω₀(x,y,z) = γB₀(x,y,z). In CSI, a gradient magnetic field is not used as this would mix the spatial and chemical shift information, both of which affect the Larmor frequency. Instead, the spatial information is encoded using spin phase as a function of location in space, φ(x,y,z). Since this is a time-consuming process it is normal to increase the voxel size and/or reduce the image dimensionality, relative to MRI, to maintain good signal to noise and acceptable acquisition time.
### 3.3.2 Signal to noise considerations in MR

The signal-to-noise for MR varies as,

\[ S/N \propto \frac{V_s N_0 \eta^2}{\sqrt{T}} \]

where \( V_s \) is the sample volume; \( N \) is the number of spins per unit volume; \( \eta \) is the filling factor (the fraction of the RF coil volume that is occupied by the sample) and \( T \) is the temperature. Signal-to-noise is most sensitive to \( \omega_0 \) and \( \eta \), and optimization should start with these variables. Since \( \omega_0 \) is increased for large magnetic fields and for low mass nuclei, it is recommended that micro MR imaging techniques use \(^1\)H under a strong magnetic field. The filling factor should be maximized by reducing the size of the RF coil to match the dimension of the sample. Since \( S/N \) varies with \( T^{-1/2} \), sample heating by induction resistance in the RF should be minimized \( \text{via} \) active cooling. If analyzing nuclei other than \(^1\)H, isotopically enriched samples can be used to increase \( N \).

### 4. Spectral microscopy of biofilms

Biofilms consist of microbes living within a protective extracellular polymeric matrix (EPS). Enhanced microbial survival rates within the EPS, make biofilms among the most abundant form microbial life form on Earth. In addition to biologists and ecologists, the medical community is also very interested in biofilms due to their adverse effect on human health when infecting implanted devices [9, 10]. Increasingly, research into biofilms is expanding beyond these areas, into potential technological applications, including green chemical synthesis, environmental remediation and energy production [10-12]. However, it is recognized that studies of biofilms for technological purposes are still in their infancy, largely because they are complex living materials with heterogeneous properties that change in space and time, making them both interesting and challenging to study analytically [13, 14].

#### 4.1 Chemical compatibilities

**Raman and IR**: Raman and IR are good methods to sense typical biofilm-relevant molecules such as proteins, polysaccharides, metabolites and different hydrogen bonding states of water [15-17]. In particular, vibration-based spectroscopic microscopy is useful for imaging biofilm hydration, \( \text{CH}_2 \) and \( \text{CH}_3 \) in lipids, phosphate groups in lipids and phospholipids, carboxylic acid in certain polysaccharides and amide bands from proteins (Table 2). Biofilm maturity is often tracked \( \text{via} \) polysaccharide to protein ratios [18]. Multivariate statistical analysis can help find correlations in complex data sets [19]. Raman has an advantage that it is not incompatible with aqueous systems, so it can be used in hydrated samples. Infrared, on the other hand should either be used on dehydrated samples or biofilms can be grown on IRE surfaces. However, the low penetration depth for IRE can also be a drawback if portions of the biofilm are outside of the detection volume. For example, active bacteria are usually at the outer surface of the biofilm, far from the anchoring surface where sensing occurs. Still, ATR-IR offers the opportunity to detect the growth and biochemical analysis of films in real-time [20, 21]. Therefore, we anticipate the use of ATR-IR microscopy for real-time studies of early stages of biofilm development including initial bacterial attachment, the addition of biochemical conditioning films and early bacterial growth stages.

**Magnetic resonance**: MR of \(^1\)H and \(^{13}\)C spins in biofilms is typically utilized due to their prevalence in the biofilm hydrocarbon materials and cells. Chemical information \( \text{via} \) CSI can reveal cellular metabolites such as lactates, acetates, pyruvate, succinate, UDP-glucose, AMP, glutamate, and lysine [22]. It can also differentiate between planktonic and biofilm-bound bacterial, as well as those bacterial in different environments, such as nutrient rich vs. nutrient depleted zone, or active vs. dormant bacteria in different locations within the biofilm [22-24]. Mechanical properties can be determined by analysis of spectral line shapes and relaxation times of \(^{13}\)C [25]. Specific pulse sequences, separated by different time delays, can report on the diffusion of \(^1\)H in water molecules to differentiate between water molecules which range from hydrogen bonded/immobile within enclosed areas, and bulk water that flows through the biofilm [26].
<table>
<thead>
<tr>
<th>Raman Band (cm⁻¹)</th>
<th>IR Band (cm⁻¹)</th>
<th>Polysaccharide</th>
<th>Proteins</th>
<th>DNA/RNA</th>
<th>Carotenoids</th>
<th>Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>1750-1540</td>
<td>1760-1600</td>
<td>C-C, C-O</td>
<td>Amide I</td>
<td>-</td>
<td>-</td>
<td>C-C, C-O</td>
</tr>
<tr>
<td>1617-1600</td>
<td>1600-1585</td>
<td>Aromatic ring</td>
<td>C-C Tyr, Trp</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1607</td>
<td>1500-1400</td>
<td>-</td>
<td>C-C Try, Phe</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1580-1575</td>
<td>1550</td>
<td>-</td>
<td>Amide II, Trp</td>
<td>G, A ring</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1460-1314</td>
<td>1450-1398</td>
<td>CH₂, COO⁻, CH</td>
<td>CH₂, CH</td>
<td>-</td>
<td>-</td>
<td>CH₂, CH₂</td>
</tr>
<tr>
<td>1304-1200</td>
<td>1450,1250</td>
<td>-</td>
<td>Amide III</td>
<td>-</td>
<td>-</td>
<td>C-H, CH₂</td>
</tr>
<tr>
<td>1160-1125, 1095-1000</td>
<td>1152–900</td>
<td>C-C, C-O, C-O-C, C-O-H, CH</td>
<td>C-N, C-C, C-N</td>
<td>PO₂⁻</td>
<td>C-CH₃</td>
<td>C-C, PO₂⁻</td>
</tr>
</tbody>
</table>

* Tyr = tyrosine; Trp = tryptophan; Phe = phenylalanine; G = guanine; A = adenine.

4.2 Spectral microscopy of biofilms

4.2.1 Imaging the EPS

The EPS is a fundamental biofilm component, which modifies the physiochemical environment, enabling, among other things, cell-to-cell signaling, gene exchange and protection against detachment. The EPS can also trap extracellular enzymes and metabolites near to the cells, enabling an initial biodegradation stage to occur outside of the bacteria. This can help the bacteria obtain nutrients or survive in chemically challenging environments. In fact, the EPS is so fundamental, that a certain type of microbe living within it is physiologically distinct from the same type in the planktonic state. Biofilm EPS is made from extracellular macromolecules such as nucleic acids (including DNA and RNA), polysaccharides and proteins, which can be detected with spectral microscopy. In addition, studies of EPS distributions and physicochemical properties can improve our understanding of overall biofilm properties related to diffusivity, solubility and structural stability [27]. For example, Raman spectral microscopy on multispecies biofilms confirmed a strong correlation between polysaccharides bands in the range 1200-1000 cm⁻¹ (see Table 2) and the structural appearance from different spherical and filament features seen using optical and fluorescent microscopy.

Increased sensitivity can be achieved using SERS to generate high-resolution chemical maps of biofilm EPS by, for example, immersing the biofilm in a colloidal silver suspension followed by in situ measurements [28]. This method was used to rapidly acquire a SERS map (figure 7) with spatial resolution 3μm x 3μm for the visualization of the spatial distribution of COO⁻ stretches in polyanionic polysaccharides (1383 cm⁻¹) from polyanionic polysaccharides (c) and the amide III band at 1280 cm⁻¹ from proteins (d). Adapted with permission from (Ivleva NP, Wagner M, Szkola A, Horn H, Niessner R, Haisch C. Label-Free in Situ SERS Imaging of Biofilms. J. Phys. Chem. B 2010;114(31):10184-94). Copyright (2010) American Chemical Society.
4.2.2 Imaging of microbes and their metabolites within biofilms

In addition to the spatial distribution of the EPS, the distribution of bacteria within it is also critical. For example, spatial distributions of the most active bacteria can result from differential access to nutrients at different depths within the biofilm. The existence of so-called persister cells in biofilms, which are dormant bacteria, are important in medical research because their limited metabolic activity strongly limits their ingestion antimicrobial drugs. Bacteria can be detected and differentiated from the EPS background by their distinct spectral signature arising from either composite biomolecules or their metabolites, which are generated during growth or other biological functions.

An important class of biomolecules produced within bacteria are pigments called carotenoids. Carotenoids protect microbes in biofilms by absorbing damaging high-energy blue light. They also have an anti-oxidant role. They are found predominantly in photosynthetic microbes as light harvesting centers. Moreover, dietary carotenoids are being investigated as aiding in the prevention of macular degeneration and cancer. Carotenoids are split into two classes: oxygen containing (xanthophylls) and non-oxygen containing (carotenes). The use of TERS microscopy with simultaneous AFM imaging for the chemical detection of β-carotene bands enabled the differentiation of cyanobacteria cell clusters and isolated cells from EPS [29]. As seen in figure 8a, the TERS spectra of one segment of a biofilm sample was dominated by C-C, C-H and C=O vibrational bands from β-carotene (1521 cm$^{-1}$ and 1160 cm$^{-1}$, respectively). A third band for the Si-Si stretching at 520 cm$^{-1}$ could be seen, which originated from the silicon support materials from a silver-coated AFM tip. Strong signal enhancement from the 30 nm silver coating on the AFM tip allowed a short (8 sec.) acquisition time for each pixel with an impressive 10 nm lateral resolution.

Raman microscopy was demonstrated as a useful tool in studying biofilms containing the pigmented bacteria *Rhodococcus sp.* SD-74. This biofilm has promising applications in bioremediation, due to the ability to degrade alkanes in some strains. A study monitored the accumulation of carotenoid pigments inside the bacteria along with the simultaneous imaging of other biofilm components such as lipids, proteins and nucleic acids over 9 days [30]. Unlike with β-carotene in cyanobacteria biofilms previously discussed, carotenoids in this study were observed to be dispersed evenly in the biofilms as can be seen in figure 9, with no strong accumulation of “carotenoid pools”. In addition, a three-fold increase in carotenoid concentration was observed within one week, whereas the lipid and protein concentration stayed relatively constant. The amount of carotenoids was also observed to be more important for bacteria in biofilms than in planktonic state which could explain the enhanced persistence of biofilm microbes.

Another Raman microscopy study of biofilm formation used the spectral bands from carotenoids and EPS constituents to monitor biofilm growth for wild *Cronobacter sakazakii*, a pathogen, as well as different mutant strains [31]. The wild strain showed higher biofilm production than for the mutant strain for the same growth conditions. In addition the relative amount of EPS and carotenoid production in the biofilm was lower for the mutant species. Similar to *Rhodococcus sp.* SD-74 biofilms, this study confirmed that the carotenoids did not accumulate in pockets, but were evenly distributed on the surface of the biofilms, leading the authors to suggest that they are components of both the bacteria membranes as well as the EPS. The two works above demonstrate that the accumulation of high concentrations of carotenoids in biofilms can be a survival strategy to protect against harsh environmental conditions such as UV radiation, high temperatures, and the presence of reactive oxygen species.
Leucine is an amino acid metabolite that can be used to monitor protein synthesis by bacteria. *Escherichia coli* microcolonies in mature biofilms were identified by Raman spectral microscopy of the local accumulation of leucine via its 922 cm⁻¹ absorption band [32]. The Raman spectra of the biofilm outside of the colonies were substantially different, including a strong DNA peak, such as the one at 1097 cm⁻¹, which was used for chemical imaging of the EPS. The exact biological role the leucine pool is still unknown, but it is speculated that it can be involved in triggered biofilm disruption.

4.2.3 Interaction of biofilms and their environment

Given the role of biofilms in pathogenesis, there is strong push to understand their response to antibiotics. Synchrotron IR microscopy was used in transfectance mode to study the spatial distribution of antibiotic uptake of an in open microfluidic channels, under laminar flow conditions [33]. The channels were open to avoid interaction of the synchrotron light with device wall material and the channels were shallow (10 µm) to prevent complete attenuation of the transflected synchrotron beam. The antibiotic was mitomycin-C (MMC), which targets DNA in *Escherichia coli* bacteria. Mitomycin-C did not interact with extracellular DNA in the EPS because it only becomes activated within the bacteria. After exposure for eight hours, strong MMC-DNA adducts were observed, in areas closer to the source (beginning of the channel) and in areas with strong protein amide III bands (largely localized in bacteria). A correlation between the reduction in strong amide III regions and the increase of DNA-MMC adduct signal indicated MMC uptake by the bacteria and their death due to MMC exposure. Interestingly, growth of amide III continued in some regions, suggesting that biofilm structure resulted in internal flow diversions, localizing MMC to some regions and not others. Additionally, bacterial diversification could have played a role in their survival in some locations within the biofilm, via metabolic changes that limited effects of MMC toxicity and/or migration to regions of low MMC concentration. The authors also studied the evolution of the EPS composition during biofilm development, which monitored biofilm-associated biomacromolecules during the first 100 minutes of growth. It was observed that after a 30 minute lag time, the quantities of polysaccharides, glycoproteins, DNA/RNA and bacterial amide III sequentially underwent exponential increases, each approximately 10 minutes following the previous, before reaching a stationary phase.

Synchrotron IR spectral microscopy, was also used to study the uptake of sulphur into biofilms from underground sulfidic aquifers [34]. These biofilms contained both bacteria and archaea, the latter being single cell microbes with no nucleus or organelles. Regions of higher S=O stretches in sulphate (1240 cm⁻¹) were observed due to uptake by the biofilm. In order to determine the significance of the sulphate accumulation zones, visualization of archaea and bacteria was undertaken by the spectral microscopy of the C-H stretch band in lipids (2960–2850 cm⁻¹) and C-O-C and C-O ring vibrations in polysaccharides (1200–900 cm⁻¹), which are different for each microorganism. A correlation between bacteria locations and higher sulfate band intensity demonstrated that sulphate uptake by bacteria was significant compared to archaea.

There is great potential to use biofilms for bioremediation, particularly of hazardous sites, thanks to the resistance from harsh environments provided by the protective EPS. Utilization of SERS for the study of bioremediation of chromate, sulfate and nitrate from *Shewanella oneidensis* biofilms was undertaken using gold nanoislands which were produced *in situ* within bacteria after they were exposed to a PBS buffer containing HAuCl₄ [35]. The authors simultaneously determined the spatial distribution of chromate, sulfate and nitrate using their spectral signature. Bioreduction of Cr(VI) into chromate was also monitored. This allowed studying the inhibiting effect of sulfate and

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*Fig. 9* Micrograph *Rhodococcus* sp. SD-74 biofilms after 1, 3, 5, 7 and 9 days of growth (a). Raman spectral images showing the distribution of carotenoids (b-d), proteins and lipids (e and f), and nucleic acids (g). Image size is 16 x 16 µm². Same colour scale applies to the Raman images in each column. Reprinted from (Zheng YT, Toyofuku M, Nomura N, Shigeto S. Correlation of Carotenoid Accumulation with Aggregation and Biofilm Development in Rhodococcus sp SD-74. Anal Chem. 2013;85(15):7295-301). Copyright (2013) American Chemical Society.
nitrate on Cr(VI) uptake and the bacterial induced enzymatic chemical reduction to Cr(III). As sulfate and Cr(VI) competed for entrance in the cells via sulfate transport system, less Cr(VI) and its reduction product, Cr(III) were observed in cells when sulfate concentration was higher. As nitrate inhibits the reduction of Cr(IV), higher concentration of nitrate led to a decrease of Cr(III) in cells, but had no effect on Cr(IV) uptake. Bioremediation of Cr(VI) has also been studied using MRI. Beauregard et al. used this technique to study the reduction of Cr(VI) to Cr(III) using a Serratia sp. biofilm containing in situ reduced Pd(0) catalyst [36]. As Cr(III) complexes are paramagnetic, they have a concentration dependent T1. In addition, Cr(VI) and Cr(OH)3 are diamagnetic, so their T1 relaxation times were concentration independent. Imaging of the concentration of the species inside the biofilm-palladium catalyst allowed identification of heterogeneities in the in the system. This would allow monitoring and guided optimization of these systems.

A new class of flow-templating microfluidic bioreactors are being developed to control and pattern biofilm growth for more accurate analytical studies and future technological applications [37]. Special SERS surfaces were imbedded into the microchannels to enable one-dimensional chemical images of the template nutrient solution, enabling visualization of the template stream dimensions, local concentration gradients and eventually, the effect of biofilms on them. [38]

4.2.4 Diffusion through biofilm

Diffusive mass-transfer slows substrate penetration into, and breakdown products out of, the biofilm and is therefore, responsible for large concentration gradients. In effect, diffusion is one of the most important factors from the perspective of growth kinetics, metabolic activity, catalysis and resistance to toxins and anti-microbial drugs. A major contributor to the diffusion is the biofilm porosity and density. In one study, Raman microscopy was used to determine the structure of a Streptococcus mutans biofilm in water by mapping the ratio of EPS/water concentrations, via CH and OH spectral intensity, respectively [39]. Different criteria were established to characterize the biofilms. For example, the mean CH/OH ratio was used to quantify the total biomass density of the sampled area. Since biofilms are heterogenous, defining porosity is important. For this purpose, the authors considered as voids, pixels where the density was under 17% of the maximal value for the entire area. By dividing the number of pixels corresponding to voids by the total number of pixels in the map, the areal porosity was determined. The authors also studied the diffusion of HOD and polyethylene glycol (10 kDa) through biofilms. They observed that HOD was able to diffuse everywhere in biofilm, whereas the penetration of polyethylene glycol was limited and depended on the local biomass density.

Chemical shift imaging (CSI) was used to make concentration measurements of solutes at different depths within the biofilm to study diffusion and consumption of lactate and fumarate in Shewanella oneidensis biofilms [40]. The analytes included lactate and fumarate and their breakdown products, succinate and acetate. The biofilm was grown under hydrodynamic flow in anaerobic conditions, where fumarate was the electron acceptor and lactate as electron donor and carbon source. Simultaneous MRI of the biofilm allowed determination of the three-dimensional structure of the biofilm, in addition to probe water diffusion in the vicinity of the biofilm (Figure 10a). A one-dimensional CSI measurement was recorded in order to determine steady-state depth-dependent concentration profiles (Figure 10b). The authors observed that lactate was depleted near the biofilm/fluid boundary indicating that lactate is rapidly oxidized by cells near the top layer of the biofilm. The dual CSI/MRI was also utilized to study the effect of the uranium-containing contaminant UO2Cl2 on the metabolism of Shewanella oneidensis biofilms [41]. Using depth-resolved CSI measurements, the authors observed a sharp decrease in the consumption of lactate upon exposure to UO2Cl2. While the consumption of lactate is normally higher on top of the biofilm, with UO2Cl2 exposure, the lactate consumption was observed to be higher at the bottom of the biofilm. In addition, in these conditions, no acetate was produced even if acetate is the normal break-down product of lactate. This suggests that lactate was used as a carbon source to enhance the EPS production as a response to the UO2Cl2 exposure. These studies emphasized the importance of dual systems like MRI/CSI to study electroactive biofilms in applications such as microbial fuel cells, where bacteria on top of the biofilm have a better access to the nutrient solution than those which are closer the electrode.

As biofilms grow, their effect on liquid flow in reactors becomes important. As the biofilms are porous, water can go through them, but is slowed. Using MRI to study Shewanella oneidensis biofilms, it was observed that the effective diffusion coefficient for water gradually decreased from the top to the bottom of the biofilm due to changes to depth-dependent morphological changes. [42]
Fig. 10  Water-selective MRI and depth-resolved CSI measurements for a *Shewanella oneidensis* biofilm constantly fed in a flow reactor with lactate as an electron donor and fumarate as an electron acceptor. (a) Shows a water-selective MRI image in the x-z plane. The biofilm is directly under the bottom coverslip. (b) Depth-resolved MRSI spectra of the biofilm. Each row in the spectral image correspond to the NMR spectra of a 2500 x 2500 x 31.2 µm³, the sample being scanned along the z axis. The color scale for the spectral image goes from green to red, with the latter corresponding to a higher intensity. The dashed line crossing the two parts of the figure marks the biofilm-fluid interface. Adapted by permission from Macmillan Publishers Ltd: ISME Journal (McLean JS, Ona ON, Majors PD. Correlated biofilm imaging, transport and metabolism measurements via combined nuclear magnetic resonance and confocal microscopy. ISME J 2008;2(2):121-31). Copyright (2008)

5. Conclusion

In this chapter, Raman, infrared and magnetic resonance spectral microscopy was reviewed and their application to the study of biofilm was extensively reviewed. We extensively reviewed exciting studies using spectral microscopy for chemical and structural evaluation of the biofilm extracellular polymeric substance (EPS); the distribution, metabolites and biochemical composition of bacteria within the EPS; as well as physiochemical properties, such as diffusivity and uptake of foreign molecules. The different techniques and applications presented are already having a positive impact on the advancement of knowledge on biofilms and other areas of microbiology. However, with the recent explosion of interest in spectral microscopy, we expect a rapid expansion of these studies in the future.

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