Detection Techniques for Extracellular Polymeric Substances in Biofilms: A Review

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Extracellular polymeric substances (EPS) are one of the main components of biofilm, prompting biofilm to form a cohesive three-dimensional framework. Numerous methods are available to help characterize the properties and the structural, chemical and physical organizations of EPS during the biofilm formation process. This review highlights key techniques from different disciplines that have been successfully applied *in-situ* and non-destructively to describe the complex composition and distribution of EPS in biofilm, especially microscopic, spectroscopic, and the combination of multi-disciplinary methods that can provide new insights into the complex structure/function correlations in biofilms. Among them, confocal laser scanning microscopy (CLSM) is emphasized, and its principles, applications, advantages, and limitations are summarized. Multidisciplinary techniques have been developed and recommended to study EPS during the biofilm formation process, providing more in-depth insights into the composition and spatial distributions of EPS, so as to improve our understanding of the role EPS plays in biofilms ultimately.

Keywords: Biofilm; Extracellular polymeric substance; Detection technique; Multidisciplinary methods

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INTRODUCTION

As a dominant microbial lifestyle, biofilms are structured, highly dynamic communities of sessile microorganisms formed by cells embedded in a matrix of extracellular polymeric substances (EPS) produced by them (Watnick and Kolter 2000; Battin *et al.* 2007). They can occur at nearly all interfaces (solid–liquid, solid–air, liquid–liquid, and liquid–air) (Ivleva *et al.* 2010). Among of them, growing appreciation of the importance of biofilms occurring at solid–liquid interface (such as stream and marine biofilm), has recently led to the recognition of an urgent need for an ecological theory that can contribute to our understanding of them (Battin *et al.* 2016). In nature, they usually may be in the form of microbial mats as well as river sediment biofilms, aquifer, soil biofilms, or plant roots and foliage biofilms. In industrial systems, biofilms may be present as biofouling layers. In medicine systems, biofilms are an important issue on tissues as well as on biomaterials including invasive devices and implants (McDougald *et al.* 2012; Neu *et al*. 2015). Accordingly, Karunakaran *et al*. (2011) evolved the related studies of biofilm into an independent discipline. Biofilm has also been called "City of Microbes," when Watnick compared it with a human city (Watnick and Kolter 2000). Then, the EPS matrix was hailed as the "House of Biofilm Cells" by Flemming *et al.* (2007), which can be attributed to the scaffold of the three-dimensional (3D) polymer network that accounts for more than 90% of biofilms (Ivleva *et al.* 2010; Kavita *et al.* 2013). The EPS is exported

from the intracellular space, to form an extracellular polymeric matrix (Battin *et al.* 2016). In fact, in an immobilized but dynamic microbial environment (Sutherland 2001b), EPS mediate the transition from reversible to irreversible adhesion of single cells, consequently forming a cohesive, 3D polymer network that interconnects and transiently immobilizes biofilm cells. EPS are also validated in the degradation and sorption of organic and inorganic compounds (Pal and Paul 2008) and barrier system of cells resistant to hostile environments, and serve as sources of carbon and energy for biofilm growth (Wu *et al.* 2012).

Knowledge of the structure and functional properties of EPS is crucial for understanding the role of biofilms. Even though carbohydrates and proteins have been validated as the main components of EPS, the biochemical characteristics of these compounds remain obscure because of their complex structures and unique linkages (Jiao *et al.* 2010). Moreover, defining the composition of EPS is critical for the elucidation of structure–function relationships that can facilitate the development of chemical strategies to disrupt biofilms. Battin *et al.* (2007) summarized some new paths to biofilm research and concluded that the present is the best time for biofilm research. Accordingly, numerous analytical techniques have been advanced to help characterize the components and spatial distribution of EPS in biofilms. Currently, microscopic and spectroscopic techniques, which are devoted to the isolation and characterization of EPS from different systems, are the most widely used. Furthermore, an increasing number of researchers have devoted efforts to a comprehensive study of the mechanism of EPS interaction, resulting in a fixed structure and specific functional properties of biofilm. New approaches that are needed to convert biofilm descriptors into quantitative and qualitative parameters of chemical and molecular compositions require both morphological and chemical characterizations. Some studies have attained a more comprehensive understanding of biofilms by implementing different combinations of techniques (Wagner *et al.* 2009; Yu *et al.* 2011; Paquet-Mercier *et al.* 2014). The aim of this review is to present a summary of recommended analytical technologies which help to acquire a better understanding of the complexity and structural, chemical and physical organizations of EPS. The advantages and limitations of such technologies are also presented. The investigation of EPS is beneficial to the implementation of methods that are appropriate to analyze. Gradually, the application of improved analytical methods will expand on our current, perhaps incomplete view of what biofilm structures really are and the extent to which they are affected by EPS. This review also highlights future areas of study, emphasizing the potential of further inter-disciplinary research.

DEFINITION, CHARACTERISTICS, AND SPATIAL DISTRIBUTION OF EPS

EPS are situated at or around the bacterial cell surface and are often regarded as glycocalyx or slime, which facilitate and accelerate bacterial adherence to the substratum. EPS mostly contain bacterial secretions, shedding of materials from the cell surface, cell lysates and hydrolysates, and the adsorption of organic constituents from the survival environment (Sheng and Yu 2006; Pal and Paul 2008). EPS are a complex mixture of biomolecules (proteins, polysaccharides, nucleic acids, lipids, and other macromolecules) that are secreted by microorganisms and that hold microbial aggregates together (Wingender *et al.* 1999; Stewart and Franklin 2008). Proteins and exopolysaccharides represent the key components of macromolecules, accounting for 40% to 95% of EPS

(Karunakaran and Biggs 2010). However, the composition and quantity of EPS vary depending on the type of microorganisms (Kavita *et al.* 2013), age of the biofilms (Zhang *et al.* 2010), and environmental conditions under which the biofilms exist (Vu *et al*. 2009; Wagner *et al*. 2009; Villeneuve *et al*. 2011) and constantly mediate the adhesive and cohesive properties of the biofilms during biofilm formation. For instance, it has been shown that the highest productivity of EPS is observed during the early stages of biofilm formation (Zhang *et al*. 2010). Generally, the production of EPS is significantly increased under so-called adverse conditions. For example, Jiao *et al*. (2010) found that substantially higher carbohydrate-to-protein ratios were observed for the acidophilic microbial biofilms than the previously reported ratios. And more than twice as much EPS was derived from a mature biofilm as from a mid-developmental-stage biofilm (approximately 340 and 150 mg of EPS per g [dry weight] for a mature biofilm and a mid-developmental-stage biofilm, respectively). Thus, EPS production can to some extent reflect the physiological state of the biofilms (Sabater *et al*. 2007).

Exopolysaccharides are high-molecular polymers with molecular masses of 500 to 2000 kDa (Sutherland 2001a; Denkhaus *et al*. 2007). Microbial exopolysaccharides are long molecules that are either linear or branched (Flemming and Wingender 2010). They are either homopolysaccharides or heteropolysaccharides (Czaczyk and Myszka 2007); most are heteropolysaccharides. They are responsible for both adhesive and cohesive interactions (Ahimou *et al*. 2007a) and play a key role in maintaining the structural integrity of biofilms (Sutherland 2001a; Chen and Stewart 2002; Denkhaus *et al*. 2007; Wang *et al*. 2014); thus, they have been termed "adhesive polymers."

Another main component of EPS, protein, is primarily classified into two types: enzymatic proteins and structural proteins. Enzymatic proteins have a significant role in metabolism and are even considered to function as an efficient external digestive system (Flemming and Wingender 2001, 2010). Proteins have also been shown to contribute to the anionic properties of EPS and even act as the electron donor or acceptor in redox reactions in biofilms. The negative charge of proteins is ascribed to the presence of diacid amino acids, such as aspartic acid (Denkhaus *et al*. 2007). Some studies have established that structural proteins determine the process of microbial attachment to different solid surfaces. Karunakaran *et al*. (2010), for example, suggested that attractive electrostatic forces between charged proteins in EPS could impart cohesive stability to the biofilm matrix. Similarly, Ahimou *et al*. (2007b) found that the calcium absorption of biofilms has a considerable effect on the cohesive energy of the EPS matrix, which may be attributed to the anionic properties of protein. Some scholars have even shown that the predominance of protein compositions rather than polysaccharides leads to greater biofilm stability (Sheng *et al*. 2010). Proteins are of great nutritional value and directly participate in the chemical processes essential to life.

The high diversity of polysaccharide and protein components in the biofilm matrix is an emerging theme. Zhang and Bishop (2003) suggested that EPS polysaccharides can be utilized faster than EPS proteins if microorganisms are in a starved state. Chen *et al*. (2013) reported that the higher yield of EPS would promote the biofilm growth. Future studies will have to probe deeper into the molecular mechanisms that regulate the synthesis of the matrix (Branda *et al*. 2005).

The distributions of various EPS components are also heterogeneous. According to their spatial distribution, EPS can be subdivided into soluble EPS (weakly bound with cells or dissolved into the solution) and bound EPS (closely bound with cells) (Nielsen *et al*. 1999; Barranguet *et al*. 2004; Sheng *et al*. 2010). Furthermore, bound EPS have been

shown to be a dynamic double-layered EPS structure that includes loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS) (Poxon and Darby 1997; Yu *et al*. 2009; Chen *et al.* 2013).

TB-EPS surround cells and are closely integrated with cell walls, whereas LB-EPS are distributed outside TB-EPS and have a loose structure and low density (Yu *et al*. 2009; Zhang *et al*. 2010). LB-EPS are sensitive to the environment, and such sensitivity is considered a protective response of bacteria under fluctuating conditions (Zhang *et al*. 2010). The response actually occurs in a coordinated fashion using cell-to-cell signaling known as quorum sensing (Vu *et al*. 2009; Shrout and Nerenberg 2012). The contents of LB-EPS and TB-EPS influence the bioflocculation, settleability, and de-waterability of sludge. Thus, most of the studies concerning LB-EPS and TB-EPS have focused on their characteristics in activated sludge. However, their contents in biofilms directly affect the migration and transformation of nutrients and pollutants; thus, further study is needed on the differences in their combination with nutrients and pollutants (Kang *et al*. 2009), which will help us to track the bioremediation process in biofilms and its role in biofilm biology.

EXTRACTION AND DETECTION TECHNOLOGIES FOR EPS

The components, quantity, and function of EPS vary considerably, which further affects the structure and function of biofilm. Thus, an in-depth study of EPS is imperative. However, the *in-situ* chemical analysis of EPS components remains a challenge because the different types of polymers cannot be analyzed using a simple and straightforward analytical approach. Accordingly, improved methods and techniques are continually being developed. These methods and techniques are generally classified into two types: nondestructive *in-situ* techniques for monitoring time-resolved biofilm EPS accumulation, and techniques that analyze the EPS extracted from disrupted biofilms (Karunakaran *et al.* 2011). A summary of the advantages and limitations of both types of techniques are presented in the following sections to clarify when these methods are recommended.

EPS Extraction and Chemical Analysis Methods

Extraction methods

Extraction as a simple and feasible sample pre-treatment technique has been employed for the quantification of EPS in biofilm. A number of methods have been developed and applied to extract EPS from biofilms.

Methods of extracting EPS are important in the study of the physicochemical properties of EPS and their impact on contaminants in aquatic environments. The extraction of EPS from biofilms can be realized by employing appropriate physical or chemical extraction methods or their combinations. Physical extraction methods, such as low- and high-speed centrifugation, ultrasonication, steaming extraction, and heat treatment have often been applied to biofilms as well as activated sludge. Chemical extraction methods include the use of ethylene diamine tetraacetic acid (EDTA), cation exchange resins (CER) (Romaní *et al*. 2008), NaOH, and NaCl. However, a universal EPS isolation method is not yet available, and the extraction yield, composition, and physicochemical properties of EPS vary significantly with different extraction methods.

The efficiency of these methods is based on numerous factors, such as cell lysis, extraction yield, extraction specificity, and the chemical residuum from the extraction solution to the EPS extracts. The greatest problem with extracting EPS occurs when

methods are too harsh, where intracellular materials are released into the extract (Flemming and Wingender 2010). Hence, this aspect is typically validated (or not) depending upon the confidence given by a measure of cell-lysis. Both DNA and ATP measurements have previously been used as indicators of lysis (Takahashi *et al*. 2010). However, it has been recently acknowledged that DNA is an integral component of the EPS matrix itself (Cheng *et al*. 2011).

Some of the advantages and limitations of representative extraction techniques are presented in Table 1. Generally, more EPS were extracted using chemical methods than using physical methods; however, the chemicals used for extraction possibly react with EPS and therefore affect their structure (D'Abzac *et al*. 2010; Sheng *et al*. 2010). The optimal method should be selected carefully. Thus, the extraction procedure has to be adapted to the specific type of EPS under study. For example, for soluble EPS, centrifugation is most favored, whereas for bound EPS, various extraction methods have been developed. LB-EPS and TB-EPS may be extracted separately to study the compositions and functions of the two types of bound EPS in biofilms. In general, the original or modified CER method was still the most widely accepted EPS extraction method, because of its high efficiency and low cell lysis (D'Abzac *et al*. 2010).

The main approaches are presented in Table 1.

Table 1. Relevant Extraction Techniques for EPS in Biofilms and Their Respective Main Features

	Extraction Technique	Advantages	Limitations
Physical Methods	Heating	Effective (Pal and Paul 2008)	Induces hydrolysis of EPS, disrupts cells (Denkhaus *et al.* 2007; Pal and Paul 2008)
	Steaming	High protein yield, insignificant cell lysis (Zhang *et al.* 1999)	Releases significant quantities of hexose sugar (Brown and Lester 1980)
	Centrifugation	Effective, does not cause cell lysis	Low yield of EPS, little protein (Pan *et al.* 2010), ineffective for bound EPSs (Pal and Paul 2008)
	Ultrasonication	High protein yield (Pan *et al.* 2010)	Ineffective degradation of some components of EPS (Pan *et al.* 2010)
Chemical methods	EDTA	High EPS yield (Pal *et al.* 2008; Metzger *et al.* 2009)	Low protein and carbohydrate contents; extractant affects EPS composition (Pan *et al.* 2010)
	CER	Mild, effective, low cell mortality rate (Frolund *et al.* 1996; Karunakaran *et al.* 2011), higher protein content (D'Abzac *et al.* 2010)	Low nucleic acid contents (D'Abzac *et al.* 2010), difficult to identify the specific EPS fractions involved in metal bindings (Stewart *et al.* 2013).
Combined methods	Formaldehyde + NaOH	Effective, obtains more EPS (Metzger *et al.* 2009), low cell mortality rate	Not sensitive to polysaccharide contents, reacts with amine groups of proteins or amino sugars from EPS (D'Abzac *et al.* 2010)
	Centrifugation+ formaldehyde	Greater carbohydrate yield (Zhang *et al.* 1999)	Reacts with EPS molecules (Zhang *et al.* 1999)

The approaches listed in Table 1 have the following limitations: (i) the extraction techniques (*e.g.*, CER) appear to be unsuitable for very thin films (three- and six-day-old biofilms) because of the lack of sufficient biomass (Barranguet *et al*. 2004); and (ii) no consensus exists on EPS extraction techniques, and the complete extraction of all EPS components from a biofilm remain a challenge due to the intracellular contamination and the extracellular contamination (Pal and Paul 2008; Takahashi *et al.* 2010; Redmile-Gordon *et al.* 2014). Thus, extraction techniques should be normalized.

Chemical analysis methods

A number of methods, such as conventional ultraviolet-visible spectrophotometry, mass spectrometry, chromatography, and combinations thereof, as well as Fourier transform infrared spectroscopy (FTIR) and three-dimensional excitation–emission matrix fluorescence spectroscopy (3D-EEM), have been applied to characterize the EPS extracted from biofilms (Sheng and Yu 2006). The characterization of polysaccharides and proteins is performed because of their importance in biofilm formation and metabolic and regulatory activities.

The anthrone–sulfuric acid colorimetric method (Johnson and Fusaro 1966) and the phenol–sulfuric acid colorimetric method (DuBois *et al.* 1956) have been used for the determination of total polysaccharide contents extracted from biofilm. Chromatographic methods have been recognized as a vital technique for carbohydrate analysis (Denkhaus *et al.* 2007). High-performance liquid chromatography (Churms 1996) and combined gas chromatographic–mass spectrometry (GC–MS) (Domozych *et al.* 2005) have been used to qualitatively and quantitatively analyze monosaccharides intensively.

Extracted protein contents can be determined by the Lowry Foline-phenol method using bovine serum albumin as the standard (Lowry *et al*. 1951), which was modified continually (Redmile-Gordon *et al.* 2013). In many laboratories, the Bradford Coomassie brilliant blue dye method has become the recommended method for quantifying protein, mostly because it is simpler, faster, and more sensitive than the Lowry method (Bradford 1976). Moreover, the Bradford method introduces less interference by common reagents (Kruger 1994). However, if the protein content in an EPS sample is low, it is barely detected by the Bradford method. In such cases, 3D-EEM is a more sensitive method for detecting low contents of protein or protein-like substances (Pan *et al*. 2010). Furthermore, 3D-EEM can be used to distinguish fluorescent compounds that may exist in the complex EPS mixtures (Sheng and Yu 2006); however, because of its insensitivity to polysaccharides, the fluorescence signals of EPS are primarily attributable to proteins or humic substances (Laspidou and Rittmann 2002). Furthermore, environmental factors, such as solvent effect, solution pH value, and temperature, can affect the fluorescence intensity of the EPS examined. Her *et al*. (2003) suggested that future studies employing other analytical techniques, such as pyrolysis GC–MS, should compare the results against 3D-EEM results to fully confirm their hypotheses.

In-situ Characterization of Extracellular Polymeric Substances in Biofilm Systems

An optimal method should allow for real-time analysis and make the best possible reflection of real-process conditions of interest. In this review, the more popular approaches used to investigate the EPS of biofilms *in-situ* non-destructively are presented. Compared with the methods mentioned above, "*in-situ*" here means the characterization of EPS without extraction from biofilms and with no or limited other sample preparations.

However, the term "*in-situ*" is not intended to imply that the biofilm is in exactly the same condition as was originally found, especially in the case of biofilms occurring in river sediments, hull bottoms, and drinking water pipes. However, in some indoor or outdoor experiments, samples that occur at some specific materials, such as microscope slides (Proia *et al.* 2012), metallic substrates (Ivleva *et al*. 2010), or crystal surfaces (Bhargava 2012), can be directly observed by using the accordingly techniques. These approaches mostly originate from spectroscopy and microscopy, as well as combinative spectral microscopy techniques. Such materials each have their own advantages in the analysis of EPS. Spectroscopic techniques are well-established techniques for identifying functional groups in molecules. They are of outstanding importance for online, non-invasive biofilm monitoring, especially when coupled with for spectral calibration and pattern recognition (Reuben *et al.* 2014). Furthermore, spectroscopic techniques could be used to qualitatively and quantitatively analyze EPS compositions. In contrast, microscopic techniques, coupled with image analysis, are especially advantageous in extracting biofilm structural and architectural parameters (Barranguet *et al*. 2004). Spectral microscopy can be used to attain a global understanding of structure–function relationships by requiring both morphological and chemical characterizations simultaneously (Paquet-Mercier *et al*. 2014).

Spectroscopic Technologies

Several spectroscopic methods suitable for biofilm monitoring, including infrared (IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy, are outlined in this subsection.

Fourier transform infrared spectroscopy

FTIR spectroscopy is a popular nondestructive technique for monitoring time-resolved EPS variation (Karunakaran and Biggs 2010; Chen *et al.* 2013). This technique is used as a preliminary screening procedure to identify the nature of the EPS components. An IR spectrum provides a highly specific vibrational fingerprint of the sample under investigation. Infrared radiation is absorbed at frequencies at which the molecule can be promoted to an excited state. Spectral fingerprints are then obtained, with the contributions of the functional groups of all biochemical molecules in the sample combined. Samples must be dried before FTIR analysis because of the strong absorption of water in the mid-IR region (Reuben *et al.* 2014).

Nuclear magnetic resonance spectroscopy

NMR is a technique based on the absorption of radio frequencies in the presence of magnetic fields (Wolf *et al.* 2002). Slight variations in magnetic fields resulting from the electrons orbiting the nuclei induce a shift in energy level and appear as resonance signals, which is characteristic of the chemical bond of a given nucleus. The aforementioned chemical shift allows the chemical analysis and structure determination of large molecules (such as EPS). The ^{1}H nucleus (proton) is the most commonly used nucleus because of its high natural abundance and high MR sensitivity (Neu *et al.* 2010b).

Similar to FTIR, NMR spectroscopy is employed to generally distinguish and identify the types of chemical functionalities in biofilm samples, *e.g.*, carbonyls, peptide bonds, and aromatics. NMR data provide the key quantitative parameters of the intact matrix, including the percentages of EPS components by mass. In order to provide more detailed characterization of the EPS functional groups, the exact chemical mechanism of metal binding should be revealed further (Jiao *et al.* 2010). To date, solid- and liquid-state

NMR techniques have been applied to study the chemical composition and molecular mobility of biofilm EPS. This technology was particularly motivated by the demand for the fundamental transformation of biofilm descriptors into quantitative parameters of chemical and molecular composition. McCrate *et al.* (2013) determined the chemical composition of a bacterial biofilm using solid-state NMR and biochemical analysis. Reichhardt and Cegelski (2013) implemented solid-state NMR to deliver quantitative insights into the composition and structure of biofilm systems. Jiao *et al.* (2010) applied solid-state NMR and linkage analysis to characterize the polysaccharide composition and yielded limited but promising information, such as, they found that solid-state NMR cannot distinguish between the β-O-4 and β-O-3 linkages of glycosidic carbon atoms. Thus, a more in-depth analysis of purified EPS fractions is needed to illuminate the structures molecular distribution of polymers (Reichhardt *et al.* 2015).

The advantages of NMR are its noninvasive and nondestructive qualities. Its drawbacks, however, include its low signal-to-noise ratio (SNR) and time-consuming data acquisition (Wolf *et al*. 2002; Kirkland *et al*. 2015). Given that the energies of these transitions are low compared with the thermal fluctuations, there is only a small amount difference among the populations in the excited and non-excited states. Therefore, NMR is considered a relatively insensitive method compared with optical methods. Furthermore, NMR for the proton resonance requires labelled substrates by using isotope or non-isotope, and the label-requiring technologies may affect the biofilm physiology (Reuben *et al*. 2014).

Microscopic Technologies

A range of microscopic technologies, which allow the imaging of labeled or unlabeled EPS at high spatial resolutions, have been developed over the last few decades. These technologies, including scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM), confocal laser scanning microscopy (CLSM), and atomic force microscopy (AFM), have become highly regarded because of their high potential in the analysis of biofilms. This section primarily focuses on the principles and applications of CLSM and summarizes its advantages and limitations.

SEM and ESEM

The EPS and amorphous-phase surrounding cells in a biofilm can be directly observed from a two-dimensional image generated by using SEM technology. However, a high vacuum is needed to evaluate the samples. Due to the fact that biological samples have non-conductive properties, prior to SEM observation, biofilm samples must be subjected to rigorous processing steps including fixation, dehydration, and then sputter-coating with a conductive metal such as gold to ensure the electrical conductivity (Weber *et al.* 2014). The intensive dehydration is carried out with a series of ascending concentrations of acetone and ethanol. In other words, the water is replaced by the organic solvents having lower surface tension and less or no hydrogen bonding ability (Hannig *et al.* 2010). The morphology of the biofilm may even be altered by the dehydration process. Alternatively, the samples can be freeze-dried (FD), critical point–dried (CPD) using transitional fluid, such as liquid or solid carbon dioxide (Alhede *et al.* 2012), or hexamethyldisilazane dried (HMDS). Finally the specimens have to be coated with a kind of conductive material, for example sputtered with gold. Hazrin-Chong and Manefield (2012) proved that the use of HMDS drying was preferred over the more commonly used CPD method as the former is safer, cheaper, and more practical. Conversely, Ratnayake *et*

al. (2012) concluded that conventional glutaraldehyde fixation followed by CPD was superior to the non-fixed control, FD, and the glutaraldehyde fixation with HMDS drying methods in terms of preserving the EPS better.

An SEM image of an aquatic biofilm, which was subjected to the conventional chemical fixation followed by the intensive ethanol dehydration, is depicted in Fig. 1, and some fragments of algae and EPS can be clearly observed. The SEM results can provide good comparative information demonstrating clear differences in the structures of biofilms generated under different experimental conditions. Consequently, SEM images are useful for describing biofilm morphotypes (Simões *et al*. 2007; Wang *et al.* 2014). Although this technique presents a very detailed morphological image, it does not provide any chemical information and can analyze only dried samples (Sandt *et al.* 2007; Hannig *et al.* 2010).

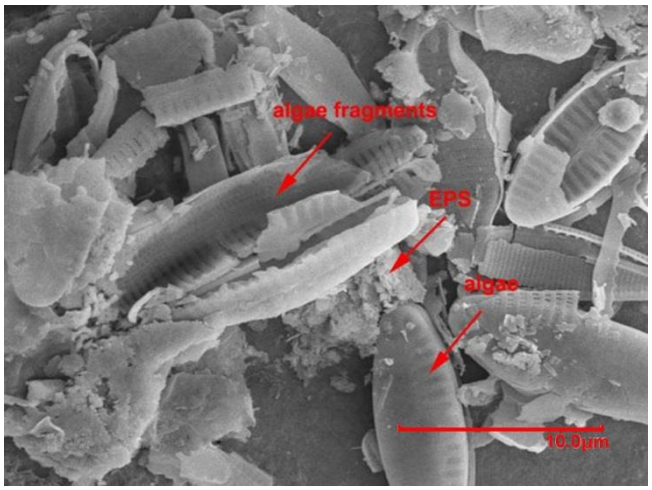

Fig. 1. SEM micrographs of a biofilm formed on glass slides in an urban river. Scale bar = 10 µm

To overcome the shortcomings of SEM, wet-mode ESEM can be performed under a moderate vacuum and without the prior fixation, dehydration, or conductive coating of the biofilm. If completely untreated, however, EPS are not electron-dense and thus do not resolve well in ESEM. Furthermore, the three-dimensional visualization of the structures is sometimes limited (Hannig *et al.* 2010). Therefore, Priester *et al*. (2007) introduced staining methods into ESEM analysis to map the EPS in biofilms. This combination allowed for increased image contrast; however, only the part of the EPS was well discriminated. Accordingly, subsequent staining, imaging, and image analysis procedures were added to this combination technology. However, time-resolved online and nondestructive biofilm visualization by ESEM is still infeasible during the process of biofilm formation.

Multiple fluorescence staining and CLSM

As a commonly applied analytical tool for biofilm investigations, CLSM can be performed in real time and in a nondestructive manner (Lerchner *et al*. 2008). CLSM allows the visualization and quantification of three-dimensional (3D) structures of living and fully hydrated biofilms (Neu *et al.* 1997; Lawrence *et al.* 1998; Beyenal *et al.* 2004). CLSM can be used in a multichannel mode, in which the different channels map individual biofilm components. The 3D reconstruction image of a biofilm is obtained by combining

a series of optical sections taken at different depths in the biofilm by image analyses with software (Savidge and Pothoulakis 2004).

The multiple color staining technique and CLSM can together visualize the distribution of components of EPS in a biofilm. Based on staining with lectins and imaging with CLSM, the qualitative and quantitative analysis of various EPS components in a biofilm can be achieved, and said quantification is based on fluorescence intensities (Schlafer *et al.* 2016). In particular, CLSM has been demonstrated to be more sensitive than the chemical extraction of EPS in young biofilms (< 1 week old, Barranguet *et al.* 2004). However, a fluorescence labeling approach depends on the specificity of the selected stains and is constrained by a lack of understanding of EPS composition and structure.

In recent years, the simultaneous use of multiple color stains has been increasingly adopted to characterize various EPS components in biofilms (Neu *et al*. 2002; Battin *et al*. 2003; Chen *et al*. 2006; Adav *et al.* 2010). Accordingly, more and more fluorochromes (typically purchased from Sigma, Molecular Probe, and Life Technologies) have been tested and selected to probe *in-situ* the corresponding content distribution of EPS. A list of vital dyes that many researchers have found to be the most useful for CLSM imaging are compiled in Table 2 together with their labeled objects and the associated parameters. Their selection mainly depends on the research need, sample pH, and excitation/emission properties (Adav *et al*. 2010). Clearly, there is a desire to have a single probe for EPS of the overall biofilm (Neu. *et al*. 2014).

Table 2. Stains Used in Sample Staining Schemes (One-Photon LSM)

Stains	Labeled Objects (Targets)	Excitation (nm)	Emission (nm)	Channel
Fluorescein-isothiocyanate (FITC) (Chen *et al*. 2006; Adav *et al*. 2010)	Protein	488	520	Green
TMR-ConA (Chen *et al.* 2006; Adav *et al*. 2010)	α-Mannopyranosyl and α-Glucopyranosyl residues	555	580	Green
Concanavalin A (ConA)-Texas red (Battin *et al*. 2003)	α-d-Glucose and α-d-mannose	561	570 to 590	Green
TRITC (Zippel and Neu 2010)	Ficoll and glucan	568	590 to 610	Red
Calcofluor White	β-d-glucopyranose polysaccharides	400	410 to 480	Green
DiD (Adav *et al.* 2010; Baird *et al.* 2012)	Cell membranes and lipids	644	665	Far-red
SYTOX Blue (Adav *et al*. 2010)	Dead cells	458	460 to 480	Green
SYTO 63 (Chen *et al*. 2006)	Total cells	633	650 to 700	Far-red
None-1(Auto fluorescence-1) 1) (Zippel and Neu 2010)	Chlorophyll a	647	665	Far-red
None-2 (Auto fluorescence-2) (Zippel and Neu 2010))	Cyanobacteria (phycoerythrin and phycocyanin)	567	630	Red

The general principles in designing a multicolor staining scheme should be continuously presented and developed in practice. First, the criteria for selecting fluorochromes should be established. Next, an appropriate pretreatment method for staining, which mainly includes fixation and immobilization, should be selected (Nosyk *et*

al. 2008; Adav *et al*. 2010). The specimens are then stained; in this step, the order of staining, selection of buffer, incubation time of staining, and washing steps need to be set optimally (Chen *et al*. 2007; Adav *et al*. 2010). Subsequently, the specimens are examined using CLSM. Finally, the recorded CLSM images are analyzed with the appropriate software, including three different aspects: visualization, quantification, and deconvolution (Neu *et al.* 2015).

The key consideration in multiple fluorescent experiments is the use of highly specific fluorochromes with minimum spectral peak interference, as mentioned by Chen *et al*. (2007). The experiments should also meet at least one of the following conditions: (i) If there is no overlap of the excitation spectra of all the fluorochromes, then the fluorochromes will be excited one by one under an adequate light source; and (ii) If parts of the emission spectra of all the fluorochromes do not overlap, then the emitted spectra can be observed one by one using a limited observation wavelength band. For example, because of the overlapping excitation and emission wavelengths, Con A, Nile Red, and tetramethylrhodamine isothiocyanate (TRITC) cannot be applied to a sample simultaneously. In particular, the application of Nile Red has been shown to interfere with the application of many other stains (Adav *et al*. 2010). DAPI shows a very broad emission signal and thus should not be employed in multiple staining (Savidge and Pothoulakis 2004). In addition, the excitation of DAPI requires expensive UV or two-photon lasers, and the UV excitation wavelength can result in high autofluorescence (Fig. 2); therefore, simultaneous multichannel imaging using DAPI is challenging (Palmer *et al*. 2006). To detect the corresponding emission signals of multiple fluorochromes, CLSM usually has three channels: green, red, and far red (blue), which allows for the direct observation of the development of individual biofilm components (Neu *et al*. 2004). However, the drawback of applying multiple fluorochromes on the same specimen is that the simultaneous multiple color staining might cause serious channel interference.

Some studies have shown that the thickness and density of a biofilm are major influencing factors that can result in light attenuation and limited dye penetration (Barranguet *et al*. 2004; Wagner *et al*. 2009). The maximum observable depth in biofilms reaches up to hundreds of μm (Barranguet *et al*. 2004; Wagner *et al*. 2009; Halan *et al*. 2012). Nevertheless, CLSM can provide an accurate representation of EPS in young biofilms, assessing the qualitative and quantitative changes in the early stages of development. As a result, for dense or thicker biofilms, which have been embedded and physically sectioned, embedding may be done using nanoplast, epon, paraffin, or a so-called tissue freezing medium, and subsequent sectioning may be carried out using a normal microtome or a cryotome (Battin *et al*. 2003; Savidge and Pothoulakis 2004). Furthermore, obtaining higher-resolution images of thick biofilm samples by two-photon LSM instead of conventional single-photon laser microscopy has proven possible if appropriate excitation wavelengths and fluorochromes are used (Neu *et al*. 2004). Two-photon LSM, which is an emerging technique with real potential for examining biofilms (Lawrence and Neu 2003; Neu *et al*. 2010a), provides advantages over the conventional confocal microscopy with potentially increased resolution, reduced phototoxicity and photo-bleaching of the fluorescent probes (Choi *et al*. 2010), and also reveals the improved imaging performance of two-photon excitation in terms of the 3D point spread function and the 3D optical transfer function (Gu and Sheppard 1995; Neu *et al*. 2002; Garrido-Baserba *et al.* 2016). It is necessary to note that in the detection of EPS in biofilms in river or sea water, the autofluorescence of phototrophic organisms (cyanobacteria and green algae) results in strong signals in the entire excitation range (Neu *et al*. 2002; Zippel and

Neu 2010), generally with imaging characterized by fluorescent green, which particularly interferes with extracellular proteins (Fig. 2). The minimal autofluorescence detected during scanning is used as a reference spectrum that is subtracted from the lambda spectra during linear unmixing (Baird *et al*. 2012). Moreover, lambda scanning settings can be implemented to eliminate spectral cross-talk (Adav *et al*. 2010; Baird *et al*. 2012).

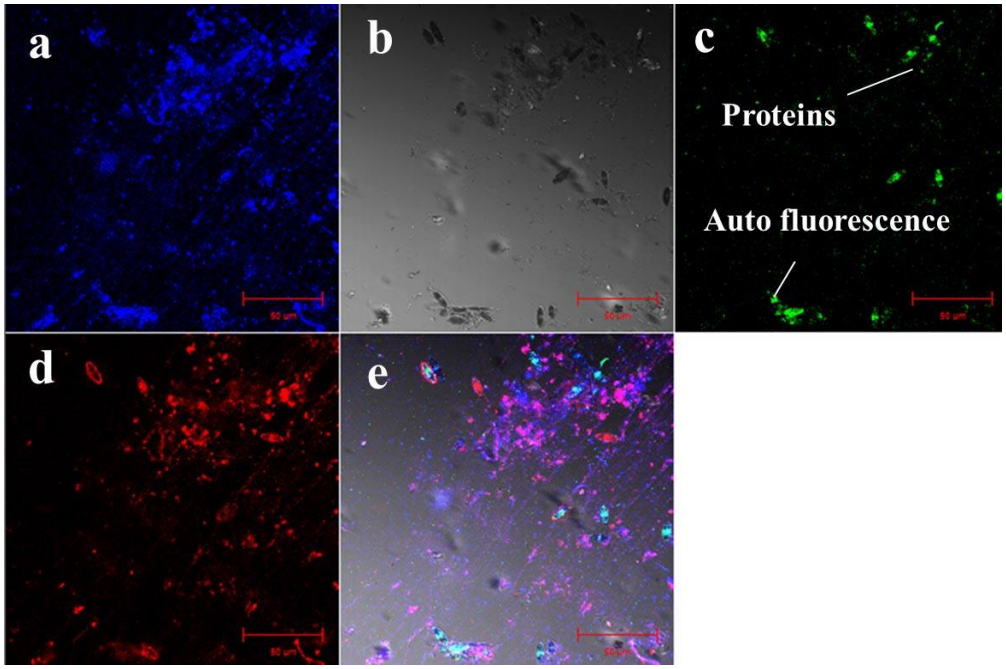

Fig. 2. The maximum intensity projection of a lotic biofilm examined by CLSM (one-photon excitation). (a) Blue (DAPI) total cells; (b) phase contrast image; (c) green (FITC) proteins; (d) red (ConA-TMR) α-mannopyranosyl and α-glucopyranosyl residues; (e) the resulting overlay. Scale bar = 50 μm

To obtain reproducible and reliable image data by CLSM multiple fluorophore staining, many challenging problems must be solved, including the selection and development of high-specificity stains to optimize the staining protocol, the expense, and the toxicity of some of the fluorochromes. Staining may be of low specificity, and given that some components of EPS cannot be stained, CLSM can only provide information on the distribution and amount of stainable EPS components. With the main components of EPS unknown, several issues, including whether the makers used can specifically bind to the target substances and whether the more comprehensive biomarkers are accessible to mark the various components of the EPS, remain unsettled (Yu *et al*. 2011). Particularly, the operator should be aware of such limitations and be able to collect the data in the most appropriate mode to minimize these effects.

Atomic force microscopy

The production of EPS by bacterial cells has been observed by electron microscopy, but this technique cannot provide information about samples in the hydrated state and often requires complicated preparation procedures. In contrast, AFM can be used in ultra-high vacuum, liquid phase, gas phase, and electrochemical environments. AFM imaging can be performed in contact, non-contact, or tapping modes. Scanning probe measurements of many biological samples have successfully been performed in air, but only in contact and

not tapping mode. The tapping mode has obvious advantages in detecting biological samples (Jalili and Laxminarayana 2004). In consequence, tapping mode AFM has superiority in imaging the surface morphology of biofilms and unraveling the intermolecular forces at the nanoscale level both in air and fluid environments, without necessitating metal-coating or staining (Hansma *et al*. 2000; Jalili and Laxminarayana 2004; Dufrene 2008). In particular, AFM can render 3D images with a nanoscale resolution (less than 1.0 nm) to clearly show the EPS secretion and the entrapment of bacteria cells within the EPS matrix (Beech *et al*. 2002; Pradhan *et al*. 2008). Van der Aa and Dufrêne (2002) used AFM to characterize the supramolecular organization of bacterial EPS attached to a solid substratum. AFM topographic images and force–distance curves were used to characterize the morphology and molecular interactions of the substratum during the formation of bacterial biofilms. They concluded that proteinaceous EPS accumulate at the solid substratum surface in the form of a thin, continuous layer from which supramolecular assemblages protrude. Meanwhile, AFM topographic images also reveal the nature of adsorbed EPS. Ahimou *et al*. (2007b) employed AFM to measure *in-situ* EPS/EPS and cell/EPS interactions within a well-defined volume of biofilm. The *in-situ* measurement of the cohesive energy levels of moist biofilms revealed a stronger effect of calcium absorption on the cohesive energy of the EPS matrix and a weaker effect of calcium absorption near the microbial cell surface. This finding could indicate that outer EPS layers are more loosely associated with one another; then more opportunities will be provided for calcium absorption and crosslinking in outer layers. By contrast, deeper EPS layers are more tightly associated with cells and therefore contain less calcium. This phenomenon further verifies that LB-EPS and TB-EPS have different capabilities in combining with calcium.

AFM provides information about the morphological details, but little data on the chemical composition of biofilm. Other limitations of the technique include relatively long imaging time, expensive equipment, inability to obtain large-area survey scans before increasing the magnification, and low-light efficiency. Furthermore, soft biofilm samples are easily damaged by the tip even when the forces used lie within the nano-Newton range (van der Aa *et al.* 2002; Halan *et al*. 2012).

Spectral Microscopy Techniques

In this section, we review the use of spectral microscopy for the chemical and structural evaluation of biofilm EPS. Spectral microscopy extended the utility of standard spectroscopic tools to enable the collection of spatially resolved spectra, thus filling the information gap in pure microscopy. Each analyte has its own unique absorption spectrum; thus, spectral microscopy can be used to identify different absorbers at the molecular and atomic levels and visualize their distribution in space.

Raman microscopy

Raman microscopy (RM) is a nondestructive spectroscopic technique based on the Raman scattering of monochromatic laser light that provides fingerprint spectra with the spatial resolution of an optical microscope. The common integration of Raman spectroscopy with a microscope enables spectral analysis at a micrometer spatial resolution. Thus, RM can simultaneously reveal the chemical composition and the structure of EPS at diverse biofilm formation stages (Janissen *et al*. 2015). Specifically, RM has great advantage in detecting the analyte molecule with the symmetrical modes of molecular motion, which are not sensed by typical infra-red spectroscopy (Neugebauer *et al.* 2002).

Ivleva *et al*. (2008) and Wagner *et al*. (2009) used RM to monitor the chemical composition of different types of EPS during the biofilm formation process at selected Raman bands, which confirmed that RM can effectively supplement CLSM analysis. It can reproducibly reveal changes in the chemical composition of the biofilm matrix, even changes that are not detectable by CLSM. It requires no or limited sample preparation, providing information about the label-free EPS components of fully hydrated biofilms *in-situ*. Moreover, compared with CLSM, RM does not require a tunable excitation source, because the whole spectrum can be collected by excitation with a fixed laser wavelength (Ivleva *et al*. 2008).

Raman spectra are characterized by a high specificity. However, Ivleva *et al*. (2008) revealed that the binding of cations induced several changes in the Raman spectra of polysaccharides, and they applied algal alginate as a model polysaccharide to determine the frequency regions in the Raman spectra that can be used for the analysis of the influence of metal cations. Furthermore, the effect of photo bleaching should be handled (Wagner *et al*. 2009). RM is also time-consuming because it stays on a single point for a considerable time and then scans the sample point by point. To improve the speed of RM, confocal Raman microscopy (CRM), which allows for high-speed scanning, was developed. Compared with CLSM, CRM does not need to filter or eliminate the autofluorescence of the sample. Given its desirable characteristics, CRM is an ideal technique for investigating the effects of various environmental factors on biofilm growth (Sandt *et al*. 2007). Virdis *et al*. (2012) demonstrated that CRM allowed monitoring of biofilm development at different growth stages, without impacting its structural or metabolic activity. Li *et al*. (2015) presented CRM for in situ, real-time imaging of the biomineralization in biofilms, through which it was shown that Pseudomonas aeruginosa biofilms could produce morphologically distinct carbonate deposits that substantially modified biofilm structures.

FTIR and ATR microscopy

Coupling FTIR and attenuated total reflection microscopy (ATR-FTIR) extends internal reflection spectroscopy to the microscopic scale (Buffeteau *et al*. 1996). ATR-FTIR has been successfully applied to the *in-situ* nondestructive study of biofilms in real time and under fully hydrated conditions (Ojeda *et al*. 2008). In this technique, the accumulation of various EPS-associated functional groups and the structural changes in EPS polymers can be monitored by growing the biofilms directly on the ATR crystal (Humbert and Quilès 2011). Because of the high refractive index of the ATR crystal, ATR-FTIR imaging typically uses multichannel detectors to achieve spatial localization (Bhargava 2012) and provides a high numerical aperture, resulting in a higher spatial resolution (Chan and Kazarian 2003). However, ATR-FTIR is not suitable for thick biofilms because the penetration depth of the evanescent wave is below 1.0 μm (Kavita *et al*. 2013), and is a zero-dimensional measurement technique that captures only information from the molecules near the surface (Paquet-Mercier *et al*. 2014). Furthermore, some questions have yet to be addressed, for example, what part or which layers of the biofilm contribute to the recorded ATR spectrum? The individual spectral features of FTIR often overlap because of the extreme heterogeneity of biofilm constituents. Consequently, ATR-FTIR is suitable for analyzing the EPS extracted from biofilms (Reuben *et al.* 2014). Notably, the interpretation of spectral changes measured at the molecular level is sometimes subtle and complex, requiring knowledge and experience of ATR-FTIR bacterial fingerprints to be able to identify and differentiate the spectral changes induced by changes in environmental conditions (Humbert and Quilès 2011).

The quality of images obtained with an IR microscope is traditionally constrained by throughput and SNR (Reddy *et al*. 2013). In a review, Bhargava (2012) focused on the science of IR microspectrometry, especially on recent developments in the mid-2000s that can potentially transform imaging spectroscopy. He pointed out that a microscope based on planar array infrared (PA-IR) spectrometers could rapidly examine small regions with exceptionally small signals, *e.g.*, mapping of monolayers, a capability that is not easily achievable by FTIR microscopes. Such spectrometers can hopefully detect EPS in biofilms.

FUTURE PERSPECTIVES AND CONCLUSIONS

The production and distribution of EPS reflect the attachment and aggregation process, provide an optimal environment for the exchange of genetic material between cells, and maintain a spatial arrangement for microorganism consortia, which dramatically influence the structure of biofilm over a prolonged period. An increasing number of studies have focused on the specific components of biofilm EPS, as well as their spatial differentiation and stability at different growth stages. To gain a chemical and structural evaluation of biofilm EPS, this article has extensively reviewed studies using techniques from various fields such as microscopy, spectroscopy, biochemistry, and their combination. However, some of these promising techniques, such as AFM or ESEM, require costly equipment, while for others, such as SEM and CLSM, extensive preparation of the samples is necessary. And all the techniques mentioned above have not been fully utilized to date.

However, much is yet to be learned regarding the roles of EPS in the functions and characteristics of biofilm to systematically elucidate the effects of EPS on biofilm growth, structure, and function. Further efforts should also be devoted to the integration of multidisciplinary technologies to study the behavior of EPS in the biofilm growth phase. A theoretical framework, which can perfect the "biofilmology" discipline, should also be established. Moreover, with such high expectations, hardware developments are likely to spur the development of faster algorithms and signal-processing strategies to store data, improve spectral corrections, and extract information with high-definition imaging.

The existing research methods to date may provide new knowledge about the structure–function correlations in biofilm. Overall, integrated technologies must be developed to overcome the multidimensional challenges in understanding EPS in biofilms at different growth phases, including the initial attached bacteria, colonies, and mature biofilm. Multidisciplinary approaches should be developed to study EPS during biofilm formation, provide more in-depth insights into the composition and spatial distribution of EPS, and ultimately improve our understanding of the role EPS play in biofilms.

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