Position Paper:

Extracellular polymeric substances of biofilms: suffering from an identity crisis

Thomas Seviour[1,*], Nicolas Derlon[2], Morten Simonsen Dueholm[3], Hans-Curt Flemming[1,4], Elisabeth Girbal-Neuhauser[5], Harald Horn[6], Staffan Kjelleberg[1], Mark C.M. van Loosdrecht[7], Tommaso Lotti[8], M, Francesca Malpei[9], Robert Nerenberg[10], Thomas R. Neu[11], Etienne Paul[12], Hanqing Yu[13], Yuemei Lin[7,*]

[1]Singapore Centre for Environmental Life Sciences Engineering, Nanyang Technological University, 637551, Singapore. [2]EAWAG, Swiss Federal Institute of Aquatic Science and Technology, Department of Process Engineering, CH-8600 Dübendorf, Switzerland. [3]Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark. [4]University of Duisburg-Essen, Faculty of Chemistry, Biofilm Centre, Essen, Germany. [5]Laboratoire de Biotechnologies Agroalimentaire et Environmentale (LBAE), Universite Paul Sabatier, Toulouse, France. [6]Karlsruhe Institute of Technology (KIT), Engler-Bunte-Institut, Water Chemistry and Water Technology and DVGW Research Laboratories, Karlsruhe, Germany. [7]Department of Biotechcnology, Delft University of Technology, Delft, The Netherlands.[8]Department of Civil and Environmental Engineering – DICEA, University of Florence, Florence, Italy. [9]Dipartimento di Ingegneria Civile e Ambientale, Politecnico di Milano, Milan, Italy. [10]Department of Civil and Environmental Engineering and Earth Sciences, University of Notre Dame, Notre Dame, USA. [11]Department of River Ecology, Helmholtz Centre for Environmental Research - UFZ, Magdeburg, Germany.[12]Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, Université de Toulouse, Toulouse, France. [13]Department of Chemistry, University of Science and Technology of China, Hefei, China.

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Abstract

Microbial biofilms can be both cause and cure to a range of emerging societal problems including antimicrobial tolerance, water sanitation, water scarcity and pollution. The identities of extracellular polymeric substances (EPS) responsible for the establishment and function of biofilms are poorly understood. The lack of information on the chemical and physical identities of EPS limits the potential to rationally engineer biofilm processes, and impedes progress within the water and wastewater sector towards a circular economy and resource recovery. Here, a multidisciplinary roadmap for addressing this EPS identity crisis is proposed. This involves improved EPS extraction and characterization methodologies, cross-referencing between model biofilms and full-scale biofilm systems, and functional description of isolated EPS with *in situ* techniques (e.g. microscopy) coupled with genomics, proteomics and glycomics. The current extraction and spectrophotometric characterization methods, often based on the principle not to compromise the integrity of the microbial cells, should be critically assessed, and more comprehensive methods for recovery and characterization of EPS need to be developed.

Introduction

Often described in a cursory manner as the slime, the extracellular polymeric substances (EPS) are key to the formation, persistence and physicochemical behavior of microbial biofilms across clinical, environmental and industrial settings (Seviour et al. 2012b). Moreover, increased tolerance to antimicrobials is the result of the ability of certain pathogens to produce EPS, which hence constitutes a global threat to the consequences of multidrug resistance (Frieri et al. 2017).

EPS also play significant roles in the successful implementation of water reclamation and purification technologies that have arisen to meet increasing demands for water of different purities, water scarcity (predicted by the United Nations to be the biggest global problem in the coming decade), land shortage and the water-energy nexus. EPS provide structure for anaerobic and aerobic granular sludges, which have emerged over the last thirty years, along with activated sludge and fixed biofilm systems (i.e. trickling filters), as alternatives for biological treatment of industrial and domestic used waters with lower land and energy footprints (Bengtsson et al. 2018). Advances in membrane technologies have made it possible to create drinking water either from sources that were previously considered not available for drinking water production (i.e. brackish water seawater, or wastewater) (Le and Nunes 2016), or without the addition of chemical disinfectants (Derlon et al. 2012, Madaeni 1999). However, the hydraulic throughput of these technologies is often limited by membrane fouling, which in many instances is due to biofilm growth.

Biofilms, therefore, feature prominently in many of the challenges facing water technology implementations. As the number of antimicrobial-resistant strains increases, and the range of water reclamation and purification technologies grows, so too does the need to control or predict EPS production. Yet, despite decades of research, we know very little about the molecular composition and function assigned to individual EPS components, and we are not in a position to control the formation and composition with any meaningful predictable outcome. This limits our ability to manage biofilms effectively. We need to enhance our efforts to deliver improved analytical methods and unravel biochemical production pathways, and most importantly, discontinue the use of methods that misrepresent the roles and significance of EPS. The current practice of dismissing EPS, or relegating them to

merely a perfunctory study as a footnote in process optimization, should be abandoned. It is essential to identify and reveal how EPS composition determines the microscopic and macroscopic behavior of biofilm systems.

We propose that identifying functional biofilm EPS is the critical path to address key questions in biofilm control. This will not be possible if we persist with the current practice of applying general, superficial and correlative characterizations alone. However, prior to suggesting a roadmap for achieving an in depth understanding of EPS, it is first necessary to explain why so little progress has been made in identifying and characterizing extracellular polymers present in biofilms.

The extracellular matrix

The EPS of biofilms are a complex mixture of interlaced biological polymers. They provide mechanical stability and scaffolds that allow biofilm cells to establish synergistic microconsortia, enhance water retention and nutrient sorption, provide protection against viruses, predation, antimicrobials and disinfectants, and ultimately act as nutrient recycling yards (Flemming and Wingender 2010). These functions can be provided by a large variety of biopolymers, particularly polysaccharides, proteins and nucleic acids. EPS compounds originate from different community members and a specific organism can produce different polymers as a function of time or condition. Moreover, EPS produced by a given microbial population can persist long after the population producing it has disappeared. All of these different components contribute to the function and organization of the matrix. Additionally, many of the biopolymers produced by the cells are processed by extracellular enzymes embedded in the extracellular matrix (Whitfield et al. 2015). It is currently not possible to track the production of specific EPS components over time or attribute them to the specific host

organism in mixed species biofilm communities, nor do we have the means to effectively manipulate EPS quantity or composition. A better understanding of the EPS would derive from metabolic labelling approaches (Liang et al. 2017). For example, EPS biosynthesis compounds could be tracked to identify the organisms producing them, when and where they are released, and their fate over time. This could be monitored in real-time using state-of-the-art laser microscopes and nanoscopes to generate high-resolution three-dimensional image data sets. Limitations in our current understanding of the EPS, however, render such methodologies presently beyond our reach.

Structural and functional assignment of key biofilm EPS is confounded by their compositional complexity, but also by the challenges in processing and isolating EPS components. The diversity of biofilm EPS is described in Figure 1, in terms of the number of types of molecules observed across a range of biofilms (i.e. rather than in any single biofilm). See Box 1 for a description of each EPS. Biofilms and many of the EPS described in Figure 1 are poorly soluble in aqueous systems. Unless methods are developed to extract the entire spectrum of biofilm EPS, our understanding of EPS will be skewed by solubility and characterization biases. Mechanical and chemical methods have been applied for EPS extraction from these biofilms with the objective of maximizing extraction yield and minimizing cell lysis (Ras et al. 2011). In most cases these methods have not been verified to assess whether they extract the structural polymers from the biofilms. While potentially effective on some biofilms, these extraction protocols are often only partially or marginally effective, which results in the characterization of EPS that are not important for the biofilm structure (Felz et al 2016). This is particularly the case for stratified and dense aggregates such as fixed biofilms or granular sludge.

A solution for the insoluble?

The range of techniques required to extract and solubilize known biopolymers, such as the polysaccharides cellulose, chitin and alginate (examples of neutral, cationic and anionic polysaccharides respectively), highlights the need for even harsh extraction methods (i.e. non-aqueous, extreme pH or temperature) (Zhang et al. 2017). Combinations of mechanical pre-treatments (grinding, ultrasonication, homogenizers), acidification (demineralization), enzymatic hydrolyses, alkalinization (for deproteination or deprotonation), novel solvents like ionic liquids and heat treatments are typically invoked in order to extract such polysaccharides (Kumari and Rath 2014, Seviour et al. 2015b, Younes and Rinaudo 2015). While cytosolic protein extraction is possible through cell lysis, the task is far more problematic for structural proteins. These are often large (Julio and Cotter 2005) and/or have a tendency to fibrillate, whereby alkalization or acidification may be required to solubilize them, often in conjunction with enzymatic treatments (Le et al. 2016).

Given the analytical challenges of identifying and characterizing functional EPS of biofilm assemblages, we should sometimes be prepared to apply methods that damage cells rather than prioritizing cell integrity (Felz et al. 2016) in order to resolve the contributions of a broader range of key extracellular polymers. This approach would then include the subsequent step of retrospectively identifying whether extracted polymers are extracellular, as accomplished by microscopic techniques (Neu and Lawrence 2014, Wagner et al. 2009). The target extracellular polymers can be recovered from solution by fractional precipitation (e.g. using anti-solvent addition or pH neutralization), and purified further by, for example, electrophoretic or chromatographic techniques (Seviour et al. 2010a). Complementary biophysical assays can then be undertaken on biofilm and isolates to elucidate function (Seviour et

al. 2015a). Detailed structural and functional characterization of novel relevant extracellular polymers requires significant quantities of a sufficiently pure compound, which is a common and often insurmountable hurdle to achieve the ultimate goal of resolving more precisely the identity of key extracellular polymers.

Do the same extracellular polymers provide the same functions across systems?

Despite the complexity and diversity of EPS in multi-species biofilms, we assume that particular roles performed by EPS are conserved across biofilms, e.g. gel formation and adhesion (Lin et al. 2013). The more information we acquire on the mechanical, biophysical and structural aspects of the extracellular polymers contributing to these functions, the easier it will be to identify and monitor their expression. This could involve information derived from metaproteomic analysis, specific labelling of functional groups in polymers (e.g. by lectins) and observation by microscopy (Neu & Kuhlicke 2017), or quantifying polymers with greater accuracy. The list of reference polymers is limited to those isolated from a relatively small number of models, often clinical organisms (e.g. *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*) (Colvin et al. 2012, Marvasi et al. 2010). These bacteria are uncommon in biofilms found in water treatment biofilms and their polymers are unlikely to be representative of biofilm EPS in water treatment systems. Another approach for understanding whether EPS perform common functions across different biofilm system, currently neglected in EPS research, could be to screen interactions between EPS and known glycan-binding proteins in order to infer function and identity (i.e. glycomics) (Cummings and Pierce 2014, Lipke 2016). This would create a database

for EPS comparison, identification of new sugar-binding proteins for visualization of novel sugars, and potentially facilitate the identification and analysis of glycoproteins.

Agreeing on model biofilms for EPS characterization

Full-scale biological systems in the water sector are often represented by highly diverse microbial communities (Saunders et al. 2016). We would expect the EPS to be similarly complex at a molecular level. Hence, full-scale systems may not be the ideal starting point for isolating and characterizing reference polymers. We should therefore improve the resolution of characterization of EPS from biofilms comprising organisms known to contribute to key water and wastewater biofilm functions, such as nitrification, enhanced biological phosphate removal, floc and filament formation and the Anammox process. The microbial community composition of model systems can be tracked and compared to full-scale systems. Biomass samples from these model systems should be made broadly available to the water sector and act as a common reference point for initial EPS characterization. There are a few examples of EPS isolated from bacteria found in biofilms related to wastewater treatment, including granulan (Seviour et al. 2012a), alginate-like exopolysaccharide (ALE) (Lin et al. 2010), acid soluble polysugars (Pronk et al. 2017) and glycosylated proteins (Lin et al. 2018). However, we still need to understand how widespread these EPS are in biofilms, as well as identify new extracellular polymers from other key systems to expand our database of identified, characterized and relevant EPS.

Sequencing approaches for EPS characterization

The application of next generation DNA-sequencing methods in conjunction with bioinformatic analyses may allow for the identification of signature extracellular polymers across a vast number of environmental biofilms, and to elucidate their

198 regulation. Metagenome assembled genomes (MAGs) representing individual
199 community species can be described relatively inexpensively (Albertsen et al. 2013),
200 and when coupled with long-read sequencing technologies, such as PacBio and
201 Nanopore sequencing, closed genomes from mixed communities can be constructed
202 (Hao et al. 2017, McIlroy et al. 2017). MAGs provide blueprints for the proteins
203 (enzymes, transporter, and chaperones) that are involved in the biogenesis of all
204 cellular components and EPS. In the case of proteinaceous EPS, MAGs provide the
205 exact recipe for how to synthesize them. Genetically encoded systems for EPS
206 biogenesis can be predicted by bioinformatic approaches such as genome annotation
207 and pathway modeling. However, EPS identified purely through bioinformatics and
208 molecular methods remain theoretical extracellular polymers only. Hence, validation
209 through biophysical and chemical characterization of isolated reference compounds
210 will be required.

211 Sequencing and molecular techniques can enable recombinant model systems to to be
212 designed to produce extracellular proteins for chemical and biophysical
213 characterization, where the proposed extracellular proteins are expressed and isolated
214 from bacteria with little or no biofilm production, as is the case for common
215 laboratory strains of *E. coli* and *B. subtilis* (Dueholm et al. 2010). Such proteins can
216 even be used to generate antibodies that can be applied for *in situ* analyses.
217 Furthermore, identifying the genetic blueprints for the synthesis of reference polymers
218 would allow us to identify related systems by homology searches (Dueholm et al.
219 2012) and employ transcriptomics to determine how such genes are regulated in
220 response to environmental factors. Liquid chromatography combined with tandem
221 mass spectrometry (LC-MS/MS) could confirm that theoretical extracellular proteins
222 are expressed in complex samples (Cox et al. 2014). LC-MS/MS may also provide

information on chemical modifications, which could be relevant for their functions. However, while methods for high throughput protein identification are well-established, the same advances have not been achieved for extracellular polysaccharide analysis due to the structural diversity of carbohydrates (Wang et al. 2017, Zhao and Jensen 2009). Furthermore, the reliability of current methods, e.g. for polysaccharide quantification by colorimetric methods, is impaired by other chromogenic compounds (i.e. interference) and non-representative reference sugars (Le and Stuckey 2016).

In situ approaches have an important role to play

New and combined imaging techniques offer the opportunity to link the production of specific EPS components with specific bacterial groups *in situ*, as well as validate whether the isolated polymers are indeed extracellular. Imaging provides a link between genomic information and how the EPS are distributed throughout the biofilm (i.e. with regards to location), whereby changes in microbial cells and matrix composition can be monitored over time and together with changes in environmental parameters. Advanced imaging techniques can be combined with increasingly sophisticated computational analyses to describe microbial behavior quantitatively with greater precision (Neu et al. 2010). Laser scanning microscopy coupled with fluorescent staining has proven to be the most flexible approach for imaging biofilm EPS (Neu and Lawrence 2014). Key fluorescence approaches include selective fluorogenic staining (e.g. TOTO-1 for DNA (Okshevsky and Meyer 2014), NileRed for lipids (Rumin et al. 2015), Sypro/NanoOrange and epicocconone for proteins (Randrianjatovo et al. 2015, Zubkov et al. 1999), lectins for analysis of EPS glycoconjugates (Neu and Kuhlicke 2017), and EPS specific antibodies, e.g., WO1

247 for amyloid proteins (Poul et al. 2007)). By combining EPS microscopy with
248 fluorescence *in situ* hybridization (FISH), EPS production can potentially be linked to
249 specific bacterial taxa (Bennke et al. 2013, Tawakoli et al. 2017).

250 Finally, chemical imaging could become a key tool for analyzing complex microbial
251 communities, and bridging isolation and *in situ* characterization studies. Particularly
252 relevant techniques include FTIR imaging, Raman microscopy, nanoSIMS and ToF-
253 SIMS as well as synchrotron-based imaging such as STXM, although some problems
254 still need to be addressed, such as correlated imaging (suitable mounting and probes)
255 and scale of observation (covered area and depth) (Gowen et al. 2015, Lawrence et al.
256 2003, Marshall et al. 2014).

257 Can EPS recovery help us to move towards a circular economy?

258 A better understanding of the EPS matrix will lead to improved strategies for both
259 resource recovery and biofilm management in water and wastewater treatment
260 systems. The growing interest in renewable resources highlights a focus on the
261 production of EPS from waste biomass, and their conversion into bioproducts and
262 biomaterials, as an appealing route for contributing to a reduced economic
263 dependence on fossil fuels (More et al. 2016) and enhanced sustainability and
264 economics of wastewater treatment (Lin et al. 2015). EPS-like polymers
265 (hydrocolloids) cannot, in general, be derived from oil-based chemicals, and hence
266 supply relies solely on natural resources. Wastewater derived hydrocolloids could be
267 an important new supply route. A better understanding of the metabolic pathways
268 involved in EPS biosynthesis, molecular composition, interactions with other
269 materials and structure-function relationships would lead to the identification of new
270 applications and markets for EPS, ensure stable and cost-effective production of

biopolymers from waste biomass and wastewater, and provide a step towards successful development of extracellular polymer-based bioproducts.

Improved bioprocess control through EPS management

The optimum strategy for biofilm control depends largely on whether EPS production is beneficial (e.g. granular sludges) or detrimental (e.g. membrane bioreactors, infections or biofouling). For both outcomes, altering the mechanical properties of biofilms may improve the process management. Changing either the EPS constituents that are present or how they interact with each other, will modify biofilm cohesive strength, viscosity or elasticity. This can allow for easier removal of biofilms from filters by backwashing or to select for rapid settling of granular sludge in high throughput wastewater processes. There are several strategies available to change the mechanical stability of biofilms, including the use of enzymes, (e.g., lipases, hydrolases, proteases), oxidants (e.g., Cl_2), chelators (e.g., EDTA), or temperature (Jones et al. 2011, Stewart 2014). The current shortcomings in our understanding of EPS make these approaches highly empirical and less effective. A better understanding of the EPS composition, configuration, and interactions among constituents will inform on more effective and targeted chemical interventions.

If we understood more about which EPS are present, what they are doing and how their expression is regulated, another strategy targeting biofilm mechanics could be to modulate EPS secretion. This would allow for biofilms to be engineered to have more desirable properties, such as reduced adhesion and increased permeability. Thus, membrane reactor performances are improved. EPS secretion could be regulated by applying different growth or operating conditions. Certain growth conditions, such as nutrient-limitation, feast-famine or extended solid retention time, may increase

exopolysaccharide secretion. In membrane biofilters, excessive exopolysaccharide production reduces biofilm permeability and thus throughput of drinking water (Desmond et al. 2018). Supplementing process waters with phosphorus can increase biofilm permeability and reduce membrane head loss (Lauderdale and Brown 2010). In conventional membrane systems, however, phosphorus limitation may prevent microbial growth and biofouling (Vrouwenvelder et al. 2010). While hydraulic conditions are known to influence biofilm morphology (Fish et al. 2017, van Loosdrecht et al. 1995), the exact relationship between reactor hydraulics and EPS production has not yet been elucidated. A better understanding the genomic regulation of EPS formation and the factors that influence it could yield a real breakthrough. This might allow for advanced control of mixed microbial communities with respect to EPS, as is currently under development for pure cultures (Ha and O'Toole 2015).

Establishing the means to control biofilm EPS is thus crucial for improved management of our water resources and to stave off the emergence of multi-drug resistant pathogens. Before we can benefit from better control and engineering of biofilm-based systems in water treatment, identify alternative antimicrobial therapies, and recover EPS as a bioresource, we need to go beyond describing the EPS in terms of the exopolymer classes present and identify exactly which molecules contribute to specific biofilm functions. This involves an integrated, multidisciplinary approach on biofilms and molecular isolates (summarized in Figure 2). Improved EPS extraction methods, advanced imaging, chemical characterization, and genetic and biophysical analyses, need to be applied to biofilms and EPS isolates alike.

Conclusions

A better understanding of the EPS will increase the breadth of strategies available for controlling biofilms in water, wastewater and medical systems alike, which are currently unreliable, empirical and binary (at best). A variety of complementary approaches is required, to overcome extraction and analysis biases, as well as knowledge constraints regarding, for example, exopolymer references in databases. Required developments include:

- Extraction methods targeting full solubilization of key structural and functional EPS, with a preparedness to use harsh methods if necessary, contingent on using methods to verify the intra- or extra-cellular origin of the analyzed molecules;
- Chemical characterization methods to identify the exact molecular structure;
- *In situ* methods for verifying the identity, distribution and function of the EPS (biophysical, imaging with fluorescent or nanoparticle-based probes and chemical profiling); and
- Model biofilm systems to cross-reference industrially and medically-relevant systems.

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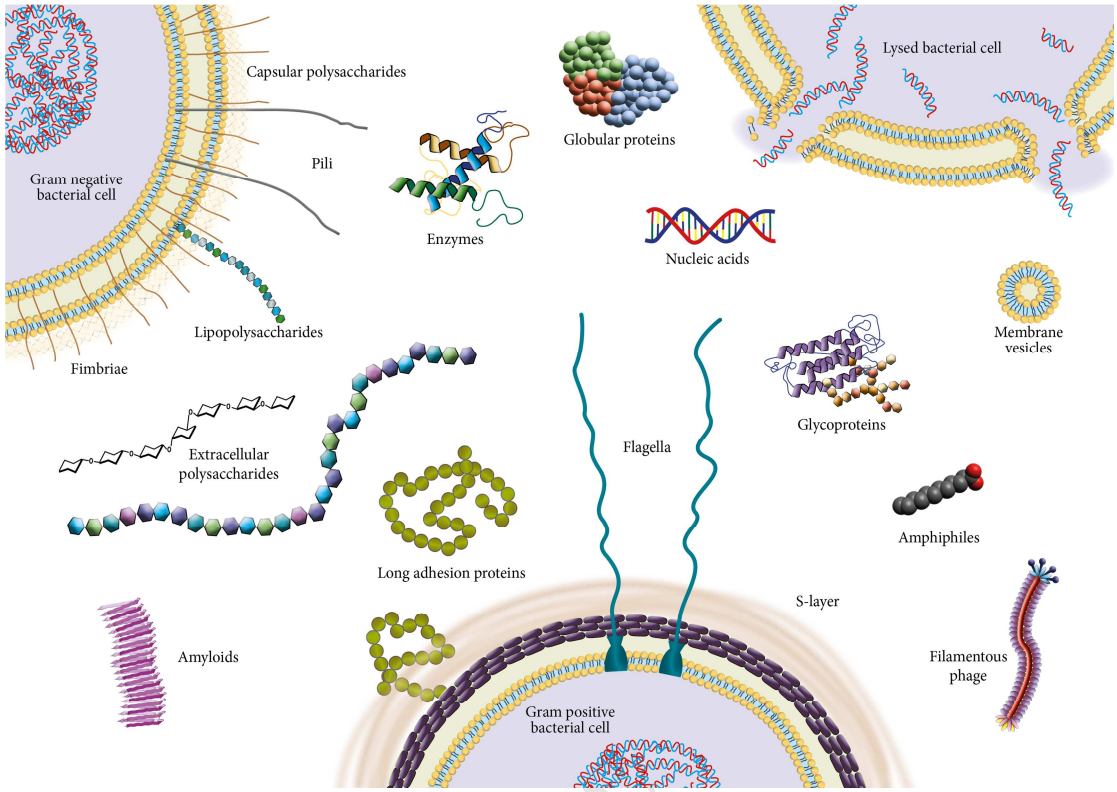

Box 1: Description of EPS found in the extracellular matrix of various biofilms

Amphiphiles (Neu 1996, Sand and Gehrke 2006): glycolipids (e.g. emulsan) and lipoproteins (Hiroshi et al. 2012), which along with microbially-derived humic-like compounds play key roles in interface interactions (Ogawa et al. 2001, Rosenberg and Ron 1999, Schurig et al. 2013).
Long adhesion proteins e.g. CdrA of *Pseudomonas aeruginosa* (Borlee et al. 2010), Biofilm associated protein of *Staphylococcus aureus* (Taglialegna et al. 2016).
Extracellular proteins: Exoenzymes e.g. lipase (Tielen et al. 2013), polypeptides.
Amyloids: e.g. Functional amyloids of *Pseudomonas* (Fap) (Dueholm et al. 2010), TasA of *Bacillus subtilis* (Romero et al. 2010) and curli of *Eschericia coli* (Dueholm et al. 2012).
Extracellular polysaccharides: anionic e.g. alginate-like exopolysaccharides (Lin et al. 2010), cationic e.g. Pel (Jennings et al. 2015), neutral e.g. cellulose (Serra et al. 2013), amphiprotic e.g. granulan (Seviour et al. 2010b).
Membrane vesicles: Enzyme-filled blebs from the outer membranes of G(-)(Turnbull et al. 2016) and G(+)(Liu et al. 2018) cells.
Nucleic acids: i.e. extracellular DNA (Turnbull et al. 2016).
Lipopolysaccharides: Involved in cell recognition and immunity (Nakao et al. 2012).
Filamentous phage: e.g. Pf4 bacteriophage in *Pseudomonas aeruginosa* (Secor et al. 2015).
Glycoproteins: e.g. Glycosylated amyloid-like proteins (Lin et al. 2018).
Capsular polysaccharides: i.e. surface-attached polysaccharides (Wang et al. 2015).
Pili: Hair-like appendage on bacterial surface composed of pilin proteins.
S-layer: external layer of cell envelope consisting of proteins or glycoproteins (Sleytr et al. 2014).

Figure 1: Illustration of exopolymers typically found in the extracellular matrix of biofilms. Note, such constituents have been identified from a range of biofilms, and not all matrices contain each of these components. Refer to Box 1 for a description of each exopolymer.

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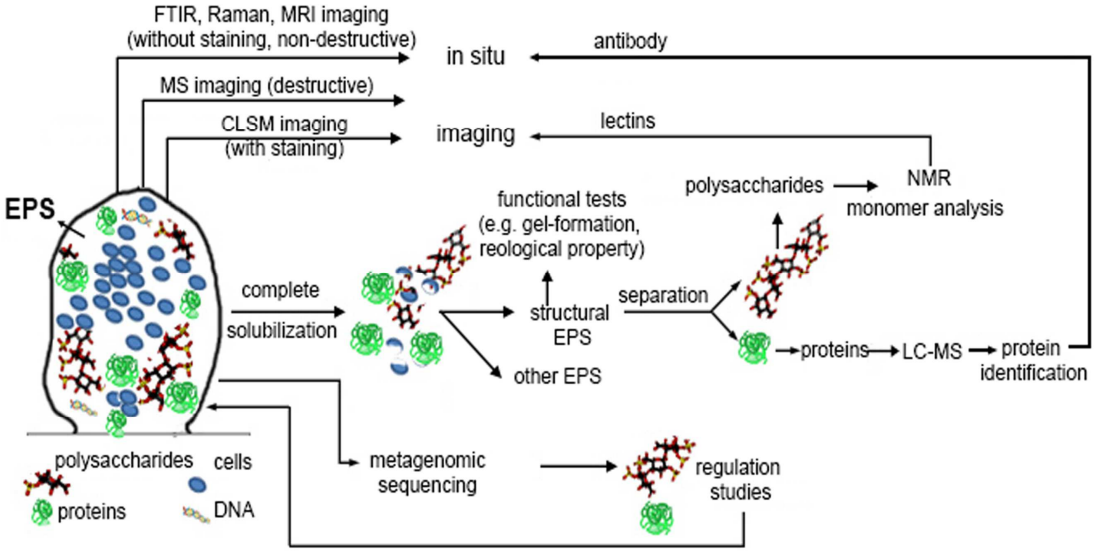

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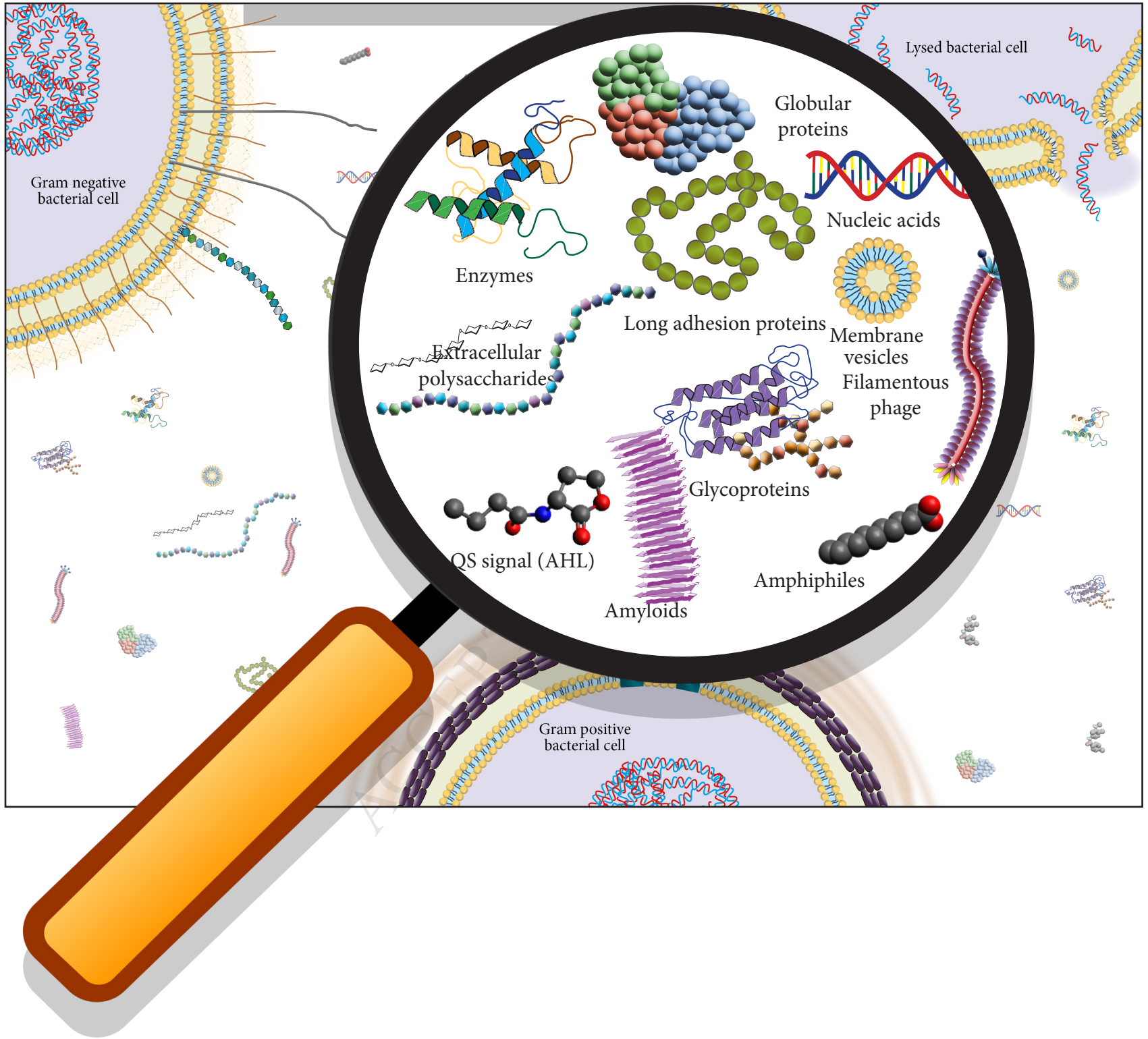
638 **Figure 2: Proposed multidisciplinary roadmap for resolving the identities and functions of**
639 **extracellular polymeric substances in biofilms, involving complementary chemical, biophysical**
640 **and 'omic' analysis of biofilms and isolated constituents.**

Highlights

- Extracellular polymeric substances feature in key societal problems (clinical, environmental)
- Methods and standards of EPS recovery and characterization need to be critically assessed
- More emphasis should be placed on methods that enable identification (chemical and function)
- Integrated and multi-displinary analyses are required on biofilms and EPS isolates
- Will improve biofilm management and enable a more circular economy in water and waste


Gram negative bacterial cell
Lysed bacterial cell
Globular proteins
Nucleic acids
Enzymes
Long adhesion proteins
Membrane vesicles
Extracellular polysaccharides
Filamentous phage
Glycoproteins
QS signal (AHL)
Amphiphiles
Amyloids
Gram positive bacterial cell