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Review

Rethinking characterization, application, and importance of extracellular polymeric substances in water technologies

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Biofilms play important roles in water technologies such as membrane treatments and activated sludge. The extracellular polymeric substances (EPS) are key components of biofilms. However, the precise nature of these substances and how they influence biofilm formation and behavior remain critical knowledge gaps. EPS are produced by many different microorganisms and span multiple biopolymer classes, which each require distinct strategies for characterization. The biopolymers additionally associate with each other to form insoluble complexes. Here, we explore recent progress toward resolving the structures and functions of EPS, where a shift towards direct functional assessments and advanced characterization techniques is necessary. This will enable integration with better microbial community and omics analyses to understand EPS biosynthesis pathways and create further opportunities for EPS control and valorization.

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Introduction

Extracellular polymeric substances (EPS) are complex mixtures of biopolymers secreted by microorganisms consisting mainly of polysaccharides, proteins, lipids, nucleic acids, and humic substances. The EPS play crucial roles in the formation and stability of biofilms. For water and wastewater engineering biofilms, such as activated sludge flocs and granules, the EPS provide structural integrity, protection, and a matrix for nutrient and waste exchange. However, despite the critical role played by the EPS in water and wastewater biofilms (e.g. promoting flocculation of activated sludge), the exact composition and identities of EPS in environmental and water biofilms largely remain a mystery (i.e. EPS 'identity crisis'). This was previously flagged as a critical knowledge gap in water and wastewater engineering [1] as it hinders the process optimization and the development of circular economies targeting EPS recovery. The lack of understanding on EPS composition contrasts to extensive knowledge regarding the microbial ecology of biofilms in water technologies, which has been made possible by advancements in DNA and RNA sequencing technology. Furthermore, the EPS were recently identified as a recoverable resource from biological wastewater treatment processes. Resolving the identities and structures of EPS and elucidating their behaviors and functions would increase opportunities for application and inform processing and formulation of EPS biopolymers.

Here, we submit our report card on efforts subsequent to the previous call-to-arms to address this critical knowledge gap. A general shift has been noted towards direct rather than indirect EPS functional assessment, with biophysical and physicochemical characterizations on extracted, or in some cases even purified EPS, rather than retrospectively assigning phenotypic changes to EPS type. EPS characterizations also tend to be more focused now, with a tightening of classifications, the introduction of new EPS subcategories, and even full identification. Furthermore, recent advancements in microbial community surveys and

omic analyses such as long-read sequencing, metagenomics combined with advanced bioinformatic tools have significantly enhanced our understanding of bacterial diversity and EPS biosynthesis. This has occurred concurrently with an increasing number of EPS applications, such as wood adhesives, corrosion inhibitors, and hydrogels, showcasing the versatility and potential economic benefits of EPS in various industrial and environmental contexts. This review article focuses on recently applied EPS characterization studies, which specifically enhance our understanding of EPS structures, identities, and structure–function relationships. These studies are taking place in parallel with developments in 'omics approaches that pave the way for understanding how EPS are synthesized, which bacteria are responsible, and how expression is regulated. Finally, we discuss new valorization strategies where EPS functionality can lead to biomaterials with enhanced properties.

New extracellular polymeric substance characterization strategies provide greater structural insight

New characterization strategies and a greater focus on the EPS in recent years have provided more precision in what the EPS contain and how they behave [2]. It is now understood, for example, that glycosaminoglycans (GAGs), including hyaluronic acid–like and sulfated GAGs-like polymers, are present in aerobic and anammox granules [3] and sialylated and sulfated glycoconjugates are abundant in both anaerobic and aerobic sludges [4]. The presence of amyloid-like structures [5] and surface (S-) layer proteins has been observed to provide structure to granular sludges [6,7]. Macromolecular degrading catabolic proteins such as polysaccharide-degrading hydrolases, lyases, proteases, and nucleases are also present, however, along with restructuring proteins such as oxidoreductases and transporter and ion-binding proteins [8,9]. Together these findings further suggest that the EPS are more than a structural scaffold but an extracellular digestion and preprocessing system [10]. Finally, extracellular lipids have also been attributed to a structural and mechanical function in activated sludge, although their concentrations and compositions in activated sludge are yet to be fully explored [11]. Here, we discuss several novel EPS characterization methodologies that have provided additional valuable insight into the EPS of water biofilms.

Although the limitations of basic quantification methods using colorimetry for proteins and sugars for absolute quantification have been described previously [12,13], they are still applied to generate valuable information, such as describing relative abundance between samples or changes over time. This can then be used to support mathematical models of EPS regarding its production and consumption. For example, Xing et al. correlated a reduction in loosely bound exopolysaccharides (LB-EPS) and tightly bound exopolysaccharides (TB-EPS) with increased zeta potential and decreased sludge volume index and presented a model for LB-EPS and TB-EPS generation and consumption [14], which they suggested could be used to regulate sludge settleability.

Resolving the abundance of individual EPS compounds can better inform EPS production dynamics and functionality [15]. For example, in a polyphosphate accumulating organism (PAO)-enriched biofilm, ^{13}C-labeled extracellular and intracellular proteins, and exopolysaccharides were quantified by mass spectrometry–based proteomics and liquid chromatography-high-resolution mass spectrometry (LC-HRMS), respectively. Using a secretion-signal prediction tool, extracellular proteins could then be distinguished from intracellular proteins and turnover rates in intracellular and extracellular biopolymers then calculated. It could thus be concluded that the EPS were degraded by general decay of biomass rather than a preferential EPS consumption by the flanking populations [16].

Biophysical characterizations have been undertaken to resolve EPS behavior through an understanding of their higher order structures. Circular dichroism (CD) spectroscopy, transmission electron microscopy, atomic force microscopy, and rheology are just some of the methods available for EPS biophysical characterization. EPS functionality can be informed from higher order structure. Therefore, particularly for extracellular proteins that are more easily identified than sugars (i.e. by proteomics), higher order structure and function can be suggested by the primary structure using an increasing number of online tools for predicting structural features such as PONDR and AlphaFold. For example, with EPS from anammox granules, the structural units forming the 3D network in concentrated EPS dispersions were suggested to be functional amyloids [17]. Seviour et al. isolated a structural protein from anammox granules [18] homologous to that described by Lotti et al. [19]. The prediction of β-sheet structural motifs by computational structure prediction was confirmed for both by CD spectra. This protein was also determined *in silico* to contain intrinsically disordered domains, which were shown by single molecular droplet assay on isolated and recombinant expressed protein fragments to promote phase transition of the protein into liquid condensates. These condensates adhered to cells, and this was proposed as a mechanism for how it could promote biofilm formation [18].

Identifying and resolving the structure of EPS remain challenging due to the complexity of the molecules involved, resulting from low solubility and purity, structural heterogeneity, and microbiological variability [15,20]. Nonetheless, significant progress has been made toward better resolution of characterization through a combination

of improved analytical methods and a broader suite of fluorescence-binding proteins coupled with microscopy. Fluorescence lectin-binding analysis, enzymatic quantification, and mass spectrometry demonstrated the presence of different nonulosonic acids in the extracellular matrix of a '*Candidatus* Accumulibacter' biofilm, which was further suggested by the presence of nonulosonic acid synthase genes in the metagenome-assembled genome of *Ca.* Accumulibacter, and NuIO-specific receptors, permeases, and transporters in its biofilm proteome [21]. These nonulosonic acids seem to be an integral aspect of most microbial EPS [21,22]. By combining sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Alcian Blue staining, fourier-transform infrared spectroscopy (FTIR), mammalian hyaluronic acid and sulfated GAG analysis kits, selective enzymatic digestions, and specific *in situ* visualization by Heparin Red and lectin staining, Felz et al. could identify GAGs, including hyaluronic acid–like and sulfated GAGs-like polymers in their aerobic granular sludge EPS [3]. Finally, Wong et al. raised antibodies against the same β-sheet-rich extracellular protein [7] described by Seviour et al. [18] (i.e. homologous to that described by Lotti et al. [19]). Using immunofluorescence coupled with FISH microscopy they then described its distribution throughout the biofilm relative to the major population. This led to the conclusion that it was an S-layer protein secreted by *Ca.* Brocadia sinica but also that it served a secondary function in facilitating the assembly of another population into a 3D biofilm scaffold.

Extensive characterizations have also been performed to evaluate and optimize the application potential of EPS. Mechanical properties of EPS gels were investigated using dynamic and static rheology [23], showing that strain-hardening and syneresis of the EPS occur depending on calcium concentration. Molecular-level analysis combined with FTIR and Inductively Coupled Plasma— Atomic Emission Spectrometer (ICP-AES) were employed to demonstrate that extracellular polysaccharides from anammox biofilms and proteins can bind heavy metals [24,25]. Various approaches used to characterize EPS are outlined in Table 1. Nonetheless, while some extracellular proteins, and types of sugars, have been identified for biofilms, the challenge remains to identify individual components of EPS comprehensively in biofilms, which is only possible by enabling spectroscopic analyses such as FTIR, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry following extraction and isolation.

Omics pave the way for species-resolved extracellular polymeric substance biosynthesis predictions

Recent global-scale microbial community surveys have provided detailed insights into the diversity of bacteria in wastewater treatment systems [29]. Notably, the MiDAS global project introduced the MiDAS 4 16S rRNA gene reference database with placeholder names for the many uncultured microbial species lacking an official taxonomy, providing a common language for microbes in the wastewater field [30]. It was further revealed that less than 2000 bacterial species constitute most of the biomass in global wastewater treatment systems [30]. By understanding the EPS production of these species, we might be able to predict the overall EPS properties with high precision.

Since genomes encode everything a bacterium can do, we can gain unique insights into the EPS potential of individual species via their genomes if we understand the genes involved in EPS production. Recent advancements in long-read sequencing technologies enable us to obtain high-quality (HQ) metagenome-assembled genomes (MAGs) from complex communities, which importantly contain 16S rRNA genes that can be directly linked to amplicon-based microbial community surveys. This was first demonstrated on a large scale for activated sludge through hybrid assemblies of Illumina and nanopore reads to recover over 1000 HQ MAGs [31]. However, the improved accuracy and throughput of nanopore and PacBio sequencing now allow long-read-only assemblies [32], simplifying the process of obtaining HQ MAGs and paving the way for sequencing all common bacteria in the global wastewater microbiome.

With the genomes available, we can examine their potential for EPS production. The AntiSMASH framework uses Hidden Markov Models (HMMs) and gene proximity to identify biosynthetic gene clusters (BGCs) encoding secondary metabolites [33]. A similar approach, although using simple BLAST searches, was developed for detecting BGCs encoding 16 different exopolysaccharides (exoPS) in activated sludge [34]. It was found that many of the bacteria encode known exoPS, such as bacterial cellulose and Pel polysaccharide. However, to gain a full picture of the genetic potential for EPS biosynthesis, we need to be able to detect not only BGCs encoding known exoPS but also novel types. This may be achieved by targeting conserved key genes for the four common pathways used to produce all known exoPS [35], for example, by applying broad HMM models and detection rules that fit the specific pathways [35]. Furthermore, additional information about the exoPS biosynthetic potential of individual species can be obtained by investigating which activated sugar monomers they can synthesize as building blocks for the exoPS. This strategy has been applied to anaerobic granular sludges treating papermill and brewery wastewater and revealed a high content of uronic acids in the EPS, which could be linked to specific community members [36]. Because the EPS also contain large amounts of extracellular proteins, including pili, fimbriae, amyloids, and adhesins, we also need to be able to

Table 1

Overview of current techniques used for EPS characterization.

Approach	Details	Biofilm	Outcome	References
Staining and microscopy	Immunofluorescence (antibodies)	*Candidatus* Brocadia sinica	Distribution of extracellular proteins	[7]
	Lectins Heparin Red	Aerobic granular sludge	Distribution of polysaccharides in the extracellular matrix	[3]
	Lectins	*Candidatus* Accumulibacter		[21]
Mechanical	Dynamic rheology Static rheology	Aerobic granular sludge	Strain-hardening and syneresis of EPS	[23]
	Rheology	Activated sludge	Changes in biopolymer structure	[26]
Biophysical	Transmission electron microscopy (TEM) Atomic force microscopy (AFM) Small-angle X-ray scattering (SAXS) Circular dichroism (CD) FTIR Ultraviolet-visible (UV-Vis)	Anammox granular sludge	Functional amyloids as putative structural units	[17,19]
	FTIR Nuclear magnetic resonance (NMR) High-performance gel-permeation chromatography (HP-GPC)	Bacteria from biofouled reverse osmosis (RO) membrane	Characteristics of EPS that thrive on biofouled RO membranes	[27]
	FTIR NMR Excitation-emission matrix (EEM) CD	Activated sludge	Biopolymers evolution under hydrothermal treatment	[26]
Chemical	FTIR Inductively coupled plasma–atomic emission spectrometer (ICP-AES)	Anammox granular sludge	EPS as heavy metal biosorbents with high capacities	[24]
Mathematical model	Unified model-TL1 and expanded unified model-TL2	Activated sludge	Generation and consumption mechanism of EPS	[14]
Mass spectrometry	High-resolution mass spectrometry (HRMS)	*Candidatus* Accumulibacter	Types of polysaccharides in the extracellular matrix	[21]
	Tandem mass spectrometry (MS/MS)	*Candidatus* Brocadia sinica	Identification of extracellular structure proteins	[28]
	Liquid chromatography high-resolution mass spectrometry (LC-HRMS)	PAO-enriched sludge	Turnover of EPS in granules	[16]

identify these compounds. Although this can already be done based on manual homology searches, it would be relevant to create a user-friendly tool that can detect both exoPS BGCs and genes encoding extracellular proteins without expert knowledge.

Insight into the homologs of known exoPS BGCs has revealed variations in gene conservation and synteny across taxa [34]. This suggests potential differences in exoPS processing that could lead to altered physicochemical properties. To examine the impact, we need representative bacterial pure cultures from wastewater treatment systems to study individual EPS components under controlled conditions. Unfortunately, very few cultured species are available from activated sludge, including the floc-forming *Zoogloea* [33] and *Thauera* [37]. Accordingly, there is a pressing need to apply culturomics to wastewater treatment research [38]. To ensure that the isolation efforts focus on common and abundant species in the wastewater treatment plants, we can use the results from global microbial surveys and genomic analyses. First, genome annotations may offer insights into the autotrophies of specific taxa, aiding in tailoring growth mediums for targeted isolation [39,40]. Second, microbial profiling can be used to rapidly screen for media and growth conditions that support the growth of common taxa. This strategy was recently employed to demonstrate that most activated sludge bacteria require specific components in the sludge fluid for growth [41]. Finally, as most species occur in low relative abundance, it may be relevant to enrich for the target species before isolation. This can be achieved using high-throughput cell sorting techniques, as recently demonstrated for *Ca.* Accumulibacter, *Nitrospira* [42], and Patescibacteria [43].

Since genomes only provide insights into the genetic potential for EPS production, it is important to stress that transcriptomic and proteomic analyses are required to determine if the identified genes encoding EPS are expressed and significant in wastewater treatment systems. Additionally, these analyses can reveal how these genes are regulated in response to environmental conditions, including operational processes in wastewater treatment. Such information is currently lacking but will be instrumental in developing improved treatment strategies in the future.

Innovative applications driven by extracellular polymeric substance functionality and composition

Turning biomass into value-added product and deriving new materials from waste biomass can be beneficial to the environment and transform the wastewater treatment plant into a resource recovery plant. Thus, the various potential applications of EPS recovered from sludge have been explored. The frequently studied applications for EPS are as flocculant or adsorbent [44–47]. Excitingly, studies on new EPS applications are constantly popping up. Xu et al. made sewage sludge extracts using deep eutectic solvents followed by glycerol triglycidyl ether processing and applied it as wood adhesive [48]. Having a wet shear strength of 0.93 MPa, the resulting adhesive reached to the Chinese national standard. This approach has a lower environmental impact and higher economic efficiency compared to incineration and anerobic digestion of sewage sludge. Go et al. investigated the corrosion inhibition potential of EPS from waste-activated sludge. EPS could adsorb on the metal surface, forming a film that acted as a protective barrier against corrosion on both anode and cathode sites of metal surfaces [49]. Due to the rapid development of aerobic sludge granulation biotechnology, significant amount of research has been oriented on the EPS extracted from aerobic granular sludge. Inspired by the hydrogel property of the aerobic granule itself, EPS ionic hydrogel was made by letting the structural EPS react with divalent ions. The mechanical properties of this hydrogel were comparable to ι-carrageenan [50], suggesting a potential application of EPS as hydrogel carriers. EPS-based flame retardant has been developing since 2020. It improved the fire performance of natural fiber such as flax and plastics such as polylactic acid and polypropylene by enhancing the char formation [51]. In addition, a special focus was put on the enrichment and application of the EPS containing negatively charged groups (e.g. nonulosonic acids and sulfated glycoconjugates) [52]. With increased charge density, the enriched fractions can strongly bind positively charged proteins such as histones involved in sepsis and fibroblast growth factor 2, demonstrating the possibilities for EPS potential application in the medical field (e.g. raw material for sepsis treatment drugs) and chemical field (e.g. column material for proteins purification). The recovery of EPS from sludge means that a fraction of the influent COD leaves the wastewater treatment process as biopolymers. If a significant amount of EPS (several tons per day) can be recovered, this could generate substantial economic revenues for the wastewater treatment plant, changing the economics of wastewater and sludge treatment by providing a high-value biobased raw material while reducing secondary sludge treatment requirements [53]. In order to push the development of EPS application forward, it is significantly important to connect the EPS property with its application.

Conclusion and future directions

EPS characterization requires expertise from many different research fields because EPS are not a single molecular entity. Unlike other waste biomasses (e.g. lignocellulosic), EPS are produced by multiple organisms. Resolving EPS structures, therefore, necessitates integrating biopolymers chemistry, glycomics, proteomics, genome-resolved metagenomics and transcriptomics, microscopy, culturomics, and biophysics. To address the EPS identity crisis, our research has moved closer to the state-of-the art in such areas, as outlined in this review paper. It is important that this continues, as progress in relevant disciplines continues to enrich EPS characterization, enabling the resolution of the structure and identities of more and more EPS. This is necessary to demystify the EPS, which will accelerate the uptake of EPS as a recoverable resource, inform the development of new EPS biomaterials, and improve bioprocess controls.

CRediT authorship contribution statement

Sasmitha Aulia Zahra: Conceptualization, Writing – original draft preparation. **Rozalia Persiani:** Conceptualization, Writing – original draft preparation. **Morten Kam Dahl Dueholm:** Conceptualization, Writing – original draft preparation. **Mark van Loosdrecht:** Conceptualization, Writing – review & editing. **Per Halkjær Nielsen:** Conceptualization, Writing – review and editing, Funding acquisition. **Thomas William Seviour:** Conceptualization, Writing – original draft preparation, Funding acquisition. **Yuemei Lin:** Conceptualization, Writing – original draft preparation.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- •• of outstanding interest

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