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Review Article
Electrochemical roles of extracellular polymeric substances in biofilms

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Most microbial cells in nature are surrounded by extracellular polymeric substances (EPS), which are fundamental components and determine the physiochemical properties of a biofilm. This review highlights the EPS properties of conductivity and redox ability from an electrochemical perspective, emphasizing recent findings that EPS play important roles in microbial extracellular electron transfer (EET). Basic information regarding EPS structure and components is required to better understand their effects on EET; future directions are briefly discussed.

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Introduction

Biofilm is one of the most important forms of microorganisms and is involved in the transformation of environmental pollutants and biogeochemical cycle of many elements [1,2]. Accounting for up to 90% of the dry weight biomass of a biofilm, extracellular polymeric substances (EPS) are produced to protect the microorganisms from unfavourable environments [3,4]. Previous experiments revealed a layer of EPS surrounding bacterial cells. For example, cryo-transmission electron microscopy showed that an EPS layer up to 1 μm in thickness formed and enveloped *Shewanella oneidensis* MR-1 cells [5]. In a recent study [6••], we removed the EPS from *S. oneidensis* MR-1 after 48 h of static culture; morphological results from atomic force microscopy and laser particle size analyser indicated that the cells were covered with an EPS layer of 35 ± 15 nm (Figure 1). Therefore, the EPS matrix is referred to as the "house of cells" [3].

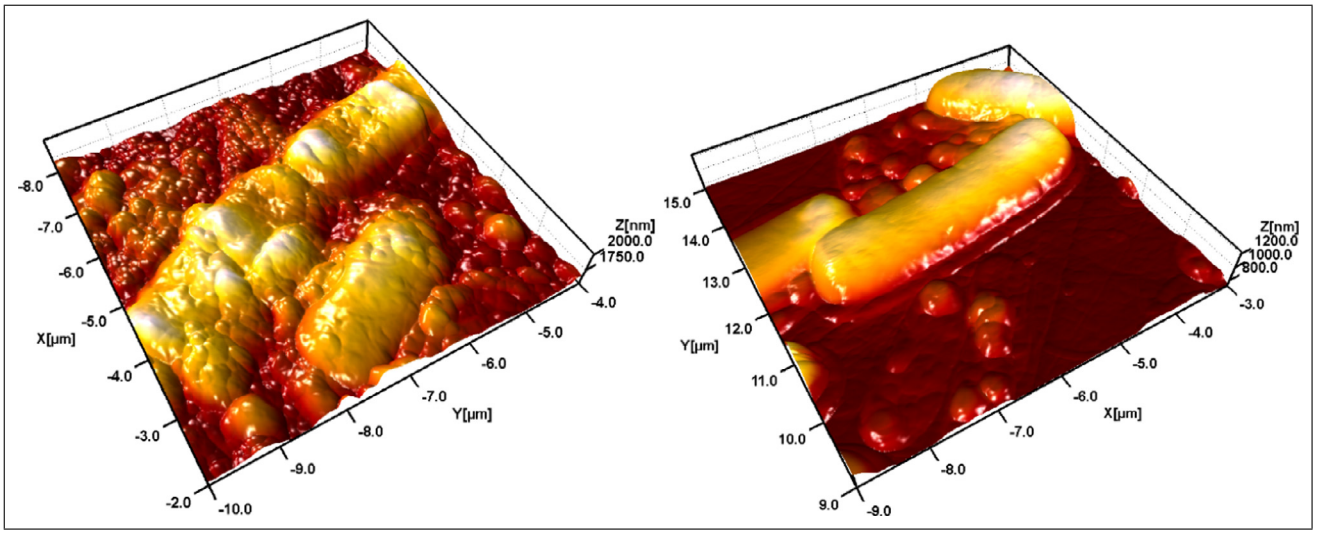
Electron transfer is one of the most important processes for life, as metabolism processes involve redox reactions of substrates [7]. Many microorganisms have been reported to transport electrons directly to external electron acceptors such as magnetite, hematite, uranium dioxide, and even electrodes [8–13]. Such electron transport processes are designated as extracellular electron transfer (EET) and are important in the biogeochemical cycles of various elements, environmental remediation, and bio-energy production [8].

Most microbial cells in biofilms are enveloped by EPS and do not directly contact the external environment. EPS serve as carbon and energy sources for biofilm and can adsorb and degrade organic compounds from the surrounding environment [2]. However, in previous studies of microbial EET mechanisms, the roles of EPS have not been examined in detail from an electrochemical perspective. The aim of this review is to describe inter-disciplinary research and highlight the electrochemical function of EPS on EET in biofilms.

Extracellular electron transfer pathways

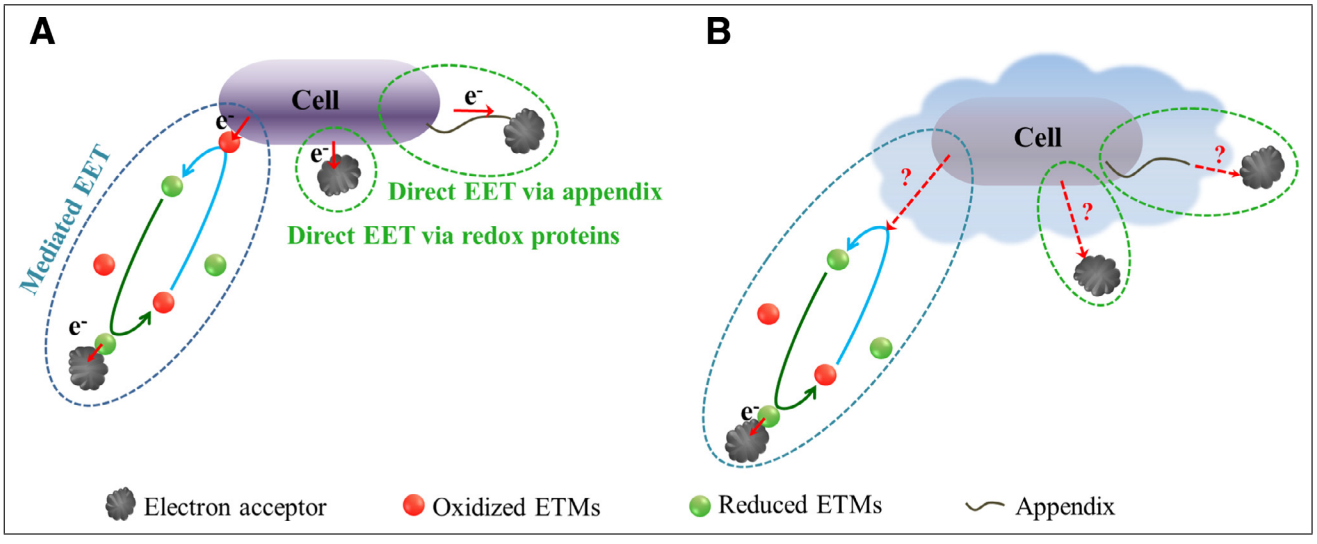
Microorganisms use EET processes to communicate with other cells or interact with external environments. Redox molecules such as flavins [14•] and phenazine [15] were reported to conduct EET, while the mediator content affects their EET processes [16–18]. Membrane-bound cytochrome *c* is a group of redox proteins involved in electron transfer [19] and play key roles in microbial EET processes [20]. Furthermore, microbes may use conductive nanowires [21–24••] or conductive pili [25••] to transport electrons extracellularly; the redox proteins of MtrC/OmcA and metallic-like conductive protein PliA are the main components on nanowires from *S. oneidensis* MR-1 [26••] and pili from *Geobacter sulfurreducens* PCA [25••,27], respectively. Based on these studies, two microbial EET pathways have been proposed: direct EET and indirect EET (Figure 2A). In the direct EET pathway, microorganisms use redox proteins in the outer membrane or extended conductive nanowires/pili to transport electrons directly to extracellular acceptors. In the indirect EET mechanism, microorganisms transport electrons indirectly to external acceptors involving self-secreted or exogenetic electron mediators [9,28]. However, what happens when a layer of EPS envelops microbial cells

Figure 1



Three-dimensional images of *Shewanella oneidensis* MR-1 cells from atomic force microscopy [6]. (A) MR-1 cells treated at 30 °C, the same temperature as that for cultivation, are enveloped by EPS, and the substrate (Pt sheet) is covered by EPS. (B) MR-1 cells treated at 38 °C show few EPS on the surface, and the substrate is very clean.

Figure 2



Microbial EET mechanisms (assuming a cell functions as an electron donor). (A) Previous studies with proposed direct and indirect microbial EET mechanisms [6]. (B) What will happen to EET processes when the cell is enveloped by EPS? ETMs: electron transfer mediators.

(Figure 2B)? Cells enveloped by EPS cannot contact the external environment directly, while EPS contain mostly microbial secretions, including electron mediators.

EPS conductivity properties

Although electron transfer between microbial cells and electron acceptors/donors in the external environment apparently must pass through the EPS layer, whether and how EPS participate in EET processes remains unclear [29]. In a modelling study, Marcus *et al.* developed a dynamic, one-dimensional, multi-species model to describe the rate of electron donor oxidation by anode biofilm in microbial fuel cells and found that the biofilm should possess a conductivity higher than 10^{-6} S cm^{-1} [30]. Further modelling studies by Torres *et al.* suggested biofilm from anode-respiring bacteria as a semiconductor with conductivity ranging from 10^{-9} to 10^{3} S cm^{-1} [31].

EPS include polysaccharides, proteins, glycoproteins, glycolipids, humic substances, and, in some cases, extracellular DNA [3,4]. A large amount of experimental data has shown that these substances possess some semiconductive properties. For example, hydrated or ionized polysaccharides function as semiconductors in the form of polymer electrolytes or conducting polymers [32]. Some reports showed that DNA can act as a semiconductor with a conductivity of 10^{-9} to 10^{-3} S cm^{-1} [33–35]. Natural proteins are typically poorly conductive, but cytochrome protein-rich nanowires from *S. oneidensis* MR-1 are on the order of 1 S cm^{-1} at 100 mV of applied bias [21]. Additionally, the type IV pili from *Geobacter* consists of PilA proteins and may be a metal-like conductor with 0.005 S cm^{-1} at pH 7 [25••,36]. Humic substances, commonly consisting of humic acids, fulvic acids, and humic, are very important components of EPS, particularly in biofilms after a long period of culture [2,4]. Humic acids are the most important type and have been reported as semiconductors with conductivity ranging from 10^{-7} to 10^{-3} S cm^{-1} [37]. Conductivity can be ionic or electronic. Given the characteristics of these main components, the EPS matrix likely acts as an ionic semi-conductor, which does not involve metallic-like electron transfer.

EPS redox properties

Many components of the EPS matrix have been reported to be redox active or electrochemically active. For example, the nucleotides guanine and adenine were shown to be electrochemically redox active [38], DNA has been also widely used as an electrochemical sensor [39–41]. Because of their key roles in cell metabolism processes, many proteins are redox active and engage in electron transfer. Some of these proteins are still highly active after being released into the external environment, e.g. cellulases. Proteomics analysis of EPS from *Shewanella* sp. HRCR-1 biofilm revealed the presence of 58 extracellular and outer membrane proteins, including *c*-type cytochromes of MtrC and OmcA [42•]. Recent studies further confirmed that the EPS matrix extracted from *S. oneidensis* MR-1 are electrochemically active and redox peaks of cytochrome *c* can be detected by voltammetry measurement [6••,43••]. Although saccharides rarely show electrochemical activity, Kang *et al.* reported that saccharides in EPS from *Escherichia coli* play a key role in reducing Au^{3+} to nanoparticles [44••]. Possible mechanisms for how saccharides reduce ions of heavy metals may be that hemiacetal groups of reducing sugars in EPS act as reducing agents or saccharides may adsorb some redox molecules to reduce the metals ions [44••,45].

With a large number of functional groups such as carboxylic acid, phenolic and alcoholic hydroxyls, and ketone [46], the EPS matrix can adsorb and/or bind secreted redox molecules such as flavins. Differential pulse voltammetry measurements on EPS from *S. oneidensis* MR-1 clearly showed the redox peaks of flavins [6••]. Humic substances consist of quinone groups as one of the main functional groups [46], which allows humic to bind both hydrophobic and hydrophilic materials and is involved in redox reactions [47,48]. Microorganisms can use humic substances as electron mediators for anaerobic respiration of organic compounds [49], an indirect EET pathway. Humic substances, together with their analogue of 2,6-anthrahydroquinone disulphonate, have been reported to widely engage in metal reduction [50,51] and dissimilatory azoreduction reduction [52].

Given the characteristics of semi-conductor, redox activity, and electron mediators, the EPS matrix, or "house of cells", likely affects extracellular electron transfer in biofilm, but additional studies are needed to gain a fundamental understanding in this research field.

Roles of EPS in EET processes

When electrons are transported between the cells and extracellular solid electron acceptors, they must pass through the EPS layer, which may be up to 1 μm thick [5]. As described above, EPS possess semi-conductive properties and consist of electrochemically active components, which may have affected the proposed EET pathways in previous studies, i.e. both direct EET and indirect EET modes, from an electrochemical perspective.

For direct EET mode, there are dramatic differences in electrical and electrochemical properties between polysaccharides, proteins, and DNA, which accordingly change the electrical and electrochemical performance of EPS matrix under different environments [29]. However, the EPS tightly bound to cells may have greater effects than those that are weakly bound to cells, given the spatial distribution; EPS weakly bound to cells may be spaced too far to achieve metallic-like electron transfer. Hopping and tunnelling are generally recognized mechanisms in long-range electron transfer in biological systems [53]. Notably, the distance between the active centre of proteins and electron acceptors/donors should be less than 2 nm because of the single-step tunnelling limit [54]; i.e. direct EET can occur only when the distance of substances is very close. The presence of the EPS layer, sometimes with a distance at the micrometre level [55], may block direct electron transfer if the EPS does not affect the process. A recent study found no electrochemical signal of flavin/MtrC from EPS in *S. oneidensis* MR-1 [56•], but the signal was detected after EPS depletion [6••]. However, direct electron transfer from *S. oneidensis* MR-1 to an electrode still occurs in EPS-retaining cells under poised potential after substrate addition [6••], indicating that EET successfully occurs through the EPS layer. Thus, hopping may enable direct EET processes of microorganisms given the distance and semiconductor properties, and the redox gradient is a key factor driving multistep electron hopping in EPS. Conductive nanowires or pili commonly acquired from cells

growing in oligotrophic chemical defined media are thought to help microorganisms achieve long-range electron transfer [21,22•,25••]. However, nutrients in these media are limited for microbial growth, and microorganisms are unlikely to produce a large mass of EPS, which is more easily produced in eutrophic media. Herein two questions are raised: can conductive nanowires or pili be obtained under eutrophic conditions such as Luria–Bertani medium? If so, will the EET along conductive nanowires or pili be affected when they meet electron mediators/redox cytochromes in the EPS layer (i.e. is there a reaction when the electrons meet redox mediators before reaching external electron acceptors)? These questions may be answered by further studies of *S. oneidensis* and *G. sulfurreducens*, which are very important for understanding biofilm behaviours during redox reactions.

Given the effects on indirect EET, EPS contain microbial secretions and adopt redox molecules with various functional groups in the biopolymer matrix. Previous studies confirmed the existence of cytochromes *c* and flavins in EPS from electrochemically active microorganisms by electrochemical measurements [6••,43••] and proteomics technology [42•]. Humic substances are well-known as electron mediators affecting microbial growth and community diversity through redox reactions [57]. Therefore, the redox molecules in EPS do not easily diffuse to the bulk solution, and a high concentration of redox molecules can be maintained in the gap between cells and electron acceptors/donors. The electron mediators in EPS may be very useful for cell growth and microbial metabolism via EET function.

Although direct and indirect EET mechanisms were mainly determined in studies of Gram-negative bacteria, some Gram-positive bacteria and yeast were also reported to transfer electrons extracellularly [16,17,58]. A recent study explored the effects of EPS on EET processes in Gram-positive/Gram-negative bacteria and yeast, and electrons were found to pass through the EPS layer of all three types of microorganisms [6••]. After EPS extraction, electrochemical signals of flavins were strengthened in Gram-positive bacteria and yeast, but disappeared in Gram-negative *S. oneidensis* MR-1. These results indicate that flavins do not act only as electron mediators as previously reported, and the important roles of EPS in EET processes should be considered.

Present studies of EPS and their electrochemistry mainly depend on EPS extraction and electrochemical measurements such as voltammetry. Different extraction methods can affect the yields and components of the EPS extract [4,59]. The detection limit varies for different voltammetric techniques from ppm magnitude to ppt magnitude [60]. Other intercellular redox molecules such as NADH may be detected in EPS, as they can be released into the surrounding environment following cell lysis, but the complexed EPS matrix will dramatically reduce their detection limit; some mutants that cannot produce EPS may be helpful for understanding the roles of EPS in EET processes.

Perspectives

EPS layer is not a solid barrier separating cells and external electron donors/acceptors, and these redox molecules can diffuse in the EPS and are involved in transferring electrons between cells and electron donors/acceptors. Much remains to be learned regarding the roles of EPS in EET, as well as their effect on conductive nanowires/pili or interspecies electron transfer. Further studies should be devoted to the biogeochemical cycling, bioenergy generation, and fate and biomineralisation of environmentally related pollutants.

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