Relevance of microbial extracellular polymeric substances (EPSs) – Part I: Structural and ecological aspects

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Abstract Extracellular polymeric substances are the construction materials for microbial aggregates such as biofilms, flocs ("planktonic biofilms") and sludge. Their major components are not only polysaccharides but also proteins and in some cases lipids, with minor contents of nucleic acids and other biopolymers. In the EPS, biofilm organisms can establish stable arrangements and function multicellularly as synergistic microconsortia. The matrix facilitates the retention of exoenzymes, cellular debris and genetic material; it can be considered as a microbial recycling yard. Gradients can develop due to the physiological activity and the fact that diffusive mass transport prevails over convective transport in the matrix. Biofilm cells tolerate higher concentrations of many biocides. The EPS matrix sequesters nutrients from the water phase. In photosynthetic communities, EPS molecules can function as light transmitters and provide photons to organisms located deeper in a microbial mat. The EPS matrix is a dynamic system, constructed by the organisms and responding to environmental changes. It enables the cells to function in a manner similar to multicellular organisms.

Keywords Biofilms; extracellular polymeric substances; EPS; microbial ecology

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Introduction

The vast majority of micro-organisms on earth live in aggregates such as films, flocs ("planktonic biofilms") and sludges. An everyday feature of such aggregates is addressed by the term "slime" which refers to highly hydrated, thick biofilms. They are slippery and can have different effects. A chapter on diffusion in biofilms owes its existence to that property, explicitly acknowledged by the author: "This review was written because of a biofilm that caused me to lose my grip and be carried down a small waterfall. The result was adequate to immobilise me sufficiently and for a long enough period to organise the diverse body of material presented" (Koch, 1990). Microbial aggregates are kept together by means of biopolymers of microbial origin, so-called extracellular polymeric substances (EPSs). The abbreviation "EPS" is used as a more general and comprehensive term for different classes of macromolecules such as polysaccharides, proteins, nucleic acids, (phospho)lipids, and other polymeric compounds which have been found to occur in the intercellular space of microbial aggregates (Wingender *et al*., 1999a). They are responsible for the cohesive forces which keep these aggregates together, i.e., biofilms, flocs and sludge (Wingender and Flemming, 1999). Although somehow inexactly used, the expression "biofilm" is commonly expanded to all these forms of microbial aggregates. The EPSs fill and form the space between the cells; they are responsible for the architecture and morphology of the matrix in which the cells live. Thus, the EPS can be considered as the "house" of the micro-organisms.

Composition of EPS

The production of EPS is a general property of micro-organisms in natural environments and has been shown to occur both in prokaryotic (Bacteria, Archaea) and in eukaryotic

(algae, fungi) micro-organisms. Biofilms containing mixed populations of these organisms are ubiquitously distributed in natural soil and aquatic environments, on tissues of plants, animals and man as well as in technical systems such as filters and other porous materials, reservoirs, plumbing systems, pipelines, ship hulls, heat exchangers, separation membranes, etc. EPSs are mainly responsible for the structural and functional integrity of biofilms and are considered as the key components that determine the physicochemical and biological properties of biofilms. The EPSs form a three-dimensional, gel-like, highly hydrated and often charged biofilm matrix, in which the micro-organisms are embedded. The EPSs create a microenvironment for sessile cells, which is conditioned by the nature of the EPS matrix. In general, the proportion of EPS in biofilms can vary between roughly 50 and 90% of the total organic matter (Christensen and Characklis 1990, Nielsen *et al*. 1997). The composition of EPS as analysed largely depends upon the method used for isolation. In general, no method has been developed which reliably yields complete extraction of EPS without contamination of intracellular components. Nielsen *et al*. (1999) have compared many different methods and the resulting quantities of EPS. The best-investigated component of EPS is the polysaccharide moiety (Sutherland, 1994; 1999a). However, the matrix is composed also of more components such as proteins and nucleic acids (Palmgren and Nielsen, 1996; Jahn *et al*., 1999) and lipids (Gehrke *et al*., 1998). Table 1 gives an idea about data from various natural and technical systems:

The EPS of *Pseudomonas aeruginosa* may serve as an individual example, as this is a model organism with which many biofilm investigations have been carried out and alginate is known as the main EPS component. The data are summarised in Table 2.

Interestingly, even in *P. aeruginosa* biofilms, more than 45% of the overall protein mass is found in the EPS where they represent more than 30% of the EPS mass. Using the activity of glucose-6-phosphate-dehydrogenase, a strictly intracellular enzyme, as a marker for contamination of intracellular components (Platt *et al*., 1985), it could be shown that the cells remained intact during the entire extraction procedure. Thus, the proteins found in the EPS matrix were not of intracellular origin. Preliminary investigations revealed that they are of relatively small molecular mass, i.e. between 5,000 and 150,000 Dalton. Strong protease activity was found which leads to a rapid breakdown of extracellular proteins (Broekman *et al*., unpublished results).

Considering the matrix as a whole and in non-laboratory systems, not only polymers of

Table 1 Composition of EPS and range of component concentration

Component	Content in EPS
Polysaccharides	40–95%
Protein	<1–60%
Nucleic acids	<1–10%
Lipids	<1–40%

Table 2 Composition of EPS from agar-grown biofilm of *Pseudomonas aeruginosa*, data related to 10^9 cells (after Wingender *et al*., in press)

Component	Biofilm (µg/109 cells)	EPS[a] (µg/109 cells)	Proportion found in EPS
Total carbohydrates	1005.8	766.6	76.2%
Uronic acids (alginate)	473.8	402.8	85.0%
Proteins	585.0	266.4	45.5%

[a]The EPS components were quantitated in cell-free solutions obtained after removal of biofilm bacteria by centrifugation and membrane filtration of the supernatants.

microbial origin are retained in the matrix. Due to its adhesive properties, particles can be trapped and integrated thus influencing the microenvironment and possibly providing nutrients (Decho, 1990, 2000). Common particulate components of the matrix are debris and humic particles, clay, silt and calcium sulphate. Substances dissolved in water can also be sorbed to the EPS molecules.

Ecological function of EPS

Adhesion and cohesion

A major ecological advantage of the biofilm form of life is that consortia of various organisms can establish and maintain their position over a long period of time, compared to the planktonic form of life. This applies not only to biofilms but also to flocs (Zartarian *et al*., 1994; Stehr *et al*., 1995) and allows for the development of synergistic relationships. A classical example is nitrification which takes place in biofilms and allows the spatial closeness of ammonia oxidisers to nitrite oxidisers. The EPS molecules which keep the organisms together and, if they form a biofilm, are responsible for adhesion to a given surface, provide this advantage. There is literally no surface material which cannot be colonised sooner or later, but there are strong differences in the colonisation kinetics. Paul and Jeffrey (1985) demonstrated that one organism can adhere to hydrophobic and hydrophilic surfaces by means of different EPS components. Busscher and van der Mei (2000) have emphasised the role of the forces responsible for the adhesion to surfaces because the entire biofilm can detach if these forces are exceeded either by external shear forces or by a decay of the molecules performing the binding. In some cases, attachment was found to stimulate the synthesis of EPS (Vandevivere and Kirchman, 1993). Both adhesion and cohesion are based on weak physico-chemical interactions and not on covalent bonds. Three major kinds of forces can be distinguished: electrostatical interactions, hydrogen bonds and London dispersion forces (Mayer *et al*., 1999; Flemming *et al*., 2000). The individual binding force of any type of these interactions is relatively small compared to a covalent C-C bond. However, the total binding energies of weak interactions between EPS molecules multiply with the large number of binding sites available in the macromolecules and add up to bond values exceeding those of covalent C-C bonds. As mentioned above, hydrophilic and hydrophobic properties of EPS have been demonstrated; on the basis of these results, all three types of binding forces are expected to contribute to the overall stability of floc and biofilm matrices, probably to various extents. The result is the formation of a three-dimensional, gel-like network of EPS, whose composition, structure and properties may vary dynamically as the micro-organisms respond to changes in environmental conditions.

The matrix network is formed by fluctuating adhesion points and the resulting matrix can behave as a gel as long as a certain shear stress is not exceeded. In this phase, the adhesion points flip back to their original arrangement. Above that point ("yield point"), new adhesion points assemble and the matrix behaves as a highly viscous fluid; fluidized biofilms could be demonstrated by Stoodley *et al*. (2000). The mechanical stability of a *P. aeruginosa* biofilm was investigated by a film rheometer in which the force for compression of a flat biofilm is measured. It allows for the determination of an apparent elasticity modulus which can be taken as a parameter for biofilm stability (Körstgens *et al*., 2001). A typical compression diagram for a *P. aeruginosa* biofilm is given in Figure 1.

It could be demonstrated that in this biofilm matrix which is dominated by polysaccharides with carboxyl groups, calcium acts as an important bridging ion which increases the stability of the network significantly. This is also the case for copper and iron but not for magnesium. In such cases, surfactants will not contribute to the dissolution of biofilms. However, if other biopolymers dominate, it is possible that surfactants have a more signifi-

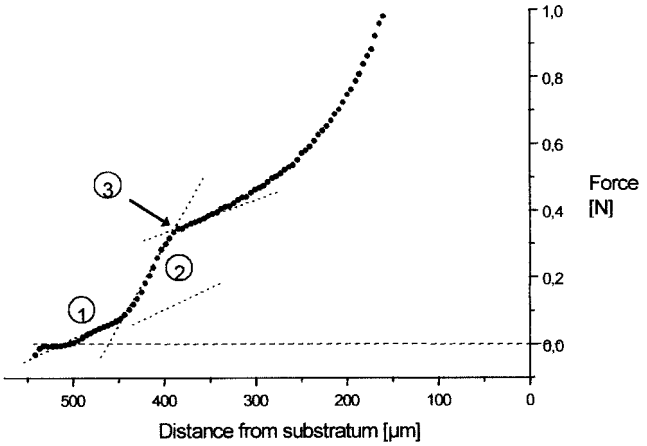

Figure 1 Compression diagram of a *P. aeruginosa* biofilm. 1: Adjustment phase, 2: linear phase (apparent elasticity modulus calculated from slope), where the matrix behaves as a gel, 3: yield point – from here on, the matrix behaves as a highly viscous fluid (after Körstgens *et al.*, 2000)

cant effect. Hydrogen bonds are also part of the overall binding force. They can be influenced by so-called chaotropic agents which have a high affinity for water, thus interfering with the water shell around the biopolymers. In some cases, this type of bond dominates the binding forces. The extent to which each bond contributes to the cumulative binding force depends strongly on the nature of the EPS molecules. As different strains can produce different EPS, the variety is considerable, suggesting that not all biofilms can be dissolved by means of only one cleaning formulation. This coincides well with observations from practice. EPSs are not totally insoluble in water. A certain amount of EPS is continuously lost to the water phase. In wastewater, this contributes to process parameters such as chemical oxygen demand (COD, Houghton and Quarmby, 1999).

Matrix architecture

The architecture of the EPS matrix influences the processes within biofilms profoundly. Costerton *et al.* (1994) have shown that pores and channels occur in which convective transport is possible to a certain extent (Lewandowski *et al.*, 1994). Hoffman and Decho (1999) postulated areas of different density of the matrix, which have been observed experimentally. These features result in an extremely heterogeneous structure. This structure is dynamic; Schmitt *et al.* (1995) showed in a *P. putida* biofilm which was charged with toluene that a rising concentration of toluene caused the formation of more polysaccharide, and furthermore, these compounds contained more carboxyl groups. Heise and Gust (1999) showed that the EPSs which were formed in the exponential growth phase of a marine isolate were different from those which were formed during the plateau phase.

An important question is whether the EPS matrix acts as a diffusion barrier. The major component of that matrix is water. It could be shown by NMR measurements that the self-diffusion coefficient of water within the biofilm is only 15% less than in free water, and that only a very small fraction, less than 0.1%, displays a significantly lower diffusion coefficient (Vogt *et al.*, 2000). There is evidence that non-charged molecules up to a molecular mass of around 10,000 Dalton experience practically no diffusion limitation. However, if they are consumed, as is the case with oxygen, gradients arise because oxygen consumption by aerobic organisms can occur faster than oxygen can follow the diffusion gradient. This is how anaerobic zones in biofilms arise, and why anaerobic organisms can find suitable habitats directly below respiring aerobic colonies. Charged molecules may interact with

charged groups of the EPS which may slow down their respective mobilities to a certain extent. This makes perfect sense from an ecological point of view because the mobility of nutrients, products and exoenzymes is not restricted within the matrix, which is of great importance for cells located in the center of clusters.

Mass transport is influenced not only by the internal architecture of biofilms but also by their interface to the water phase. Some biofilms have a highly filamentous appearance while others are smooth. It is obvious that a large number of filaments will increase the surface at which interactions with components of the water phase are possible.

Investigations on the molecular mass of *P. aeruginosa* alginate revealed a mass between 1 and 2 Mio Dalton (Grobe *et al.*, 1995; Windhues *et al.*, unpublished results) which corresponds to a length of up to 5 µm of the thread-like molecule. According to a gross estimate, based on a water content of approximately 95% in the EPS matrix, a volume element of 1 μm^2 and 10 nm thickness contains around 10 molecules of alginate and 300 protein molecules with an average molecular mass of 30,000 Dalton. With these data, realistic size relations of alginate and proteins such a volume element can be schematically depicted as in Figure 2.

The data given in Table 2 refer to *P. aeruginosa* as a model system. In other systems, other organisms will produce other EPS molecules in other concentrations. As biopolymers generally tend to form hydrogels, the ecological advantage of a matrix can be provided by a wide variety of different EPSs. This provides a certain level of protection against enzymes specific for certain types of EPS because then, organisms producing other types of EPS can protect their matrix against such enzymes. While alginate is a relatively stable EPS component, proteins obviously undergo a more rapid turnover. The metaphor of the EPS matrix as the "house" of biofilm organisms can be further expanded: polysaccharides may represent "walls, ceilings and floors" while proteins represent the "furniture" of the "house". This system arranges the proteins in proximity to the cells and prevents washing out of exoenzymes which would be lost easily under conditions of convectional flow.

An interesting aspect of the matrix architecture is the general interaction between exoenzymes and exopolysaccharides. Wingender *et al.* (1999b) showed that the heat stability of an extracellular lipase of *P. aeruginosa* was significantly increased in the presence of bacterial alginate. The same was observed for pH stability. It is quite probable that there are many more interactions of this kind. Such phenomena support the view of the EPS matrix

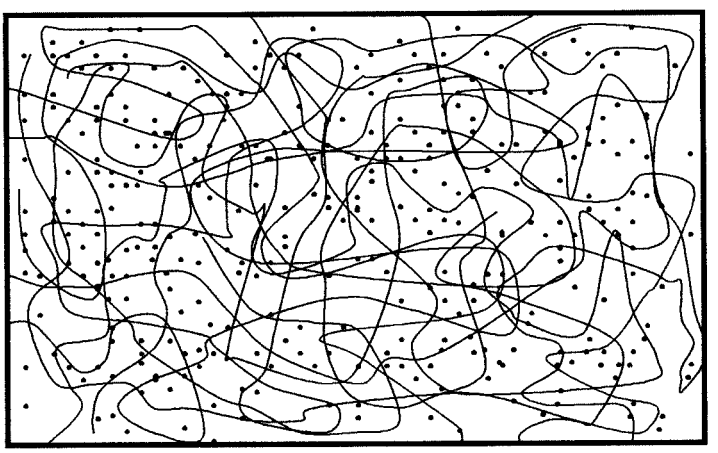

Figure 2 Size relationships of alginate and proteins in a volume element of an area of 1 μm^2 and a thickness of 1 nm, containing 10 molecules of alginate (2 Mio Da) and 300 protein molecules (30 kDa)

as an important component in the view of biofilms as a tissue, as suggested by Costerton *et al*. (1994).

In photosynthetic systems such as microbial mats or stromatolites, the EPSs play an important role in light transmission. At the biofilm surface, the prevailing irradiance and spectral composition were found to be significantly different from the incident light. The EPSs were suspected as photon traps. Multiple scattering leads to an intensity maximum for photic light (400–700 nm) of ca. 120% of incident quantum irradiation at the biofilm surface. At the bottom of the eutrophic zone in the biofilm, light was attenuated strongly to <5–10% of the incident surface irradiance (Kuhl *et al*., 1996).

Whether or not EPSs are used as a carbon source is still an open discussion. Sutherland (1995) has given an excellent overview about polysaccharide lyases, which are capable of cleaving a hexose-1,4- or ß-uronic acid sequence by ß-elimination. This author assumes that EPSs usually are not degraded by the organisms which have produced them (Sutherland, 1999b). However, EPS must be biodegradable by other species sooner or later, otherwise it would persist forever, but it appears as if the polysaccharide matrix is relatively stable and not degraded in the short term, contrary to the EPS protein moiety. Again, this makes sense from an ecological point of view because many different organisms can use the "house" once the energy and carbon is spent to build it, even if this has been performed by a predecessor. Evolution has obviously not generated organisms which readily degrade EPS generally, otherwise such organisms would have one of the largest collective carbon sources imaginable. The generation of a strain capable of degrading any given EPS matrix in a short time would lead to an environmental disaster because it would destroy natural microconsortia which perform a crucial role in the biogeochemical cycles of carbon, oxygen, nitrogen, sulphur and others. It seems as if nature has prevented such a threat by the vast diversity of EPS molecules which all are capable of fulfilling the structural role required for the biofilm mode of life. Enzyme preparations used for the dispersion of biofilms in biofouling cases (e.g., Wiatr, 1990) are ineffective for these reasons. This has been clearly demonstrated for paper-making processes (Klahre and Flemming, 2000).

Outlook

It is justified to speculate that the functions of the EPS for the ecology of microbial aggregates are much more complex than it appears at first glance. Possibly, the matrix is not just an arrangement of randomly associated biopolymers but it is synthesised and degraded in a way which supports the life of the organisms in such aggregates. It is evident that the matrix is heterogeneous in composition, architecture and over time and represents a dynamic system. Possibly, an important parameter such as cell surface hydrophobicity may be regulated by surface active EPS as predicted by Neu (1996). Recent research indicates that alasan, a surface active exocellular emulsifier produced by a strain of *Acinetobacter radioresistens* could be transferred horizontally to *Acinetobacter calcoaceticus* when both strains were grown together, changing the surface properties of the latter (Osterreicher-Ravid *et al*., 2000). The EPS matrix and the properties which it determines represent important and complex aspects when considering biofilms as a first step of the evolution of multicellular organisms.

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