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From molecules to multispecies ecosystems: The roles of structure in bacterial biofilms

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Abstract

Biofilms are communities of sessile microbes that are bound to each other by a matrix made of biopolymers and proteins. Spatial structure is present in biofilms on many length scales. These range from the nanometer scale of molecular motifs to the hundred-micron scale of multicellular aggregates. Spatial structure is a physical property that impacts the biology of biofilms in many ways. The molecular structure of matrix components controls their interaction with each other (thereby impacting biofilm mechanics) and with diffusing molecules such as antibiotics and immune factors (thereby impacting antibiotic tolerance and evasion of the immune system). The size and structure of multicellular aggregates, combined with microbial consumption of growth substrate, give rise to differentiated microenvironments with different patterns of metabolism and gene expression. Spatial association of more than one species can benefit one or both species, while distances between species can both determine and result from the transport of diffusible factors between species. Thus, a widespread theme in the biological importance of spatial structure in biofilms is the effect of structure on transport. We survey what is known about this and other effects of spatial structure in biofilms, from molecules up to multispecies ecosystems. We conclude with an overview of what experimental approaches have been developed to control spatial structure in biofilms and how these and other experiments can be complemented with computational work.

Introduction

Biofilms are communities of interacting microbes that are embedded in a matrix of polymer and protein [1–4]. For purposes of this review, a biofilm may consist either of one contiguous region of matrix-embedded microbes or of multiple discrete aggregates that may not directly touch each other but are contained in and connected through the same larger environment, such as an infected wound or lung [5, 6]. Different aggregates in contact with the same environment have the potential to interact, for example by diffusion of chemical signals and metabolic products. In the human body, biofilm infections cause morbidity and mortality, and substantial healthcare costs [7–13]. Outside the body, biofilms damage civic and industrial infrastructure, *e.g.*, by clogging systems for water treatment [14–18],

biocorrosion of oil and water pipelines and other liquid-immersed structures [19–24], and fouling shipping vessels [25–29] – thereby decreasing efficiency and increasing running costs, such as fuel, and harm to the environment. Understanding how the structures of biofilms contributes to the harms they do will allow the development of new, structure-targeting approaches to reducing or eliminating the effects of harmful biofilms.

Biofilms also have the potential to do good in the form of bioremediation and bioproduction [30–35]. In these cases, understanding the roles of structure in biofilm function will allow the development of tailored biofilms with structure optimized for a particular function. This is especially likely to be important for cases where microbial consortia – ecosystems of more than one microbial species – are needed to produce the desired function [36–40]. Optimizing the spatial arrangement of species, and therefore the concentrations of chemical substrates and metabolic products that reach microbes of each species, should optimize consortial function.

Biofilms are physically distinct from free-swimming or free-floating systems of microbes (in what is commonly called the “planktonic” state) in at least two properties – mechanics and structure. In the case of mechanics, the matrix provides cohesive force between embedded microbes (and, if the biofilm is attached to an external surface, adhesion to that surface). In the case of structure, as a result of the matrix holding microbes in place, biofilms have a spatial structure that varies only very slowly in time. This is unlike the case for planktonic. When microbes are suspended in fluid, their spatial positions rapidly rearrange due to Brownian motion, fluid flow, and microbial motility. Therefore, the system typically cannot be thought of as having a spatial structure, other than the most general fluid structure characterizing thermalized or super-thermalized suspensions of colloids – in many real-world cases, planktonic bacteria will be at low densities [41, 42]. If planktonic bacteria are at high density, dynamic structures may appear [43, 44], but these do not have the fixed positions that characterize biofilm structures.

We and others have recently reviewed what is known about biofilm mechanics and the associated measurement techniques [45, 46]. Here, we review what is known about biofilm structure, which is overall an understudied characteristic. Structural properties that are important for biofilm biology and mechanics range in size over orders of magnitude. Molecular lengthscales alone can range from the ~1 nm scale of individual sugar moieties in matrix polysaccharides to the millimeter length of genomic bacterial DNA, which can be released by cell lysis to become a matrix constituent [47]. Single microbes themselves are each approximately 1 micron in size, and this lengthscale also characterizes each cell’s region of adhesion to and local interaction with the matrix. A multicellular biofilm can be macroscopic in size if growth conditions permit – a common laboratory example of this is a biofilm grown in a flow cell, which can be a few hundred microns in thickness, but millimeters in width and centimeters in length [48]. Important real-world biofilms are often smaller than laboratory models – for example, the multicellular aggregates comprising biofilm infections in soft tissue are ~100 microns in diameter [49, 50]. It is important to note that environmental conditions in the laboratory can lead to very specific structures that have not been seen in infections or other real-world scenarios [51].

Structure on all lengthscales matters for diffusive transport. Indeed, one physical way to distinguish between planktonic and biofilm systems of bacteria, absent any biological signature, might be to set a threshold timescale characterizing the time needed for diffusive transport of materials of interest across the size scale of interest, and to compare that threshold with the timescale characterizing significant structural rearrangement. If the rearrangement timescale is greater than the diffusion timescale, the system is effectively structured and biofilm-like; if the diffusion timescale is greater than the rearrangement timescale, the system effectively has little or no structure and is planktonic-like. To our knowledge, this is the first time this metric has been proposed. Although in many cases other measures, such as the presence of large amounts of matrix or altered gene expression states, make it very obvious whether a system is in the biofilm or the planktonic state, this “rearrangement *versus* diffusion timescale” metric might allow more nuanced understanding of liminal states, such as when bacteria are transitioning from the planktonic to the biofilm state, or when the system has features of both planktonic and biofilm bacteria (*e.g.*, bacteria are motile but confined in a small volume at high density).

The presence of spatial structure in the organization of bacteria, in combination with bacterial metabolism and inter-species “warfare”, gives rise to microenvironments that impact biology and disease course – for example, antibiotic resistance [52]. Thus, how structure and diffusive transport give rise to microenvironments will be a pervasive theme of this review. Because biofilm structure is under-studied, there is specific information on only a few microbial species available to include in this review. However, we expect the physical principles involved to be widely important across biofilms of many species. Therefore, we begin each sub-section of the review with a summary of what structural characteristics, and related physics, we expect to be important at each size scale. We will then give specific examples where these are known. *Pseudomonas aeruginosa* is an opportunistic human pathogen that readily forms biofilms with disease impact, and has therefore been more widely studied, in vitro and in vivo, than other biofilm-forming organisms. As a result, more is known about the structural properties of *P. aeruginosa* biofilms than about other types of biofilms. Therefore, the examples given below will over-represent *P. aeruginosa*. This should not be misconstrued to mean that the general properties described are exclusive to *P. aeruginosa*. Rather, this should be thought of as an incomplete scaffold which can be used to guide studies of other organisms, as well as further investigation of *P. aeruginosa*.

As a secondary model organism, we will discuss *Bacillus subtilis* biofilms. These are not medically relevant but have been the subject of many basic-science studies, including studies of transport and structure. These also have the advantage of having a lower level of safety classification than *P. aeruginosa* (*B. subtilis* is BSL-1, whereas *P. aeruginosa*, because it is a human and animal pathogen, is BSL-2). This could make *B. subtilis* a good choice for researchers who want to investigate the type of ideas discussed in this review, but who are constrained to, or prefer to, work in laboratories that do not meet BSL-2 standards.

We conclude with a discussion of the need for new approaches to studying biofilm structure, with a particular focus on the need for physically-based experimental techniques for controlling biofilm structure, so that the effects of specific structural features can be determined.

Microenvironments – why they matter

In the context of biofilms, at least two types of microenvironment are important: the microenvironment of the biofilm itself, and the microenvironment surrounding the biofilm, consisting of the colonized area (e.g. implants or tissue) and the surrounding fluid and/or local host environment. For the purpose of this review, we focus on the first. However, we note that biofilm-driven alterations in the extra-biofilm microenvironment can also be important – for example, changes to the host metabolic microenvironment (as a result of antibiotics) can alter infection and immune function [53].

Within biofilms, microenvironments are characterized by low amounts of available oxygen or other growth substrate – this is due to consumption of growth substrates by microbes near the biofilm surface as growth substrates are supplied from outside [54]. As a result, microbes deeper within the biofilm often have lower metabolisms and growth rates [55, 56]. Because many antibiotics are most effective against actively-growing bacteria, for example antibiotics that interfere with accurate protein synthesis [57], lower metabolism also results in lower susceptibility to antibiotics [52]. This is a phenotypic antibiotic resistance that results from one type of microenvironmental property, namely limited access to growth substrate, and resulting reduction in microbial metabolism. However, microenvironments also help facilitate evolution of genetic antibiotic resistance due to concentration gradients between microenvironments [58, 59]. Furthermore, horizontal gene transfer, which can include genes conferring antibiotic resistance, is facilitated by bacteria being held in close proximity to each other in biofilms [60–63].

Intercellular signaling, in the form of quorum sensing, uses diffusible molecules called autoinducers [64]. When the sensed concentration of autoinducers is sufficiently high, the gene expression of quorum-sensing-responsive bacteria changes, predominantly to increase the production of collective goods [65, 66]. Spatial structure in the form of high local density of bacteria can result in high concentration of autoinducers and thereby the activation of quorum sensing [67, 68].

Molecular Structure

The matrices of biofilms, in which constituent microbes are embedded, contain different species of polymers and proteins. These extracellular polymeric substances (EPS) are primarily polysaccharides and, at least in the case of *P. aeruginosa*, extracellular DNA (eDNA). Each of the polymers has unique molecular structures such that the biofilm matrix has a wide array of chemical and mechanical properties. Interactions between polymers and protein in the matrix give rise to physical structure in the biofilm as well as mediating bacterial adhesion and cohesion. Figure 1 shows a cartoon of the biofilm matrix, schematically indicating possible components and interactions.

Molecular Structure in Polymer Interactions

For gels made of a single polymer species, structural properties such as polymer length and polydispersity, the degree of branching (or the lack thereof), and the charge and charge distribution of the polymer will all impact gel mechanics, as will the density of polymers; for

microbial biofilms, the volume fraction of microbes can also impact mechanics, as in a colloid-polymer mixture. If the gel is made up of more than one polymer species, and/or if the polymers interact via binding to each other or via crosslinkers, the resulting microstructure of the biofilm will also impact mechanics. Such inter-polymer interactions are controlled by molecular structure, such as crosslinking sites or electrostatic attraction or repulsion. Compared with what is known about structure in biofilms specifically, a great deal of information about the physics of polymers and polymer gels is well-established and easily accessible [69–71], so we will not cover this in depth here.

For *P. aeruginosa*, for example, mannose sugar groups on the EPS Psl likely allow this polymer to be cross-linked by the protein CdrA [72]. Also for *P. aeruginosa*, the negative charge sign of eDNA (resulting from phosphate groups on the “side rails” of the DNA “ladder”) and the positive charge sign of the EPS Pel (resulting from partial acetylation of the two amino sugar types comprising this polymer) allow these two polymers to bind [73]. We have shown that these interactions likely contribute to the elasticity and the yield strain, respectively, of the biofilm [74]. In addition, researchers have theorized that the mannose in Psl may trigger oxidative bursts in neutrophils, immune cells that readily clear individual bacteria but cannot clear many types of biofilm infections [75]. Alginate, another *P. aeruginosa* EPS material, can be crosslinked by multivalent cations, such as calcium, and the mechanical properties of alginate gels depend on the concentration of crosslinking ions. The interplay of electrostatic interactions between the polymers in the *P. aeruginosa* biofilm proves to be important for resulting mechanical integrity when the matrix is exposed to altered salinity by the addition of calcium cations [76, 77]. Also in *P. aeruginosa* biofilms, simple chemical perturbations to the biofilm environment such as ions, polyelectrolytes, pH changes, and common organic molecules are only minimally effective in compromising biofilm elasticity, revealing that these molecular polymer associations, once formed, are resistant to many simple disruption mechanisms [78].

Molecular Structure in Matrix-Cell Association

Polymer and protein in the extracellular matrix continue to interact with the cells in the matrix after having been secreted. Some polymers localize near cells, and may even bind to them, using the cells themselves as a crosslinking point. In *P. aeruginosa*, secreted CdrA protein may act as a cellular tether this way [79]. CdrA binds to mannose groups on the EPS Psl, and we have found that interbacterial adhesion mediated primarily by Psl is characterized by a more-localized force maximum than is interbacterial adhesion mediated primarily by Pel, which is not known to have a cellular tether [74].

In addition to CdrA, *P. aeruginosa* produces two carbohydrate-specific binding proteins, lectins called LecA and LecB that are secreted and outer membrane bound, respectively. While which specific extracellular polysaccharides the lectins interact with is still unknown, both lectins promote biofilm adhesion and growth. While not being cell-bound like LecB, LecA likely acts to bind cells closer to one another, enhancing aggregate formation [80–82].

Lipopolysaccharides (LPS) are another class of polysaccharide present in the biofilm matrix of gram-negative bacteria; however, LPS are at least an order of magnitude smaller than EPS. LPS are chains of polysaccharide embedded in the outer membrane of the bacteria and

therefore mediate many interactions of bacterial cells with their environment, in addition to being a major component in activating the immune response of the host. In *P. aeruginosa*, alteration in LPS structure causes dramatic changes in cellular adhesion and cohesion and the viscoelastic properties of the bacterial biofilm [83] and help determine the hydrophobicity and the surface charge of the cell membrane [84]. LPS structure also changes in Cystic Fibrosis infections [85], and so the structural properties of LPS in biofilm formation is relevant for studying the course of CF infections. While association of LPS with the main EPS components in *P. aeruginosa* is not entirely clear, alginate, Pel, and Psl genes are important to the production of LPS [86–88], showing that LPS and EPS are most certainly intertwined in the structure of the bacterial biofilm.

Another lipid species, surfactants called rhamnolipids, also affect the hydrophobicity and therefore the cohesion and adhesion of bacteria within the biofilm. The correct balance of this surfactant impacts the entire biofilm lifecycle, from biofilm initiation by surface attachment to the resulting superstructure of the biofilm by helping maintain channels in the biofilm, which are vital for nutrient diffusion [89, 90].

Molecular Structure and non-Biofilm Components

The molecular structure of matrix constituents also determines their chemical interactions with non-matrix, diffusing molecules that might be introduced to the biofilm from the outside or be released by metabolizing microbes inside the biofilm. For example, it has been suggested that electrostatic binding between cationic antibiotics and anionic matrix polymers, such as alginate and eDNA, may slow or even prevent the diffusion of antibiotics into the biofilm [91, 92]. If the diffusion of antibiotics into biofilm is hindered, the constituent bacteria will experience a slower increase in antibiotic concentration and will therefore have more time to activate adaptive resistance mechanisms. If the antibiotics have sufficient binding energy with the matrix that they never enter the biofilm, then the internal bacteria are never exposed to antibiotic at all.

Matrix components can protect bacteria from the immune response of the host by blocking many of the pathways by which the host immune system acts to identify and kill bacteria. Opsonins are diffusible molecules, produced by the host's immune system, that promote phagocytosis by host immune cells such as macrophages and neutrophils. It is thought that EPS materials can prevent opsonins from binding to bacteria, and therefore help biofilm bacteria evade phagocytosis [93, 94]. In *P. aeruginosa*, O-acetyl groups on alginate bind non-antibody opsonins. Strains with acetylated alginate were able to survive in a solution of complement and leukocytes, and stop activation of antibody-independent complement [95]. Psl also protects mucoid *P. aeruginosa* biofilms from opsonization as well as complement-mediated killing [96]. There is also an enzyme that binds to Psl in the matrix that degrades the bactericidal enzyme elastase produced by neutrophils [97].

EPS components (and capsular polysaccharides) mask surface epitopes (the area on the antigen that antibodies bind to) so that antibodies do not bind to the microbe surface, preventing recognition. They also prevent the attachment of the opsonic complement proteins that facilitate engulfment by cells like macrophages and neutrophils. Bacteria also can secrete enzymes that destroy complement proteins, which are inhibitory proteins that

prevent activation and chemotaxis of immune cells, and GTPase-activating proteins that impair the actin cytoskeleton of neutrophils. The transport of these chemicals should also be mediated by diffusion.

Disrupting Molecular Structure as Biofilm Treatment

Briefly, we note that one approach to removing harmful biofilms is to attack and break down the biofilm matrix. This often is a molecule-specific approach, targeting specific characteristics of EPS or other matrix materials. Thus, not only can a better understanding of the molecular structure of biofilm matrix components lead to better treatment methods, but learning what chemical specificity results in matrix disruption will also lead insight into what molecular structures are important for matrix integrity. Disrupting matrix integrity as a treatment method both makes the physical removal of the biofilm easier, as well as in many cases allowing for easier diffusion of antibiotics thereby increasing the efficacy of existing treatments.

Enzymes are a particularly common method to attack the proteins and polymers that maintain the structure of a bacterial biofilm. Some of these enzymes act to break down the polymers into smaller segments, compromising the network structure. Enzymes such as deoxyribonucleases (DNases) and glycoside hydrolases break down the large polymer EPS chains into smaller segments, compromising network integrity. DNase hydrolytically cleaves the phosphodiester linkages in the backbone of DNA, and glycoside hydrolases break down the glycosidic linkages in polysaccharide chains. In *P. aeruginosa*, extracellular DNA in the matrix breaks down when DNase is added to the biofilm [98]. Pel-, Psl-, and alginate-specific glycoside hydrolases are successful in degrading each of their respective polymers [99, 100]. Even generic glycoside hydrolases have proven successful against *P. aeruginosa* biofilms: α -amylase and cellulase disrupt the biofilms in a wound model [101]. Proteolytic enzymes are also potential targets for biofilm treatment, and these enzymes are successful at disrupting biofilm integrity by compromising the proteins in the matrix [102].

Other potential biofilm disruptors include antimicrobial peptides or other molecules that lead to biofilm dispersal. While not acting on the biofilm EPS components themselves, by interrupting the cascade of signals that trigger biofilm initiation and growth, the biofilm can be compromised at the level of molecular signaling [103].

Multicellular, mesoscale structure

The biofilm matrix contain many materials which can interact chemically with molecules diffusing in from the environment and out from the biofilm. They also contain microbes whose metabolic activity consumes growth substrate (primarily oxygen and carbon) and releases metabolic by-products. Both of these properties impact the development of differentiated microenvironments in the biofilm. In addition, fundamental principles of diffusive transport indicate that structural characteristics of biofilms, on the scale of multicellular aggregates, will impact the development of microenvironments. Therefore, in this section we consider how whole-biofilm shape, size, and distribution of EPS and cells affect transport of molecules. Figure 2 is a cartoon that schematically indicates different features that could describe the mesoscale structure of aggregates.

Shape

Studies of *P. aeruginosa* biofilm infections in soft tissue – in wounds and in lungs – show that these infections consist of many multicellular aggregates, each roughly spheroidal and on the order of 100 microns in diameter [49, 50]. The best geometric approximation for such a biofilm is a sphere, which is the shape that minimizes the ratio of surface area to volume. Thus, for a given total biomass of biofilm, this shape would be expected to minimize transport of materials, such as growth substrate or antibiotic, into or out of the biofilm aggregate.

Biofilms also grow on implanted medical devices, such as catheters, heart valves, and joint implants. Images of such biofilms show shapes that conform to the surface of the infected device [104, 105]. Since these biofilms arise from microbial attachment to and proliferation on device surfaces [106], which have a roughly planar geometry, and since removal (for example by flow and by immune cells) should tend to limit the biofilm thickness that can develop, we expect that device-associated biofilms should be associated with an approximately planar shape – *i.e.*, a rectangular prism with height less than width and length. This shape has a much higher ratio of surface area to volume than does a sphere. Even allowing for one face being occluded by the device (which should not be significantly permeable to transport), from purely geometrical considerations such a biofilm should have the potential for more transport relative to volume, with consequent impact such as increased antibiotic susceptibility. Transport between the body and the biofilm will also be impacted by host factors, such as the development of dense tissue and host material around the implant site [107]. Furthermore, biofilms grown in non-trivial geometries can form streamers, which are regions of biofilm that are suspended in flow and not directly adjoining a surface; as they grow, these streamers can span gaps and eventually block fluid flow [108], thus greatly reducing transport in some scenarios.

Size

The overall size of biofilm communities impact their development and subsequent structure by dictating the timescale for materials to be transported into the biofilm interior or for materials to be transported from the interior to the outer surface. Unlike flowing fluid environments where convective flow drives solute transport, material movement in biofilms is mediated by diffusion. Diffusion in the simplest sense is the spreading of molecules as a result of thermally driven random walks. This results in the commonly observed macroscopic phenomena that substances under diffusive transport undergo a net movement from regions of high to low concentrations, becoming uniformly distributed over a sufficient time course. Fick's first law describes the number of particles flowing per unit area and time, and is represented commonly by the variable J . In three dimensions it takes the form:

$$J = -D\left(\frac{\partial c}{\partial x} + \frac{\partial c}{\partial y} + \frac{\partial c}{\partial z}\right)$$

where D is the diffusion coefficient, c is the particle concentration, and x , y , and z are the spatial coordinates, thereby making $\frac{\partial c}{\partial x}$, $\frac{\partial c}{\partial y}$, and $\frac{\partial c}{\partial z}$ the concentration along the directions parallel to the x , y , and z axes, respectively. For the idealized approximation of spheroidal or

slab shaped biofilms, the time taken for a given substance to diffusively penetrate the core or bottom surfaces of each respective geometry (and reach a concentration that is 90% of the solute concentration found in the surrounding fluid media) is given by:

$$t_{90} = \alpha \frac{L^2}{D}$$

with $\alpha_{sphere} = 0.37$ and $\alpha_{slab} = 1.03$ [109]. Here D will be set by properties of the diffusing material and by its interactions with matrix materials, while L will be set by the biofilm size – taking on the value of radius for a spheroid, or thickness for a planar slab biofilm. There exist several studies which determine the diffusion coefficients of various solutes through biofilms [109–112]. However, some studies are limited by considering pure diffusion only, and neglecting any binding or other specific interactions between the diffusing solute and the matrix.

This is an important limitation, as illustrated by antibiotic transport into biofilms. In many cases, antibiotics have been found to readily penetrate biofilms. However, for positively-charged antibiotics, such as the aminoglycoside tobramycin that is a front-line treatment for *P. aeruginosa* infections, binding to the negatively-charged EPS alginate in the biofilm matrix has been shown to slow the penetration of tobramycin into the biofilm interior [113]. Decreasing the rate at which antibiotic concentrations rise inside the biofilm could allow interior cells more time to adjust their physiology to achieve phenotypic tolerance to the antibiotic [114–116]. Many other EPS materials have been shown to bind to tobramycin and other ionic antibiotics, often by unidentified binding sites, such that the antibiotic is essentially confined to a small thickness on the biofilm periphery [117–120]; as a result, the size and three-dimensional structure of the biofilm keeps non-peripheral bacteria from being exposed to high concentrations of antibiotic.

Heterogeneity

Biofilms on the mesoscale, from one to thousands of microns, as accessible by optical microscopy, are far from uniform. There are many types of spatial heterogeneity within a biofilm, including in the metabolic action of cells, in the distribution of EPS [121], and in the distribution and density of cells. Heterogeneity can modulate the local transport of materials within the biofilm – *e.g.* by increasing the local concentration of binding sites for diffusing material or by blocking the transport of larger materials by increased local density of matrix polymers or cross-linking sites [122]. Heterogeneity in the distribution of matrix materials can impact antibiotic resistance by creating a “shield” of antibiotic-binding EPS around the periphery of the biofilm [73]. Heterogeneity in the distribution of EPS could also allow specific materials to be localized in ways that increase benefit to the biofilm – for example, by localizing adhesive materials to the region where the biofilm adjoins a surface, and by localizing cohesive materials to the biofilm interior. Furthermore, it has recently been shown that biofilms can, using biomineralization, create a “shield” of calcium carbonate that prevents diffusion of substances from the biofilm exterior into the inner region of the biofilm [123].

Heterogeneity in the metabolic activity of cells constituting the biofilm can also lead to non-trivial growth dynamics, as cells at the periphery compete with cells in the interior for growth substrates such as carbon source or oxygen. In the case of *B. subtilis*, metabolic coupling between fast-growing peripheral cells and growth-limited interior cells can give rise to oscillations in growth rate [124] that are controlled by inter-cellular signaling that propagates in the form of waves of potassium that reduce the electrostatic potential across the cell membrane [125]. This can be extended to allow the growth rates of different colonies, coupled through a shared fluid culture medium, to oscillate either in-phase or out-of-phase [126]. In the case of *P. aeruginosa*, a growth instability can arise that elongates three-dimensional aggregates in the growth-favoring direction [55].

Bulk, Macroscopic Structure

On a macroscopic scale, millimeters and larger, and seeable by the naked eye, *Pseudomonas aeruginosa* biofilm colonies in general often appear to be fairly smooth with a homogeneous surface. However there are strains of *P. aeruginosa* and other species whose colonies have a wrinkled morphology [127–131]. Such strains are referred to as rugose small colony variants (RSCVs); they are associated with high production of some matrix polymers and infections with RSCVs typically correlate with poor patient outcomes [132, 133]. The degree to which the wrinkles themselves may serve a function in the biofilm is, to our knowledge, largely unknown. It may be that in many cases the wrinkles are an outgrowth of high matrix production, which is known to provide chemical and mechanical benefits to the biofilms, but that these structures themselves do not have a function in the biofilm.

However, one additionally noteworthy bacterial species, *Bacillus subtilis*, can form remarkable macroscopic biofilm structures at liquid-air and solid-air interfaces [134]. When cultured on an agar surface, biofilms of this organism develop a radial wrinkled structure that give rise to a fluid tunnel network; wrinkles in *B. subtilis* biofilm colonies can originate when localized cell death concentrates mechanical forces [135]. Studies have found that these fluid channels help transport nutrients into a large biofilm structure but not necessarily into the center [136]. Furthermore, *B. subtilis* biofilm colonies can both impose an elastic deformation on a nutrient gel substrate, (*i.e.*, produce a structural change in their environment) and increase the flow of fluid and nutrients through the gel substrate to their constituent bacteria [137]. Similar transport benefits may arise to RSCV strains of other species. If so, this would constitute yet another example of spatial structure impacting biofilms *via* changes in transport.

Spatial arrangements of multiple species

Most laboratory studies of biofilms use one or, at the most, two species of microbes. However, in real-world scenarios multi-species biofilms are widespread – and inter-species interactions can strongly influence the biology of multispecies biofilms. Most inter-species interactions are mediated *via* diffusible molecules. The concentration of diffusing molecules at steady state depends on the distance r from the source as $\sim \frac{1}{r^2}$. In this section, we discuss

how spacings between different species in biofilms are related to the diffusive transport of key molecules.

In the multispecies oral biofilm, species co-aggregate in a way suggesting that spatial proximity of one species benefits another species [138, 139]. However, for at least one pair of oral bacteria, the story is more complicated. As a metabolic by-product, *Streptococcus gordonii* produces both a sugar that is consumed by *Aggregatibacter actinomycetemcomitans* and hydrogen peroxide, which at sufficiently-high concentrations kills *A. actinomycetemcomitans* (and other microbes). When the trade-off between higher sugar concentration and lower hydrogen peroxide concentration is optimized, *A. actinomycetemcomitans* aggregates are located between 4 and 13 μm distant from *S. gordonii* aggregates [140].

P. aeruginosa can sense peptidoglycan from *Staphylococcus aureus* or other Gram-positive bacteria and, in response, produce diffusible factors that lyse and kill *S. aureus*. Since the same chemical factors also damage host tissue, co-infection with both species can result in worse outcomes [141]. *P. aeruginosa* and *S. aureus* are often co-infecting organisms, but they are not indiscriminately intermingled. In chronic wounds, *S. aureus* aggregates are found, on average, 20–30 μm below the surface of the wound, and *P. aeruginosa* aggregates in the same wounds are found, on average, 50–60 μm below the wound surface [142]. This spatial separation of species likely result both from *P. aeruginosa*'s ability to chemotax toward diffusible chemicals delivered by the bloodstream below the wound bed [143] (*S. aureus* is non-motile) and to killing of *S. aureus* by *P. aeruginosa* if the two species are too close to each other.

To our knowledge, other specific cases of spatial structure in multispecies biofilms are not known. This is largely because investigations of real-world biofilms are more difficult than investigations of laboratory biofilms, and investigations of multispecies biofilms are more difficult than investigations of single-species biofilms. However, in our opinion, of the many types of spatial structure discussed in this review, many of which are under-investigated, the study of structure in multispecies biofilms is the likeliest to yield significant new advances in knowledge.

Research needs and Opportunities

To move beyond the current state-of-the-art, the community of biofilm researchers needs more and better measurements of what structures (from molecular to multispecies distributions) arise in real-world biofilms, and better ways to understand how these structures matter. For understanding the effects of molecular-level structure, there is a large body of extant work in chemical engineering and physical chemistry upon which biofilm researchers can build. For understanding the effects of larger-scale structures, there is much less extant work on which biofilm researchers can build. Therefore, there is a critical need for ways to control biofilm structure on the cellular and multi-cellular scale and to measure the effects of altered structure [144]. In addition, studies that determine how the microstructure of biofilms give rise to bulk structure and mechanical properties would be of interest and use for the biofilm community for their ability to reveal hitherto-unexpected

biological and biophysical features. These experiments can be complemented and better understood using modeling. Figure 3 is a cartoon illustrating different types of experimental approaches to structuring biofilms.

Several approaches to experimentally controlling biofilm structure, or the structure of microbial populations not necessarily in the biofilm state, have been developed. Microfabricated containers allow bacterial response to be probed using microscopy [52, 68, 154] or more sophisticated chemical probes [67, 155]. Patterning the surface can control microbial adhesion on a size scale of many cells [147–149] or a few cells [150–152]. Microbes can be patterned directly to a surface on the size scale of many cells [153] or on the single-cell level [145, 146]. Although it has not been used to modulate and study the effects of structure specifically, a biofilm model in which bacteria are encapsulated in alginate beads has the potential to be used to create “biofilm” aggregates with a well-defined, user-determined distribution of sizes [156]. It should be possible to build on this technique to control the spacing between aggregates of the same and different species.

Other techniques have been developed for modeling biofilm structure and its effects. Individual, agent-based modeling, such as iDynamics [157], allows the intrinsic “graininess” of biofilms made up of many discrete bacteria to be captured better than does continuum modeling. Agent-based models have been used to examine the relationships between biofilm structure, diffusion of resources, and the development of microenvironments [55, 56, 158–162]. One early model combined individual agents with continuum modeling [163]. Continuum modeling may sometimes be preferable over agent-based modeling if the system to be considered is very large in terms of the number of individual agents, or if for some other reason the questions to be considered are intractable or take too much computational time to resolve using individual-based modeling. Like agent-based models, continuum models have been used to examine the relationship between biofilm structure, resource transport, and microenvironments [164–166].

Conclusion

It is trivially obvious that biofilms are spatially structured, as the biofilm matrix holds constituent microbes in place. It is also clear, from fundamental principles of material transport and microbial metabolism, that the spatial structure of biofilms must impact their biology. There are a number of empirical examples of the importance of spatial structure for biofilm biology, for structures ranging from nanometers to hundreds of microns in size. However, as size and complexity increase, the amount of concrete knowledge available decreases, and the potential scope for new discoveries concomitantly increases.

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Figure 1.

Schematic cartoon of possible components and interactions in the biofilm matrix. For the sake of clarity, this cartoon shows a low density of matrix material and a limited number of polymer and protein types. These are not necessarily characteristic of real biofilms. Empty spaces should be thought of as filled with water molecules, not shown.

Figure 2.

Schematic cartoon of different types of spatial heterogeneities that could exist in biofilms on the mesoscale. Here, L could range from a few microns to hundreds of microns. The degree to which any of these heterogeneities exist within any specific biofilm will depend on structural factors such as size, matrix composition, and cell density. Heterogeneities in matrix composition could, by altering the local transport of materials, contribute to heterogeneities in the concentrations of diffusible growth substrate and antibiotics. Cell density and metabolism of growth substrate, including carbon source, oxygen, and materials such as iron, can also give rise to heterogeneities in these diffusible factors. In turn, regions that have limited access to growth substrate will contain cells that have a slower-growing and, especially in the case of limited oxygen concentration, anaerobic metabolism.

Figure 3.

Different experimental approaches to controlling the structure of biofilms. From top to bottom, the position of initial, seeding cells on a surface can be controlled either by manipulating individual cells using laser trapping (or micro-contact printing, not shown) [145, 146] or by controlling the adhesion of cells to specific patterns on the surface by functionalizing well-defined regions [147–152]. Not included in this figure for regions of space, but in the same thematic class as placing individual cells, is “stamping” many cells onto a surface using a structured transfer material [153]. Alternatively, groups of cells can be confined within microfabricated structures [52, 67, 68, 154, 155] or within gel droplets [156].