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Topical Review

Biofilms and mechanics: a review of experimental techniques and findings

Vernita D Gordon^{1,2,3}, Megan Davis-Fields^{1,3}, Kristin Kovach^{2,3}
and Christopher A Rodesney^{2,3}

¹ Institute for Cellular and Molecular Biology, The University of Texas at Austin, 2500 Speedway, Mail Stop A4800, Austin, TX 78712, United States of America

² Department of Physics, The University of Texas at Austin, 2515 Speedway, Mail Stop C1600, Austin, TX 78712, United States of America

³ Center for Nonlinear Dynamics, The University of Texas at Austin, 2515 Speedway, Mail Stop C1610, Austin, TX 78712, United States of America

E-mail: gordon@chaos.utexas.edu

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Abstract

Biofilms are developmentally-dynamic communities of sessile microbes that adhere to each other and, often, to other structures in their environment. The cohesive mechanical forces binding microbes to each other confer mechanical and structural stability on the biofilm and give rise to biofilm viscoelasticity. The adhesive mechanical forces binding microbes to other structures in their environment can promote biofilm initiation and mechanosensing that leads to changes in biological activity. Thus, physical mechanics is intrinsic to characteristics that distinguish biofilms from free-swimming or free-floating microbes in liquid culture. However, very little is known about the specifics of what mechanical traits characterize different types of biofilms at different stages of development. Even less is known about how mechanical inputs impact microbial biology and how microbes can adjust their mechanical coupling to, and interaction with, their environment. These knowledge gaps arise, in part, from the challenges associated with experimental measurements of microbial and biofilm biomechanics. Here, we review extant experimental techniques and their most-salient findings to date. At the end of this review we indicate areas where significant advances in the state-of-the art are heading.

Keywords: biofilm, bacteria, surface sensing, surface adhesion, bacterial cohesion, rheology, microrheology

(Some figures may appear in colour only in the online journal)

Introduction

Biofilms are communities of interacting microbes that are embedded in a matrix of extracellular polymers and protein. Annually, biofilm infections affect 17 million Americans, cause at least 550 000 American deaths, and cost the US healthcare system billions of dollars [4–6]. In addition to health concerns, biofilms damage civic and industrial infrastructure—for example, by clogging systems for water treatment [7, 8], biocorrosion of oil and water

pipelines and other liquid-immersed structures [9, 10], and fouling shipping vessels [11, 12]—thereby decreasing efficiency, increasing fuel usage and running costs, and causing harm to the environment.

Mature biofilm infections have higher resistance to antimicrobials and the host immune defence than do their genetically-identical planktonic counterparts [13–17]. The mechanical integrity of the biofilm matrix contributes to the difficulty of removal and harmful effects of the biofilm. Many harms done by biofilms arise from the mechanical integrity of

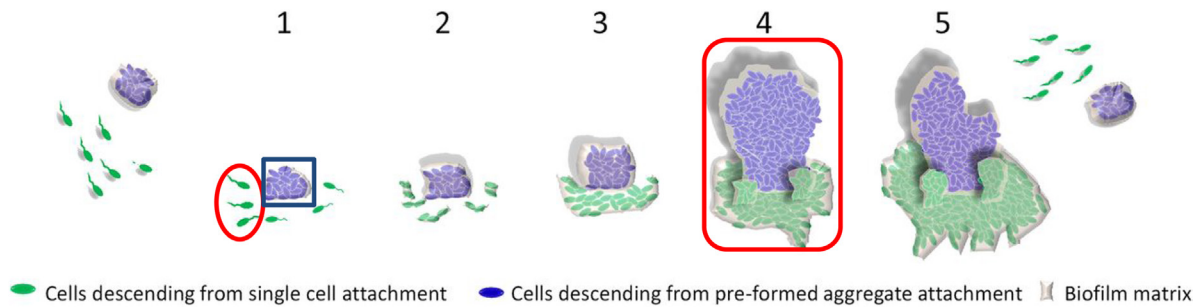


Figure 1. The biofilm life cycle. In the canonical view of the biofilm life cycle, biofilms initiate when single cells attach to a surface (1, circled in red) and, as a result, begin to change their gene expression from the planktonic to the biofilm state. Thus, both the adhesive forces binding single bacteria to a surface and the biological sensing and response to the surface are intrinsic to the biofilm life cycle. *This is the focus of the first, single-cell portion of this review.* As the biofilm grows, bacteria are embedded in a matrix of polymers and proteins. The cohesive forces conferred by the matrix result in viscoelastic biofilm mechanics. *The mechanics of mature biofilms (4, boxed in red) is the focus of the second portion of this review.* Furthermore, there is an unexplored link between the mechanics of mature biofilms and the initiation of new biofilms, as follows: We and our collaborators have recently shown that, when biofilms initiate, pre-formed aggregates (blue box) can have a growth advantage over single cells [3]. As a result, we have proposed a revised view of the biofilm life cycle: (1) The surface may be seeded by single cells and/or aggregates. (2)–(4) Descendants of initially-seeding single cells and aggregates grow; when competition for growth resources is high and the environment is spatially structured, aggregates can produce more progeny per initial cell than single cells do because the height of cells at the top of the aggregate gives them better access to growth resources. (5) Bacteria disperse, as both single cells and aggregates, and can initiate biofilm growth in new areas. Aggregates may come from existing biofilms shedding pieces. If so, understanding biofilm mechanics, how environmental forces detach pieces of the biofilm, and what size distributions, number densities, and biological properties of detached pieces result, can be essential to understanding biofilm initiation. Figure from [3] (figure 8 in the original publication) published in mBio (<http://mbio.asm.org/>), and modified for this review by adding boxes and circles to highlight key aspects of biofilm development. Used under the Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0/>).

the biofilm matrix. By holding bacteria in place, the matrix controls intercellular associations and differentiation of microenvironments [18]. Differentiated microenvironments, in turn, lead to enhanced intercellular signaling, increased virulence, and increased resistance to antibiotics. Mechanical breakup of biofilms can render bacteria more susceptible to antibiotics [19]. Detached, smaller pieces of biofilms are thought to have greater susceptibility to antibiotics than the larger, original biofilm because larger biofilms will have more limited diffusive transport of growth substrate and antibiotics—many of which act specifically on actively-growing bacteria. Debridement, the mechanical scraping-away of biofilm, is part of the standard-of-care for chronic wounds and has been shown to induce a brief window of increased antibiotic susceptibility for the biofilm remaining in the wound [20]. Indeed mechanical removal is an important component of most approaches for treating biofilm infection [13–15]. Thus, biofilm mechanical integrity and response under mechanical agitation are tightly linked to medical impact and possibilities for treatment. For biofilms outside the body—for example, on ship hulls or in pipelines for oil or water—mechanical removal is also the first line of action [21, 22].

For the cases discussed in the preceding paragraph, the biofilm's response to mechanical input is passive. Much less is known about how microbes, particularly biofilm-forming bacteria, respond actively to mechanical cues. Yet, bacteria live in, migrate between, and must adapt to a wide range of mechanically-differentiated and -changing environments [23]. For biofilm development in particular, the transition from suspension in a fluid environment to adhesion to a solid substrate is associated with radical changes in signaling and gene expression. A handful of recent papers have suggested that bacteria may be able to sense mechanical changes in their

environment through mechanosensitive proteins in their cell envelopes [24–27]. In higher eukaryotes, mechanosensing and mechanotransduction are well-established to be of widespread importance for a diverse set of processes including both normative development and disease [28–30].

Figure 1 gives an overview of the biofilm life cycle and indicates the conceptual linkages between the first part of this review, on single-bacterium mechanics and biomechanical response, and the second part of this review, on the mechanics of mature biofilms. Determination of the biofilm developmental cycle, and extant understanding of biofilms more generally, has been dominated by the model organism *Pseudomonas aeruginosa*. As with any biological model system, it is an error to over-generalize from one type of biofilm-forming organism to all biofilm formers. In our view, a more appropriate approach is to elucidate the roles of mechanics in different types of biofilms and different stages of biofilm development and mechanical challenge, with the long-term hope of establishing a sufficiently-broad substrate of knowledge for many species that common themes are able to emerge. The present state of the art is not sufficient for sweeping, cross-species themes to be revealed. It is our hope that this review may inspire broader investigation such that a more comprehensive understanding of biofilm mechanics may be established in future.

Background: *Pseudomonas aeruginosa* specifics

P. aeruginosa is an opportunistic human pathogen that is by far the best-characterized, most widely-studied model organism for biofilm formation. The widespread use of *P. aeruginosa* as a model organism for biofilm formation has two principle causes. First, *P. aeruginosa* readily forms biofilms on a large

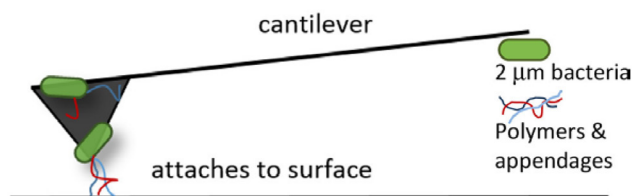


Figure 2. AFM can be used to probe the forces attaching bacteria to surfaces. Attachment forces can be mediated by polymers coating the bacterial cell body and by the outer surface of bacterial cell envelope and attached appendages. Reproduced with permission from [1].

variety of surfaces (and multicellular, biofilm-like aggregates in liquid suspension [31]) and under a wide range of growth conditions, including on contact lenses, in water treatment facilities and pipes, and on oil spills. Second, *P. aeruginosa* biofilm infections are an important factor that detrimentally affect the outcome for patients with implants or medical devices, chronic obstructive pulmonary disease, chronic wounds, and cystic fibrosis [32, 33]. Thus, near-ubiquity and high practical impact together have led to the selection of *P. aeruginosa* as a model organism in the majority of biofilm related research.

Background: surface sensing, and biofilm initiation

Cyclic-di-GMP is an intracellular chemical signal used by many species to regulate gene expression for biofilm initiation [34]. Levels of cyclic-di-GMP increase when planktonic *P. aeruginosa* adhere to a surface [35–37]. Without cyclic-di-GMP, bacteria that otherwise form robust biofilms within 12 h instead make no biofilm whatsoever over at least 72 h of culturing [38].

Review: experiments that measure single-cell mechanics and biological response

In the canonical picture of biofilm development, the first step is the initial adhesion of individual planktonic cells to a surface [39] (figure 1). Upon adhesion, bacteria change their gene expression profiles to initiate the transition to the biofilm state. Thus, the mechanics of single-cell adhesion and the ability of individual cells to sense the surface to which they are attached are intrinsic to biofilm development.

Mechanical coupling of bacterial cells to a surface

Intriguingly, Professor Howard Stone's group at Princeton has shown that shear stress applied by fluid flow increases the residence time of surface-adhered *P. aeruginosa* cells [40]. The mechanical interaction between an individual bacterium and a surface can be probed using atomic force microscopy (AFM) (figure 2). AFM uses the deflection of a cantilever to image the height features of a surface and to measure forces of interaction between a surface and the cantilever. We and others have used AFM to study how bacterial cells interact with the surface via extracellular polysaccharides (EPS), pili, and other bacterial appendages in pathogens such as *P. aeruginosa*,

S. epidermidis, *S. aureus*, and *S. salivarius*, *S. xylosum*, and *E. coli* [41–46]. AFM is a powerful tool for studying planktonic cells and how small numbers of cells interact with surfaces. There are many measurements that can be carried out using AFM, as exemplified below.

In previous work in our lab, using AFM, we characterized the unique EPS which causes symmetric surface adhesion in *P. aeruginosa* by measuring the characteristics of surface adhesion forces in bacteria with differing EPS expression [44]. Single *P. aeruginosa* cells were able to apply up to 3 nN of force [44]. With tip-less cantilevers, Zeng *et al* attached single cells of multiple species of bacteria to a cantilever each to be used as a probe itself. From this study, *S. xylosum* and *S. epidermidis* mediated the strongest surface attraction with forces up to nearly 15 nN on clean glass, while *P. fluorescens* could apply around 1 nN of force. *E. coli* was the weakest, with the force of attachment less than 0.5 nN [41]. Xu *et al* and Touhami *et al* both investigated the force mediation by pilus of *E. coli* and *P. aeruginosa*, respectively, using AFM. For the *E. coli* study, many bacteria were attached to a tipless cantilever. In this configuration, the bacteria were able to exert 1.5 nN of force on a mica surface. However they found that this surface attachment was highly dependent on the surface type used; the surfaces tested were fluorosilane, aminosilane, PEG, mica, and a silicon wafer. The strongest attachment of *E. coli* cells was on fluorosilane, while the weakest attachment was on the silicon wafer. When measuring *P. aeruginosa* pilus attachment, only a few cells were attached to the cantilever. The force of attachment was measured to be as high as 0.3 nN on mica [42, 45]. In a more specific chemical attachment experiment, El-Kirat-Chatel *et al* investigated the interaction of a single biofilm adhesin produced by *P. fluorescens* with different kinds of surfaces in order to characterize the chemical and mechanical character of its interaction with a surface [43]. Chen *et al* described the interaction with the surface caused by soft, adhesive polymer coating the bacteria, using the classic Kelvin–Voight model to characterize the viscoelastic nature of the bond in multiple bacterial species [46]. This model allows for the mechanical interaction to be described as having solid-like and liquid-like components, or elastic and viscous components, respectively. Such information is useful in both determining strength of the material of interest and for determining mechanics at time scales of relevance to biological processes.

The Kelvin–Voight model and other basic linear viscoelastic models consist of a combination of a pure spring, with a spring constant E , and a pure dashpot, with viscosity η . Different materials will be described with different arrangements of these elements, and each material will have a model that describes it best depending on the material of interest. The two most basic arrangements are given in figure 3 [46, 47]. These kinds of models work well for simple mechanical probing of the surface, as with an AFM or microcantilever device.

Mechanical sensing of a surface by bacterial cells

Although delicate and requiring specialized expertise and equipment, using AFM to probe the mechanical coupling between bacteria and a surface is relatively straightforward. The relationship between mechanical input and biological

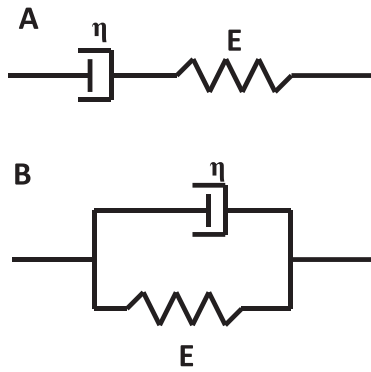


Figure 3. Spring-dashpot models for viscoelasticity. (A) The Maxwell model places the purely viscous dashpot and the purely elastic spring in series with one another. As they are in series, the strain of the material will be the addition of each element, $\epsilon_{\text{total}} = \epsilon_{\text{spring}} + \epsilon_{\text{dashpot}}$, while the stress will be equal throughout the material $\sigma_{\text{total}} = \sigma_{\text{spring}} = \sigma_{\text{dashpot}}$. (B) The Kelvin-Voigt model consists of a purely viscous dashpot and a purely elastic spring in parallel. As the dashpot and spring are in parallel, they will experience identical strain, $\epsilon_{\text{total}} = \epsilon_{\text{spring}} = \epsilon_{\text{dashpot}}$, while the stress of the material between the two elements will add, $\sigma_{\text{total}} = \sigma_{\text{spring}} + \sigma_{\text{dashpot}}$. Thus the time-dependent stress of the viscoelastic material time will be given by $\sigma(t) = E\epsilon(t) + \eta \frac{d\epsilon(t)}{dt}$.

response is much more complex and difficult to disentangle. One group making headway in this area is that of Professor Zemer Gitai at Princeton University. His group and collaborators have observed that surface attachment, in concert with quorum sensing, is necessary to elicit a virulence response in *P. aeruginosa* [48]. Harnessing *P. aeruginosa*'s ability to kill *Dictyostelium discoideum* using virulence factors, they have used a fluorescent readout for *D. discoideum* viability to show that surface-grown *P. aeruginosa* cells are more virulent than broth-grown planktonic cells. The increase in virulence depends on *P. aeruginosa* being attached to a solid substrate but not on chemical specifics of the surface. The membrane- and pilus-associated protein PilY1 contains a region homologous to the von Willebrand factor A domain, a mechanosensitive region commonly involved in mechano-sensing by eukaryotic cells; PilY1 is involved in *P. aeruginosa*'s response to surfaces, including surface-activated expression of virulence factors [26, 48, 49]. In another linkage between mechanical input and virulence response, Professor Anne-Marie Krachler's group (now at University of Texas Health Science Center at Houston) has recently found that surface-attached *Escherichia coli* can respond to shear by increasing virulence [50].

In another recent paper, Gitai and collaborators have found a mechanochemical response to surface adhesion mediated by type IV pili (TFP). TFP are long thin appendages protruding from many different species of bacteria that are involved in surface motility. Persat *et al* show that TFP have an integral role in a surface-associated signaling response [51].

Summary: impact of single-bacterium mechanics and mechanosensing on biofilm mechanics

It is clear that adhesion to a surface, by single bacteria or pre-formed multicellular aggregates, is the essential point of

initiation for surface-dependent biofilms (figure 1). Bacteria use appendages (such as pili and flagella) and sticky polymer coatings to promote surface adhesion. Although much remains to be studied in this area, experimental techniques for probing the adhesivity of bacteria to surfaces are well-established. In contrast, we have only limited knowledge of the relationship between mechanical inputs, bacterial mechanosensing, and resulting changes in bacterial biology that could influence biofilm properties. Correspondingly, experimental techniques for probing bacterial mechanobiology are also at a less-mature stage of development.

However, there are hints in the literature that bacterial mechanosensing may impinge on the mechanical properties of the mature biofilm. For several bacterial species, biofilms that were initiated and grown under conditions of high shear have been shown to be stiffer and more dense in proteins and polysaccharides than are biofilms grown under low shear [52–54]. To what degree this results from single-cell mechanosensing and response when the biofilm is initiated, dynamic changes as the biofilm matures, or removal of softer or weaker biofilm material by strong shear flows, is not known.

Background: the biofilm matrix

Planktonic bacteria are coated in sticky EPS that promote surface adhesion [44, 55]. In a mature biofilm, bacteria are embedded in a polymer matrix, which, for *P. aeruginosa*, can contain up to three distinct EPS materials, Pel, Psl, and alginate [56–60], as well as extracellular DNA (eDNA) [61–63] and proteins. *P. aeruginosa* is a copious producer of EPS. Microscopy observations of biofilms grown in our lab and others show discrete bacteria suspended in a continuous matrix, with the bacterial volume fraction well below 50% [2, 64]. For this reason, the matrix is the primary determinate of the mechanical properties of *P. aeruginosa* biofilms and other biofilms with low volume fraction of bacteria. This is not universal and biofilms of other species, with higher microbial volume fraction, could have very different mechanics. This is because bacteria and yeast are much stiffer than aqueous polymer matrices—the measured moduli for both Gram-negative and Gram-positive bacteria typically range from a few MPa to several hundred MPa [65, 66].

Why it could be advantageous for *P. aeruginosa* to produce more than one type of EPS is not thoroughly understood and is a topic of active research. Most thought on this topic has focused on distinct chemical properties of the different EPS materials and the possibility that having redundancy in biofilm-matrix production capability could allow one EPS to act as a backup if a bacterial line lost the ability to produce a primary EPS [67–70].

Review: experiments that measure biofilm mechanics

Biofilms are viscoelastic materials, behaving similarly to polymer gels. Studying biofilms from a mechanical standpoint allows us to consider such behaviors as elastic modulus,

yielding behavior, and energy dissipation in the system. While an understudied facet of biofilm properties, the mechanical strength and malleability allows biofilms to survive and adapt to many environments, from human bodies to industrial filters and pipes. *Staphylococcus aureus*, a pathogen responsible for many medical implant infections and endocarditis, is exposed to continuous and laminar flows in these scenarios. The viscoelasticity of the *S. aureus* biofilms allows the bacteria to stay attached to surfaces even under high fluid shear [71]. *S. aureus* microcolonies can roll under flow; this may be essential for dispersal of nonmotile bacteria.

Recent reviews provide a discussion of different approaches to measuring biofilm mechanics and a list of biofilm mechanical properties for a wide variety of species, although this list is dominated by *P. aeruginosa* studies [72, 73]. Numerous methods have been applied to study biofilm mechanics. These methods can be categorized into both flow and static assays, as follows: flow assays are done in the presence of flowing nutrients, allowing for a continuous time-series of measurements that allow dynamic properties to be studied; static assays are done without an ongoing supply of nutrients so that measurements are taken at specific time points where growth is no longer occurring.

Static assay: AFM

While there is great utility in AFM for studying single cell bacteria, as we review above, this small-scale technique can also be useful for studying the mechanics of the surface of a biofilm. AFM allows measurement of the mechanical properties of biofilms with high spatial resolution, in principle much less than the size of a single bacterial cell. In a reductionist view, the smallest fundamental unit of the biofilm (distinct from single bacteria) would be two cohering cells. We have recently used AFM to measure the cohesive forces and energies between pairs of bacteria with different patterns of EPS expression and have found that the strength of inter-bacterial cohesive interactions varies greatly with the type of EPS produced [2]. However, these bacteria were not in a biofilm state, and our bulk rheology on biofilms grown from the same bacterial strains showed that the mechanical properties of isolated bacterial pairs do not always have a straightforward mapping onto biofilm mechanics (see below under *Static Assay: Bulk Rheology*). Other researchers have used an AFM, functionalized with a microbead, to measure the adhesive and viscoelastic properties of biofilms with different levels of lipopolysaccharide production [74].

AFM has also been used to study the cohesive energy of biofilms by using the cantilever tip to abrade the surface of the biofilm. By measuring the energy dissipated as the AFM cantilever is scraped over the biofilm, a total cohesive energy measurement is obtained for the volume abraded. In this study, *P. aeruginosa* biofilms were found to have increasing cohesive strength deeper within the biofilm, from $0.1 \text{ nJ } \mu\text{m}^{-3}$ on the surface to $2.05 \text{ nJ } \mu\text{m}^{-3}$ within the biofilm. Adding calcium to the biofilms during growth increases biofilm cohesive energy, is a result that has been seen in other studies of *P. aeruginosa* biofilms [75].

Static assay: microindentation

Another method similar to the AFM is the measurement of cohesive or tensile strength using a microcantilever. These devices are often custom-made for the measurement of biofilm mechanics. By measuring the deflection of a cantilever, simple stress and strain measurements can be obtained based off of the deformation of the biofilm of interest. These measurements have been done on a wide array of biofilms, such as *P. aeruginosa* [76], *S. epidermidis* [76], *P. fluorescens* [77], *S. mutans* [78], and mixed-species biofilms [79].

In *P. aeruginosa*, biofilm cohesive strength was measured from 1 kPa to 16 kPa depending on the speed of the measurement, size of biofilm growth, and whether or not the biofilm was intact [76, 79]. *S. epidermidis* biofilms were somewhat weaker, only testing from 0.9 kPa to 1.4 kPa [76]. Biofilms of *S. mutans* bacteria were stronger like *P. aeruginosa* biofilms with strengths measured from 1 kPa up to 10 kPa [78]. A mixed species biofilm of return activated sludge (RAS) was the strongest of all biofilms measured, with strengths reaching 206 kPa, while some were as weak as 0.4 kPa [79]. The strength of the *P. fluorescens* was reported differently than the other biofilm measurements. In this microcantilever experiment, the biofilms had adhesive strengths from 0.1 to 1 J m^{-2} , thus a value more classically considered as a surface tension or stiffness [77].

A benefit of the microcantilever measurement is that it can be performed on intact biofilms at an array of sizes that are clinically relevant for human infections, ~ 10 to 10^2 microns. In addition, growth is not restricted to unidirectional flow cell. Aggarwal *et al* have used a rotating disk reactor for growth, which results in more isotropic biofilm structures [76].

Static assay: bulk rheology

A classic method for studying the mechanics of viscoelastic systems is bulk rheological studies using a rheometer (figure 4). Bulk rheology uses large amounts of material (on the order of microliters to milliliters). Experiments with rheometers can be used in both steady state or oscillatory mode. Spring-dashpot models, as discussed in the first portion of this review for single-cell measurements (figure 3), also give a good conceptual basis for more complex oscillatory bulk rheology measurements. Viscoelastic materials, including biofilms, have both an elastic (or solid-like) and viscous (or liquid-like) response to strain deformation, meaning they both store and dissipate energy when a strain is applied. Oscillatory mode allows for the complex viscoelastic modulus, $G^* = G' + iG''$, which gives the elastic and viscous moduli G' and G'' , to be measured directly [80–82] (figure 4). The elastic modulus (G') describes how the material responds to an applied force and is directly proportional to the stress applied (force per unit area). The viscous modulus (G'') is a measure of the material's response to stress with time and is proportional to the rate of deformation.

Another measure of the viscoelasticity of a material is creep compliance J . The creep compliance characterizes how the material's strain deformation $\varepsilon(t)$ changes over time as a constant stress σ_0 is applied; generally, $J = \frac{\varepsilon(t)}{\sigma_0}$.

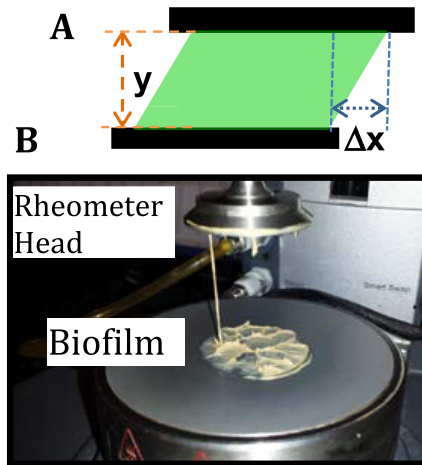


Figure 4. (A) Rheology schematic. The rheometer tool applies a shear strain $\varepsilon = \Delta x/y = \varepsilon_0 \cdot \sin(\omega t)$ to the biofilm, where $\omega =$ angular frequency of oscillation and $t =$ time. The resulting shear stress response, $\sigma = \sigma_0 \cdot \sin(\omega t + \delta) = \varepsilon_0 \cdot [G' \sin(\omega t) + G'' \cos(\omega t)]$, gives the elastic modulus G' and the viscous modulus G'' . (B) Biofilm that has been measured using a parallel-plate rheometer tool, as described in [2]. This picture was taken after the rheology measurement when the rheometer head was retracted. Biofilm adheres to both the upper and lower plates.

By measuring the elastic and viscous modulus over a range of strains and frequencies, much can be determined from a material of interest. If a material is highly time-dependent in its stress response, then the frequency sweep of the material will reveal dramatic changes over a range of frequencies. A strain sweep can be used to determine a material's yield point at a given frequency of interest. This allows measurements of energy required cause a material to yield, as energy is simply the integral of stress over strain to the point of material yield.

Two main methodologies for studying biofilms via bulk rheology have been used. In one method, the EPS components are isolated from the bacteria and their mechanics are tested. This method has been used successfully to probe the viscoelasticity of EPS material and how those polymers respond to the addition of divalent calcium ions [83]. The other method utilizes biofilm grown on many agar plates in order to achieve the amount of biofilm necessary for rheological study [82]. We have used this method to measure distinctive mechanical properties that arise from different EPS materials and show that biofilm mechanics evolve within the lungs of cystic fibrosis patients [2].

Flow assay: fluid shear

A common method for studying the growth and properties of biofilms is using flow assays with time-lapse microscopy. In such experiments, a biofilm is seeded and grown inside a channel in which nutrients are continually flowed over the growing biofilm. Flow assays allow for a continuous supply of nutrients for the bacteria as well as a way to modulate the forces experienced by a growing biofilm. Biofilm mechanics can be evaluated because the shear rate within the channel is known, and thus the response to the biofilm to varying shear rates can be determined through image analysis of the

biofilm deformation. Stoodley *et al* and Rupp *et al* have done flow rheological measurements on biofilms of *P. aeruginosa*, *S. aureus*, and mixed species [71, 84]. The results of these measurements gave shear moduli, G , in the range of a couple to tens of Pascals.

A benefit of this method of measurement is that the data is taken *in situ* as the biofilm grows in the channel, meaning the biofilm maintains any heterogeneity and complexity it creates in growth. A pitfall of this method is that the biofilm is grown under shear, and so may select for genes in response to shear flow. Also, the strain of the biofilm is measured via the deformation of 'streamers' growing off of the biofilm. As biofilms are heterogeneous, measuring the rheology of the streamer is not the full picture of biofilm mechanics or polymer contribution to mechanics.

However streamer creation itself is also of interest as it has been seen that streamers can cause more rapid clogging in a flow channel in *P. aeruginosa* biofilms, which has an impact in medical devices or industrial pipes. When biofilms form on the walls of a channel, the streamers that reach around a corner cause clogs more quickly than the biofilms that are simply growing on the flat walls of the channel. As such, a biofilm's ability to form long, ductile streamers can cause the biofilm to become more damaging in health or environmental applications [85]. Studies on multi-species biofilms show that under turbulent flow, these biofilms form streamers and ripple-like structures which are likely important in biofilm dispersal [86, 87].

Flow assay: passive and active microrheology

Another method that can utilize flow cell chambers is microrheology. Microrheology is a powerful tool for probing local mechanical response. In this method, beads are added to the biofilm throughout growth. The beads can be glass, steel, or any other material that is easily visualized within the biofilm matrix under a microscope.

Passive microrheology uses thermally-driven motion of beads to probe their local microenvironment (figure 5(A)). The motion of the beads is imaged under the microscope and software [88] identifies bead positions. For an elastic solid, the mean square displacement (MSD) $\langle r^2 \rangle$ of a bead will plateau at a value that depends on the bead size, a , and the elastic modulus of the microenvironment G_0 , thus: $\langle r(t)^2 \rangle = \frac{2k_B T}{3\pi a G_0}$, where $k_B T$ is the thermal energy [89, 90]. This approach can be extended to characterize viscous as well as elastic properties and has been used to characterize biofilms of *E. coli* and *P. aeruginosa* [91, 92]. The creep compliance of *P. aeruginosa* biofilms was found to be around 10^{-4} Pa^{-1} and to be dynamically variable with changes in what type of EPS was produced [91]. One group has succeeded in the challenging task of using the bacteria themselves as passive tracers to measure the viscoelasticity of *S. aureus* and *P. aeruginosa* biofilms [93].

Some biofilms are too stiff for passive microrheology to be an effective probe of mechanics. In such situations, active microrheology becomes the better tool. Active microrheology uses magnetic or optical tweezers to move beads imbedded

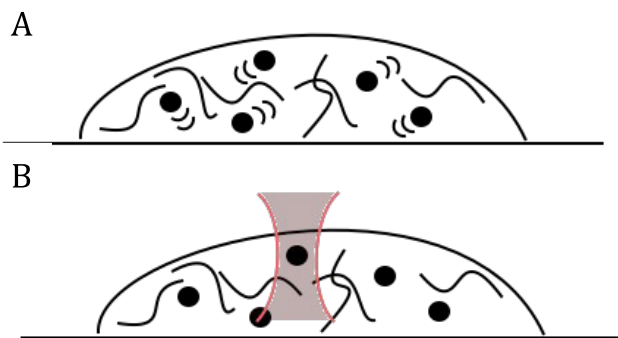


Figure 5. Microrheology measures local mechanics by measuring the displacement of tracers. (A) In passive microrheology, displacement arises from Brownian motion driven by thermal energy, $k_B T$. (B) In active microrheology, displacement arises from a directed, externally-applied force. For ease of illustration, this schematic shows an optical trap applying a force. Magnetic trapping can also be used and will not be confounded by the high optical density of biofilms, as well as being able to apply higher forces than optical trapping.

in the extracellular matrix (figure 5(B)). By applying force on the tracer bead, the strain response to this stress can be determined—this measures the mechanics of the biofilm [94].

Since microrheology probes the local region around a bead, this method can be used to probe biofilm heterogeneity. Biofilms are inherently heterogeneous, in structure, metabolism, genetics, and polysaccharide production [95–97]. As bacterial interactions with antibiotics and immune cells is primarily on a smaller scale, learning the heterogeneous distribution of mechanics and polymers is certainly of interest to efficiently attacking a biofilm via mechanical removal.

Summary, current questions, and future directions

Despite the body of work reviewed above, the role of mechanics in the formation and persistence of biofilms is still one of the most understudied aspects of biofilm research. For the earliest stages of biofilm development, little is known about single-bacterium adhesion to and mechanosensing of surfaces, but this area of research is growing rapidly. For the mature biofilm, more is known about how different matrix materials, both self-produced (e.g. EPS, proteins, and eDNA) and exogenous (added chemicals and ions) impact the biofilm's mechanical properties. However, many open questions remain here as well. Below, we sketch some of the areas most ripe for advancement.

Physical interpretation of biofilm mechanics

It is well known that increasing the concentration of polymer (c) in a gel will increase the gel's elasticity, $G' \propto c^A$, where A is a scaling factor that is 2.25 for entangled polymer in good solvent [98]. This is a physical effect that does not depend on polymer chemistry. However, we have shown that increasing Pel and alginate production *does not* increase the elasticity of *P. aeruginosa* biofilms, whereas increasing Psl production *does* stiffen biofilms [2]. Thus, understanding the molecular mechanisms by which specific matrix components

give rise to specific biofilm mechanics is essential both to understanding the physical origins of biofilm mechanics and to devising targeted strategies for disrupting biofilm mechanics. For this, it should be possible to leverage a large body of physical-chemistry work that connects the molecular chemistry of constituent polymers with the macroscale mechanical properties of bulk materials [82, 99–102].

For example, in the case of Psl, we attribute mechanical changes to cross-linking by the protein CdrA, which arises because of the specific, mannose-rich chemistry of Psl [2]. Future investigations of this system, and of other biofilm systems where cross-linking might contribute to biofilm elasticity, could be guided by rubber elasticity theory, in which $G' \sim c/M_c$. Here, c is polymer concentration and M_c is the polymer molecular weight between cross-links [103, 104]. For another example, we have shown that increased Pel production increases the yield strain of *P. aeruginosa* biofilms. Others have recently shown that cationic Pel binds with anionic extracellular DNA [105] and we have speculated that this may result in a double-network structure [2]. Rheological signatures of a double-network gel vary with the molecular interactions stabilizing the gel and can include strain hardening for 2-component systems but not for 1-component systems, non-monotonic dependence of yield stress on crosslinking density (set by the concentrations of Pel and DNA), and increase of yield strain with increasing contour length of the softer component of the network [106–108].

Potential benefits arising from better understanding of biofilm mechanics

Knowledge of the roles played by mechanics in the biofilm life-cycle has the potential to reveal mechanics-oriented approaches to preventing or remediating biofilms. Such approaches are especially appealing in the face of rising antibiotic resistance because mechanical properties and mechano-responsive biology are orthogonal to mechanisms of antibiotic activity and resistance. Some EPS types are made by more than one biofilm-forming species, such as alginate that is produced in both *P. aeruginosa* and *Burkholderia cenocepacia* biofilms [109]. This suggests the possibility of mechanics-targeting strategies for biofilm disruption that are not species-specific but rather oriented toward what type(s) of EPS dominate the matrix. The mechanics of biofilms will be an important factor in determining how biofilms break up, disperse, and seed new biofilms under mechanical perturbation, such as flow in industrial settings. Predicting and possibly controlling biofilm dispersal would allow for more efficient clearance. Knowing what mechanical cues activate virulence or biofilm initiation, and how these cues are sensed, would open the possibility for preventing bacteria from sensing the cue, thus disrupting virulence upregulation and/or biofilm formation from their inception.

Need for improved, standardized approaches to measurement

One prevalent issue with the current understanding of biofilm mechanics is that it is difficult, perhaps impossible, to meaningfully compare measurements from different research groups,

or even within the same group, that use different growth conditions, bacterial strains, and measurement techniques.

Another issue with the current state of biofilm mechanics studies is the lack of *in vivo* measurement techniques. *In vitro* studies allow for more control over experimental conditions and much easier access to the biofilm. However, *in vitro* biofilm structures are very different from *in vivo* biofilm structures [110–113]. It is likely that the mechanical properties of biofilms grown and measured *in vivo* would deviate from the mechanical properties of biofilms grown and measured *in vitro*.

There are methods, such as AFM and microrheology, which allow biofilms to remain intact and preserve structural integrity and microenvironments while probing local mechanics. There are also approaches for characterizing biofilms *in vivo* and *ex vivo*—for example, mouse models for chronic wounds and explanted human lung tissue [113, 114]. However, mechanical measurements have not yet been applied to these more clinically-relevant biofilms. Until the state-of-the-art includes measurements of the mechanics of biofilms grown *in vivo*, it will be difficult if not impossible to determine what mechanical characteristics actual biofilm infections have and to distinguish the roles of genetics *versus* environment in biofilm mechanics.

How do biofilm mechanics impact interactions with the immune system?

Knowledge of the mechanical properties of biofilm infections *in vivo*, and how mechanical properties can be altered, has the potential to reveal new approaches to treating biofilms by mechanical removal. This has the potential to intersect with another field of research on the mechanics of phagocytosis. Neutrophils are phagocytic immune cells that are first responders to infection and easily engulf planktonic bacteria [115–117]. However, mature biofilms are protected against phagocytosis despite being surrounded by continually-recruited neutrophils that do not penetrate the biofilm [112, 113]. The role of biofilm mechanics in evasion of the immune system is not known.

Because $\sim 10 \mu\text{m}$ neutrophils are an order of magnitude smaller than the $\sim 100 \mu\text{m}$ biofilm aggregates, for neutrophils to phagocytose biofilm bacteria they must be able to break off a piece of the biofilm which is small enough for them to ingest [118]. It has been estimated that neutrophils apply a stress of $\sim 1 \text{ kPa}$ during phagocytosis [119]. This stress is within the range of G' values we measure for biofilms, which suggests that the mechanical properties of a biofilm are likely to impact its resistance to phagocytosis [2]. This inference is supported by studies of other phagocytic cells: phagocytosing blood granulocytes and macrophages also exert stresses of $\sim 1 \text{ kPa}$ [120, 121]; phagocytosing *Dictyostelium* and *Entamoeba* can break off and ingest small pieces of a target [122, 123].

Biofilm mechanics are also likely to impact the timescale of phagocytosis. Changes in timescale matter because bacteria-produced virulence factors can kill neutrophils and other immune cells [124, 125].

Lack of knowledge of how polymicrobial interactions impact biofilm mechanics

Most studies of biofilm mechanics have been done on single-species biofilms. However, most real-world biofilms contain multiple microbial species. Inter-species interactions are known to produce synergies, and some reported synergies are at least suggestive of changes in the adhesive and/or cohesive properties of biofilm bacteria. Synergy in biofilms of the oral cavity includes enhanced coaggregation and protection [126, 127], and mixed-species cultures in aquatic systems grow biofilms that are more robust and protective to bacteria than are biofilms grown from single-species cultures [128]. Moreover, different species of bacteria can have both beneficial and harmful interactions, which can lead to a bacterial fight-and-flight response [129]. This leads to optimal distances between bacterial species, creating microstructures and environments within the biofilm. The changes in matrix composition, aggregation and growth, and biofilm organization that accompany a transition from single to multi-species biofilms could well lead to changes in biofilm mechanics. This area is entirely uninvestigated, yet knowledge of how polymicrobial interactions impact biofilm mechanics and mechanical vulnerabilities would be widely applicable.

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