Biophysics of biofilm infection

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Abstract

This article examines a likely basis of the tenacity of biofilm infections that has received relatively little attention: the resistance of biofilms to mechanical clearance. One way that a biofilm infection persists is by withstanding the flow of fluid or other mechanical forces that work to wash or sweep microorganisms out of the body. The fundamental criterion for mechanical persistence is that the biofilm failure strength exceeds the external applied stress. Mechanical failure of the biofilm and release of planktonic microbial cells is also important in vivo because it can result in dissemination of infection. The fundamental criterion for detachment and dissemination is that the applied stress exceeds the biofilm failure strength. The apparent contradiction for a biofilm to both persist and disseminate is resolved by recognizing that biofilm material properties are inherently heteroge-neous. There are also mechanical aspects to the ways that infectious biofilms evade leukocyte phagocytosis. The possibility of alternative therapies for treating biofilm infections that work by reducing biofilm cohesion could (1) allow prevailing hydrodynamic shear to remove biofilm, (2) increase the efficacy of designed interventions for removing biofilms, (3) enable phagocytic engulfment of softened biofilm aggregates, and (4) improve phagocyte mobility and access to biofilm.

Keywords
cohesion
viscoelastic
failure
mechanical
neutrophil
detachment

Introduction

Bacteria or fungi that aggregate in biofilms can cause persistent infections (Costerton et al., 1999). These infec-tions are typically localized, slow moving, recurrent, and poorly controlled by antibiotics or antiseptics (Parsek & Singh, 2003). The host immune response to the continued presence of microorganisms can give rise to collateral damage to neighboring healthy tissue, perpetuating a degradative, nonhealing state. A few well-known examples of biofilm-associated infections are cystic fibrosis pneumo-nia, catheter-associated urinary tract infection, prosthetic joint infection, periodontitis, and chronic dermal wounds.

One of the main reasons that biofilm infections are so persistent is the reduced susceptibility of microorganisms in biofilms to killing by antimicrobials including systemic antibiotics, topical antiseptics, and antimicrobial compo-nents of the host defense (Stewart & Costerton, 2001; Fux et al., 2005). Biofilm tolerance to antimicrobial agents has been extensively researched and discussed. The purpose of this article is to examine another likely basis of the tenacity of biofilm infections that has received much less attention: the resistance of biofilms to mechanical clearance. This topic requires engagement with structure, hydrodynamics, material properties, polymer gel physics, and adhesive and cohesive failure. The intent of this article is to outline the application of elementary biophysics concepts to the resil-ience of biofilm infections.

Structure

When thinking about mechanics, it is important to have a good grasp on the structure and geometry of the system. Much has been written about the three-dimensional archi-tecture of biofilms formed in in vitro reactors and environ-mental settings (Costerton et al., 1995; O'Toole et al., 2000). In vivo, however, biofilm structures are often quite different as has been recently articulated by Bjarnsholt et al.(2013a). The celebrated mushroom-shaped biofilm cluster observed in flow cells in the laboratory is not seen in vivo.

Instead, relatively small aggregates of biofilm cells are found intermixed or covered with extensive host-derived material (Gristina *et al.*, 1985; Baltimore *et al.*, 1989; Hall-Stoodley *et al.*, 2006; James *et al.*, 2008; Stoodley *et al.*, 2008, 2010; Bjarnsholt *et al.*, 2009). In other niches, thick biofilms form, but often contain substantial amounts of host or precipitated mineral particulates (Wright & Kirschner, 1979; Friskopp, 1983; Marrie and Costerton, 1984; Marrie *et al.*, 1987; Stickler *et al.*, 1993; Tan *et al.*, 2004). Conceptual models of *in vivo* biofilm structure are diagrammed in Fig. 1.

Biofilm composition and mechanical properties

As a material, a biofilm can be conceptualized as a dispersion of colloidal particles (microbial cells, mineral precipitates, host biological debris) in a hydrogel [microbial extracellular polymeric substances (EPS) and host extracellular polymers such as mucus, collagen, or released DNA]. In the biofilm literature, the constituents of EPS have been identified as polysaccharides, proteins, and extracellular DNA (Branda *et al.*, 2005; Flemming & Wingender, 2010). There has been less attention to understanding the composition of admixed host polymers and particulates, but these components will clearly be important in the mechanics of the *in vivo* biofilm.

Biofilms typically exhibit viscoelastic behavior when mechanically stressed (Klapper *et al.*, 2002; Stoodley *et al.*, 2002; Wilking *et al.*, 2011; Böl *et al.*, 2013). That is, they can deform in both an elastic, reversible manner and in a viscous, irreversible manner. Most biological materials, such as mucus or tissue, are also soft and viscoelastic (Levental *et al.*, 2007). There are numerous parameters that can be appropriately used to characterize the mechanical behavior of these materials. In this article, I will mention only two: G, the elastic modulus, which can be thought of as a spring constant, and σ_B, the biofilm failure strength, which characterizes the applied force per area that will cause the biofilm to break. Both of these parameters characterize the response of a material to an applied force, but they capture qualitatively different aspects. G describes the reversible stretching of the material under tension and can be thought of as the stiffness of the material. Materials with larger values of G are harder to deform. σ_B describes the breaking of the material and can be thought of as its overall strength. Materials with larger values of σ_B are harder to break. It is clearly simplistic to think of these properties as single values in the context of the systems begin discussed. The reality of a viscoelastic material is that the measured failure strength will depend on how quickly or slowly stress is applied. In addition, we know that biofilms are heterogeneous with regions that are stronger or weaker.

Biofilms resist mechanical clearance

One way that a biofilm infection persists is by withstanding the flow of fluid or other mechanical forces that work to wash or sweep microorganisms out of the body (Seymour *et al.*, 2004; Stewart, 2012). Here are four examples.

Consider the lumen of a central venous catheter, where there are typically periods of no flow punctuated by intervals of significant flow. For a biofilm to accumulate in these locations, it is critical that the shear stress applied during peak fluid flow is insufficient to remove the biofilm.

In the familiar example of dental plaque, a spectrum of forces is exerted on the oral biofilm. These forces range from mild shear associated with the flow of saliva (weak but continuous), to contact with moving tongue, cheeks, or food (intermediate and frequent), to water jets and toothbrushing (occasional but intense.) Biofilm will only build up if it is strong enough to resist disruption by the frequent forces or is able to regrow in the intervals between the intense but infrequent forces. Toothbrushing is effective precisely because it is sufficiently powerful to remove much of the biofilm. To be effective, it must be repeated regularly on a cycle that is comparable to or shorter than the biofilm regrowth time. Of course, there are locations in the dentition, such as interproximal zones, that are sheltered from the forces delivered by toothbrush bristles. These areas are prone to greater plaque accumulation.

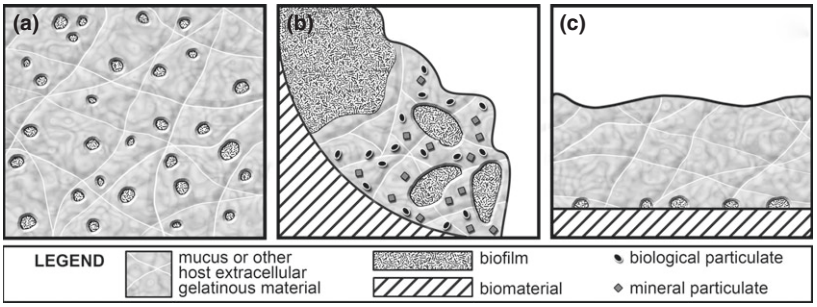

Fig. 1 Conceptual models of *in vivo* biofilm structures. (a) Small microbial aggregates (e.g. 5–50 μm in size) are distributed in a gel-like matrix which may be composed of host extracellular matrix material, dead neutrophils and released neutrophil DNA, and microbial EPS. This model could apply to biofilms in the cystic fibrosis lung or in chronic wounds. (b) Large aggregates of microorganisms comingle with precipitated mineral phases or host-derived material such as platelets and fibrinogen. This model could apply to biofilms such as dental plaque, the infectious vegetation on a heart valve, or the encrustation in a urinary catheter. In these examples, the biofilm/host material/mineral aggregation is attached to a surface. (c) Small aggregates of surface-attached microorganisms are covered by a secondary layer of primarily host-derived material.

Airborne particles, including microorganisms, are continuously deposited in the lungs. In healthy individuals, these particles are swept out of the lung by the mucociliary escalator. Beating cilia steadily push the fluid mucus layer, along with entrapped particles, up and out of the lungs. In individuals with cystic fibrosis, the mucus is thickened by the disease and the motion of the escalator is much impaired. The residence time of bacteria in the mucus layer is long enough for them to grow into biofilm aggregates and exert pathogenic effects.

In endocarditis, the biofilm attached to a heart valve persists in a turbulent, high shear environment. In this case, the flow of fluid never ceases so the intrinsic mechanical strength of the biofilm/vegetation has to exceed the maximum stress developed by the blood flow.

One can begin to develop a quantitative basis for analyzing the interactions described above by comparing the shear stress applied to the biofilm with the biofilm cohesive strength (Fig. 2). Although measurements of biofilm cohesive strength range over orders of magnitude, this comparison allows us to appreciate that biofilms are strong enough to mechanically persist in many settings. The fundamental criterion for mechanical persistence is $\tau_F < \sigma_B$ where τ_F is the applied shear stress and σ_B is the biofilm failure strength. Put another way, when the force applied is not enough to overcome biofilm cohesiveness, the biofilm persists.

Dissemination of infection

Although the discussion thus far has focused on how biofilms stay stuck in place, we also know that biofilms can shed planktonic cells or aggregates of cells. This is important in the context of infection because planktonic release events can disseminate the infection to other parts of the body or spawn episodes of acute infection (Fux *et al.*, 2004; Hall-Stoodley & Stoodley, 2005). For example, oral streptococci are sometimes recovered from heart valves.

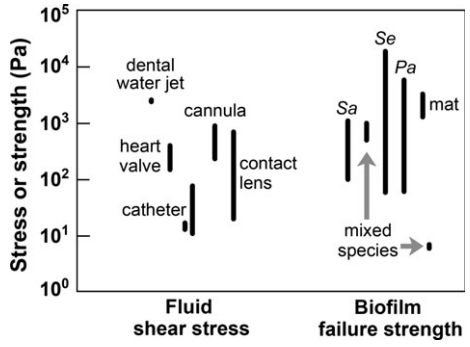

Fig. 2 Magnitude of calculated shear stresses in the human body and measured biofilm failure strengths. Sources: Day (1997), Ohashi *et al.* (1999), Bundy *et al.* (2001), Mareels *et al.* (2007), Möhle *et al.* (2007), Dasi *et al.* (2009), Aggarwal & Hozalski (2010), De Bartolo *et al.* (2011), Clark *et al.* (2012), Vignaga *et al.* (2012), Rmaile *et al.* (2014). Sa, *Staphylococcus aureus*; Se, *Staphylococcus epidermidis*; Pa, *Pseudomonas aeruginosa*.

The bacteria are thought to have travelled to the heart, where they attached, following a transient bacteremia associated with teeth cleaning. The biofilm on a central venous catheter can be asymptomatic until enough microorganisms are released at once to trigger a bloodstream infection. In cystic fibrosis pneumonia, acute exacerbations may result from biofilm dispersion events.

There is an apparent contradiction in the requirement for biofilms to be both strong enough to resist detachment and weak enough to permit release of planktonic cells. This contradiction is resolved by recognizing that biofilm material properties, such as other chemical and biological properties (Stewart & Franklin, 2008), are inherently heterogeneous. Aggarwal *et al.* (2010) have reported repeated microscale measurements of cohesive strength of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* biofilms; they find these values are distributed over more than two orders of magnitude (see Fig. 2). In other words, there are parts of a biofilm that are strong enough to remain attached even during high shear stress events and there can also be parts of the same biofilm that are weak enough to readily detach.

The fundamental criterion for detachment (dissemination) is $\tau_F > \sigma_B$, which says that for detachment to occur the applied force must exceed biofilm cohesiveness.

Biofilms resist phagocytosis

Microorganisms interact with and evade phagocytic leukocytes via a complex exchange of biochemical signals and toxins (Nizet, 2007; Sarantis & Grinstein, 2012). Biofilms may additionally thwart phagocytosis by simple mechanical means. Here, I consider this possibility. The ability of a phagocyte to engulf a biofilm aggregate likely depends on both the size of the aggregate and on its material properties, adhesive strength and cohesive strength in particular (Möller *et al.*, 2013). For example, a neutrophil may be able to engulf a sufficiently small biofilm aggregate even if it is mechanically rigid and unbreakable. The critical threshold size for engulfment of a biofilm cluster would logically be similar to the size of the phagocyte itself. Neutrophils are *c.* 10–12 μm in diameter. We can therefore speculate that any biofilm aggregate less than about 10 μm in size may be vulnerable to phagocytosis. Experimentally, neutrophils are able to phagocytose 3- and 6-μm-diameter polystyrene beads, but exhibit frustrated phagocytosis when presented with 11-μm-diameter beads, engulfing only about 50% of the bead circumference (Herant *et al.*, 2006). If a biofilm aggregate is larger than 10 μm, it may still be subject to phagocytosis, but only if it is soft enough to be fragmented by the forces applied by the engulfing cell. In other words, there is likely a critical threshold value of biofilm cohesive strength above which a biofilm is protected from phagocytic engulfment. Using a mathematical model of the phagocytosis process, Herant *et al.* (2006) suggest that the attractive force between a neutrophil and its target body could be of the order of magnitude of 10^3 Pa. Referring to Fig. 2, this could be sufficient to allow phagocytosis of some, but not all biofilms.

There is a second physical consideration in the phagocytic attack on a biofilm. This has to do with the mobility of the phagocyte. Phagocytes respond to attractant molecules released by microorganisms and migrate toward them. Such migration requires a permissive matrix. It is possible that some biofilms affected milieus, for example the thickened mucus of the cystic fibrosis lung, are so viscous that they retard or prevent leukocyte chemotaxis. This could tip the balance in favor of the biofilm. In fact, Matsui *et al.* (2005) reported exactly this effect. Neutrophils migrated in mucus constituted at concentrations in the normal range (1.5–2.5% dry weight) but exhibited little migration in any direction in thickened (6.5%) mucus representative of the airway mucus in the cystic fibrosis lung. Parkhurst and Saltzman (1994) measured leukocyte migration in cervical mucus and concluded that neutrophils move effectively in normal mucus. These authors also measured human neutrophil motility in well-defined collagen gels and found that motility decreased with increasing collagen concentrations above 0.2 mg mL^{-1} (Parkhurst & Saltzman, 1992). A conservative extrapolation of their data suggests that neutrophils would be completely immobile in a collagen gel of 1 mg mL^{-1} (0.1%).

The elementary biophysics of hydrogels suggests that modest increases in local polymer concentration, C, either biofilm EPS of host extracellular matrix materials, could lead to very large increases in the matrix strength. For example, the elastic modulus, G, of a gel increases approximately as $G \sim C^{2.25}$ (De Gennes, 1979). In other words, a doubling of matrix polymer concentration could increase gel modulus by nearly a factor of five. Such increases in gel rigidity could obviously reduce phagocyte mobility.

Together, these observations suggest that the ability of leukocytes to effectively penetrate and police mucus layers can be strongly related to the extracellular polymer concentration because the polymers determine the viscoelastic properties of the gelatinous matrix. The matrix polymers may derive from secreted mucus, necrotic tissue, DNA released from dead neutrophils or neutrophil extracellular traps, as well as from the EPS produced by microbial biofilm.

This argument, formulated here in terms of leukocyte migration in mucus, may extend to the limited penetration of leukocytes to the surface of an encapsulated implant. After implantation of a biomaterial, it is common for chronic inflammation and a foreign body reaction to ultimately conclude with fibrous encapsulation of the device (Anderson *et al.*, 2008; Bryers *et al.*, 2012). The capsule consists largely of collagen deposited by fibroblasts, and it effectively walls off the implant surface. If there is a biofilm on this surface, this process may effectively construct a fortress in which bacterial biofilm is shielded from host cellular defenses.

Of course, the EPS of a microbial biofilm itself may serve the function of excluding phagocytes. In the cystic fibrosis lung, there is a common selection over time for *P. aeruginosa* mutants that overproduce the polysaccharide alginate. Does the copious alginate gel limit physical access of neutrophils to bacterial cells? This function has been postulated (Mai *et al.*, 1993; Bjarnsholt *et al.*, 2009).

Treatments based on weakening biofilm

The discussion of issues above naturally leads to the possibility of alternative therapies for treating biofilm infections based on weakening biofilm cohesion. Reducing biofilm cohesive (or adhesive) strength could (1) allow prevailing hydrodynamic shear to remove biofilm, (2) increase the efficacy of designed interventions for removing biofilms, (3) enable phagocytic engulfment of softened biofilm aggregates, and (4) improve phagocyte mobility and access to biofilm.

A wide variety of chemical, biochemical, and enzymatic strategies can be envisioned for effecting biofilm weakening and dispersion (Chen & Stewart, 2000; Landini *et al.*, 2010; McDougald *et al.*, 2011; Bjarnsholt *et al.*, 2013b; Kostakioti *et al.*, 2013). I present a sampling of such approaches here for sake of illustration; this listing is far from comprehensive. The examples below focus on targeting the biofilm extracellular matrix.

A direct chemical attack on the biofilm extracellular matrix may be behind the relative success of halogens and other oxidizing biocides as antibiofilm disinfectants. Free chlorine caused erosion of a *S. epidermidis* biofilm that was not observed with other antimicrobials (Davison *et al.*, 2010). The strong oxidant periodate is sometimes used to diagnose the presence of polysaccharides in the biofilm matrix based on its ability to oxidize and degrade these macromolecules (Chaignon *et al.*, 2007).

Enzymatic degradation of biofilm EPS holds promise as a biofilm removal approach (Johansen *et al.*, 1997; Marcato-Romain *et al.*, 2012). For example, an enzyme discovered based on its involvement in the natural dispersion of aggregates of *Actinobacillus actinomycetemcomitans* (Kaplan *et al.*, 2003), Dispersin B, cleaves a linear polymer of *N*-acetylglucosamine, a common biofilm extracellular polysaccharide. Treatment with this enzyme can remove biofilms of multiple bacterial species (Itoh *et al.*, 2005) and has been shown to alter the mechanical stability of *S. epidermidis* biofilm under hydrodynamic challenge (Brindle *et al.*, 2011). The intriguing strategy of delivering the coding potential for this same enzyme in the DNA of an engineered bacteriophage has been demonstrated (Lu & Collins, 2007).

Because biofilm EPS often contains proteins and extracellular DNA, proteases and DNAases are also candidate enzymes for breaking down biofilms (Whitchurch *et al.*, 2002; Chaignon *et al.*, 2007; Boles & Horswill, 2008; Hall-Stoodley *et al.*, 2008; Lequette *et al.*, 2010; Nijland *et al.*, 2010).

It should also be possible to alter biofilm matrix cohesion by interfering with cross-linking interactions between matrix polymers. Chelants that compete for multivalent cations such as calcium or iron have been shown to remove biofilms (Turakhia *et al.*, 1983; Banin *et al.*, 2006; Raad *et al.*, 2008). The efficacy of these agents may derive from disrupting electrostatic interactions between bridging cations and strands of negatively charged polymers (Chen & Stewart, 2002). Concentrated urea, which disrupts hydrogen bonding, has been shown to facilitate removal of

S. epidermidis biofilm (Brindle *et al.*, 2011). Thus, hydrogen bonding may contribute to matrix integrity.

One can also imagine a new class of drugs that inhibit the biosynthesis, export, or anchoring of EPS important for biofilm cohesion. These drugs, which are mostly hypothetical, would likely not be bactericidal. One potential example of such a strategy is a cocktail of chemistries reported to suppress exopolysaccharide synthesis in the cariogenic organism *Streptococcus mutans* (Falsetta *et al.*, 2012).

A fourth way to target the biofilm matrix is through the regulatory mechanisms that control elaboration of EPS. The leading example of this possibility involves interfering with a regulatory mechanism now understood to be common in bacteria centered on the secondary messenger molecule cyclic di-GMP (Hengge, 2009; Römling *et al.*, 2013). In overly simplistic yet consistent terms, high levels of cyclic di-GMP induce synthesis of EPS constituents and promote biofilm formation, whereas low levels of cyclic di-GMP lead to breakdown of the matrix and stimulation of motility and cell release. Chemical inhibitors of cyclic di-GMP metabolism might someday be useful in forcing the transition toward dissolution of matrix polymers and upregulation of motility (Sambanthamoorthy *et al.*, 2012). Other chemical signals tied in with cyclic di-GMP, such as nitric oxide, might also induce this switch (Barraud *et al.*, 2009; Li *et al.*, 2013).

One caution with these approaches is that they must be performed in such a way so as to avoid dissemination of large numbers of planktonic microorganisms. Bacteria detached from a catheter would probably need to be deliberately withdrawn from the device and captured rather than allowed to escape into the bloodstream, for example. It may be necessary to simultaneously treat with antibiotics to control dispersed cells. It is also possible that in some instances, the immune defenses may be adequate to neutralize the released microorganisms on their own.

Conclusion

It should not be surprising that existing antimicrobials fail to remove or weaken biofilms: these agents have been discovered and developed based on their ability to kill germs without consideration or measurement of biofilm removal. Some biocides and antiseptics, such as glutaraldehyde or chlorhexidine, may even cross-link biofilm and make it less prone to removal (Simões *et al.*, 2005; Brindle *et al.*, 2011). Progress in developing new antibiofilm therapies will follow when the biofilm is better understood as a mechanical structure and measurement of biofilm material properties and biofilm removal becomes more routine (Jones *et al.*, 2011; Lieleg *et al.*, 2011; Böl *et al.*, 2013). Mathematical and computer modeling of biofilm fluid–structure interactions also has an important role to play (Duddu *et al.*, 2009; Taherzadeh *et al.*, 2010; Vo *et al.*, 2010; Lindley *et al.*, 2012).

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