Review

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The *in vivo* biofilm

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Bacteria can grow and proliferate either as single, independent cells or organized in aggregates commonly referred to as biofilms. When bacteria succeed in forming a biofilm within the human host, the infection often becomes very resistant to treatment and can develop into a chronic state. Biofilms have been studied for decades using various *in vitro* models, but it remains debatable whether such *in vitro* biofilms actually resemble *in vivo* biofilms in chronic infections. *In vivo* biofilms share several structural characteristics that differ from most *in vitro* biofilms. Additionally, the *in vivo* experimental time span and presence of host defenses differ from chronic infections and the chemical microenvironment of both *in vivo* and *in vitro* biofilms is seldom taken into account. In this review, we discuss why the current *in vitro* models of biofilms might be limited for describing infectious biofilms, and we suggest new strategies for improving this discrepancy.

Growing awareness of biofilms

Amongst microbiologists, the period between 1880 and up until the end of the last century is popularly referred to as 'the pure culture period' [1]. Bacteria were largely viewed as independent, free floating, or swimming organisms, which are now referred to as the planktonic phenotype. Most work on characterizing the physiology of bacteria including their molecular biology as well as the development of antibiotics has been based on planktonic bacteria grown in liquid media. Such ventures have functioned well in the development of drugs against acute infections that are often dominated by planktonic bacteria. Most acute infections can now be cured with antibiotics within days if the right treatment is initiated appropriately [2]. However, a new challenge is posed by another category of bacterial infections, which are much harder to successfully treat with available antimicrobials. Microscopic investigations of numerous chronic infections have revealed that the infecting bacteria are physically aggregated in clusters and surrounded by a polymer matrix, a phenomenon referred to as the biofilm phenotype [3]. Interestingly, it appears from the scientific literature that scientists have observed the biofilm phenotype of bacteria for several hundred years. The term biofilm, which refers to bacterial aggregation, was introduced in 1981 [4], but in environmental microbiology, bacterial aggregation has been observed and regarded important for community function for a much longer time [5]. The classic example is the observation of aggregated bacteria in the 'scurf of the teeth' by Anthony van Leeuwenhoek (published in 1684), which refers to the plaque development later described by many dentists. In fact, dentists were the first to acknowledge the problems of aggregating bacteria in relation to the development of caries and other dental infections including periodontitis. The famous microbiologist Louis Pasteur (1822–1895) also observed and sketched bacterial aggregates causing wine to become acetic [6], which ultimately led to his discovery of pasteurization.

In medicine, the first real observations linking the etiology of a persistent (chronic) infection to the aggregation of bacteria were reported in the 1970s in the lungs of patients suffering from cystic fibrosis (CF). Aggregated bacteria were observed in sputum of CF patients chronically infected with mucoid strains of *Pseudomonas aeruginosa* [7,8]. It has now been recognized that biofilms readily colonize foreign objects inserted into the human body, as well as in many body compartments, when the host defense is compromised, for example, in CF (compromised mucociliary activity) and chronic wounds (reduced vascularity). The number of infections and conditions, where biofilms are known to be involved is growing each year, and the definition of the term 'biofilm' is constantly being refined to accommodate new knowledge. For example, it has been found that pathogenic biofilm aggregates can form without attachment to a surface and that the biofilm matrix does not necessarily need to be produced by the bacteria of the biofilm itself (for reviews, see [9–13]). The major hallmarks of *in vivo* biofilms are thus aggregated bacteria, which tolerate the host defense and high concentrations of antimicrobial agents even over longer times (Figure 1).

When it comes to treatment strategies both for dental plaque and aggregated bacteria in wine and beer, the

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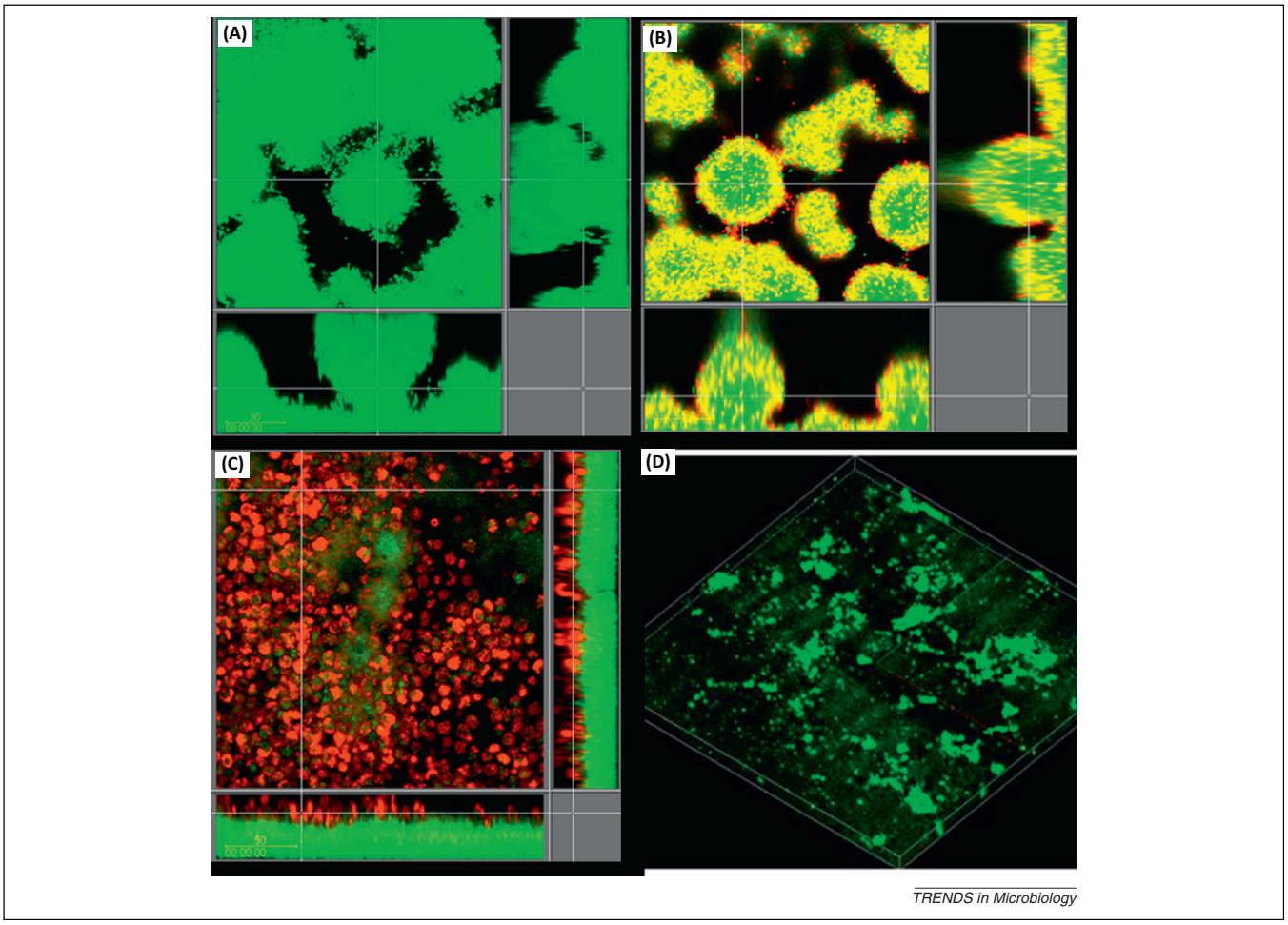

Figure 1. *Pseudomonas aeruginosa* biofilms. (A–C) *In vitro* flow cell biofilms of *P. aeruginosa* visualized by confocal laser scanning microscopy. **(A)** Wild type *P. aeruginosa* biofilm untreated and **(B)** wild type *P. aeruginosa* biofilm treated with antibiotics (tobramycin, 100 μg/ml for 24 h). Notice that the majority of the bacteria survive the antibiotic treatment. Green bacteria are alive and red/yellow are dead. **(C)** A persisting *P. aeruginosa* biofilm (green) is protected against the activity of surrounding freshly isolated live human polymorphonuclear leucocytes (red). Reproduced, with permission, from [83]. **(D)** *P. aeruginosa* biofilm microcolonies on a silicone implant 3 days post-infection in a mouse model. Reproduced, with permission, from [62].

solutions are simple and efficient. Dentists advise frequent mechanical removal of the plaque by tooth brushing and Pasteur simply devised heating of the specimen. However, in most human biofilm infections it is more difficult to devise an efficient eradication therapy. Antibiotics are less effective [14], and biofilm infections on foreign bodies such as artificial joints or osteosynthetic material often require surgical removal or replacement of the foreign material for complete eradication as physical or chemical disinfection methods are rarely available outside dental medicine [15,16]. To efficiently treat and prevent biofilm infections, we thus need to understand how bacterial biofilms are established in the human body. This review summarizes how bacterial biofilms develop and form *in vitro* as studied in simple model systems. By contrast, little is known about biofilms in chronic infections and much more research on *in vivo* biofilms is needed. Such research will also help the design of new and optimized *in vitro* models to better fit *in vivo* observations. In the following sections, we discuss central aspects of biofilm research and how our current knowledge is shaped and limited by available model systems. We largely focus on biofilm studies of the opportunistic pathogen *P. aeruginosa*; however, most if not all bacteria are capable of forming biofilms and many other pathogens are studied *in vitro* using similar approaches.

The classical view of biofilms based on *in vitro* observations

Even though the first publications of aggregating bacteria were *in vivo* observations, our later knowledge of the developmental processes of medical biofilms is largely derived from surface growth *in vitro* systems [17–20] such as simple flow cells [21]. The most studied bacterium in this context is the opportunistic pathogen *P. aeruginosa*, a Gram-negative rod-shaped bacterium, which is found in soil, water, and most man-made environments throughout the world and forms biofilms on almost any wetted surface. It grows both in oxic and hypoxic environments and can use oxygen or nitrate as an electron acceptor for its heterotrophic respiration.

In vitro biofilms consist of bacteria in microcolonies encapsulated by exopolymeric substances (EPS) excreted by the bacteria themselves. A large part of the biofilm is made up of water channels, which are thought to operate as a distribution system of nutrients and O_2 [22,23]. Despite this porous architecture, steep gradients of O_2 and other

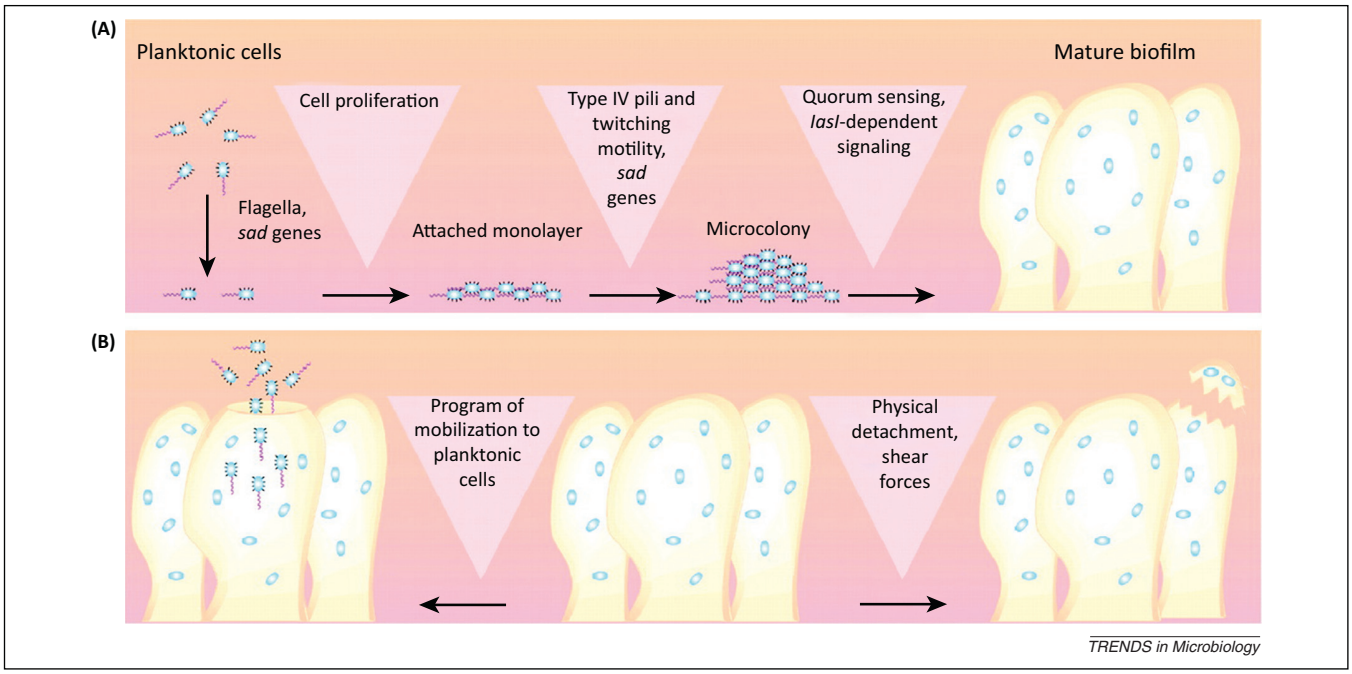

Figure 2. Formation of *in vitro* surface-attached *Pseudomonas aeruginosa* biofilm. Initial reversible attachment is followed by irreversibly attached bacteria forming the base of microcolonies. Mature, mushroom structures then develop and this is coordinated by bacterial signaling. Finally, mature biofilms can disperse either by physical forces or by a specific program to mobilize planktonic cells. Adapted, with permission, from [26].

substrates and nutrients are present in the biofilm matrix towards the deeper parts of the biofilm driving pronounced microenvironmental and physiological heterogeneity [24,25].

The development of *in vitro*, surface-attached biofilms can be divided into three different stages: (i) attachment; (ii) maturation; and (iii) dispersion [17,19,26] (Figure 2).

The *in vitro* biofilm is initiated by reversible binding of bacteria that prime the surface for subsequent irreversible attachment of bacteria to a surface. The surface is initially covered with bacteria capable of twitching motility, which enables *P. aeruginosa* and many other bacteria to move over moist surfaces [27]. The migration of the bacteria and subsequent biofilm formation is almost unlimited as long as nutrients are available. From these irreversibly attached bacteria, bacterial microcolonies emerge by clonal growth [18]. An interesting feature often seen with *in vitro* biofilms is a characteristic network of mushroom structures and open voids that develop over time (Figure 1). The mushroom-shaped structures are composed of a stalk and a cap. The stalk is formed by clonal growth, whereas motile bacteria climbing the stalks by help of type IV pili form the caps of the mushrooms [17]. Biofilm researchers have put great emphasis on understanding the development of these biofilm structures. Klausen *et al.* [18] showed that the formation of the structure is influenced by nutrient availability and other environmental conditions.

Apparently, motility is a key factor in the 3D microcolony formation of *P. aeruginosa* biofilms *in vitro* but such structure formation is also highly dependent on the carbon source [18].

Many recent reviews propose that biofilm formation, from planktonic cells to the sessile growth, is a complex and highly regulated process [28], possibly dependent on the expression of a specific genetically encoded biofilm program [28,29]. However, the existence of such a specific genetically encoded switch in growth mode is still debated. Close examination of microscopic biofilm images from published *in vitro* studies indicates a high variability in successional biofilm growth patterns, such as timing of mushroom development and surface coverage, which can also be explained by temporal events reflecting adaptation to nutritional and environmental conditions [30–32]. A lack of a biofilm-specific program and the presence of a number of adaptations in *P. aeruginosa* was supported by a recent analysis of transcriptomic data [24]. This study indicated that *P. aeruginosa* biofilms become iron and O_2 limited, whereby the bacteria expressing type IV pili-related genes exhibited a growth pattern characteristic of stationary phase growth. Thus, biofilm formation and the successive growth and development patterns seem to depend on default aggregation of bacteria with an increasingly slow growth rate and subsequent adaptation to a microenvironment characterized by steep chemical gradients and mass transfer limitations for O_2, nutrients, and substrates. Unfortunately, how these aspects of the microenvironment affect biofilm development are rarely considered or measured in most *in vitro* studies, although new imaging techniques are available to facilitate combined microscopic imaging of the structural and chemical landscape of biofilms [33,34].

The last stage of the *in vitro* biofilm developmental cycle is believed to be dispersal of cells or small aggregates [26,35,36]. The mechanisms of dispersal are not fully understood, but the dispersion of single planktonic cells is thought to rely on a genetically programmed process or on enzymes such as dispersin B [37], whereas cell clusters can also be removed by hydrodynamic shear forces [26] and/or by prophage-mediated cell death [38,39].

For patients with chronic infections, such dispersal can have severe implications, as it would allow biofilm bacteria to spread throughout the infected organ or to colonize other parts of the human body. Dispersal of biofilms during chronic infection can cause an acute bloodstream infection, as sometimes seen for patients with colonized catheters and implants [14], or suffering from other biofilm infections such as endocarditis [26,40–42]. Remarkably, in CF patients, only *Burkholderia* species are able to cause systemic infections [43].

Importance of using the right *in vitro* model

In addition to the flow cell system [21], other *in vitro* model systems have been developed for both high throughput screening and in-depth investigations. For high throughput screening, the most common methods are the static microtiter plate assay [44] and the Calgary Biofilm Device [45] with 96 (or more) pegs that fit into microtiter plates. These assays are used to test for biofilm growth, by staining the biomass with crystal violet or stains to distinguish between live and dead cells (LIVE/DEAD *Bac*Light Bacterial Viability Kit, Invitrogen). More comprehensive investigations of biofilm growth rely on continuous flow cell systems [21,46], bacterial colonies on agar plates [47], drip flow reactors [48], or rotating disk reactors [49]. For all these methods, it is important to realize which parameters are tested (e.g., dispersal, direct killing, anti-virulence, etc.). It can thus be difficult to distinguish between different modes of action and to tell whether an observed antibiofilm effect is due to dispersal, growth inhibition, or killing. In flow cell systems, the structural dynamics of biofilms can be directly visualized by microscopy but the throughput is low and most are limited by the prerequisite of a surface, although non-surface dependent methods have been developed [50,51]. The extensive use of abiotic surface-dependent biofilm assays may bias our current view and might lead to false negative results of biofilm formation because non-surface attached aggregates are lost.

In general, *in vitro* biofilm models range from approximately 1 cm^2 in the static microtiter plate assay to 10 cm^2 in the flow cells of bacterial biofilms attached to a stable surface, where biofilms can be up to 300 μm thick. In all cases, the upper biofilm layer is either exposed to atmospheric air or a continuous supply of liquid media – a situation that differs significantly from conditions in the host. Although all of the above *in vitro* systems can be reproducibly used for testing the effects of simple parameters on biofilm structure and growth, they fail to mimic the complexity of the host environment, as discussed below.

Animal models of *in vivo* biofilm infections

Acute bacterial infections typically involve planktonic bacteria and are generally treatable with antibiotics, although successful treatment depends on accurate and fast diagnosis. However, in cases where the bacteria succeed in forming a biofilm within the human host, the infection often turns incurable and will develop into a chronic state [52–55]. Biofilms have been found in most chronic infections and, as mentioned earlier, both *in vitro* and *in vivo* models of many infections have been developed. However, for improved understanding of infectious biofilms it is important that these models resemble the *in vivo* conditions in the body.

Many chronic diseases have their own animal models, such as CF [56–58], otitis media [59], chronic wounds [60], and implant-related infections [61,62]; such *in vivo* biofilm models are thoroughly reviewed elsewhere [63]. One drawback of animal models is that even though they reflect the ongoing battle between bacterial pathogens and host immune response, the long-term inflammatory response and substantial antibiotic treatment is difficult to mimic. In the CF lung, this interplay can last up to 30 years and is known to result in both phenotypic and genotypic variants of the infecting bacteria [64].

In an attempt to mimic persistent infections, the majority of animal models for chronic *P. aeruginosa* lung infection use embedded bacteria (in either seaweed-derived agar or agarose or native alginate harvested from the bacteria). However, even these models have a limited lifetime of 1–3 weeks [56]. In other models, this problem is overcome by repeated exposure of bacteria, but the mice either die or become resistant to this procedure [56,57,65,66].

Experimental animal models investigating the interplay of foreign bodies and bacteria have been used for a long time. Biofilm models using pre-colonized implants are generally preferred, simply because these models have the advantage of controlling the inoculum on the implant prior to insertion. A model using pre-colonized silastic subdermal implants in rabbits was used, for example, to investigate the evolution and organization of a *P. aeruginosa* biofilm [67]. Another model in mice was used for investigation of *Staphylococcus aureus* biofilms in implant-associated osteomyelitis [68]. A third type of foreign body infection model was established as a treatment model [62] using pre-colonized flat silicone implants inserted in the peritoneal cavity of the mouse.

To monitor the interplay between immune cells and the biofilm, the silicone implant model was further developed to use pre-colonized silicone tubes [61]. In such tubes, the biofilm is exposed to immediate contact with immune cells but protected from the encapsulation of secreted (peritoneal) fluids, pus, proteins, etc., which would otherwise block and interfere with direct microscopy. This makes it possible to obtain a specimen containing the interaction between inflammatory cells and biofilm. Such devices provide new opportunities to investigate the development of the inflammatory and continuously host response against biofilms and the interaction *in vivo* over longer times [61]; such studies are not achievable *in vitro*.

Chronic biofilm infections: the *in vivo* biofilm

Two rarely discussed properties of *in vivo* biofilms are their actual amount and size. When observing biofilms *in vivo*, we have noticed two striking differences for *in vitro* biofilms: (i) we have never observed the classical mushroom biofilm structure often seen *in vitro*; (ii) *in vivo* biofilms generally appear significantly smaller in spatial extension (aggregate diameter) than *in vitro* biofilms. We therefore questioned whether the size of *in vivo* biofilms in chronic

Table 1. Biofilm size in chronic infections in otherwise sterile parts of the human body[a]

Biofilm demonstrated in	Visualization method	Approximate diameter size	Refs
Lung infections (CF)	Light microscopy	~4–8 μm	[7]
	Light microscopy	~5–100 μm	[98]
	FISH[b]	~5–100 μm	[54]
	FISH	~5–50 μm	[99]
Chronic wounds	FISH	~5–200 μm	[52]
	FISH	~5–100 μm	[78]
	Light and electron microscopy	~35–55 μm	[100]
Soft tissue fillers	FISH	~5–25 μm	[53]
Otitis media	FISH	~15–25 μm	[101]
	FISH	~10–80 μm	[102]
	FISH	~4–40 μm	[55]
Implant associated	Electron microscopy	~500 μm	[103]
	FISH	~50 μm	[104]
	Electron microscopy	~5–15 μm	[105]
	FISH	~5–30 μm	[106]
Catheter and shunt associated	Electron microscopy	~5–1000 μm	[107]
	Electron microscopy	~20–500 μm	[108]
	Fluorescence microscopy	~20–1200 μm	[109]
	FISH and electron microscopy	>1000 μm	[110]
Chronic osteomyelitis	Electron microscopy	~25 μm	[111]
	Electron microscopy	~25 μm	[112]
	Light and electron microscopy	~5–50 μm	[113]
Chronic rhinosinusitis	Electron microscopy	~5–30 μm	[114]
	Fluorescence microscopy	~5–20 μm	[115]
Contact lenses	Electron microscopy	~50–100 μm	[116]

[a]The biofilm aggregate size was estimated by measuring the longest diameter or length directly on the micrograph images in the source articles.

[b]FISH, fluorescence *in situ* hybridization.

infections is random and if the current *in vitro* models contain biofilm aggregates with similar features? To address these questions, we measured the cell aggregate or patch diameter size of biofilms as shown on micrographs in publications describing chronic biofilm infections (Table 1); most published micrographs are 2D representations and we measured the longest axis of each microcolony of aggregated bacteria (Figure 3).

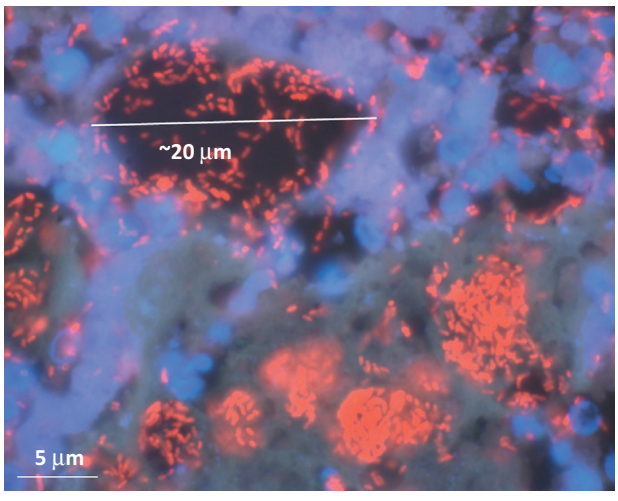

Figure 3. Biofilm aggregates of *Pseudomonas aeruginosa* in a chronic infected cystic fibrosis (CF) lung. Using a specific *P. aeruginosa* PNA fluorescence *in situ* hybridization (FISH) probe, the bacteria are visualized in red, whereas the inflammatory cells surrounding the biofilm patches are counterstained with DAPI (blue). Reproduced, with permission, from [54]. The white line depicts how the biofilm/microcolony size was measured in the articles in Table 1. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; PNA, peptide nucleic acid.

The observed *in vivo* biofilm patches were all similar in sizes between the different types of chronic infections (Table 1) and mostly ranged from ~5 to 200 μm in diameter; the median for the smallest and largest biofilm diameters were 5 and 50 μm, respectively. However, catheter- and shunt-associated infections reached larger biofilm patch sizes up to 1200 μm, possibly due to the presence of a large solid abiotic surface easily accessible to bacterial colonization. Even with the somewhat larger catheter and shunt-associated biofilms, *in vivo* biofilms are generally much smaller than the *in vitro* model systems, which can produce several cm^2 of up to several hundreds of micrometer-thick bacterial biofilms attached to uniform abiotic surfaces. By contrast, the size of the biofilm patches observed in experimental animal models is very similar to the estimated size for human infections [62]. Thus, many small biofilm patches can be present in an infection but they are segregated by host material indicating that size is an important parameter.

Apparently, biofilm aggregates in chronic infections have an optimal size spectrum or at least an upper size limit of approximately 200 μm. What determines this size limitation of *in vivo* biofilms remains to be studied in detail, but we speculate that both substrate and nutrient availability as well as the host immune response and antibiotic treatment play a key role for such apparent limitation of the growth of individual biofilm patches in chronic infections.

The consequences of the inflammatory response to biofilms

An intimate interaction between the immune system and biofilm aggregates has been demonstrated *in vitro*. The host responses include activation of both the adaptive [69]

as well as the innate [70–74] immune systems, and bacteria seem to respond to the presence of immune cells [74,75]. Such *in vitro* experiments have provided some insights into the responses raised in *in vivo* biofilm infections, but to fully understand the immune response to biofilm infections we argue that *in vivo* models or host material must be used.

Persistent biofilm-forming *P. aeruginosa* in the airways of CF patients are thus believed to induce an ongoing and self-reinforcing coactivation of both the innate and the adaptive immune response leading to persistent inflammation during chronic *P. aeruginosa* lung infection [13]. It is this prolonged inflammatory response, dominated by polymorphonuclear neutrophils (PMNs) and not the bacteria *per se*, which causes the tissue damage, necrosis of the lung tissue, and eventual death of the patient [76,77]. Similar biofilm-induced activation of the immune response causing continued inflammation is probably involved in other biofilm infections, such as in chronic wounds [52,78,79], but such activation remains to be demonstrated and studied in detail.

According to *in vitro* biofilm experiments, the outcome of the engagement between the immune system and biofilm, including *P. aeruginosa*, *S. aureus*, and *Streptococcus epidermidis*, depends on which components of the immune system are involved [72,80–82] and the virulence of the biofilm [58,71,72,83,84]. These *in vitro* observations comply, for example, with the higher susceptibility of the *P. aeruginosa* biofilm seen in lungs of mice with a fast endobronchial accumulation of PMNs as compared to mice with a slow endobronchial accumulation of PMNs [81]. Further confirmation of the validity of observations obtained from *in vitro* studies involving host response components and biofilm is provided by the higher tolerance of infectious *P. aeruginosa* biofilms in mice when *P. aeruginosa* is able to produce rhamnolipid that lyses PMNs as seen *in vitro* [61,85]. *In vitro* studies of the interaction of biofilm with host response components has also been useful to demonstrate that mature *in vitro Candida albicans* biofilms are able to evade PMNs by engaging beta-glucans as decoys to prevent activation of PMNs [86]; a mechanism that may explain the observed long-term coexistence of *in vivo* mucosal *C. albicans* biofilms and accumulated PMNs without intensive annihilation of the PMNs [87]. Likewise, biofilm formation by *S. epidermidis* leads to *in vitro* evasion of phagocytosis mediated by complement opsonization, which may aid to colonization of the human nasopharyngeal tract [88]. Also, the ability of *Mycobacterium abscessus* to exploit added PMNs for enhanced *in vitro* biofilm formation adds to the understanding of how *M. abscessus* exists in biofilms surrounded by PMNs during chronic infection *in vivo* [89].

Persistence in chronic infections

Although *in vitro* biofilm studies have provided valuable explanations for early events in biofilm infections, the mechanisms enabling the observed persistence of biofilms in chronic infections have not yet been addressed successfully with conventional *in vitro* systems. Such mechanisms may encompass both phenotypic and genotypic adaptations enabling pathogenic bacteria in the biofilm to coexist with the host response for a prolonged time as seen in CF lungs [54] and chronic wounds [52].

The *in vivo* challenges of bacteria in biofilms associated with chronic infections include long-term exposure to ongoing host response attacks including intense O_2 depletion by PMNs [90] and lactate accumulation [91] for the formation of reactive oxygen species (ROS). Bacteria must not only combat or resist the bactericidal activities of the immune response, they also need to adapt their metabolism to microenvironmental changes resulting from the activity of the host and the nutritional consequences of aggregate formation as discussed above. These challenges could affect the growth of the microorganisms and restrict the size of the *in vivo* biofilm during infection.

Concluding remarks

In vitro model systems have revealed important insights into biofilm physiology, antibiotic tolerance, and many other aspects of surface-associated bacterial growth. Most current hypotheses and mechanisms of biofilm formation and dispersal, as well as tolerance to antibiotics and phagocytic predators, have originated or have heavily relied on observations of defined *in vitro* biofilms in flow cells. Yet, it is clearly difficult to extrapolate from such defined settings to the more complex and still largely uncharted host microenvironment experienced by pathogenic bacteria in chronic biofilm infections. Thus, it is important to know the limitation of the current *in vitro* systems and to ask the right questions when correlating *in vitro* observations to *in vivo* biofilms. An important link between *in vitro* and infectious biofilms may be established based on compliance between observations from *in vitro* biofilm and from *in vivo* observations in representative animal models, where more variables can be controlled, while maintaining a dynamic interaction with the host.

In vivo studies have shown that biofilm aggregates are often numerous and small, and small size does apparently not matter when it comes to persistence and induced continuous inflammation. However, there are important differences between *in vitro* biofilm structures and *in vivo* biofilms associated with chronic infections in humans and animal models. In animal models, we mostly find undifferentiated small biofilm aggregates reminiscent of, for example, small aggregates in aquatic environments [92]. One exception is the animal model using hollow tubes where much larger biofilm accumulation can be observed [61], possibly due to abscess formation creating a barrier that detains the innate host defense to these biofilms. Similar mechanisms seem relevant for the larger biofilm accumulation observed in catheter- and shunt-related infections.

The most obvious reason for the differences between *in vitro* biofilms and biofilms associated with chronic infections is the total lack of defense mechanisms in *in vitro* biofilm systems. In most biofilm habitats (both environmental and medical), grazers, phagocytes, and even bacteriophages may be present, along with the presence of pus and other excreted fluids and polymers. Although these dynamic factors result in a patchy occurrence of *in vivo* biofilms, these are generally unwanted and avoided in *in vitro* model systems where they are difficult to control or reproduce.

Another reason for the differences in biofilm structure found *in vitro* and *in vivo* could be due to differences in the

microenvironment, that is, different levels of substrate and mass transfer limitation imposed by the biofilm structure and the conditions in the host versus in the flow cell. Unfortunately, we know very little about the chemical landscape and microenvironments inhabited by biofilms in chronic infections but the presence of oxygen-depleted zones *in vivo* has been demonstrated in a few cases [93]. Similarly, and maybe somewhat surprisingly, the actual chemical microenvironment during *in vitro* biofilm experiments in flow cells remains largely unstudied, although a close connection between bacterial cell cluster size and the O_2 microenvironment in such flow cell biofilms has been demonstrated in a few cases [25,34].

Although bacterial infection and biofilm formation may initiate the patient's symptoms, there are strong indications that the immune system may actually be the strongest contributor to disease and pathology in chronic infections [94]. We know very little about how the host immune system possibly restricts biofilm aggregate size and even how biofilms initially form during the development of chronic infections. It has, however, been shown that early IgG response and *P. aeruginosa* mutations, which lead to hyper production of alginate (mucoid phenotype) are both necessary and indicative for development of chronic lung infection in CF patients [95,96]. We need to (i) further improve *in vitro* models to alleviate their current limitations in mimicking observed *in vivo* conditions, (ii) improve our insight to the microenvironmental conditions associated with chronic infections, and (iii) improve experimental means of mimicking *in vivo* conditions in optimized *in vitro* models. To accomplish such optimization, new imaging techniques [33] and development of more advanced flow cells taking advantage of microfluidics [97] seem very promising. Another strong technological advancement encompasses the full implementation of modern genomics approaches in the study of chronic biofilm infections. This will allow insights to *in vivo* transcriptional activities as well as to adaptive responses of pathogenic bacteria enabling their colonization and long-term persistence in the ecological micro niches associated with chronic infections. In conclusion, today's *in vitro* biofilm models do not mimic biofilms in chronic infections. *In vivo* biofilms are smaller in physical dimensions, they lack mushroom-like structures, are embedded in host material, and are continuously exposed to host defense reactions. Especially, the effects of host immunity over weeks, months, or years has to be considered in future experimental approaches, and this requires the development of much more complex models of chronic infection than flow cell biofilms *in vitro*.

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