Chapter 7

General overview and perspectives

Antimicrobial penetration in infectious biofilms: from qualitative microscopic imaging to quantitative, structural analysis of biofilms

This chapter represents the contribution of Brandon Peterson to a review paper, jointly prepared with He Yan, and to be combined with a similar chapter with different emphasis to be included in his PhD thesis to form a comprehensive manuscript for later submission.

ABSTRACT

It is estimated that 60% of bacterial infections are due to biofilms. Biofilms are recalcitrant to antimicrobials as a result of phenotypic changes of the inhabiting bacteria, biofilm structure and composition. Qualitative imaging of biofilms date back to the 17th century, when Antonie van Leeuwenhoek reported on the poor penetration of vinegar into oral biofilm. Unfortunately, the general understanding of the limited penetration of antimicrobials into biofilms is still based on qualitative imaging. In this review, we summarize progress made in the qualitative understanding of antimicrobial penetration through advances in microscopic techniques and our recently discovered quantitative relations between visco-elastic properties of biofilms and antimicrobial penetration. Therewith the role of visco-elasticity of biofilms extends beyond its protection offered against mechanical removal forces. The dual role of biofilm visco-elasticity in the protection of biofilm against mechanical and chemical attacks revealed in this review warrants identification of visco-elasticity as a biofilm virulence factor. Pathways are discussed to reduce the virulence of bacterial biofilms by intervening with its visco-elastic properties.

It is currently estimated that over 60% of all human infections treated by physicians are due to biofilms (21), examples being oral biofilms ("dental plaque") and biofilms involved in a variety of conditions like osteomyelitis, chronic otitis media, the diabetic foot or chronic bacterial prostatitis. In their biofilm mode of growth, bacteria adhering on a surface, embed themselves in a matrix of extracellular polymeric substances (EPS) that not only offers physical protection against antimicrobial penetration, but also yields bacterial properties that can be different from the ones of their planktonic or free-swimming counterparts (20). Biofilm organisms can become inherently resistant to antimicrobials through mutation, formation of antibiotic degrading enzymes, endogenous oxidative stress, phenotypic changes or low metabolic activities (41). The recalcitrance of bacteria in biofilms to antimicrobial treatments therewith can become thousands of times stronger than of planktonic bacteria. (3,31,41). Unfortunately, the general understanding of the limited antimicrobial penetration in biofilms is still based on qualitative imaging of biofilm structure and subsequent mathematical modeling (5,15).

Qualitative imaging of biofilms and the realization that biofilms are little penetrable by antimicrobials date back to the 17th century, when Antonie van Leeuwenhoek commented in a report to the Royal Society of London "that the vinegar with which I washt my Teeth, kill'd only those Animals which were on the outside of the scurf, but did not pass thro the whole substance of it". Despite this early identification of the biofilm mode of growth by Van Leeuwenhoek, technological advances enabling scientists to study biofilm structure and composition have only been around for a few decades (14), although mostly still based on qualitative microscopic imaging (43).

In this review, we first pay attention to the general mechanisms through which bacteria in a biofilm mode of growth may resist the action of antimicrobials and secondly focus on the difficulties encountered by antimicrobials in penetrating infectious biofilms. We summarize progress made since Van Leeuwenhoek in the qualitative understanding of the penetration of antimicrobials into biofilms through advances in microscopic techniques, the most common being confocal laser scanning microscopy (CLSM). It is advocated that through a relation between structure and composition with visco-elastic relaxation of a biofilm under external stress, macroscopic physical properties of a biofilm can be derived that facilitate explanation of antimicrobial penetration into a biofilm on a quantitative basis. Therewith the role of visco-elasticity of biofilms extends beyond its protection offered against mechanical removal forces, which warrants identification of visco-elasticity as a biofilm virulence factor. Finally, this review identifies new pathways to reduce the virulence of bacterial biofilms by intervening with its visco-elastic properties.

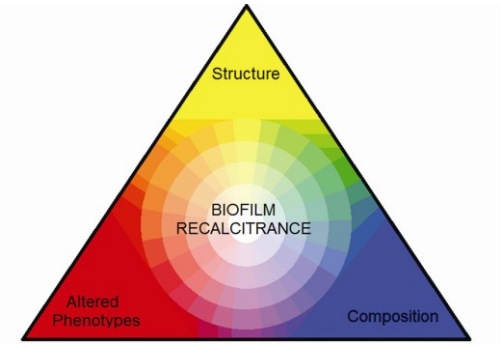

Figure 1. Key-properties of biofilms that govern biofilm recalcitrance toward antimicrobials.

Mechanisms of biofilm recalcitrance toward antimicrobials. The biofilm mode of growth protects individual bacteria from a variety of environmental challenges, including bacteriophages, chemically diverse biocides, host immune responses and antimicrobials (78). In Figure 1 we identify three key-properties of biofilms that govern the mechanisms through which biofilms become recalcitrant to antimicrobials (3,18,38,68,72). Biofilm structure determines many transport processes within a biofilm. Penetration of antimicrobials and nutrients into a biofilm depend on the degree of channelization of the biofilm and the presence of a suitable medium for molecular transport through the biofilm. Usually transport of antimicrobials and nutrients is limited, based on whether the composition of both the EPS matrix and the bacterial cell surfaces adsorb these compounds on their way in or not. Importantly, biofilm structure is dynamic, adapting in both space and time to its environmental conditions (17), amongst which pH, temperature, fluid shear, nutrient availability and host defenses. As a result of nutrient deprivation, bacterial phenotypes in a biofilm can be altered, leading to the formation persister cells (40,52), that can remain dormant without causing disease for prolonged periods of time. Moreover, most antimicrobials target marcromolecule synthesis inside bacteria (15) during active metabolism, thus the presence of a slow metabolism contributes to antimicrobial recalcitrance of biofilms in general, and persister cells can tolerate higher concentrations of antimicrobials than nearby recalcitrant biofilm organisms (33,66). Taken together, whereas planktonic organisms have ample access to nutrients and by the same token, are highly susceptible to antimicrobials, structural and compositional features of a biofilm form the major impediments for

nutrient deprivation, the development of altered phenotypes and antimicrobial recalcitrance.

Qualitative understanding of the recalcitrance of biofilms toward antimicrobial penetration using microscopic techniques. Over the past decades, microscopic techniques have greatly aided to yield a general description of biofilm structure and to a limited degree its microbiological and biochemical composition. Whereas early electron microscopic techniques enabled imaging of organisms in a biofilm, high vacuum conditions have long obscured the role of the EPS matrix (see Table 1), that appeared as a dark rim of condensed matter on the bacterial cell surfaces in a biofilm (69). Scanning electron microscopy (SEM) can provide high resolution of the bacterial cell surface, visualizing membrane damage from antimicrobials. However, internal cellular integrity remains elusive using SEM (49). Transmission electron microscopy (TEM), on the other hand, can observe interior bacterial structures and internal damage caused from antimicrobials (81). Still, these electron microscopy techniques corrupt the natural state of biofilms. Through the years, many microscopic techniques have been developed, amongst which the environmental SEM, that enable to study biofilms in their native hydrated state (7).

CLSM has become the most employed technique to study biofilms as it offers many more possibilities than just simple imaging (see Table 1). Many key components of biofilm structure have been determined using CLSM in association with chemically binding fluorochromes, including 3D architecture (59,62,74), mushroom-shaped colonies (19,75), stratification (36), establishment of water

Table 1. Microscopic imaging techniques used to study biofilm structure, together with their advantage and disadvantages and the contributions these techniques have forwarded with respect to our understanding of the limited penetration of antimicrobials into biofilms.

Microscopic Technique	Advantages (+) / Disadvantages (-)	References
Scanning Electron Microscopy (SEM)	+ High resolution - Operates under vacuum	(6,49,67),
Transmission Electron Microscopy (TEM)	+ High resolution + Intracellular features visible - Requires ultra-thin samples - Operates under vacuum	(58,67)
Confocal Laser Scanning Microscopy (CLSM)	+ Operates under hydrated conditions + FISH mode allows species identification + Fluorescence labeling allows visualization of specific molecules + Allows visualization of membrane damage after appropriate staining - Stain penetration is limited - Laser penetration is limited - Fluorescence quenching during operation	(1,12,36,64,70, 80)
Environmental SEM	+ Operates under hydrated conditions + No coating required - Limited working focal distance	(7,13)
Acoustic Microscopy	+ Operates under hydrated conditions + Non-invasive and non-destructive - Low resolution of surface structures - Provides only estimates for thickness	(24)
Focused Ion Beam Scanning Electron Microscopy (FIB-SEM)	+ High resolution + Due to milling of the surface, 3D reconstruction possible - Operates under vacuum	(81)
X-ray Scanning (Scattering) Microscopy	+ Operates under hydrated conditions + No staining - Natural contrast restricted	(37)
Optical Coherence Tomography (OCT)	+ Operates under hydrated conditions without sample preparation + Deep penetration in biofilms + Non-destructive - Limited resolution	(29,80)
Electron Microscope Tomography	+ High resolution - Destructive sample preparation	(48)

channels (17,68), presence of extracellular polymeric substances (EPS) (37,39,63), bacterial cell surface damage and viability (30) as well as biofilm failure under compression (11). The establishment of water channels, however, has never been directly established, but rather has been indirectly established by the absence of stain uptake by bacteria and matrix components. Chemically reactive lectins provide information about the molecular composition of sugars within a biofilm (1). Alternatively, fluorescent *in situ* hybridization (FISH) differentially stains different bacterial species providing information of the location of bacterial species within multispecies biofilms (82). A limiting factor in the use of CLSM is that the penetration ability of the fluorochromes is typically confined to 20 to 40 μm (77), and it is impossible to see much deeper within a biofilm structure. Moreover, the penetration capability of the laser itself limits the depth of view within a biofilm structure. Two-photon laser systems have helped to increase the penetration depth of the laser light, yet they still require the use of fluorochromes that may have harmful effects to the biofilms (42).

These drawbacks of CLSM have stimulated the use of other microscopic techniques that still allow to image biofilm structure, but that do not rely on staining (see Table 1). Optical coherence tomography (OCT) for instance, relies on the scattering of light rather than stain fluorescence and is used to accurately describe voids water channels. CLSM easily overestimates the presence of voids, because they also appear due to lack of penetration of the stains. OCT, not relying on stain penetration, has demonstrated that heterotrophic biofilms had a porosity under 70%, when 98% porosity was found using CLSM (80). Focused ion beam scanning electron

microscopy (FIB-SEM) uses ion-sputtering of biofilm mass with repetitive imaging in between to observe the organisms within biofilms at a high resolution (81), and it can clearly visualize cell wall damage inside a biofilm due to antimicrobial attack (see Figure 2). Meanwhile, X-ray scanning microscopy can obtain detail on smaller biofilm structures than CLSM (37), specifically the EPS matrix. The different energies of biological components are compared against standards and converted to absorbance to obtain images. Similarly, structural re-arrangements of extracellular polysaccharides were observed as a function of pH using small angle X-ray scattering (16), though unlike X-ray scanning microscopy, no converted image is possible. As a major advantage, X-ray scanning microscopy can be used to interpret chemical interactions between biofilm components and antimicrobials. A non-staining method capable of determining mass transfer rates is acoustic microscopy. The ultra-high frequency ultrasound waves (70 MHz) are able to penetrate deep into biofilms detailing surface interfaces and are sensitive enough to differentiate localized changes in composition (24).

In summary, advances in microscopic techniques have yielded undisputed structural information on voids and water channels in biofilms. Staining and wave-energy scattering have enabled the visualization of different chemical environments in a biofilm. Yet further advances from here on have been lacking. Structural features can be used in computer programs to yield an estimate of antimicrobial penetration, but lack reliable input parameters and do not include specific interactions between inhabiting bacteria, biofilm matrix composition and the antimicrobial. Moreover, the main difficulty with microscopic techniques is the highly limited field of view

in combination with observer bias toward nice images. This calls for an urgent need for more quantitative, observer-independent techniques to characterize biofilms in terms of structure and composition.

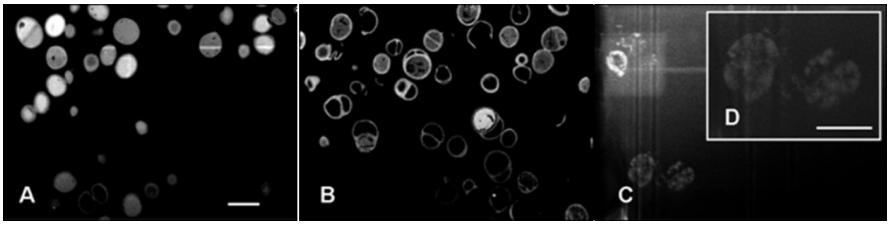

Figure 2. FIB-SEM cross section-images of OsO4-stained, FIB-sectioned *S. epidermidis* ATCC 35984 biofilms prior to and after exposure to quaternary-ammonium solutions, demonstrating the development of holes in the bacterial cell wall due to the interdigitization of the hydrophobic tail of the quaternary ammonium molecules.
A) control (exposure to TSB),
B) 1 x MBC of a quaternary-ammonium solution (Ethoquad C/25 (Cocoalkyl methyl (polyoxyethylene) ammonium chloride),
C) 3 x MBC,
D) Higher magnification of C.
Note that after exposure to 3 x MBC, the number of adhering staphylococci is rather low, but the few bacteria that could be imaged appear highly damaged. The scale bar denotes 1 µm.

Stress relaxation to assess biofilm structure and composition. Biofilms are visco-elastic materials comprised of an initial elastic response followed by viscous relaxation when subjected to external stress. Importantly, whereas microscopic techniques in general do not yield quantitative descriptions of structure and composition of a biofilm, visco-elasticity is determined by an interplay of structure and composition and can be quantitatively assessed. Measurement of the visco-elasticity of a material either involves measuring deformation under constant stress (“creep” (35)) or measuring the stress required to maintain a constant deformation (“stress relaxation” (11)), as

summarized in Figures 3a and b. Although creep and stress relaxation measurements are thought to be caused by different processes (26), they can be analyzed using similar mathematical models resulting in similar time constants of their response processes (53).

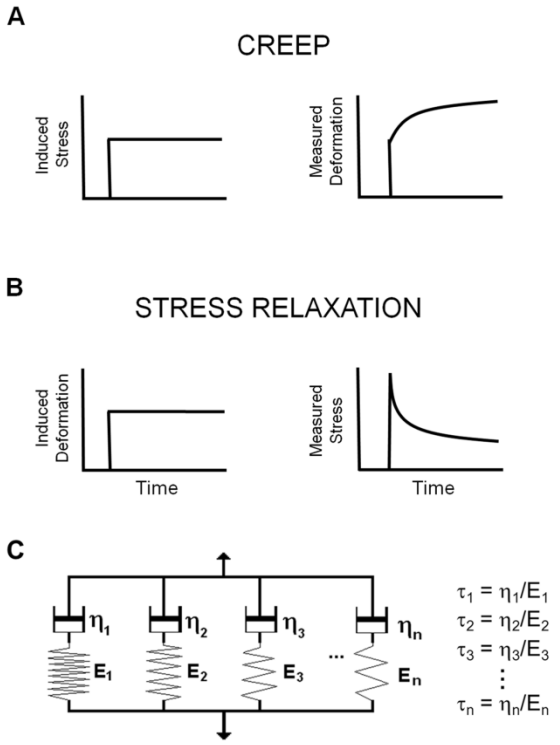

Figure 3. Visco-elastic measurements of "creep" and "stress relaxation".
A) During creep measurements a constant stress is induced while the deformation of the biofilm is recorded over time.
B) During stress relaxation measurements, a constant deformation is induced and the stress required to maintain that deformation is recorded over time.
C) The response to an induced deformation can be mathematically modeled using multiple Maxwell elements, each with their characteristic time constants representing the viscous part of the response ("the dash-pots", η_i) and an immediate elastic response component ("the springs", E_i). Characteristics time constants of each response process follow from τ_i as indicated in the graph.

Stress relaxation can, amongst other methods, be derived from uniaxial compression (34). In uniaxial compression, a plunger deforms the biofilm over a specified distance or strain and the stress relaxation is recorded as a function of time. Since stress relaxation results from multiple re-arrangement processes in a deformed biofilm, the mathematical description of stress relaxation possesses multiple terms. Generally, stress relaxation is described by a number of so-called Maxwell elements (see Fig. 3c) with each element containing a time constant, characteristic to the underlying time-dependent re-arrangement process and a spring constant, representing the immediate elastic response. Generally, three to four Maxwell elements are required to describe the stress relaxation of biofilms. Since bacteria in a biofilm constitute the heaviest masses, their re-arrangement upon an induced deformation will be slow, and the relative importance of the slow Maxwell element has been intuitively associated with bacterial re-arrangement in a biofilm. On the other hand, water has the smallest viscosity in a biofilm, and therefore the fast Maxwell element has been associated with the flow of water through a biofilm, which leaves an association between the behavior of EPS with the intermediate Maxwell element(s). Principal component analysis of Maxwell elements describing the stress relaxation exhibited by biofilms with different matrix chemistries has pointed out that in general three principal Maxwell elements suffice to describe stress relaxation of biofilms. The fastest principal element was associated with the outflow of water and soluble polysaccharides, while a second principal element was associated with the EPS matrix as a whole, with a distinct impact of the presence of eDNA included in a third principal element with a

narrowly confined time constant range within the range of the second element. The presence of bacteria themselves as the heaviest masses is included in the first principal component with an inverse impact of bacterial prevalence with respect to the presence of water and soluble polysaccharides, i.e. the free space in a biofilm available for bacterial re/arrangement processes (50). Importantly, herewith there exists a relation between quantifiable visco-elastic properties of a biofilm that relates with their structure and composition that goes beyond the possibilities of microscopic imaging techniques.

Role of visco-elasticity in biofilm survival. Visco-elasticity of biofilms provides a protection mechanism allowing survival of its inhabitants under mechanical attack, while recently visco-elastic properties have also been related to the penetration of antimicrobials into a biofilm. Below, we will briefly discuss the role of visco-elasticity in protecting a biofilm against mechanical and chemical challenges.

Survival of biofilms on intra-vascular catheters in the human blood stream. Catheter-associated blood stream infections are common after long-term catheterization of patients and result from catheter-associated biofilms. Biofilms on intra-vascular catheters have to withstand the fluctuating shear from the pulsatile blood flow, using their visco-elastic properties to adjust to local changes in fluid flows while maintaining their structural integrity. In an initial attempt to withstand increasing shear forces, biofilms extend in the direction of flow through re-arrangement into so-called streamers, as shown in Figure 4a (60,61). When the visco-elastic properties of a biofilm are overloaded, biofilm failure and detachment from a surface occur (73). The visco-elasticity of biofilms allows these streamers the possibility

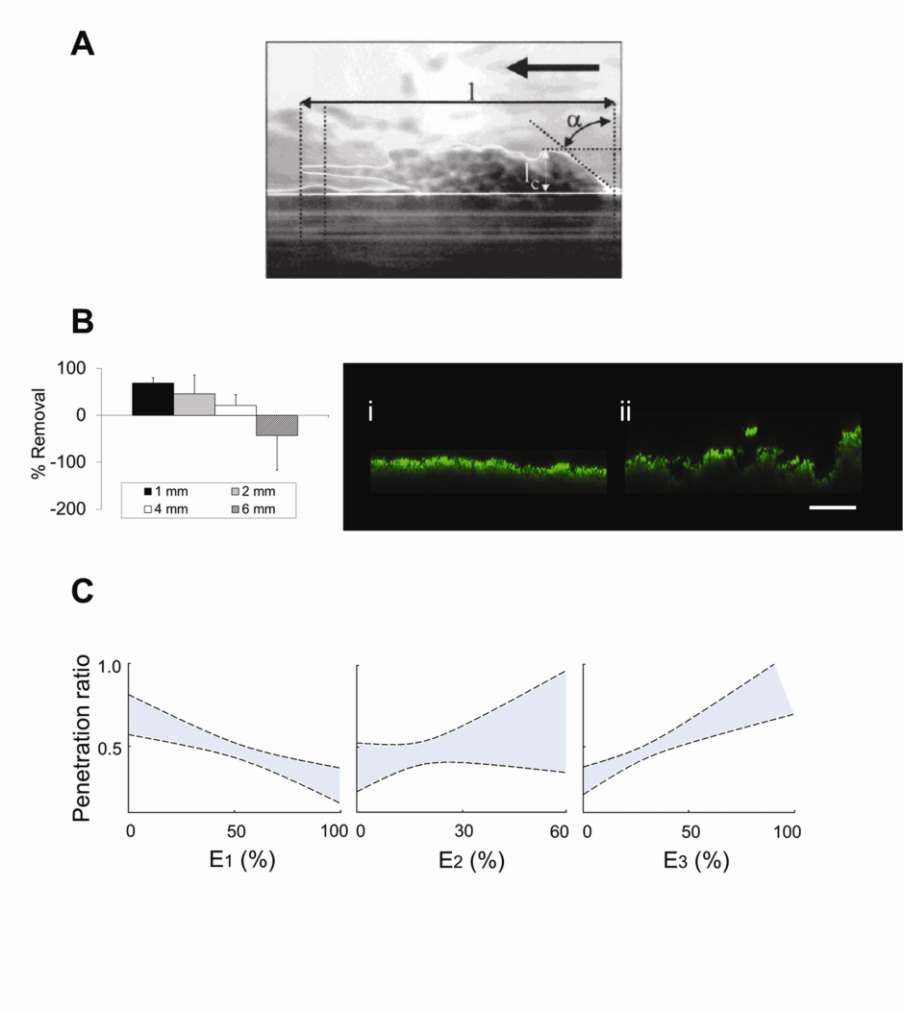

Figure 4. Role of visco-elasticity of biofilms in their survival under mechanical and chemical challenges.
A) Side view of a streamer in a biofilm growing under an applied wall shear stress. The edge of the streamer has been outlined for clarity, and the direction of fluid flow is indicated by the thick arrow (adapted from 71, with permission).
B) Biofilm removal or expansion (negative removal) for different distances between a biofilm and the bristle tips of a powered toothbrush (adapted from 8), together with CLSM images of biofilms prior to (i) and after (ii) non-contact brushing showing volumetric expansion (scale bar indicates 75 μm).
C) Penetration ratio of antimicrobials as a generalized function of the relative importance of the three Maxwell elements E_1, E_2 and E_3, denoting the fast and slow relaxation components, respectively. Dashed lines represent 95% confidence intervals (adapted from 27).

to extend and therewith gain time till more favorable flow conditions develop. How and when biofilms in the blood stream fail is arguably the most important biofilm property (25).

Mechanical removal of biofilms in the oral cavity. In the oral cavity, like in the blood stream, biofilms are mechanically challenged by naturally occurring fluid flows and tongue movements, but also by daily toothbrushing. Toothbrushing removes oral biofilm through direct contact with toothbrush filaments, but powered toothbrushes also do so in a non-contact mode (76), provided the energy output of the brush is high enough (8). The visco-elasticity of oral biofilms under non-contact brushing causes the biofilm to expand rather than to directly disperse prior to disruption (8). Moreover, if toothbrushing is arrested before detachment, biofilms are left in an expanded state (see Figure 4b (28)).

Visco-elasticity of biofilms and antimicrobial penetration. A major advance in our understanding of the limited antimicrobial penetration into biofilms has been made by the realization that biofilm structure and composition are reflected in the visco-elastic response of biofilms after deformation (34). Penetration of chlorhexidine into oral biofilms increased with increasing relative importance of the slow and decreasing importance of the fast relaxation element (see Figure 4c (27)). Involvement of slow relaxation elements suggests that biofilm structures allowing extensive bacterial re-arrangement after deformation are more open, allowing better antimicrobial penetration. Involvement of fast relaxation elements suggests that water dilutes the antimicrobial upon penetration to an ineffective concentration in deeper layers of the biofilm (27). Note that these results could also

have been expressed in one principal component, since the more open structure allowing bacterial re-arrangement inversely relates with the presence of water filled channels (50).

Visco-elasticity: a new biofilm virulence factor. Bacterial adhesion to a substratum causes a phenotypic change of expression that makes removal more difficult (47,79) and as a consequence the ability of a bacterial strain to adhere is considered as a virulence factor (23,32). The biofilm state of growth is also known as a virulence factor due to its increased recalcitrance to antimicrobials (2,46). The above examples, as summarized schematically in Figure 4 demonstrate that visco-elasticity is a pivotal property of biofilms facilitating their survival under mechanical and chemical attack. Therefore, we here propose that visco-elasticity should be regarded as a new biofilm virulence factor.

Future perspective on clinical applications. The realization that visco-elasticity is a biofilm virulence factor opens new pathways for the control of biofilms in disease and understanding of ill-understood phenomena like the bio-acoustic effect (56). The CLSM images in Figure 4c clearly demonstrated that biofilms expand upon the transfer of energy by non-contact, powered toothbrushing (8,28). This effect was accompanied by an increase in the slow and a decrease in the fast Maxwell relaxation of these biofilms. These changes facilitated increased penetration of oral antimicrobials (28).

Pulsating waves of energy have been shown to amplify antimicrobial efficacy against biofilms, and is referred to as the “bio-acoustic effect” (51,65). Gentamicin has been shown to have enhanced killing in *Pseudomonas aeruginosa* and *Escherichia coli*

biofilms *in vitro* and *in vivo* in rabbits after ultrasound treatment with frequencies ranging from 30 – 500 kHz and energies ranging from 10 – 3000 mW/cm^2 (9,10,55,57). Although biofilms of Gram-positive bacteria were initially thought to be resistant to enhanced killing from ultrasonic waves (51), with enough energy (3000 mW/cm^2) Gram-positive bacteria were also found to be susceptible (56). Reducing the frequency of the wave to 2 – 3 Hz increased efficacy of antimicrobials in biofilms of *Staphylococcus aureus* and single and mixed species oral biofilms (22,44,45). Since there is an analogy between non-contact, powered toothbrushing usually done at frequencies between 260 and 750 Hz with the bio-acoustic effect (4), it is likely that ultrasound will change the structure of biofilms to cause better penetration of antimicrobials into a biofilm and therewith increased killing. Visco-elastic measurements have not yet been done however on ultrasonically treated biofilms. Interestingly, low frequencies were more efficient in increasing the killing efficacy of antimicrobials than higher ones (54). The characteristic time constants derived from the stress relaxation of deformed biofilms, ranging from < 5 s, 5 – 100 s, and > 100 s for the fast, intermediate and slow elements respectively, suggest that the main effects of pulsating energy waves are through effects on the fast principal Maxwell element and thus associated with the presence of water in the biofilm. At the low frequencies associated with more efficient killing, the pulsating energy waves are closer to cutoff between the frequencies of the fast and intermediate Maxwell elements.

Taken together, these considerations point to a new pathway to increase the efficacy of pulsating waves of energy by fine-tuning the frequencies of these treatments to the resonance frequencies of the

different constituents in biofilm matrices, which will have a major impact on both their mechanical stability and antimicrobial penetration through a direct effect on the visco-elasticity of biofilms.

Changes in visco-elastic properties of biofilms that enhance antimicrobial efficacy can not only be achieved through pulsating waves of energy but also chemically. After exposure to detergents, *Staphylococcus aureus* biofilms grown on stainless steel had increased expression of the slowest Maxwell element, or bacterial re-arrangement. *Pseudomonas aeruginosa* biofilms on stainless steel were affected by N-acetyl-L-cysteine, as both the fast and intermediate Maxwell elements were reduced by over half while the slowest Maxwell element nearly doubled. *P. aeruginosa* biofilms on membrane filters were similarly affected by both N-acetyl-L-cystiene and DNaseI, reducing both the fastest and intermediate principal elements (50). Thus also the pre-treatment of biofilms by chemicals directly affects their visco-elastic properties and changes them into a direction favorable for subsequent antimicrobial treatment.

Conclusions. Increasing antimicrobial resistance has become a serious concern in modern medicine. The development of new antimicrobials is being outpaced by the increasing antimicrobial resistance of different bacterial strains. Bacterial biofilms provide added protection against antimicrobials and the host immune system, providing a diverse set of microenvironments increasing the chance of survival under mechanical and chemical attack. This review identifies the visco-elasticity of biofilms as a pivotal property of biofilms in dealing with mechanical and chemical challenges and proposes to consider visco-elasticity as a new, biofilm virulence

factor. Accepting visco-elasticity as a biofilm virulence factor, allows better understanding of a variety of studies done on different biofilms demonstrating that sufficient energy input into biofilms at the right frequency enhances the efficacy of antimicrobials. More importantly, considering visco-elasticity as a virulence factor opens new pathways to reduce the virulence of bacterial biofilms by intervening with its visco-elastic properties that rely on a combination of physical and chemical intervention. This may elongate the life-time of current generations of antibiotics in an era where the development of new antibiotics seems to stall, yet being direly needed.

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