Biofilms in wounds: a review of present knowledge.

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Abstract

Following confirmation of the presence of biofilms in chronic wounds, the term biofilm became a buzzword within the wound healing community. For more than a century pathogens have been successfully isolated and identified from wound specimens using techniques that were devised in the nineteenth century by Louis Pasteur and Robert Koch. Although this approach still provides valuable information with which to diagnose acute infections and to select appropriate antibiotic therapies, it is evident that those organisms isolated from clinical specimens with the conditions normally used in diagnostic laboratories are mainly in a planktonic form that is unrepresentative of the way in which most microbial species exist naturally. Usually microbial species adhere to each other, as well as to living and non-living surfaces, where they form complex communities surrounded by collectively secreted extracellular polymeric substances (EPS). Cells within such aggregations (or biofilms) display varying physiological and metabolic properties that are distinct from those of planktonic cells, and which contribute to their persistence. There are many factors that influence healing in wounds and the discovery of biofilms in chronic wounds has provided new insight into the reasons why. Increased tolerance of biofilms to antimicrobial agents explains the limited efficacy of antimicrobial agents in chronic wounds and illustrates the need to develop new management strategies. This review aims to explain the nature of biofilms, with a view to explaining their impact on wounds.

Keywords: wound chronicity, EPS, immune evasion, biofilm detection, anti-biofilm strategies,

Biofilm properties

The focus on bacterial biofilms has increased in the last twenty years. Until recently, microbiologists have emphasized the planktonic state over the biofilm state. However the number of conditions where biofilms are known to be involved are growing each year and it has now been put forward that bacteria predominantly grow as sessile communities rather than as single cells.[1-3]

Biofilms have traditionally been studied in simple models in the laboratory. Paul Stoodley and colleagues presented a five-phase model of biofilm formation *in vitro* under continuous flow conditions.[4] In the first stage planktonic cells reversibly attach to a surface. Irreversible binding follows this attachment and then multiplication into microcolonies. These microcolonies produce EPS, which in turn surrounds the colonies. After a couple of days the microcolonies attain tower- or mushroom-like structures measuring up to 150μm in the flow-cell.[2,4,5] The extracellular matrix contains a mixture of polysaccharides, proteins and DNA.[6-8] When the biofilm grows to a size not beneficial for bacterial survival and growth (e.g. due to nutrient limitations), focal areas of the biofilm are liberated. It is hypothesized this enables the otherwise sessile biofilm bacteria to spread

and colonize to form a new biofilm. Hence it seems that the biofilm lifecycle is a dynamic process capable of renewing itself.[2,4,5]

However, it has been shown that biofilms *in vitro* (Fig. 1) have little to do with biofilms found in nature in terms of size and shape.[3,9] It seems that biofilms causing harm in the human body are rarely anchored to a solid surface but rather found in a semi-solid state in the tissue. Furthermore the size of the infecting biofilms never reaches diameters larger than 100µm, unless the biofilm habitats an undisturbed surface (e.g. catheter).[3,9]

The reason for the augmented interest in bacterial biofilms is their inherent tolerance towards antimicrobial agents and inflammatory responses of the host. The ability to withstand antimicrobials is divided into two subtypes. Traditionally antibiotic resistance has received most attention, however it is antibiotic tolerance which is the prominent player of biofilm survival. Whereas resistance covers the inherited features that directly impede the efficacy of the antimicrobial, tolerance is the ability to sustain with the antibiotic due to the physical state of the bacterium.

Several resistance traits are found in the biofilm mode of growth and there are reports of increased mutation rates in biofilms which enhance resistance development.[10-13] The active export of antimicrobials (including aztreonam, gentamicin, tetracycline and tobramycin) by efflux pumps, such as the MexAB-OprM efflux pump, has been characterized in *Pseudomonas aeruginosa* biofilms and other biofilm forming pathogens.[14-19] By actively exporting the antimicrobial molecules lethal concentrations are never reached within the bacterium and the bacterium will able to survive.

Another resistance trait found in biofilms is the production of antibiotic degrading enzymes such as beta-lactamase.[13,20,21] The presence of beta-lactamase in a biofilm has been shown to change the pharmacokinetics of β-lactam antibiotics from time-dependent killing to a dose-dependent and thus further decreases the efficacy of the antibiotic. [22-24]

However as mentioned above, probably the most important trait of the biofilm is the innate tolerance to antimicrobials. Here the slow growth rate and the presence of accumulated matrix molecules are of utmost significance.

The biofilm matrix is composed of macromolecules including proteins, extracellular DNA and polysaccharides. Although its composition is variable, the most prominent matrix molecule for *P. aeruginosa* is probably the exopolysaccharide alginate, whereas exported cytoplasmic proteins composed of N-acetylglucosamine are important in *Staphylococcus aureus* and glucans in *Streptococcu*s species. Evidence has shown that alginate and cyclic glucans in the periplasm of the

bacteria may protect biofilms from aminoglycosides by binding the antibiotics.[25,26] Also, another of the major polysaccharides in the *P. aeruginosa* biofilm matrix (known as Psl), has been shown to provide a physical barrier toward various antibiotics during the initial stages of biofilm development.[27] It was found that Psl sequestered antibiotics (such as polymyxin B) to the matrix by electrochemical interactions and thereby limited their access to the cell surface.[27] Another important matrix molecule is extracellular DNA (eDNA). eDNA offers stability to the structure and has been shown to enhance biofilm development.[7,28.29] Furthermore eDNA has been shown to bind and decrease penetration of certain antibiotics (e.g. aminoglycosides) into biofilms.[30-32]

Additionally the growth rate and gene expression within a mature biofilm has been shown to resemble a stationary phase culture and can thus explain the lack of efficacy by traditional antibiotics, which is limited in such cultures.[9,33,34] The slow growth has been suggested to be a result of reduced nutrient and oxygen availability caused by the matrix molecules.[35,36] However, a study of Alhede et al. showed that induction of growth, by disrupting the biofilm mechanically, left the biofilm more sensitive to high concentrations of tobramycin when compared to the non-disrupted biofilm. Interestingly, this was not the case when exposing the disrupted biofilm to colistin.[9] The authors suggested that this difference could be explained by the fact that some of the antibiotic resistance traits are metabolically taxing, e.g. the efflux pumps, thus that the low levels of nutrient and oxygen within the biofilm couples the resistance properties with those of tolerance (i.e. the slow growth). Pamp et al. proposed that antibiotics that target biosynthesis (e.g. tobramycin) preferentially kill the cells facing the surface of the biofilm, while colistin killed the dormant cells residing inside the biofilm.[17]

Intriguingly, the tolerant biofilms are also able to evade the host defense. The matrix components offer a fortifying shielding effect and the production of detrimental extracellular products, such as proteases, toxins and lipases, leads to a severely impaired host defense.[37,389] The importance of this capability to kill immune cells is stressed by the fact that bacteria utilize cellular components released from the immune cells (e.g. DNA and actin) to strengthen their biofilms.[29] *P. aeruginosa* has two proteases, alkaline protease and elastase, which have been shown to inhibit chemotaxis, oxidative burst, phagocytosis and other microbicidal activities of phagocytic cells (including PMNs).[39] Furthermore it has been shown that alkaline protease and elastase are able to inhibit the biological activity of cytokines, such as IL-1, IL-2, IFN-γ and TNF[38-41] to cleave human IgA and IgG,[42] and to inactivate the complement system.[38]

An historical review of the discovery of biofilms in wounds.

The concept of biofilms in wounds has only recently been coined. However, biofilms have certainly existed historically and wounds containing biofilms have surely been successfully treated before the concept was born. When the drawings of wound tissue harbouring bacteria are viewed today, it is tempting to speculate that Sir Alexander Ogston may have unwittingly drawn a biofilm in 1880 (Fig. 2).[43] Another unrecognised clue was found by Bigger in 1944 who observed that soldiers' infected wounds treated with penicillin during World War II often seemed to respond to treatment, but then relapsed with recurrent infections.[44] Today this might arouse suspicion of a tolerant biofilm in a wound.

However, the first recorded observation of a biofilm in a wound is attributed to Gristina and colleagues following the examination of sutures and staples removed from healed wounds by scanning electron microscopy and the discovery of several kinds of bacteria in close proximity embedded within fibrous material. [45] *Staphylococcus epidermidis* was isolated from all of the wounds examined; yet healing had been accomplished uneventfully without infection or inflammation. The importance of coagulase negative staphylococci in wounds was later revised from unimportant skin flora to opportunist pathogens, and their presence in biofilms was associated with delayed, recurrent and persistent infections associated with indwelling medical devices.[46]

Speculation that biofilms might exist in wounds[47] was largely founded on animal experiments conducted during the 1990s[48-51] and from laboratory models were bacteria isolated from wounds were shown to form biofilms relatively quickly under suitable conditions.[49,52]

Irrefutable evidence of biofilms in wounds came from studies published in 2008. In one study specific bacteria were located in sections of chronic wound tissue using peptide nucleic acid (PNA) probes and fluorescent in situ microscopy (FISH). *P. aeruginosa* was detected in some instances as single cells, but also as aggregates or microcolonies surrounded yet not invaded by host cells[53]. In another study epifluorescent microscopy and scanning electron microscopy was utilised to visualise large aggregates of bacteria in wound biopsies. Gram-positive cocci within an amorphous EPS were most frequently observed, although some biofilms were composed of diverse species and this was confirmed by molecular analysis. Whereas biofilm was only demonstrated in 1 of 16 acute wounds, it was found in 30 of 50 chronic wounds. Hence biofilm was linked to wound chronicity ($p > 0.001$).[54]

Wounds are a well-suited habitat for bacteria, as the loss of skin integrity provides a moist and often nutrient-rich setting. The microbiota of the deep dermal tissues of chronic wounds is well described and harbours multiple bacterial species.[54-57] The use of specific fluorescent probes and confocal laser

scanning microscopy (CLSM) has been used to detect biofilms in chronic venous leg ulcers,[58-60] burns, [61,62] malignant wounds associated with breast cancer[63] and tissue filler infections.[64]

The use of molecular techniques to characterise wound flora has revealed the presence of diverse microbial species within chronic wounds. These mixed communities (Table 1) may indicate biofilms, but do not actually provide information on the structural or physiological parameters of the constituent member species that would indicate a biofilm phenotype.

Most studies agree on the almost universal presence of *S. aureus*, but another usual suspect found in chronic wounds is *P. aeruginosa,* which is present in approximately half of the investigated wounds. The organization and distribution of these two species has been elucidated by employing specific PNA probes for FISH analysis.[58,59] These observations revealed that the different bacterial species might be present in the same wound but they do not integrate. Very few aggregates of different bacteria in close proximity to each other were observed and never as part of a truly mixed population. Based on available evidence it seems that bacteria in chronic infections aggregate mostly as single species.[3, 58,59,65-67] This is in contrast to when bacteria aggregate in other natural environments such as the floccs in wastewater treatment plants and the soil where several species co-aggregate. This co-aggregation could be explained by the beneficial catabolism and anabolism of compounds among the different bacteria.[68] The plausible reason why multispecies biofilms are not common in chronic infections is that the nutrient availability is high and that symbiosis between different species is not a crucial requisite for growth. The key challenge for colonizing bacteria is rather whether they can survive the encounter with the defence system.

Impact of biofilms in wounds

Based on the evidence above, the concept of bacterial biofilms in chronic wounds is supported, but whether these biofilms play a role in the lack of healing is another question. The biofilm phenotype enables protection of the bacteria from both antibiotics and other antimicrobial agents such as silver and the host defence. This implies that if the bacteria succeed in forming a biofilm in the wound bed, the bacteria will be extremely difficult to eradicate. Data suggest that the presence of certain bacteria (e.g. *P. aeruginosa*) can induce ulcer enlargement, delay healing[66] and failure of split skin transplantation.[69] It has also suggested that bacteria (i.e. *P. aeruginosa*) located in the deeper regions of the wounds might play a role in keeping the wounds arrested in a stage dominated by inflammatory processes.[70] Evidence that biofilm contributes to chronic inflammation in a wound exists, but how that influences wound healing is unclear. We know that biofilms are not the cause of chronic wounds, but they might keep the wound from healing.[53]

The role of biofilms in wound colonisation and infection was explored with an animal model and *S. aureus*.[71] Wounds created on the pig were inoculated with *S. aureus* and treated with either one or two antibiotic preparations within 15 minutes (to simulate an acute infection caused by planktonic bacteria) or after 48 hours (when a biofilm had established), respectively. Using electron microscopy and fluorescence microscopy biofilms were observed in untreated wounds after 48 hours. Wounds treated with antibiotics within 15 minutes of introducing bacteria had no biofilm, showing that planktonic bacteria had been inhibited and that biofilm formation had been prevented. Antibiotics applied to wounds 48 hours after inoculation (i.e. after a biofilm had been established) failed to eradicate the biofilm. Decreased susceptibility of biofilms to antimicrobial agents is well documented[72] and largely accounts for the persistence previously observed by Bigger. In fact the term 'persister' was derived by Bigger.[44]

The difficulties of diagnosing biofilms in wounds

When biopsies from chronic wounds of 22 different patients (all allegedly infected by *P. aeruginosa*) were investigated, the samples were processed by both standard culturing methods and peptide nucleic acid-based fluorescence in situ hybridization (PNA FISH) for direct visualization and identification of bacteria.[58] The classic culturing methods revealed *S. aureus* to be present in the majority of the wounds, whereas *P. aeruginosa* was cultured less frequently. In contrast, using PNA FISH, *P. aeruginosa* was visualized in biofilms in almost half of the wounds. These *P. aeruginosa* biofilms were detected inside the wound bed, whereas *S. aureus*, when present, was detected on the surface of the wounds. Thus, it seems that, although being the gold standard, culturing is not successful for diagnosing biofilms of *P. aeruginosa* in wounds due to its deep localization. This is supported by the observations by other observers demonstrating *S. aureus* in microcolonies on the surface of the wound bed.[59,71] It was shown that the distance of the *P. aeruginosa* biofilm to the wound surface was significantly greater than that of the *S. aureus* biofilms, suggesting that the distribution of the bacteria in the chronic wounds was non-random.[59]

As described above, the microbiota in chronic wounds has been investigated for several years. In one study Gjødsbol et al investigated the microbiota by standard culturing.[57] Several different bacterial species were found in chronic venous leg ulcers, such as *S. aureus* (in 93.5% of the investigated ulcers), *Enterococcus faecalis* (71.7%), *P. aeruginosa* (52.2%), coagulase-negative staphylococci (45.7%), *Proteus* species (41.3%), and anaerobic bacteria (39.1%). Another study also investigated the flora in chronic wounds by culturing and found the most common bacteria to be

Staphylococcus (65%), *Enterococcus* (62%), *Pseudomonas* (35%) (Table 1). Molecular techniques have also been used to establish the microbiota and in several studies it has been shown that standard culturing of bacteria from wound samples does not reveal on the true bacterial diversity in the wounds.[56,58,] As mentioned above, the localization, the presence and slow growth of biofilms makes culturing difficult. Additionally a large population of anaerobic bacteria in wounds has been identified,[56] and these bacteria are also difficult to culture.

By using molecular techniques, even small populations of a specific bacterium can be detected. The drawback is that these techniques are qualitative which means that they do not reveal the relative proportions between the different bacteria or how they are organized and distributed in the wounds, as microscopy can do. Another just as important drawback is that these techniques cannot be used to identify which bacteria play a key role in the impairment of the wound healing process. Most importantly the bacteria in chronic wounds are very small and heterogeneously distributed.[55,70] This means that sampling from a chronic wound, especially using biopsies, might show false negative results.

In summary swabs from chronic wounds are not representative for the microbiota and biopsies might give false negative results. Therefore it is suggested to combine a thorough swab covering the whole wound surface with several biopsies, which should be investigated by both molecular techniques and culturing (aerobically and anaerobically).[73]

<u>Biofilm control</u>

Whereas planktonic cells are largely implicated in acute wound infections and control depends on systemic antibiotics, the increased antimicrobial tolerance of microbial cells within established biofilms[72] requires novel control strategies. One approach is to prevent biofilm formation by interfering with either the mechanisms of microbial attachment or the processes involved in biofilm maturation. The other is to remove or disrupt mature biofilm. To date neither strategy has met with unmitigated success; the range of cells with differing physiological and functional variations within a mature biofilm suggests that multiple inhibitory assaults are likely to be more effective than a single antimicrobial intervention.

Interference with attachment

Lactoferrin is part of the human innate immune response; it is found in tears, saliva, mucous and milk. It binds to components in the cell walls of Gram-negative bacteria to cause destabilisation, leakiness and ultimately bacterial lysis. It also binds avidly to iron, which is needed for bacterial motility during the initial stages of adherence to surfaces.[74] Xylitol is an artificial sweetener that

binds to the cell surface of Gram-positive bacteria that blocks adherence.[75] Disruption of *P. aeruginosa* biofilm *in vitro* with either lactoferrin or xylitol alone or in combination has been reported.[76]

In the laboratory honey has also been shown to impede attachment of *P. aeruginosa* to the surface of erythrocytes[77] and inert surfaces.[78] Also, it interferes with binding of *Streptococcus pyogenes* to inert surfaces.[79]

Interference with quorum sensing

One of the most studied strategies is quorum sensing inhibitors (QSIs). Most bacteria regulate a range of behaviours including metabolism, virulence and motility by sensing small secreted molecules in their surroundings (signal molecules). This cooperative behaviour is maintained through inter- and extracellular chemical crosstalk comparable to higher organisms.[80] This type of bacterial communication was termed quorum sensing (QS).[81] QS systems allow bacteria to "sense" bacterial density in the environment and respond by changes in gene expression.[82] By specifically targeting the QS system the idea is not to kill or detach the biofilm directly but to render the biofilm more susceptible to antibiotics and prevent expression of harmful virulence factors.

The first compounds showing good inhibition of the QS system were the synthetic furanones C-30 and C-56.[83,84] *In vitro P. aeruginosa* biofilms were significantly less tolerant to 100 μg/ml tobramycin when treated with furanone C-30.[83] In addition, *in vivo* studies in a pulmonary mouse model confirmed the potential of the furanones by demonstrating that bacteria were cleared faster in furanone-treated versus untreated mice.[83,86] Two QSIs from natural sources have recently been isolated: iberin from horseradish and ajoene from garlic.[85,86]

Using bacterial reporter assays three studies have demonstrated the ability of different honeys to interfere with quorum sensing in Gram-negative bacteria.[87-89] Manuka honey has also been shown to down-regulate three of the four genes essential for functional quorum sensing in MRSA, with knock-on effects on virulence and biofilm genes.[90]

Biofilm disruption

The use of sharp debridement is one way to reduce biofilm within a wound, but it rarely offers a permanent solution because, as with dental plaque, any remaining cells are able to regenerate the biofilm. Degradation of biofilm matrix with either cocktails of enzymes (e.g. DNAse) or maggot secretions has been reported.[91,92] Generation of hydrogen peroxide by enzymes within an alginogel disrupt biofilms *in vitro*[93]and several honeys can also disrupt biofilms.[94-97]

Ultrasound as antibiofilm treatment

A lot of research has thus been invested in finding non-invasive applications to overcome the problem of antibiotic resistance and tolerance. Promising studies show that exposing bacteria to ultrasound enhances the antibiotic efficacy. However, the underlying mechanisms of this effect are yet to be elucidated. Additionally, recent studies suggest that any mechanical force (e.g. ultrasound or shear) can be applied to re-sensitize biofilm bacteria by tearing the biofilm and stripping off the sessile cells.[9] Back in the planktonic state, the bacteria lose the tolerance provided by the biofilm. Such disruption of the biofilm by ultrasound is denoted destructive ultrasound.

Studies show that exposing *P. aeruginosa* simultaneously to low intensity ultrasound and aminoglycosides (e.g. tobramycin) improves the antibiotic efficacy.[98-105] The authors found that ultrasound alone did not affect the cell viability and that the synergistic effect was only observed if the antibiotics were applied during the ultrasonic exposure. However in another study, Qian et al. could not detect any structural difference in the biofilm by CLSM during ultrasonic exposure,[106] and further documented that the effect was also evident on planktonic *P. aeruginosa* as well.[100,101,104] An explanation for the antibacterial efficacy of this type of ultrasound was put forward by Liu et al.,[107], Runyan et al.,[108], and Nikaido,[109] who documented that low intensity ultrasound increased the permeability of *P. aeruginosa* to several tagged molecules. This ultrasonically induced permeability displayed the same frequency and peak pressure dependence as the above experiments. In addition, studies by Pong et al showed a similarly increased permeability of phospholipid vesicles.[110] Runyan et al. concluded that the effect was due to increased penetration of the antibiotics through the cell membrane of *P. aeruginosa*.[108]

In addition to the resulting transient permeability, much attention has been addressed to the destructive ultrasound in order to remove biofilms from implants and wounds.[106, 111-113] By showing that disruption of biofilms by mechanical force yields an enhanced effect of applied antibiotics, it was proven that biofilm tolerance is reversible.[9] This had been hypothesised to be due to disruption of matrix molecules and induction of growth by exposing the cells to nutrients. This inference was supported by the findings of Pitt et al.[114]

From published studies it seems that the mode of action by ultrasound has given rise to confusion and that both the terms “destructive” and “bioacoustic effect” have been used inconsistently. However, given that the above hypotheses are valid, both destructive ultrasound and the bioacoustic effect enhance the antibiotic efficacy, albeit in entirely different ways: one acting on the biofilm, the other directly on the individual bacterium.

Ultrasound debridement of wounds

Treatment of chronic wounds with ultrasound therapy has been used with seemingly good results.[115, 116] It has been suggested that the positive effect comes from a multitude of factors such as cellular recruitment and stimulation, collagen synthesis, angiogenesis, fibrinolysis.[117,118] Recently the knowledge of biofilms in non-healing wound has led to the hypothesis that the ultrasound, in addition to the above mentioned parameters, aids biofilm disruption and thereby wound healing.[119] Measuring wound healing and quantifying the presence of biofilms/bacteria is extremely difficult (if not impossible) and therefore the literature is very limited in this perspective. Escandon and colleagues found a non-significant decline in individual and total bacterial counts when treating refractory venous leg ulcers with non-contact ultrasound therapy.[116] It should be noted that biofilms able to prevent wound healing are smaller than 100µm in diameter and often situated deep in the wound bed and thereby hard to find by traditional means.[3, 58,59]

More data and possibly also better experimental setups are needed to prove the hypothesis claiming ultrasound to be an efficient antibiofilm strategy. However, the regimen seems safe and the above-mentioned indications are not to be neglected.

Phage therapy

One innovation with the potential to control wound infections is the topical use of lytic bacteriophage (or phage). These naturally occurring predatory viruses are obligate intracellular parasites that rely on bacteria for their replication. Infection of an appropriate bacterial cell usually leads to rapid viral replication within that host, followed by lysis and bacterial cell death to release viral progeny without affecting mammalian cells. However temperate phage can infect a host bacterial cell, integrate into the host DNA and remain latent for some time; their therapeutic potential is therefore low. Bacteriophages were independently discovered in 1915 by Twort in London and by d'Herelle in 1917 in Paris. The antimicrobial potential of lytic phage in treating infections was immediately recognised, particularly by d'Herelle, and several infections were successfully controlled, such as dysentery, cholera, wound infections and urinary tract infections. However the antibiotic era saw the demise of bacteriophage therapy, except in eastern European countries such as Georgia, Poland and the former Soviet Union. It is relatively recently that the continued emergence of antibiotic-resistant species has prompted a renewed interest in phage and translations of Georgian and Ukrainian studies have lately provided access to this largely forgotten therapeutic approach.

One of the most studied applications of bacteriophages has been in the control of *P. aeruginosa* infections in burns, where promising evidence of efficacy in animal models of acute infections and

against biofilms *in vitro* has been reported.[120] MRSA has been eradicated from diabetic foot ulcers with combination therapy of lytic bacteriophage and linezolid.[121] Most viruses are highly host-specific and treatments with a cocktail of lytic viruses targeted at mixed cultures of bacteria will probably be most effective clinically. Bacterial hosts most likely to be targeted include *P. aeruginosa, S. aureus,* MRSA, *Acinteobacter baumannii* and the multi-drug resistant Gram-negative bacteria (or ESBLs). Rat and pig models have been used to evaluate the effects of phage cocktails on bacterial counts and wound healing in diabetic cutaneous wounds, with limited success.[122]

The safety of such an approach has been tested in a phase I trial conducted on venous leg ulcers in America. Here 42 patients were treated for 12 weeks with either saline control or a cocktail of phages directed at *P. aeruginosa, S. aureus* and *E. coli.* Neither adverse events nor significant differences between the two study groups were observed.[123]

Further clinical data from phase II and III studies is needed, and formulations for delivering suitable phages to the wound bed will have to be developed. Much research is in progress and the licensing of wound dressings incorporating phage is expected within the not too distant future.

Interactions between phage and biofilms are complex and involve not only lysis of bacterial cells, but degradation of EPS by viral enzymes, which is an additional advantage.[124] A rabbit-ear model was used to investigate the ability of bacteriophage and sharp debridement to eliminate *S. aureus* from a chronic wound. Combination therapy gave better outcomes than bacteriophage or debridement alone.[125]

<u>Clinical evidence of efficacy of antibiofilm interventions (or lack of it).</u>

At present the number of clinical studies in which eradication of biofilms has been investigated is limited and will probably remain so until a routine test to detect biofilm in wound tissue is developed. A concept of biofilm-based wound care (BBWC) has been proposed in which sharp debridement to reduce biofilm is followed by antimicrobial agents to limit biofilm reformation. The rationale for this approach is based on physically removing biofilm and inhibiting the residual bacteria that actively try to reform biofilm before they return to their tolerant status. In a retrospective study BBWC of 190 patients with critical limb ischaemia were treated by sharp debridement coupled with ultrasound, followed by lactoferrin and xylitol, silver, cadexomer iodine and antibiotics. Improved healing was observed in these patients compared to a previous study, but the presence of biofilms before and after treatment was not confirmed by electron or confocal scanning laser microscopy.[126]

A wide range of model systems have been devised to study microbial biofilm biology[127] and many biofilm studies conducted in the laboratory have been applied to evaluate wound treatment strategies[128-134]. Animal models have also been utilised.[71, 135-138] Such studies are important, but the study of wound biofilms is very complicated and it is difficult to make comparisons between different studies, as demonstrated by the conflicting results obtained in evaluating some licensed antimicrobial dressings. Unlike disinfectants there are not yet standardised methods available to determine the efficacy of wound dressings on biofilms. Hence many new compounds and dressings have been evaluated on fast growing reference strains of bacteria in shaking cultures rather than on biofilm-growing bacteria commonly present in chronic wounds. Even when using a biofilm model, researchers should be aware of the false dogma stating that surface attachment *per se* makes the biofilm tolerant. This is not true, since young surface-attached biofilms still have high growth rates with only a limited matrix shield and therefore are highly susceptible to most antimicrobials. Biofilms across species and models seems to become tolerant between 20 hours and 48 hours after inoculation but continue developing this tolerance with time.[3,9] Another important limitation of *in vitro* models is that they have been developed under artificial conditions that aim to simulate the natural situations in which biofilms are normally established, and because the validity of these models is questionable, data obtained is not necessarily transferable to clinical practice.

Future prospects

Discovering biofilms in wounds has given insight into some of the reasons why wounds fail to heal. It has helped to explain the limited efficacy of antibiotics in chronic wounds and it has stimulated research into innovative anti-biofilm strategies. However, we still face a number of tasks to solve before chronic wounds are history. The range of possible treatment strategies of biofilm infections needs to be expanded and the *in vitro* models need to be more closely aligned to simulate the wound *in vivo*. *P. aeruginosa* is the test organism that is commonly used in laboratory biofilm models because it is easy to grow, its genome has been sequenced and knock out mutants are available. Testing a broader range of wound microbiota in both single species and mixed species models might provide a different perspective. Most importantly, in order to prove that biofilm plays the role it is believed to do, we need to improve diagnostic methods to eliminate false negatives. This task is especially important when evaluating treatment strategies in the clinic.

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