A review of the scientific evidence for biofilms in wounds

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ABSTRACT

Both chronic and acute dermal wounds are susceptible to infection due to sterile loss of the innate barrier function of the skin and dermal appendages, facilitating the development of microbial communities, referred to as biofilms, within the wound environment. Microbial biofilms are implicated in both the infection of wounds and failure of those wounds to heal. The aim of this review is to provide a summary of published papers detailing biofilms in wounds, the effect they have on infection and wound healing, and detailing methods employed for their detection. The studies highlighted within this paper provide evidence that biofilms reside within the chronic wound and represent an important mechanism underlying the observed, delayed healing and infection. The reasons for this include both protease activity and immunological suppression. Furthermore, a lack of responsiveness to an array of antimicrobial agents has been due to the biofilms' ability to inherently resist antimicrobial agents. It is imperative that effective strategies are developed, tested prospectively, and employed in chronic wounds to support the healing process and to reduce infection rates. It is increasingly apparent that adoption of a biofilm-based management approach to wound care, utilizing the "antibiofilm tool box" of therapies, to kill and prevent reattachment of microorganisms in the biofilm is producing the most positive clinical outcomes and prevention of infection.

Bacteria have traditionally been studied in their planktonic state, where they exist as "free-floating" entities in a liquid environment.[1–3] However, in most natural and clinical environments, microorganisms aggregate together on a surface or air/liquid interface and grow within communities where they encase themselves within extracellular polymeric substances (EPSs) composed of proteins, lipids, and polysaccharides.[4,5] Such an existence is now defined as a biofilm.[2,3] A key property of biofilms is that compared with their planktonic equivalents, they show increased resistance to antimicrobial, immunological, predatory, and chemical attack. Once established, biofilms are highly tolerant to removal and eradication. In this environment, the microbial cells exhibit an altered phenotype, particularly relating to growth rate and gene transcription.[6] In fact, *Pseudomonas aeruginosa* is known to display multiple phenotypes during development as a biofilm.[7] Biofilm-grown bacteria also produce quorum sensing signaling molecules, which enable them to act as a complex multicellular entity. Further advantages of biofilm growth include increased metabolic efficiency, substrate accessibility, enhanced resistance to environmental stress and inhibitors/antimicrobials, and an increased ability to cause infection and disease.[8–10]

It has gradually become clear that biofilms are more prevalent than originally believed particularly so in clinical situations.[11] Biofilms have been implicated in numerous chronic infections including cystic fibrosis, otitis media, dental caries, periodontal disease, and prostatitis, as well as being the cause of acute infections and the failure of in-dwelling medical devices.[12,13]

Furthermore, biofilms are found attached on skin[14] and there is evidence to suggest that biofilms also contribute to nonhealing in the chronic wound environment. Biofilm-related diseases are typified by a persistent infection that develops slowly, which is rarely resolved by the immune response[15] and responds inconsistently to antimicrobial therapy.[16,17] The potential of severe patient outcomes following infections involving biofilms has resulted in research efforts to identify the exact role of biofilms in chronic disease and a number of medical conditions, perhaps not already associated with bacterial infection.

The worldwide increase in obesity is a major cause of the concomitant rise in diabetes and cardiovascular diseases such as chronic venous leg ulcers (CVLUs) and diabetic foot ulcers (DFUs).[18] Moreover, diabetic patients are at increased risk of severe skin and bone infections. Chronic wounds including CVLUs, DFUs, and pressure ulcers are a major cause of distress and disability for patients, as well as being a drain on the resources of the health service, costing over £1 billion per annum.

Both chronic and acute dermal wounds are susceptible to infection due to loss of sterility of the innate barrier function of the skin and dermal appendages, facilitating the development of microbial communities within the wound environment.[19,20]

Bacterial biofilms are thought to occur on the surface of wounds[21] and are implicated in both the failure of wounds to heal[22] and the occurrence of chronic inflammation.[23] Biofilms in particular are postulated as the reason why venous leg ulcers, pressure ulcers, and DFUs develop into chronic states[24,25] by inducing chronic or perpetual inflammation in the chronic wound and so delaying healing.[26]

Studies have shown that many commercial topical agents and wound dressings are ineffective against biofilm infections.[27] Hence, focusing wound research into antibiofilm therapies may lead to improvements in the wound management strategies and ultimately lead to improved wound healing outcomes for patients.[28] Many papers have now been published relating to original clinical wound biofilm research. However, the following is a summary of published papers detailing biofilms in wounds: the effect they have in wound healing and details of methods employed for their detection in wounds.

IDENTIFICATION OF WOUND MICROORGANISMS

It is accepted that the number of genera and species of microorganisms recovered from chronic wounds is an underestimation of the total wound microbiota.[29,30] The limitations of culturing bacteria from wounds remain an area of great debate. Consequently, the use of debrided tissue with molecular analysis is often exploited to aid identification and recovery of "viable but nonculturable" microorganisms in wounds. In addition, novel microscopical techniques can be used to visualize and identify bacteria within tissues. Histological staining of debrided wound tissue has allowed imaging of bacteria residing deep within wound tissues and has showed the presence of bacterial clusters/microcolonies, indicative of biofilms, within both burn and chronic wound tissue.

While evidence from a number of studies suggested that the majority of microbial species residing within chronic wounds were aerobic, it is now recognized that many anaerobic bacteria predominate in deeper tissues.[31] Given the location of these bacteria, they would be thought not to be easily recovered using surface wound swabbing techniques. However, thorough deep swabbing of the wound area has previously been shown to yield equivalent prognostic information to biopsies.[32] Anaerobic species, such as *Bacteroides* sp., *Fusobacterium* sp., *Peptostreptococcus* sp., and *Clostridium* sp., are predominately identified in human and animal wounds.[33–36] In terms of the identification of anaerobic bacteria, molecular analyses are generally most effective.[37] This is not unexpected because culture methods in particular are renowned for underestimating the presence of anaerobic isolates in wounds[38–40] particularly if samples are not processed within a very short space of time (generally <2 hours postsampling).[32]

Studies have successfully used molecular techniques including denaturing gradient gel electrophoresis (DGGE) and subsequent gene sequencing of excised bands to compare bacterial populations in burn and chronic wounds.[33,36,41] However, there are limitations to these methods including band heterogeneity,[42,43] which may result in low-level sequence homologies; as often, the same species of bacteria can be represented by multiple bands.[42] In addition, bands may not be effectively excised resulting in sequencing errors.[44] Furthermore, some bacterial species such as mycobacterium are not easily identified by 16S rRNA sequencing.[45] Additionally, sequence databases are often biased toward pathogenic organisms[46] and thus many bands highlighted in DGGE profiles may represent previously unsequenced microorganisms.[30] Given these limitations, an underestimation of the true species diversity within a wound remains likely even following molecular analysis.[43] Despite this, DGGE analysis has provided useful information regarding the number of species present on swab and tissue samples derived from wounds, as well as confirming the presence of an unculturable population within these wounds.[36] DGGE has successfully been used by Dowd and colleagues[42] to show that there are variations in the multispecies nature of wound and skin sites.

Molecular sequencing methods are becoming more cost-effective and accessible, and with this increased throughput can be achieved. Recent advancements in bioinformatics including shotgun metagenomics have evolved in parallel with increased computational power.[42] These techniques can be used for the rapid identification and quantification of bacteria[47] and may represent a way forward in terms of analyzing bacterial populations.

MICROBIOLOGY OF WOUNDS: A BRIEF UPDATE

Chronic wounds often exhibit a highly persistent inflammatory phenotype, epitomized by the influx of polymorphonuclear leukocytes (PMNLs) to the wound site, elevated matrix metalloproteinases (MMPs), and an imbalance of several cytokines.[24] It has been suggested that *P. aeruginosa* may significantly contribute to this inflammation by producing rhamnolipids, which protect them from phagocytosis.[48–51] The continued presence of bacteria in the wound further exacerbates the situation by causing additional infiltration by PMNLs, together with MMP production.

As mentioned above, despite the limitations of routine microbiological culture in providing a representative picture of microbial diversity,[52] it is only relatively recently that molecular techniques have been applied to the identification of microorganisms in a wound. For example, Gjodsbol and colleagues[53] found that by exploiting molecular techniques, more than half of the chronic wounds investigated in their study were colonized with *P. aeruginosa.* Cultural techniques alone were shown to significantly underestimate the presence of this bacterium in sampled wounds.[36] Many published studies have grossly underestimated microbial populations in wounds by failing to culture any strictly anaerobic bacteria from clinical samples.[36] Furthermore, yeasts are also likely to be more prevalent in the chronic wound microbial community than the literature suggests.[54]

While many studies confirm the polymicrobial nature of most chronic wounds, there is still controversy about whether these organisms contribute directly toward nonhealing. It seems most likely that individual bacteria themselves are not directly responsible for nonhealing wounds.[55] Instead, two previous studies found a direct correlation between the pres-

ence of four or more distinct bacterial species in a wound and nonhealing,[36,55] suggesting that synergy between mixed populations of organisms causes the pathology seen in vivo.[42]

Chronic DFUs occur in 15% of all patients with diabetes and are particularly detrimental to health. Following inadvertent wounding, localized neuropathy can lead to wounds going unnoticed for long periods of time, resulting in the rapid development and deterioration of DFUs. Furthermore, poor circulation and impaired healing in these patients means that there is a higher than average risk of limb amputation with some 14–24% of diabetic patients with foot ulcers eventually undergoing amputation. DFUs also tend to have a complex bacterial microflora, including a higher incidence of anaerobes with *Bacteroides*, *Peptoniphilus*, *Finegoldia*, *Anaerococcus*, and *Peptostreptococcus* spp. ubiquitous in DFUs.[56]

Kirketerp-Møller and colleagues[39,57] collected and identified bacteria taken from 22 patients with chronic wounds. Chronic wound samples were exposed to standard culturing techniques and peptide nucleic acid–fluorescence in situ hybridization (PNA-FISH) for bacterial identification. In the majority of wounds, *Staphylococcus aureus* was the predominantly cultured organism, with less frequent isolation of *P. aeruginosa.* However, PNA-FISH showed that this was an underestimate of *P. aeruginosa*, identifying it in a large majority of the wounds, in contrast to the cultural approach. The authors concluded poor correlation between standard culture and PNA-FISH in terms of species identified. Combining molecular and culturing methods therefore appears to provide a more complete characterization of the microbial diversity of chronic wounds and can thereby expand our understanding of how microbiology impacts chronic wound pathology and healing.[58]

Dowd and colleagues[56] employed bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) to evaluate bacterial diversity in 40 chronic DFUs all from different patients. In this study, *Corynebacterium* spp. was the most prevalent genus with anaerobic bacteria also having a very high prevalence. Other bacteria isolated included *Serratia*, *Streptococcus*, *Staphylococcus*, and *Enterococcus* spp. The findings in the study again highlighted that traditional culturing methods were biased toward the most readily culturable microorganisms, particularly *S. aureus*, and often failed to isolate anaerobic species.

Application of bTEFAP to 23 chronic surgical wounds showed similar findings.[59] The study identified two previously uncharacterized *Bacteroidales* in all surgical site infections (SSIs) and highlighted this order as constituting the predominant population in the majority of these wounds. A mean of six genera were found to occur in any SSI, of which 60% were identified as anaerobic bacilli, thus adding to the growing evidence of an apparent underrepresentation of anaerobic bacteria in many wound studies, organisms that may be leading contributors to infection. A recent study by Freeman and colleagues[33] isolated and identified a range of microorganisms from horse wound biopsies and wound swabs using both culturable and DGGE polymerase chain reaction (PCR) techniques. The authors identified the common wound colonizing bacteria *P. aeruginosa*, *Staphylococcus epidermidis*, *Serratia marcescens*, *Enterococcus faecalis*, and *Providencia rettgeri*. The study emphasized the importance of combining molecular techniques with traditional microbiology culture techniques to provide a better understanding of the microbiology of chronic wounds.

Wolcott and Dowd[60] showed how rapidly *P. aeruginosa* could be identified from wounds using quantitative PCR ($\leq$2 hours) in comparison to 24 hours using traditional culturable techniques. Hence, unsurprisingly, it has recently been suggested that both fluorescent in situ hybridization and rDNA sequencing could soon be available for clinical use.[61] Two new molecular methods have been developed to aid wound pathogen diagnostics. The first is a quantitative PCR wound panel that while rapid, remains limited to major wound-associated bacteria and yeasts.[54] The second is universal fungal tag encoded FLX amplicon pyrosequencing[54] and bTEFAP,[56] which have a turn-around time of 24 hours and are able to detect species population levels of both bacteria and yeasts in chronic wound diagnostic samples.

CLINICAL MARKERS FOR EVIDENCE OF BIOFILMS IN WOUNDS

Many studies have reported the clinical features of chronic wounds suspected of containing a bacterial biofilm. Seven features have been used to indicate the presence of infection in human chronic wounds.[62] These have included indicators such as a pale wound bed, a yellow discharge, necrotic tissue, a clear slime, and a putrid smell. In addition, a number of studies have determined the biofilm-forming ability of cultured wound bacteria as a marker of wound biofilms. Two remaining features, namely, friable granulation tissue and a red wound bed, are also linked in the Gardner study to the development of a chronic wound. Being able to recognize the clinical symptoms of a wound infected with a biofilm is vital to improving the treatment of nonhealing chronic wounds in both human and animals. However, clinical diagnosis is highly subjective. Hence, due to concerns in biofilm identification, continued work is required to develop techniques that can rapidly identify biofilms in vivo. At present, unless the wound is heavily populated, tissue biopsies or swabs are required combined with microscopic identification techniques to confirm the presence of a wound biofilm. Scanning electron microscopy (SEM) has also often used to show the presence of biofilms within wound tissue.[63] Often, images that clearly show evidence of extracellular polysaccharide covering the attached bacteria are taken as a positive identification of a bacterial biofilm.

The clinical assessment of a wound biofilm is vital for diagnosis. However, to date, no clear definition is used by clinicians to indicate biofilm infection, although possible indicators are highlighted in Table 1. In DFUs, for example, diagnosis is generally based on clinical signs and symptoms of inflammation. Currently, the only true means of identifying a biofilm is through the use of molecular techniques.[61]

SCIENTIFIC EVIDENCE FOR BIOFILMS IN WOUNDS

Visualization—macroscopic

Evidence of "slough" in a wound and its microscopic examination has been proposed to be a clinical marker of a biofilm. In addition, other markers such as a "shiny or sheen" appearance of a wound may be indicative that a biofilm is present. These subjective approaches, however, are without basis, lack

Table 1. Markers for identification of a biofilm in a wound

Clinical sign	Marker	Identification method
Nonhealing wound	Slough	Visual examination
	Shiny	Visual examination
Malodor	Smell	Smell
Necrotic tissue	Necrotic tissue	Visual examination
Unresponsive/recalcitrant to antimicrobial interventions	Lack of change to antimicrobial effect/reoccurring	Visual examination Microbial bioburden test
Polymicrobial microbiology	Cultural and molecular identification	Standard culturable techniques Molecular techniques—PCR
Isolated bacteria showed a high biofilm-forming potential	Biofilm-forming potential	Use microtiter assay with crystal violet
Biopsy—visualization	Evidence of microcolonies	Microscopic examination following a Gram stain Scanning electron microscopy Light microscopy
	Evidence of extracellular polymeric substances	H&E stain, calcofluor white/ethidium bromide; Congo red/Ziehl carbol fuchsin; safranine/FITC-ConA; DAPI/PAS
	Evidence of an inflammatory response (not always evident)	H&E stain

DAPI/PAS, 4′,6-diamidino-2-phenylindole/Periodic Acid-Schiff stain; FITC-ConA, fluorescein isothiocyanate/concanavalin A; H&E, hematoxylin and eosin; PCR, polymerase chain reaction.

objective criteria, and remain unproven although it is regarded that if a biotic or abiotic surface exists together with planktonic microorganisms, then sessile microorganisms within a biofilm will also be evident.

Visualization—microscopic

Initial evidence for the presence of biofilms in chronic wounds was sparse, but there are now a plethora of studies utilizing a range of staining and molecular techniques to address this issue. Early studies did little more than show that bacterial wound isolates could successfully be grown in in vitro biofilms[64] and could be achieved in a very short space of time (<10 hours). Staining for the presence of bacterial EPSs was also used as a biofilm indicator and was shown to occur in CVLU wound swabs by light microscopy[65] and in acute wound biopsies from mice and horses by confocal laser scanning microscopy (CLSM).[33,66] However, EPS is well known not to just contain polysaccharides and as such can only be used as an initial marker for the presence of EPS.

Typical staining techniques for EPS/bacteria have included calcofluor white/ethidium bromide, Congo red/Ziehl carbol fuchsin, safranine/FITC-ConA, and DAPI/PAS.[65–69] Fluorescence microscopy confirmed the presence of a green fluorescent protein expressing *P. aeruginosa* biofilm as early as 8 hours in a rat model.[70] However, this particular model failed to show any biofilm-associated delayed healing perhaps because *P. aeruginosa* PAO1 is not a particularly virulent wound isolate.

PNA-FISH is a molecular technique that can accurately pinpoint and image microbial cells in host tissue. PNA-FISH has successfully been used on chronic wound tissue samples to locate and describe distinct biofilms.[31,57,71,72] Interestingly, observations from chronic biofilm infections suggest a high incidence of low bacterial diversity/monospecies aggregates, even in multispecies infections.[11] Evidence from FISH studies has shown that *P. aeruginosa* exists in separate and defined population pockets (i.e., microcolonies) within these wounds.[24,57,71] In contrast to this, commensal (e.g., oral and gastrointestinal tract) and environmental biofilm aggregates appear to contain multiple species and are believed to form biofilms with high bacterial and niche diversity. The reasons for this remain unclear.

While it seems likely that persistent biofilms exist in a large proportion of chronic wounds, the incidence appears to be much lower for acute traumatic wounds.

Electron microscopy of biopsies from chronic wounds found that 60% of the specimens contained biofilm features (typified by dense bacterial aggregates) in comparison to significantly fewer (6%) biopsies from acute wounds.[31] These observations could help to explain the different prognostic outcomes for chronic and acute wounds. Fadeev and Nemtseva[73] described a correlation between a microorganism's ability to form a biofilm and the duration of illness. They highlighted evidence of biofilms in wounds 3 days after surgery and suggested that the ability to form biofilms and duration of illness could be considered to be a marker for the development of a chronic state.

BIOFILM-FORMING POTENTIAL OF WOUND BACTERIA

Bacterial attachment to a surface is a prerequisite for biofilm formation. However, bacterial adhesion exists as a reversible and irreversible state[74] and the irreversible state does not guarantee that the microbe is in an "early biofilm" state. Methods to measure the biofilm-forming potential (BFP) of a bacterium can be performed using a crystal violet microtiter plate assay.[75] This assay involves a 96-well plate within which bacteria are allowed to adhere and then those attached are stained with crystal violet. Given the capacity of the microtiter plate, a high throughput assay for BFP is achievable. The crystal violet assay provides a quantitative measure of BFP and alternative models have been used to correlate its findings. Examples include the Centers for Disease Control (CDCs) bioreactor model or Congo red model. The CDC reactor method has advantages in that it has a number of coupons available for sampling different materials and for studying microbial adhesion and biofilm formation.[76]

Fadeev and Nemtseva[73] investigated the BFP of bacteria isolated from soft tissue infections. A photometric method showed that all strains of *Pseudomonas* and 80% of *Enterobacter* had the ability to form biofilms. Furthermore, Rupp and Fey[77] developed in vivo models to evaluate adhesion and biofilm formation by *S. epidermidis* with good results. Further studies on the BFP of wound isolates investigated the BFP of bacteria isolated from[68] wound and skin sites,[78] demonstrating that bacteria cultured from chronic and acute wounds had significantly ($p < 0.05$) higher BFP than bacteria isolated from skin.

The genera documented and showed to represent the greatest BFP have included *Pseudomonas*, *Staphylococcus*, *Bacillus*, and *Moraxella* sp.,[79] although these are species and strain dependent. Synergy and antagonism within biofilms must not be overlooked. They represent a growing area called "sociomicrobiology" and are very important to biofilm development in chronic wounds. It is possible that the presence of some genera may enhance the risk of biofilm formation within a wound.[80] Traditionally, accepted pathogenic organisms may not necessarily be the isolates that show the strongest BFP and these may require assistance from those bacteria, which do have a high BFP. This reflects many of the observations documented in the literature reporting that bacteria show a range of different pathogenic mechanisms.[81,82]

IN VITRO AND IN VIVO MODELS OF BIOFILMS

To be able to study wound biofilms successfully, it has been necessary to generate in vitro and in vivo wound models. These include laboratory models such as the constant depth film fermenter (CDFF),[68] a collagen gel matrix containing biofilm aggregates with serum protein mimicking the wound bed of chronic wounds, a flat-bed perfusion biofilm model,[83,84] polymer biofilm model,[85] and the Lubbock chronic wound biofilm (LCWB) model [86] to name a few. These models have been shown to simulate the functional characteristics of chronic pathogenic biofilms and can be sampled for characterization and analysis of the experimental biofilms. For example, the CDFF allows biofilms of constant depth to be generated in a flow-through fermenter system, which can be sampled periodically in response to perturbation. This model showed that "biofilm-grown" wound isolates exhibit higher antibiotic tolerance than their planktonically grown isogenic counterparts, a situation characteristic of chronic wounds. The collagen gel matrix model has also been shown to mimic important hallmarks of biofilms, such as the bacterial establishment in an extracellular polysaccharide matrix and increased antibiotic tolerance.[69] The LCWB model utilizes multispecies biofilms to evaluate the effect of antibiofilm agents, with recent studies suggesting that different treatments can target specific populations within a biofilm.[87] The limitations of models such as the LCWB and the collagen gel matrix are that they can only be run as batch systems for 24 or 48 hours, whereas other models such as the CDFF can be run for days or weeks to look at the long-term effects of biofilm therapeutic treatments, as well as allowing flexibility regarding the maturity (age) of biofilm that can be tested. The dynamic, continuous-feed growth environments of the CDFF[68] and flat-bed perfusion biofilm models[83] also more closely mimics the in vivo situation. Thorn and Greenman[83] grew *P. aeruginosa* and *S. aureus* biofilms within continuously perfused cellulose matrices and applied two antimicrobial wound dressings to the surface of mature biofilms. The model showed the antimicrobial susceptibility profiles of biofilms and enabled differentiation to be made between bactericidal and bacteriostatic effects. Other novel research tools include monitoring the viability of sessile biofilm bacteria in real time using a rapid form of CLSM over 48 hours. In one study, it was observed that 90% of all sessile bacteria within the biofilm progressively died within 24 hours in the presence of silver-containing wound dressings.[85]

While we know that strict anaerobes are an important component of the wound microflora, it is interesting to note that such models are able to maintain an anaerobic population in an essentially aerobic environment, through integration of the anaerobes into the biofilm. The LCWB model employed by Sun and colleagues[88] showed the ability of clinically significant anaerobic bacteria to thrive in aerobic conditions.

A number of animal wound studies support the presence of biofilms in wounds.[89,90] One of the early studies examined sequential biopsies from skin wounds in neutropenic mice over a 60-hour time period.[66] Infiltration of the wound by PMNLs and macrophages was noted after 36 hours (indicative of inflammation) with tissue necrosis at 60 hours being strongly suggestive of a chronic wound phenotype. In addition, the biopsies showed development of bacterial clusters over time, indicative of biofilm formation[66] with inflammatory cells strongly being associated with these bacterial clusters. A reproducible chronic wound model in diabetic mice also showed delayed healing of wounds with *Pseudomonas* biofilms.[91] Importantly, histological analysis of wound biopsies from this model showed a number of essential indicators of inflammation characteristic of chronic wounds, including extensive inflammatory cell infiltration, tissue necrosis, and epidermal hyperplasia adjacent to challenged wounds. Further direct evidence for the effect of bacterial biofilms on cutaneous wound healing was seen in another novel murine cutaneous wound system where biofilms developed in splinted cutaneous punch wounds.[92] In comparison to a biofilm-deficient mutant bacterial control strain, this study showed that delayed wound reepithelialization was directly caused by the presence of a staphylococcal bacterial biofilm. Schaber and colleagues[93] investigated the formation of *P. aeruginosa* biofilms in thermally injured mice and found

that biofilms formed within 8 hours. Confirmation of biofilm presence was achieved by light, electron, and CLSM. It was observed that the *P. aeruginosa* biofilms within burned tissue surrounded blood vessels and adipose cells.

Another animal model of porcine partial-thickness wounds inoculated with *P. aeruginosa* revealed the presence of two distinct bacterial wound populations.[65] These were a nonadherent population, easily removed from the wound by flushing with saline (indicative of a planktonic wound microflora), and a second more adherent population, only removed using surfactant and vigorous scrubbing (indicative of a biofilm). Using light microscopy, SEM, and epifluorescence microscopy on a porcine model, partial-thickness wounds inoculated with a wound-isolated *S. aureus* strain revealed the presence of biofilm-like structures after 48 hours.[63] This study showed that *S. aureus* formed firmly attached microcolonies and EPS encased bacterial colonies on the wound surface. These biofilm-like communities had increased antimicrobial resistance compared with their planktonic phenotype in vivo.

Overall, the structural and physiological results of these various models support the hypothesis that bacterial biofilms play an important role in wound colonization, infection, and nonhealing.

EVIDENCE FROM IN VITRO TISSUE CULTURE AND ENGINEERED SKIN MODELS

Simple tissue culture models using HCA2 fibroblasts and keratinocytes (both from neonatal foreskin) have been tested with bacteria isolated from chronic wounds.[94,95] In addition, bacterial supernatants from anaerobic *Peptostreptococci*[94,96] and *Staphylococci*[95] were found to impair wound healing responses in in vitro tissue culture scratch wound, cell viability, and apoptosis assays. Furthermore, Kirker and colleagues[95] created co-cultures of *S. aureus* biofilms and human keratinocytes (HKs) by growing *S. aureus* biofilms on tissue culture inserts and then transferring the inserts to existing HK cultures. After 3 hours, the biofilm reduced the viability of the HK and after 9 hours apoptosis increased significantly.

Two commercially available skin equivalents, Graftskin (Apligraf, Organogenesis, Canton, MA), and a reconstituted human oral epithelium (RHE; SkinEthic Laboratories, Nice, France) have been used as in vitro wound models to study bacterial[97] and candidal[98] biofilm infections. Graftskin is made from human neonatal dermal fibroblasts in a type I collagen matrix overlaid with a cornified epidermis (derived from neonatal keratinocytes), while RHE is derived from neonatal foreskin keratinocytes. The use of tissue-engineered skin in this way allows direct visualization of the relationship and spatial distribution between the biofilm and tissue without the need for invasive biopsy sampling of wound patients.

CLINICAL EVIDENCE FOR BIOFILMS IN WOUNDS

Biofilms have been observed in both acute and chronic human wounds. Techniques such as SEM have been used to visualize biofilms in samples taken from chronic wounds. James and colleagues[31] found microcolonies of multiple species of bacteria surrounded by amorphous EPS material in over 60% of the chronic wound samples examined with evidence of biofilms in only one out of 16 acute wound samples. It was also observed that the acute wounds healed comparatively quickly, generally within 2–3 weeks, while the chronic wounds were still open after 2–3 months. This difference in the rate of healing would seem to support the evidence from the in vitro and animal models that biofilms have an important influence on how quickly wounds can heal. This paper is considered the first to identify biofilms in both chronic and acute wounds.

Antibiotic resistance of bacterial biofilms is known to be highly elevated compared with planktonic equivalents. Bacteria such as *P. aeruginosa* are almost impossible to eradicate from chronic wounds using conventional antibiotics, a situation highly suggestive of a biofilm phenotype. Importantly though, it appears that in the early stages of biofilm formation (within the first 24 hours), the biofilm community is more susceptible to selected antibiotics.[99] Once matured beyond this (48 hours), it was shown to become increasingly resistant to antibiotics.

Interestingly, biofilms have also been identified in burn wounds. Kennedy and colleagues[100] used light-electron microscopy and routine microbiology to determine the presence of biofilms in burn wounds and utilized specific stains to detect both microorganisms and EPS. The researchers detected biofilm in the ulcerated area of the burn wound together with a mixed community of microorganisms. The recalcitrant nature of the biofilm was associated with sepsis and the authors suggested that the excision and closure of a burn wound was very important in the reduction of burn wound sepsis.

TREATMENT OF IN VITRO BIOFILMS RELEVANT TO WOUNDS

An array of different methods has been proposed for the removal of biofilms.[26,101,102] Dowd and colleagues[87] set up the LCWB model to evaluate the efficacy of biofilm effectors in inhibiting biofilm formation in vitro. The test isolates included *P. aeruginosa*, *E. faecalis*, and *S. aureus*. The following antibiofilm agents were evaluated for efficacy, xylitol, salicylic acid, erythritol, farnesol, and two proprietary technologies (Sanguitec gels). It was found that 20% xylitol, 10% erythritol, 1,000 mg/mL farnesol, 20 mM salicylic acid, and 0.1% of the Sanguitec gels completely inhibited biofilm formation. Selective inhibition was noted for salicylic acid against *S. aureus* and xylitol against *P. aeruginosa*. Erythritol at 5% inhibited both *P. aeruginosa* and *S. aureus*. Thorn and colleagues[84] investigated the antimicrobial effectiveness of silver (Aquacel Ag) and iodine (Iodozyme) containing wound dressings on preformed biofilms of *P. aeruginosa* and *S. aureus* grown in the flat-bed perfusion biofilm model. It was found that the iodine dressing was more efficacious than the silver dressing on biofilms. Sun and colleagues[86] using their LCWB model evaluated the efficacy effectors that included gallium nitrate and triclosan against biofilms. Both agents showed selective inhibition of *P. aeruginosa* and *S. aureus* biofilms. In another study, Percival and colleagues[85] investigated the efficacy of a silver-containing wound dressing on biofilms of *P. aeruginosa*, *Enterobacter cloacae*, and *S. aureus* grown in a chambered slide model. Using CLSM, it was found that following exposure of the biofilm to a silver dressing for 48 hours, total kill of bacteria within the biofilm

was achieved. Bjarnsholt and colleagues[102] investigated the efficacy of silver on *P. aeruginosa* biofilms. It was found that a concentration of 5–10 μg/mL of silver sulfadiazine eradicated the biofilm, but a concentration of 1 μg/mL had no effect. More recently, it was shown that iodine was a more effective antimicrobial on mature biofilms than silver.[68]

TREATMENT OF IN VIVO WOUND BIOFILMS

Wound bed preparation is considered significant to biofilm prevention and control[103] with sharp debridement, the most clinically and cost-effective way of removing biofilms.[104] Presently used standards of care have been shown to be only marginally effective on biofilms. Consequently, methods such as the systemic and topical application of antibiotics, antiseptics, and physical debridement of both the biofilm and devitalized tissue are being employed and often together.[105] However, biofilm-based wound care (BBWC) management strategies that aid biofilm suppression have been designed and are now increasingly in use with positive clinical outcomes.[106] In a recent retrospective single-center study, Wolcott and Rhoads[106] evaluated the frequency of complete healing in subjects with a chronic wound in a limb with critical limb ischemia when managed using BBWC. One hundred ninety patients were considered for the course of antibiofilm therapy. In total, 77% of the wounds healed completely and 23% were classified as nonhealing. The conclusion drawn from the study was that a BBWC approach significantly improved healing and it was suggested that "effectively managing the biofilm in chronic wounds was an important component of consistently transforming nonhealing wounds into healable wounds."

Antibiofilm treatment principals have also been presented in case studies on two patients.[107] Although the wounds of these patients had differing etiologies, one being a venous leg ulcer and the other an arterial wound, both showed evidence of a biofilm. Both were successfully healed after 3 and 6 months, respectively, using BBWC.

Wolcott and colleagues[99] also investigated the hypothesis that newly formed wound biofilms were more susceptible to antimicrobial treatment. A study in four different biofilm research laboratories using four different models investigated the resistance of biofilms to antimicrobial treatment. The models employed included a drip-flow model, a porcine skin punch biopsy ex vivo model, a mouse chronic wound model, and a clinical longitudinal debridement study. The findings showed that while the biofilm was susceptible to the antibiotics tested in the first 24 hours of treatment, after 48 hours, the biofilm became increasingly tolerant to the same antibiotics. In particular, a significant decrease in resistance of the biofilm to gentamicin was seen for all studies for up to 24 hours. It was concluded in this study that along with serial debridement to remove a mature biofilm, topical antibiotic treatment should follow to treat the remaining immature biofilm that is more susceptible to antibiotics.

As mentioned previously, Davis and colleagues[63] employed the use of a porcine partial-thickness wound model inoculated with *S. aureus*. The wounds were treated with either Mupirocin cream or triple antibiotic ointment after 15 minutes (representation of the planktonic phenotypic state) or 48 hours (biofilm phenotypic state) after initial inoculation of the wound. To visualize evidence of the biofilm, light microscopy, SEM, and epifluorescence microscopy were employed. Both treatments were found to be effective in reducing planktonic bacteria but had reduced efficacy when *S. aureus* was present in the biofilm phenotypic state.

The antimicrobial properties of silver have been reported for many years,[108] with silver-impregnated dressings known to be effective against biofilms. Biofilm-forming isolates show a decreased susceptibility to silver dressings compared with nonbiofilm-forming bacteria and this is supported by a previous findings from a number of studies where the efficacy of silver-impregnated wound dressings was reduced when used to treat biofilms compared with their planktonic counterparts.[109] Presently, there is a high clinical need for a wound dressing to show effectiveness against planktonic and biofilm residing isolates.[110] The effect of silver-containing dressings is known to have varying degrees of success on different genera and species of bacteria isolated from chronic wounds,[111] reflecting species differences in the way in which they interact with silver.[112] However, it is generally acknowledged that silver has the same effect on bacteria regardless of the dressings from which it is delivered.[113] As such, the physical form[108] and the type of silver present[111] are thought to contribute to the effectiveness of the dressings. While data suggest a valuable role for silver dressings as a treatment for biofilm infections, a biofilm infected wound requires a multifaceted treatment approach. This should include thorough debridement and systemic appropriate antibiotics, where antibiotic treatment is tailored specifically to each wound infection[114] together with a rotating topical antiseptic for the more extremely recalcitrant wounds.

Antibiotic resistance of microorganisms within a biofilm can have a significant influence on wound healing in mammalian medicine. When wound isolates are grown in the biofilm phenotypic state, they have been shown to exhibit enhanced tolerance to antibiotics. This tolerance of a biofilm occurs through phenotypic rather than genotypic changes. Many studies have reported the evidence of antibiotic-resistant isolates in biofilms, in particular meticillin resistant *S. aureus*, vancomycin-resistant *Enterococcus*, and multidrug resistant *Acinetobacter baumannii*.[115,116]

Bacteria behave synergistically to promote an environment that supports the development and maintenance of a chronic wound.[56] This could be achieved utilizing bacterial clumping assays[117] and aggregation assays.[118] "Cross-kingdom" interactions between bacteria and fungi also require investigation[119] as these interactions have been shown to be of significance to wound healing. Better understanding of bacterial interactions will aid in the development of novel reatment mechanisms for medical biofilms.

Both chronic and acute dermal wounds are susceptible to infection due to an inherent inability to keep wounds completely sterile, thereby facilitating the development of microbial communities within the wound environment.[65,120–122] Bacteria with a biofilm phenotype are thought to predominate on the surface of wounds and have been implicated in the inability of many wounds to heal. The studies highlighted within this paper provide evidence that biofilms reside within the chronic wound and represent an important mechanism underlying the observed, delayed healing. The reasons for this include both protease activity and immunological suppression. Furthermore, because biofilms inherently resist antimicrobial agents, it is imperative that effective strategies are

developed, tested prospectively, and employed in chronic wounds to support the healing process.[123–124]

To date, there is growing evidence to show the presence of microbial biofilms within wounds. Despite this, research is required to determine the exact role played by multispecies biofilms in terms of delaying the wound healing process. In addition, more therapies and randomized clinical trials are required, as well as better methods for the detection of a biofilm. It is only by understanding the scientific and clinical evidence and role biofilms play in wound healing that development of the next generation of wound treatment strategies for nonhealing chronic wounds can be achieved. Furthermore, it is increasingly apparent that adoption of a biofilm-based management approach to wound care, utilizing the "antibiofilm tool box" of therapies, to kill and prevent reattachment of microorganisms in the biofilm, is producing the most positive clinical outcomes.

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