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A Formidable Foe is Sabotaging Your Results: What You Should Know about Biofilms and Wound Healing

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Abstract

Learning Objectives—After reading this article, the participant should be able to: 1. Describe biofilm pathogenesis as it relates to problem wounds, 2. Understand the pre-clinical and clinical evidence implicating biofilm in problem wounds, 3. Explain the diagnostic and treatment challenges that biofilms create for problem wounds, 4. Demonstrate a basic understanding of emerging strategies aimed at counteracting these processes.

Summary—Biofilm represents a protected mode of growth for bacteria, allowing them to evade standard diagnostic techniques and avoid eradication by standard therapies. Though only recently discovered, biofilm has existed for millennia and complicates nearly every aspect of medicine. Biofilm impacts wound healing by allowing bacteria to evade immune responses, prolonging inflammation and disabling skin barrier function. It is important to understand why problem wounds persist despite state-of-the-art treatment, why they are difficult to accurately diagnose, and why they recur. The aim of this article is to focus on current gaps in knowledge related to problem wounds, specifically, biofilm infection.

Introduction

The Centers for Disease Control estimates that 65% of all human infectious disease is caused by bacteria with a biofilm phenotype and NIH estimates that this number is closer to 80% [1]. Using this information, the collective toll of biofilm infection in the US is estimated at 17 million infections and 550,000 deaths [2]. However, diagnosing biofilm infection is a significant clinical challenge. Definitive diagnosis requires visual confirmation of adherent bacteria encased in extracellular polymeric substance (EPS) [3] using imaging methodologies that are not clinically available. Additionally, biofilm producing bacteria do not grow reliably in culture [4–6]. Thus, current clinical diagnostic tools are insufficient to identify biofilm infection. Polymicrobial infection consistent with biofilm infection is common in chronic cutaneous ulcers (CCU) [7]. CCU are a major public health threat that affect 2% of the population in the US and globally [8]. The estimated cost of caring for the 6.5 million people in the US with CCU is 50 billion dollars per year [9]. Despite the prevalence, cost, morbidity and mortality for patients, there have been no new

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pharmacologic treatments for CCUs approved by the FDA for over 10 years [10]. We posit that one of the reasons that clinical trials have failed and problem wounds remain clinically challenging is occult biofilm infection.

Biofilm Biology and Pathogenesis

The formation of *in vitro* biofilm has been modeled into several stages (Fig. 1) [11]. Initially, planktonic (free floating) bacteria reversibly attach to a surface in a monolayer, followed by irreversible attachment and clonal growth into microcolony aggregates [12] (Video1) (See video, Supplemental Digital Content 1 which reviews the stages of biofilm development, the criteria for diagnosing biofilm infection and has scanning electron microscopy images to show what a biofilm infection looks like. This video is available in the “Related Videos” section of the Full-Text article on [PRSJJournal.com](https://www.prsjournal.com) or available at INSERT LINK HERE.)

This process is mediated by large shifts in gene expression patterns that regulate phenotypic changes in the bacterium. *Pseudomonas aeruginosa* is capable of altering the expression of as many as 800 proteins during this process [13]. The initiation of a biofilm can occur within a matter of hours [14]. Bacteria within the biofilm communicate through a process known as quorum sensing, and collectively secrete a matrix composed of proteins, polysaccharides and extracellular DNA called extracellular polymeric substance (EPS) [15] that encases the microcolonies. A significant portion of the biofilm is comprised of water channels, which function as a complex distribution system for oxygen and nutrients [16, 17]. The biofilm proceeds through maturation phases, where mushroom-like structures develop [18]. Once the environment is no longer optimal for bacterial survival, as in the instance of nutrient exhaustion, bacteria either actively detach and disperse from the biofilm [11, 19] or are detached by fluid shear forces and are separated from the larger structure in matrix-protected aggregates [20].

The criteria proposed by Parsek and Singh to define biofilm infection (Fig. 2) are now widely accepted [3] (Video1). These include (1) adherence of infecting bacteria to a surface (2) direct visual evidence that bacteria are encased in EPS (3) confinement of the infection to a particular location and (4) demonstration of antibiotic recalcitrance despite known susceptibility of the bacteria in the planktonic state. The fact that bacteria in a biofilm state are adherent and confined to a particular location indicates that they will not cause bacteremia or systemic manifestations of infection *while in a biofilm state*. Bacteria that are released from biofilm in a planktonic state can cause bacteremia, so the presence of a biofilm infection is not a benign finding and represents a potential risk of systemic infection. Any claims of biofilm infection or eradication should be held to the standard of meeting the 4 criteria of Parsek and Singh [3].

Clinical Evidence for Biofilm Infection in Wounds

An inciting injury or wound is not required for biofilm infection to develop. Biofilm can form in the context of a number of tissue types in the body including wounds, the respiratory and sinus tracts, gastrointestinal mucosa as well as on implantable and injectable materials. Biofilm is implicated in a number of disease states including implant-associated infections,

otitis media, cystic fibrosis, bacterial endocarditis, infectious nephrolithiasis as well as osteomyelitis and problems wounds [3, 19, 21-24].

The first evidence for biofilm formation in human wounds was described by James *et al* and Bjarnsholt *et al* in 2008 [23, 25]. The former utilized epifluorescent microscopy and scanning electron microscopy (SEM) and observed biofilm formation in biopsies from 30 out of 50 (60%) problem wounds [23]. Bjarnsholt *et al* also provided evidence for biofilm in problem wounds using fluorescence in situ hybridization (FISH) to observe *Pseudomonas aeruginosa* in the form of microcolonies, now known to be characteristic of biofilm [25]. Kathju *et al* subsequently described the presence of biofilm, utilizing confocal laser scanning microscopy and FISH on retained sutures that were removed from a problem, non-healing surgical site infection [26]. Complete debridement and removal of the foreign suture material resulted in resolution of the wound. The same authors also demonstrated that biofilm contributes to wounds associated with mesh infections in the context of abdominal hernia repair [27]. Recently, Elgharably *et al* evaluated the role of biofilm in sternal wound infections occurring after median sternotomy [5] (see video). The authors prospectively enrolled 6 patients with sternal wound dehiscence and 3 patients without infectious complications having repeat sternotomy for elective cardiac surgery. Using SEM and confocal laser scanning microscopy, staphylococcal biofilm infection was detected on the sternal wires of all 6 patients with wound dehiscence and none of the wires from the 3 patients without infectious complications (Fig. 3). Further work has demonstrated evidence for biofilm formation in burns [28], diabetic and venous ulcers [29-32], and malignant wounds associated with breast cancer [33]. It has been suggested that biofilm may contribute to oncologic transformation in the setting of gastrointestinal disease, but has not been investigated in the context of cutaneous transformation (i.e. Marjolin's ulcer).

The Clinical Challenges of Biofilm Infection

Biofilm presents specific challenges for plastic surgeons (Fig. 4) that render biofilm-infected wounds difficult to diagnose and difficult to treat.

Biofilm is Infrequently Detected by Routine Culture

Unlike infection caused by bacteria in a planktonic state, standard clinical culture techniques are insufficient to diagnose biofilm infection [34-36]. Elgharably *et al* confirmed the presence of biofilm infection on sternal wires using SEM and confocal laser scanning microscopy, but of those 6 patients, only 2 had a positive wound cultures that grew staphylococci. The other 4 patients had negative cultures (Table I) [5]. Emerging methods of molecular biofilm detection include nucleic acid amplification techniques, such as PCR, molecular detection of biofilm-associated molecules, such as quorum-sensing molecules, and fluorescent in situ hybridization to detect species-specific bacterial ribosomal DNA, amongst others [37]. Techniques that rely on the detection of bacterial elements alone are not sufficient to detect biofilm formation. For example, PCR may detect the presence of bacterial DNA, but this does not confirm that the bacteria detected are part of a biofilm structure. Hence, microscopic techniques, such as SEM, that directly visualize polymicrobial bacteria present in aggregates and indicates the extent of EPS and biofilm formation on a

surface remain the gold standard to diagnose biofilm infection [37]. Unfortunately, there are no routinely available techniques to diagnose biofilm infection in the clinical setting, and this is an important area for future research and development.

Biofilm Creates Bacterial Recalcitrance to Antimicrobials

Biofilm is clinically problematic because it reduces the susceptibility of bacteria to antimicrobials. This includes topical antimicrobial dressings, such as silver impregnated dressings [6]. There are two mechanisms that contribute to this recalcitrance: antimicrobial resistance and antimicrobial tolerance [38, 39]. Antimicrobial resistance refers to the ability of bacteria to grow despite the presence of antibiotic owing to inherent characteristics of the bacteria [39]. Examples of this include the expression of enzymes that degrade antibiotics, such as beta-lactamases, the expression of drug efflux systems that decrease the intracellular concentration of antibiotics, or through evolved mutations that modify the antibiotic's target. Resistance genes are thought to be more readily exchanged in the biofilm state because of the high concentration of extracellular DNA as well as the biofilm's structural stabilization that facilitates horizontal gene transfer [3].

Antimicrobial tolerance, however, is the predominant mechanism for antimicrobial recalcitrance in the biofilm state [39]. Tolerance refers to the ability of bacteria to avoid cell death despite known susceptibility to the antibiotic owing to the physical state of the bacterium. The slow growth rate of bacteria within biofilm decreases the efficacy of antibiotics that target rapid cell division, such as beta-lactam antibiotics that interfere with cell wall synthesis. Further, biofilms do not simply act as diffusion barriers to antimicrobials. Distinct mechanisms exist that resist antimicrobial action. Macromolecules within EPS, including extracellular DNA, are known to either bind antibiotics and interfere with their function, such as the aminoglycosides [40-42], or provide physical barriers that protect bacteria from antibiotic exposure [43]. However, antimicrobial recalcitrance due to the presence of biofilm is largely a reversible phenomenon and resolves when bacteria return to the planktonic state after biofilm disruption, e.g. following debridement [44, 45].

Biofilm Infection Evades Host Immune Response and Induces Chronic Inflammation

Non-healing wounds contain elevated levels of pro-inflammatory cytokines, proteases and excessive neutrophils. Biofilm have evolved to be directly capable of inducing these changes by manipulating the host immune response. For example, *Pseudomonas aeruginosa* quorum-sensing molecules can directly induce expression of pro-inflammatory cytokines from host cells [46]. Biofilms release planktonic bacteria, lipopolysaccharide (LPS), quorum sensing molecules and other exotoxins, and bacterial DNA into the local environment resulting in recruitment of neutrophils to the wound site[47, 48].

Neutrophils are present in abundance in chronic wounds [49, 50], but they are rendered ineffective by biofilm in multiple ways. Reactive oxidants produced by phagocytic cells do not penetrate the EPS. Further, biofilm also prevents the appropriate clearance of neutrophils by macrophages [13, 51]. Their subsequent aberrant degradation contributes to the release of proteases that are characteristically elevated in problem wounds and which negatively impact healing. Interestingly, some bacteria require the presence of these proteases to

facilitate biofilm formation [52]. The benefit gained from host-immune manipulation is the formation of a parasitic relationship allowing for a sustained growth environment in the non-healed wound and a sustained nutrient source through the influx of immune-induced protein-rich plasma exudate.

Biofilm Recurs After Debridement

In a pre-clinical porcine burn model of biofilm infection with *Pseudomonas aeruginosa*, standard of care debridement performed by a plastic surgeon was insufficient to eradicate biofilm. The authors observed a temporary decline in bacterial burden, however, biofilm can be regenerated by only a few remaining bacteria and infection returned to pre-debridement levels after 48 hours. Moreover, new microcolonies were discovered within deeper tissue layers, post-debridement, raising the concern that sharp debridement might result in inoculation into deeper tissue and persistent infection [6]. Indeed, pathogenic biofilms in tissues appear to be more likely to exist as semi-solid microcolonies within tissue rather than strictly adherent to a wound surface, and are often located deeper within wounds [18, 29, 30, 38]. A direct comparison between different debridement modalities, including sharp debridement, hydrosurgical debridement, or ultrasound-mediated disruption of biofilm, has not been performed to evaluate the efficacy of biofilm eradication and its potential effect on clinical outcomes.

Biofilm Disables Skin Barrier Function

The primary functions of skin include thermoregulation, protection against evaporative water loss and acting as a barrier from pathogenic organisms. Biofilm compromises skin integrity by interfering with skin permeability, and negatively impacts the latter two functions [6]. Despite the fact that wounds may appear closed, it is now evident that gross visual inspection does not reflect the functional integrity of the skin. Transepidermal water loss (TEWL) provides a more objective and functional measurement of skin integrity than visual assessment alone [53]. Elevated TEWL is suggestive of increased skin permeability and potentially impaired function that not only allows egress of water, but can also allow bacteria to penetrate below the skin. Biofilm producing strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, the two most common isolates from burn wounds [54], induce human keratinocyte expression of microRNAs (miR-146a and miR-106b) that downregulate the expression of tight junction proteins zona occludens-1 and zona occludens-2 causing elevated TEWL [6]. Tight junction proteins, along with gap and adherens junction proteins are primarily responsible for skin barrier function in mammals [55, 56].

Biofilm Producing Bacteria Express Proteins That Degrade Soft Tissues

Biofilm producing bacteria are known to secrete ceramidase [57-59], an enzyme that breaks down ceramide, further compromising skin integrity [57]. Ceramides are a major component of extracellular lamellar sheets present in the stratum corneum. They are a key component of the keratinization process in mammalian epidermis and function to maintain the permeability barrier and evaporative water loss functions of the skin [60, 61]. When bacterial infection is in a biofilm form it induces bacterial expression of proteases [62-64]. These bacterial derived proteases can also activate host matrix metalloproteases [63, 65-67] and stimulate neutrophil respiratory burst. The high numbers of neutrophils present in biofilm

infected wounds results in robust release of reactive oxygen species and host elastase that can degrade soft tissue. Thus, biofilm infection will promote tissue erosion. Clinical manifestations of this phenomenon are development of an open wound, such as sinus tracts or pressure ulcers, in areas overlying osteomyelitis due to biofilm infection. The diagnostic evaluation of a problem wound should include assessment for underlying osteomyelitis. Collectively, the inoculation of biofilm producing bacteria into deeper tissues after debridement with biofilm recurrence, the aggressive but dysfunctional immune response, the loss of skin barrier function, and biofilm induced tissue degradation all likely contribute to the high recurrence rate as well as flap and skin graft loss that is observed with problem wounds [68-71].

Therapeutic Strategies

Case Scenario

The patient is a 17 year old male wrestler with no significant past medical history who underwent an orthopedic procedure for a ruptured patellar bursa. Post-operatively, the patient developed a surgical site infection and subsequent non-healing wound (see video). Over the course of five months, the patient had removal of the patellar bursa and two attempts at skin grafting without resolution. Wound biopsies were obtained that demonstrated infection with methicillin resistant *Staphylococcus aureus* (MRSA). However, the wound did not respond to therapy with Bactrim, despite demonstrated sensitivity of the culture. At this point the patient was referred to the senior author for evaluation (Fig. 5). Non-healing wounds of this nature that are refractory to standard therapy are highly suggestive of occult biofilm infection. The patient responded to MRSA decolonization with 5 days of 4% chlorhexidine gluconate showers, 2% mupirocin applied to the nares and wound twice daily, oral Bactrim DS twice daily for 10 days and serial wound debridements in the office.

This simple case highlights two important features of biofilm infection. First, bacteria do not require a compromised host/patient to establish biofilm infection. The capacity of the bacterial strain to produce biofilm can overcome innate immunity even in the healthiest patient. Second, debridement disrupts biofilm and the bacteria revert to a planktonic state [13, 72, 73]. During that time they are susceptible to antibiotics. There is approximately a 48-72 hour window after sharp debridement and biofilm disruption before biofilm infection is re-established [6, 73]. A critical step in the management of problem wounds is to disrupt biofilm infection by debridement and then to take advantage of the therapeutic window by using topical and/or systemic antibiotics to eradicate the infection. Thus, the wound bed must be adequately prepared with debridement and eradication of biofilm infection before it can be closed either surgically or non-surgically.

A novel method to address biofilm is the use of resorbable antibiotic-impregnated beads after debridement. In orthopedic surgery, biofilm represents a formidable challenge in the treatment of open extremity fractures and infected arthroplasties. Antibiotic-impregnated beads, placed into the wound after debridement, have been shown to elute high local concentrations of antibiotics, which can target biofilm much more directly than intravenous antibiotics [74, 75]. They have also been shown to reduce infectious complications in open

fractures [76] and diabetic foot ulcers [77]. Our case series below illustrates the application of this concept to the treatment of pressure ulcers with osteomyelitis.

Clinical Case Series

We have applied resorbable antibiotic beads into pressure ulcers after debridement and right before flap coverage for the past several years. We have compared the pressure ulcer recurrence rates at 12 months between patients who received antibiotic beads, and those who did not. These patients were all treated by a single surgeon (GMG) and received the same pre-operative evaluations, surgical flaps for coverage and the same post-operative care.

In 104 patients who did not receive antibiotic beads, the recurrence rate at 12 months was 39.4%. In 16 sequential patients with focal osteomyelitis and pressure ulcers who received flap coverage with antibiotic beads, the recurrence rate at 12 months was 12.5%. The rate of pressure ulcer recurrence was compared between the two groups using chi-squared analysis, with $p < 0.05$ as a threshold for statistical significance. This difference was statistically significant ($p = 0.037$).

There is one FDA approved antimicrobial wound dressing (Procellera[®], Vomeris Inc.) that does not make therapeutic claims to treat biofilm, but has been shown to inhibit biofilm formation in vitro [78]. This dressing works by generating a 1 volt electric field [79] and electrodynamic forces are known to disrupt biofilm formation [80-83]. There are many other potential therapeutic strategies to address biofilm infections that have recently been reviewed [38]. Areas of research include 1) topical application of agents that interfere with bacterial attachment such as medicinal honey or lactoferrin through iron-sequestration 2) quorum sensing inhibitors such as synthetic furanones or also, medicinal honey 3) lytic bacteriophage therapy, which employs viruses that are naturally destructive for bacteria 4) mechanical biofilm disruption through ultrasound-mediated or surgical debridement. At this time there are no treatment interventions that have level I evidence to support use in wounds and meet the Parsek and Singh criteria for biofilm infection and eradication.

Conclusions

Biofilm poses significant challenges for plastic surgeons. Biofilm disrupts normal wound healing by allowing bacteria to evade immune responses, prolongs inflammation, erodes tissues and disables skin barrier function. It also complicates treatment options by causing antimicrobial recalcitrance and recurrence after debridement. There are no good ways to diagnose it, so treatment is initiated based upon the clinical diagnosis alone. Though only recently described in the setting of CCU and other problem wounds, the impact of biofilm is that it is common to ALL problem wounds regardless of etiology and occult biofilm infection may contribute to unexplained flap or graft loss. Understanding how biofilm affects the wound bed and using debridement and antimicrobial strategies to eradicate that infection will help plastic surgeons achieve better results when managing problem wounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Stages of biofilm development

Transient adhesion to a surface is followed by robust, irreversible attachment and the formation of microcolonies through clonal growth. Bacteria collectively communicate through a process known as quorum sensing to secrete extracellular polymeric substance (EPS) to form a mature biofilm. Biofilm structures can be flat or mushroom-shaped. The last stage is characterized by a return to motility where either individual cells, or cells encased in small aggregates of the EPS material are sloughed. Adapted and modified from Stoodley, P., et al. (2002). "Biofilms as complex differentiated communities." *Annu Rev Microbiol* **56**: 187-209.

Criteria for Biofilm Infection

Infesting Bacteria are Adherent
to a Surface

Direct Examination of Tissue Shows
Clusters of Bacteria Living Within a
Matrix Called Extracellular
Polymeric Substance (EPS)

Infection is Confined
to a Particular Location

Infesting Bacteria Demonstrate Antibiotic
Recalcitrance Despite Known Susceptibility
in the Planktonic State

Figure 2.

Criteria for biofilm infection as described by Parsek and Singh (Parsek, M. R. and P. K. Singh (2003). "Bacterial biofilms: an emerging link to disease pathogenesis." Annu Rev Microbiol **57**: 677-701.).

Figure 3. Scanning electron microscopy (SEM) detection of biofilm infection

Sternal wires were removed from patients undergoing elective sternotomy without wound infection (non-SWI) or with open sternal wounds due to infection (SWI) and evaluated by SEM. Non-SWI wires have patchy coating of proteinaceous strands with no bacteria seen on higher magnification. SWI wires have thick biofilm coating with evidence of bacterial cocci beneath biofilm (see arrow). Panels on left 60X magnification (scale bar= 1mm) and panels on right 10,000X magnification (scale bar= 5µm). Reprinted with author permission from Elgharably, H., et al. (2013). "First evidence of sternal wound biofilm following cardiac surgery." *PLoS One* **8**(8): e70360.

Figure 4.
The clinical challenges posed by biofilm infection.

Figure 5. Non-healing post-surgical wound treated as biofilm infection

Wound shown on presentation to the senior author (left panel) and 4 weeks later (right panel) after treatment with MRSA decontamination protocol and serial office debridements. Wound healed and did not recur.

Table 1

Demographic characteristics of patients (n = 9) and SWI status.

Subjects	SWI	Age	Sex	BMI	Associated medical conditions	Procedure	Antimicrobial therapy	Time interval between procedure and debridement	Wound culture	Blood culture	Data shown in Figure #
SW001	Yes	60	M	34.7	CAD-DM-HTN-HLD-RF	CABG	Nafcillin, Daptomycin	5 weeks	MSSA	N	3A, 4, 6
SW002	Yes	84	F	40.9	CAD-HTN-HLD-RD-COPD	Redo-MVR	Ertapenem	2 weeks	negative	N	6
SW003	Yes	61	F	18	CAD-HTN-HLD-PVD	LVAD	Vancomycin, Ciprofloxacin, Sulfamethoxazol, e-triethoprim, Linezolid	12.1 weeks	No growth	N	7
SW004	Yes	54	M	27	DM-HTN-RD-OSA	CABG	Piperacillin-tazobactam, Vanomycin	3.1 weeks	MRSA	N	3B & 5
SW005	Yes	64	M	25.1	CAD- COPD-HLD-DM	PM-Repair of RV	Piperacillin-tazobactam, Vancomycin, Daptomycin	5.4 weeks	No growth	N	3A
SW006	No	28	M	23.7	END-SEP	excision scar					Not shown
SW007	Yes	43	F	41	HTN P HTN-RHD	MVR	Linezolid	9.2 weeks	No growth	MRSA	6
SW008	No	46	F	50.2	HTN P-OSA-GERD-AKI	LRB					7
SW009	No	31	M	20.7	CGH-SVT	AVR					5

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