Chronic Wound Biofilms: Pathogenesis and Potential Therapies

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Chronic wounds are a growing medical problem that cause high rates of morbidity and mortality, costing the healthcare industry in the United States millions of dollars annually. Chronic wound healing is hampered by the presence of bacterial infections that form biofilms, in which the bacteria are encased in exopolysaccharide (EPS) and are less metabolically active than their free-living counterparts. Bacterial biofilms make chronic wounds more refractory to treatment and slow tissue repair by stimulating chronic inflammation at the wound site. Bacterial species communicate through a mechanism known as quorum sensing (QS) to regulate and coordinate the gene expression that is important for virulence-factor production, including biofilm formation. This review focuses on the relationships between chronic wounds, biofilms, and QS in the virulence of chronic-wound pathogens.

Keywords: bacteria, biofilms, chronic wounds, exopolysaccharide, polymicrobial, quorum sensing

Abbreviations:
EPS, exo-polysaccharide; QS, quorum sensing; CDC, Centers for Disease Control and Prevention; AIs, autoinducers; AI-2, autoinducer 2; AIPs, autoinducing peptide pheromones; AHLs, acyl-homoserine lactones; HSLs, homoserine lactones; PQS, Pseudomonas quinolone signal

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Chronic wounds are a severe, worldwide problem. Wounds are considered chronic when healing fails to proceed normally and the anatomic and functional integrity of the skin is not achieved in approximately 1 month. Vascular insufficiency and infection contribute to nonhealing wounds; infection occurs from microorganism multiplication in the wound bed, leading to a prolonged excessive inflammatory response, delays in collagen synthesis, epithelialization, and tissue damage.1

Chronic wounds include diabetic foot ulcers, pressure or decubitus ulcers, venous leg ulcers, and nonhealing surgical-site infections. The annual incidence of foot ulcers in diabetic patients is 1% to 4% in the United States, with a lifetime risk of occurrence of between 15% and 25%. In 2006, the total cost of treatment, amputation, rehabilitation, and long-term care of diabetic foot ulcers in the U.S. totaled $10.9 billion.2 Approximately 85% of amputations are preceded by these types of ulcers. These figures will increase as the number of diabetes diagnoses is expected to rise.2 Pressure/decubitus ulcers are a common problem in nursing home, rehabilitation clinics and home-care populations; venous leg ulcers affect as many as 1% of the worldwide population.3 Surgical-site infections occur in as many as 5% of procedures and are an increasingly common type of postoperative complication; an average of 0.5% of the total hospital budget in the United States is allocated to manage these infections in affected patients.4

Currently, acute and chronic wounds are treated using a multistep approach known as TIME, as described by Schultz et al.5 First, the nonviable tissues (T) from within and around the wound are removed by debridement. Next, infection and inflammation (I) are minimized by administering antibiotics and anti-inflammatory drugs; then, moisture (M) imbalance is corrected, generally with carefully selected dressings. Last, epithelialization (E) and tissue formation are promoted by the application of specific therapies, such as growth factors.6 Wound cleansing and debridement of chronic wounds have been shown to improve healing rates.6 Debridement can be performed surgically (mechanically removing necrotic...
tissue), enzymatically (using naturally occurring matrix-degrading enzymes such as papain or collagenase), and biologically (using debriding organisms such as maggots). Hyperbaric oxygen can also be used to treat certain types of wounds such as clostridial myonecrosis, necrotizing soft-tissue infections, and selected nonhealing problem wounds. Hyperbaric oxygen stimulates tissue regeneration by increasing oxygen tension to elicit cellular responses such as angiogenesis and collagen production. Increased oxygen has also been shown to inhibit the growth of anaerobes while increasing the respiration of aerobes, which increases the uptake and efficacy of broad-spectrum antibiotics by aerobic respiration.

Despite diligent care given to patients with acute wounds, treatment often fails in chronically infected wounds. One of the major reasons for treatment failure is that acute infections can lead to the formation of biofilms. It has been shown that less than 10% of acute wounds contain biofilm, whereas as many as 60% of chronic wounds produce this barrier to treatment.

Biofilms

A bacterial biofilm is a structured community of microorganisms encased in an exo-polysaccharide (EPS) or exo-polymeric substance, which adheres to an inert or living surface. Biofilms are polymicrobial and may consist of not only bacterial cells but also fungi, viruses, proteins, extracellular DNA, and other biogenic factors. Biofilm growth helps bacteria because it is protective and increases survival in a hostile environment. The majority of bacteria in most natural and pathogenic ecosystems compose biofilms. A hallmark of biofilm producing infections is that they are highly polymicrobial; it is thought colloquially that naturally occurring biofilms are never caused by a single organism. The polymicrobial nature of biofilm increases infection virulence and complicates treatment.

Biofilm Formation

Biofilm formation is dynamic and typically involves the following 5 steps, as outlined by Stoodley et al. The first step is reversible attachment of the microbe to a surface mediated by pili, flagella, or other surface appendages or specific receptors; the second is irreversible attachment mediated by the secretion of EPS. The third step is cell proliferation, resulting in the formation of a microcolony; the fourth step is growth and differentiation, culminating in a mature biofilm community with characteristic structural features such as water channels and towering clusters of cells. The final step is dispersion of biofilm cells, actively or by passive detachment (Figure 1).

The hallmark of a biofilm is its secretion of EPS. Biofilm EPS serves a variety of purposes to the colony, the most important of which is bacterial attachment to biotic or abiotic surfaces. The EPS layer is a type of house in which the bacteria live, which features a strong foundation and offers protection from the outside environment. EPS is highly refractory to penetration by antimicrobial agents and the immune system. Bacterial EPS is also thought to protect biofilm bacteria from desiccation; to assist in ion exchange; to house and maintain degradation enzymes; and to carry nutrients such as carbon, nitrogen, and phosphorous. Fully developed biofilms (stage 4 of development) exhibit characteristic structures such as mushroom towers and water channels that bring nutrients and water to, and waste away from, the lower layers of the biofilm. The production of many virulence factors, including biofilm formation, has been attributed to cell-to-cell communication via quorum sensing (QS).

Mechanisms of Biofilm Virulence

Bacterial cells encased in biofilm EPS are different than free-living, planktonically growing bacteria in that the former are sessile (non-motile) and have reduced...
metabolic activity. This reduced activity increases antimicrobial tolerance because many classes of antibiotics are only effective against actively dividing cells by targeting peptidoglycan produced in the cell wall (β-lactams), protein (aminoglycoside) synthesis, or DNA replication (quinolones). EPS is a mechanical barrier to antimicrobials and immune system cells, which decreases their effectiveness. Stimulation of the immune system without effectively eradicating the infection causes collateral damage to surrounding tissue and chronic inflammation, which aggravates the wound, and further slows the healing process.

Biofilms increase the opportunity for the transfer of antimicrobial-resistance genes carried on mobile genetic elements, such as plasmids. Genetic transfer can occur between bacteria of the same species or among cells of different species, which increases the potential for virulent and persistent infections. Acquisition of antibiotic-resistance genes causes irreversible genotypic changes in the bacteria (with the exception of resistance genes harbored on mobile genetic elements). Antibiotics are able to eradicate susceptible bacteria that have not acquired resistance genes; however, once antibiotic use is suspended, the remaining cells can cause a recurrent infection with microbes that retain antibiotic resistance.

Distinct from antibiotic resistance is the issue of antimicrobial tolerance unique to the biofilm environment. Antibiotic tolerance is a transient phenotype that is highly refractory to antimicrobial therapy, allowing a subpopulation of cells, termed persister cells, to be maintained in the wound environment. Tolerance is not mediated by acquisition of genetic modifications but rather is thought to result from metabolically inactive biofilm cells. When antimicrobial therapy is suspended, remaining persister cells can regenerate the biofilm with a microbial population that retains a similar susceptibility profile as the original biofilm, and so persister cells are maintained.

A vital step in wound care is to surgically remove necrotic issue and microbial bioburden by debridement. Bacterial EPS promotes strong attachment of the biofilm to the wound bed, which makes full debridement difficult. Despite having most of the biofilm mass removed by debridement, a small amount of cells remains tightly attached to the wound bed, allowing remaining cells to regenerate the biofilm, leading to a high rate of recurrent infections commonly associated with biofilms.

**Biofilm Susceptibility Testing**

Clinical microbiology uses several isolated colonies of a single bacterial species from an infection, determining the organism identification and antimicrobial susceptibility. The discovery of biofilms has revealed that a number of infections, particularly chronic wounds, are polymicrobial. A bacterial species in polymicrobial infections can have a different and higher antibiotic susceptibility—a phenomenon known as *antimicrobial synergism*—than that it would have if that species was causing an infection by itself. Also, current methods of antimicrobial susceptibility testing are performed on free-living, metabolically active planktonic bacteria, whereas biofilm bacteria are sessile and relatively inactive. This can lead to misleading results from current antimicrobial-susceptibility testing methods. In a study performed by Keays et al., 60% of bacterial isolates tested were treated with antibiotic combinations that successfully inhibited all planktonically grown bacterial isolates. However, when the same isolates were grown as biofilms, only 22% of all biofilm-grown isolates remained susceptible to antibiotics. For chronic wounds, a new method of antibiotic susceptibility testing is needed.

Many in vitro biofilm experimental models have been developed. These models are now generating interest in the clinical microbiology community for biofilm-susceptibility testing (Table 1). These methods promote biofilm growth by supporting bacteria that adhere to a surface while washing away their planktonic counterparts. Bacteria can be grown statically (microtiter plate, Lubbock chronic wounds biofilm), resembling the conditions observed in fixed, chronic biofilm infections, or under dynamic, moving conditions (Centers for Disease Control and Prevention [CDC] biofilm reactor, drip-flow reactor) to mimic infections subjected to blood flow, such as endocarditis. Using these methods, investigators can more effectively study polymicrobial infections; the resulting bacteria are much more representative of biofilm infections, making susceptibility profiles more representative of what occurs in chronic wounds.

Most studies examining the efficacy of biofilm susceptibility testing versus traditional planktonic susceptibility testing have been performed in orthopedic infections; comparative studies involving *Pseudomonas aeruginosa* have been performed in patients with cystic fibrosis. In these studies, patients treated with antibiotics
The device is a lid with 96 pegs that fit into an inclined chamber continuously drips nutrient medium onto a microscope slide; biofilm is grown on the slide. The medium runs down the surface of the slide, allowing for dynamic growth.

Biofilm grows statically within wells of the 96-well microtiter plate; biofilm grows statically on the pegs of the 96-well microtiter plate. An inclined chamber continuously drips nutrient medium onto a microscope slide; biofilm is grown on the slide. The medium runs down the surface of the slide, allowing for dynamic growth.

A pipette tip can be used as a scaffold for biofilm in a test tube, growing statically. A pipette tip can be used as a scaffold for biofilm on the slide. The nutrient medium runs down the surface of the slide; biofilm is grown on the slide. A pipette tip can be used as a scaffold for biofilm in a test tube, growing statically.

Quorum Sensing

Gram-positive and Gram-negative bacteria use QS to accomplish cell-to-cell communication. This communication is mediated by small molecules that can pass freely through membranes or through a membrane channel or protein. This process depends on cell density; when a species of bacteria reaches a critical population mass, the signaling molecules are produced at high enough concentration to alter gene expression, with as many as 5% of the genes in a given genome potentially affected by QS systems. This allows bacteria to coordinate their activity based on population size. Many proteins and virulence factors produced by bacteria are differentially expressed in response to QS, including the genes responsible for biofilm formation.

QS signaling has been discovered in more than 100 microbial species and is associated with dozens of different receptors and effector molecules. QS systems consist of constitutively expressed signal molecules, or autoinducers (AIs), and a corresponding receptor that regulates gene expression when signal concentration has reached threshold concentration. AIs passively diffuse between cells to activate intracellular receptors, which alter gene expression directly, or are actively pumped out of cells to bind to transmembrane receptors (usually 2-component systems), resulting in signal transduction and downstream changes in gene expression (Figure 2).

The universal QS molecule, termed the autoinducer 2 (Al-2), is produced by more than 50 species of gram-positive and gram-negative bacteria and can influence numerous activities such as bioluminescence, virulence-factor production, and exoprotein secretion. E. coli and Salmonella spp. are the model organisms for Al-2 QS. Al-2 is produced by the las genes in E. coli, which positively mediates QS and directly increases biofilm production. Gram-positive bacterial QS systems generally use small peptides, termed autoinducing peptide pheromones (AIPs), as signaling molecules. These AIP peptides are actively exported from the cell and bind to transmembrane receptors or intracellular regulatory proteins, which regulate the transcription of target genes. An example is the accessory gene regulator (arg) QS system of Staphylococcus aureus.

QS in Gram-negative bacteria is controlled by homologs of 2 regulatory proteins, LuxI and LuxR. LuxI is an autoinducer synthase, which produces AIs, and LuxR is the transcriptional activator protein that, once bound to AI, promotes transcription of downstream genes in neighboring bacterial cells and itself. AI small-chemical signals in Gram-negative bacteria are termed acyl-homoserine lactones (AHLs) and are synonymous with homoserine lactones (HSLs). The model organism for Gram-negative QS is Pseudomonas aeruginosa, an opportunistic pathogen that is one of the most commonly isolated organisms in chronic wounds.

P. aeruginosa has two 2-component systems, LasI/R and RhlI/R, as well as a third, quinolone-based, system, that utilizes the Pseudomonas quinolone signal (PQS). Many P. aeruginosa exoproducts that are thought to induce virulence and pathogenesis, including staphylocycin, elastase, pyocyanin, and rhamnolipids, as well as genes associated with biofilm formation, are controlled by QS.

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**Table 1. Examples of In Vitro Biofilm-Grown Models Used in Research**

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>96-well microtiter plate</td>
<td>Biofilm grows statically within wells of the plate</td>
</tr>
<tr>
<td>Calgary biofilm device</td>
<td>The device is a lid with 96 pegs that fit into the wells of a standard microtiter plate; biofilm grows statically on the pegs</td>
</tr>
<tr>
<td>Drip-flow reactor</td>
<td>An inclined chamber continuously drips nutrient medium onto a microscope slide; biofilm is grown on the slide. The medium runs down the surface of the slide, allowing for dynamic growth</td>
</tr>
<tr>
<td>Lubbock chronic wound biofilm model</td>
<td>Biofilm is grown statically in a test tube in a very rich medium containing nutrient broth, plasma, and red blood cells. A pipette tip can be used as a scaffold for biofilm</td>
</tr>
<tr>
<td>CDC biofilm reactor</td>
<td>Apparatus that contains chambers submerged in a vessel; growth media is continuously mixed and poured through the chambers, creating dynamic growth of biofilm</td>
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</tbody>
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CDC, Centers for Disease Control and Prevention.

determined from biofilm antimicrobial susceptibility have improved clinical outcomes compared with traditional susceptibility testing. These protocols require standardization and further testing in clinical settings. However, they seem to reveal superior and more representative antibiotic-susceptibility patterns, which can improve treatment efficacy of biofilm-associated infections such as chronic wounds.

QL, Elastase, pyocyanin, and rhamnolipids, as well as genes associated with biofilm formation, are controlled by QS.

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QS has been shown to be important during all 5 stages of biofilm development; however, the specific QS-controlled stages differ between microbes that use different mechanisms of QS. QS was first linked with biofilm formation in 1998 by Davies et al,\(^41\) who showed that \(P.\) \(aeruginosa\) QS-negative mutants formed extremely weak biofilms with altered architecture and were not as resistant to treatment as biofilms made by QS-competent \(P.\) \(aeruginosa\).\(^41\) \(P.\) \(aeruginosa\) contains at least 39 genes that are regulated by QS systems.\(^42\) QS-deficient mutants of \(P.\) \(aeruginosa\) are highly attenuated and less virulent in mouse pneumonia\(^43\) and burn\(^44\) models, presumably because they cannot communicate or make robust biofilms. QS has also been linked to biofilm formation in bacterial species other than \(P.\) \(aeruginosa\), such as \(S.\) \(aureus\),\(^45\) \(Vibrio\) \(cholerae\),\(^46\) and \(S.\) \(pseudomonas\).\(^47\)

**Development of Antibiofilm Therapies**

A major hallmark of biofilm is the thick layer of EPS that protects the infecting microbial population from the immune system and antimicrobials. An area of current therapeutic research is the development of agents that degrade the EPS layer so already-developed antimicrobials will be effective. These dispersing agents show promise as a future therapy to be used in combination with antibiotics. An example is the enzyme β-amylase, an enzyme produced by oral bacteria that break down polysaccharide bonds; this compound has been reformulated as a dispersing agent to target the polysaccharide bonds of the EPS with in vitro success in degrading biofilm.\(^48\) Other EPS matrix-degrading enzymes include deoxyribonuclease I, a glycoside hydrolases dispersin B,\(^48,49\) and DNAse.\(^50\) Synthetic dispersing agents have also been developed, such as 2-aminoimidazole, that show activity against \(S.\) \(aureus\) biofilms.\(^51\)

QS is considered to be important for the transition between antimicrobial-sensitive planktonic cells to antimicrobial-resistant aggregates of biofilm cells. Researchers have developed agents designed to inhibit or prevent QS, with the strategy that if QS does not occur, biofilms will not be formed, and the bacteria will remain sensitive to therapy. Some examples of these QS inhibitors include furanone, which has been effective against \(P.\) \(aeruginosa\),\(^52\) \(Streptococcus\) \(mutans\),\(^53\) \(E.\) \(coli\), and \(Salmonella\) \(spp\),\(^54\) thiophenone, which shows activity against \(Staphylococcus\) \(epidermidis\),\(^55\) and \(S.\) \(aureus\) virulence inhibitor (savarin).\(^56\)

Another approach to antibiofilm therapy is to use diagnostic procedures based in molecular biology, instead of culture-based methods, for the bacterial species in chronic wounds.\(^57\) Molecular-pathogen diagnostic applications allow comprehensive evaluation of the microbial bioburden in chronic wounds, which leads to a more comprehensive and targeted therapeutic approach to wound care. Molecular diagnostics of these wounds use a rapid screen of known, common bacterial genes (including selected known resistance markers) to diagnose common wound pathogens or general bacterial primers, such as ribosomal 16S, to sequence all bacterial species in a specimen and detect species that are difficult to grow or that represent only a small fraction of the infecting bacteria.\(^58\) Rhoads et al\(^58\) demonstrated that molecular diagnostics detect organisms that do not commonly grow under standard wound-culture techniques (especially anaerobes) and that the most common organisms detected via molecular diagnostics versus traditional culture are often extremely different.\(^58\) For example, in decubitus ulcers, culture-based diagnostics detected \(Enterococcus\) \(spp\) most frequently, whereas molecular-based diagnostic methods detected \(Corynebacterium\) \(spp\) most frequently. Also, for trauma/abscess wounds, culture methods most commonly reported no growth, whereas molecular-based methods detected \(Staphylococcus\) \(spp\) most frequently. Of the 105 specimens tested, culture and molecular results agreed only 63% of the time, and in 13% of culture-negative specimens, bacteria were detected via molecular methods.\(^58\)
Completeness of chronic wound healing can differ among treatment methods. In a study performed by Wolcott et al., 20 48.5% of patients with chronic wounds treated based on traditional culture-based methods healed completely, versus 62.4% of patients with chronic wounds who were treated based on molecular diagnostics and were fully healed; the latter patients also had significantly shorter healing times. Additionally, in response to implementing molecular diagnostics, use of first-line antibiotics declined in lieu of targeted antibiotics, a strategy that has the potential to attenuate antibiotic resistance. 20 Topical antibiotics, based on the results of molecular diagnostics, have also showed increased effectiveness in wound care. In a study performed by Dowd et al., 29 culture-driven standard-of-care treatments were compared with a commercially available treatment guided by molecular diagnostics (instead of culture) and personalized topical therapy developed in a compound pharmacy specific to the microbial burden of each patient, as determined by molecular diagnostic testing. A total of 90.4% of patients who received personalized topical therapies experienced complete healing of their wounds and had more than 200% improved odds of healing compared to patients receiving care based on other protocols. 59 The results of these studies demonstrate that personalized topical and molecular diagnostic strategies yield statistically improved outcomes for patients afflicted with chronic wounds and that these protocols provide direct, targeted approaches to wound care.

Conclusions

Interest is growing in the clinical microbiology community for adapting established biofilm analysis techniques used in research to determine antimicrobial susceptibility patterns of biofilm bacteria for use in chronic wound treatment. Preliminary data suggest that using these adapted methods to determine antimicrobial susceptibility will increase treatment efficacy. Antibiofilm research has generated many avenues of potential novel therapies, including QS inhibitors and dispersing agents, as well as the use of molecular diagnostic techniques to improve current therapies. Combining biofilm research with therapeutic development has the potential to significantly increase the ability of healthcare providers to effectively treat biofilm infections.  LM

References


