Biofilms and Wounds: An Overview of the Evidence

Steven L. Percival,[1–3,*] Sara M. McCarty,[1] and Benjamin Lipsky[4,5]

[1]Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, United Kingdom.
[2]Surface Science Research Centre, University of Liverpool, Liverpool, United Kingdom.
[3]Scapa Healthcare, Manchester, United Kingdom.
[4]Division of Medical Sciences, University of Oxford, Oxford, United Kingdom.
[5]Department of Medicine, University of Geneva, Geneva, Switzerland.

Steven L. Percival, PhD

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*Correspondence: Surface Science Research Centre, University of Liverpool, Liverpool, United Kingdom (e-mail: biofilms@hotmail.co.uk).

Significance: Microorganisms can exist both in the planktonic and biofilm state. Each phenotypic state has a role to play in delaying healing and causing infections of both acute and chronic wounds. However, the virulent biofilm state is the fundamental reason that chronic wounds do not heal in a timely manner. We hypothesize that because microorganisms attach to any surface, biofilms can be found in all chronic wounds. However, it is not the biofilm *per se* that represents the greatest obstacle to the healing of a chronic wound, but its virulence and pathogenicity.

Recent Advances: Numerous studies with animals and humans have identified biofilms in wounds. In particular, these studies have highlighted how biofilms impede host fibroblast development, inflammatory responses, and the efficacy of antimicrobial therapy. Despite this, the role biofilms play in affecting the healing of wounds is still vigorously debated.

Critical Issues: Clinicians must understand the role that *pathogenic* biofilms play in impairing the healing of chronic wounds and in increasing the risk for wound infection, with its potentially catastrophic outcomes. The composition of the biofilm, its physiochemical properties, the climaxed indigenous microbiota and their virulence/pathogenicity, microbial numbers and the host's pathophysiology, and immunological fitness will govern the sustainability of a *pathogenic* biofilm in a wound and its resistance to interventions.

Future Directions: Establishing which specific *pathogenic* biofilms delay wound healing should help guide better wound care practices.

SCOPE AND SIGNIFICANCE

Microorganisms can exist in both the planktonic (free-living) and biofilm phenotypic states, with the latter being predominant in all medical and natural environments. Both phenotypic states may play an important role in impairing healing and causing infection of both acute and chronic wounds. However, it is the biofilm phenotypic state that is more fundamental in preventing chronic wounds from healing in a timely manner. As all microorganisms are able to attach onto any surface, we propose that it is not just the biofilms *per se* that are present in all chronic wounds that retards healing, but the presence of *pathogenic* biofilms. This article will highlight the fundamental aspects of microbial biofilms and provide an overview of the scientific and clinical evidence for the role of biofilms in both animal and human wounds, including their effect on preventing or delaying the healing of wounds.

TRANSLATIONAL RELEVANCE

In all environments microorganisms naturally exist in at least one of

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two main states. These include a free-living or planktonic state, and an attached or sessile state, in which microorganisms attach to each other (often referred to as coaggregating or flocculating). The surface to which microorganisms attach does not have to be a solid; it can be an attachment of microbes between two immiscible liquids or of microorganisms at a gas–liquid interface. A third state exists, in which microorganisms survive in a quasi-sessile state; this is often referred to as the reversible adhesion or transient state.[1]

Planktonic microorganisms can exist in a wide variety of fluids, including effluent, oil, water, urine, and tissue purulence. However, microorganisms have been shown to have a preference to attach to each other, forming what are called biofilm aggregates. An example of this includes mucus specimens from patients with cystic fibrosis, where biofilm grow as aggregate bacteria within the mucus, akin to slough within a chronic wound.[2] Biofilms are defined as a community of microorganisms that are attached to a surface, or a group of microorganisms themselves forming microbial aggregates, that are encased within an extracellular matrix (ECM) of polysaccharides, proteins, and glycoproteins, referred to as the extracellular polymeric substance (EPS). Within the attached state, microorganisms have the ability to create an environment conducive for their protection and longevity.[3]

A biofilm is perhaps best understood as having the characteristics of a multicellular organism. Whilst every biofilm is unique, they have certain fundamental characteristics or traits. For example, a biofilm has channels of fluid running through it, similar to a circulatory system; it responds to outside and internal responses, like a nervous system, and it displays responses that may be called altruistic. Consequently, a *pathogenic* biofilm is an entity that presents a challenge for eradication by the host immune system and by chemotherapeutic agents.[4,5] A pathogenic biofilm is one that is upregulated genetically and biochemically when compared with the more *dormant and relatively benign* mature biofilm. Examples of relatively benign or commensal biofilms include those found on the skin or in the gastrointestinal tract. Benign or commensal biofilms protect the human body from infection and disease, i.e., colonization resistance. However, benign biofilms can revert to pathogenic or virulent biofilm. When compared with benign biofilms, pathogenic biofilms have significantly higher numbers of upregulated genes, which lead to excessive development of degrading enzymes (matrix metalloproteinases), enhanced development of EPS, enhanced generation of quorum-sensing molecules, enhanced microbial proliferation, and microbial dissemination. The enhanced genetic and biochemical effects within a pathogenic biofilm lead to the upregulated immune responses that in turn lead to chronic inflammation. Each form of biofilm requires different management strategies for eradication.

CLINICAL RELEVANCE

All open wounds, because they lack the protective covering of skin, contain microorganisms from endogenous (the patient's own flora) or exogenous sources. In the early stages of the formation of a chronic wound, these microbes are generally held in check or destroyed by the host's immune system. However, if microbes attach to the wound surface and proliferate, a biofilm will begin to develop. When the biofilm is well established, it will exhibit resistance to destruction by the host immune system and antimicrobials. At this stage, the biofilm is considered mature and more difficult to eradicate. When this occurs, the wound is defined as being in a biofilm infected state.[6] In this state, it is difficult to kill microorganisms and treatment will require specialized management practices. In these situations the risk of a wound not healing and becoming overtly clinically infected (*i.e.*, showing signs of inflammation or purulence) is increased. Consequently, preventing a biofilm in the first place is fundamental for faster and more effective treatment of chronic wounds.

BACKGROUND

Human beings at birth quickly become colonized with microorganisms. These companion or indigenous microorganisms can often be associated with the human host for life, and generally serve a symbiotic or mutualistic role. Specifically, these flora can provide an important mechanism of protection through colonization resistance,[7] in which the harmless human indigenous microflora assist in preventing the attachment of problematic, potentially pathogenic microorganisms, thereby reducing the host's risk of infection. Whilst the indigenous microflora protects the host, including on the skin, it also has the capability to cause infection. This generally occurs when the microflora colonize regions of the body in which they are not indigenous, or when they gain entry to the subcutaneous tissues.

All types of surfaces can support the attachment of microorganisms,[8] including inanimate as well as biotic surfaces, such as the skin, mouth, and gut. When microorganisms attach to a surface they exist in either reversible or irreversible adhesive

states. In both, adhesion is facilitated by a number of attachment appendages found on the microbial surface, such as fimbriae and pili. A large number of microorganisms have evolved with an array of different mechanisms that support adhesion and different methods to form biofilms. The first of the microorganisms to attach to a surface, called pioneers, are a selective group of microbes able to initiate the development of a biofilm. The pioneering microorganisms then begin to secrete EPS that creates a protective environment for the residing microorganisms. The EPS, as discussed earlier, is composed principally of polysaccharides, proteins, glycoproteins, lipids, metal ions, and extracellular DNA.[9,10] The specific composition and structure of EPS has an effect on the physiochemical characteristics of the biofilm and determines its structure and architecture, and thereby its resistance to antibacterial defenses.[10] Microorganisms within a biofilm become much more tolerant to both antimicrobial agents and the host immune cells.[5]

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

The development of a biofilm in a wound proceeds in a series of complex, but discrete and well-regulated steps. However, the exact mechanisms involved in this process differ among genera and species. Nevertheless, the stages of biofilm development are similar across a wide range of circumstances and types of microorganisms.

The sequence of biofilm development in wounds can be divided into three major stages:

(1) Microbial attachment to the wound surface.
(2) EPS production and growth of microbes forming microcolonies.
(3) Maturation and dissemination of microbial cells.

For attachment to occur, microorganisms require a surface. Once attached to a surface (abiotic or biotic–Stage 1), the microorganisms begin to produce EPS that enhances and secures their attachment. The EPS also helps to attach the microbes to each other and may provide a source of nutrients for growth and proliferation. The pioneering or primary colonizing microbes alter the microenvironment in a way that aids in the recruitment of secondary and tertiary colonizing microorganisms. As the microbial cells on the wound surface divide, they develop into cell clusters or a microcolony (Stage 2). Enhanced production of EPS by the resident microbes occurs, which helps to embed the aggregating microbes even further within the biofilm.

The evolution of the biofilm's architecture and its indigenous microbiota is constantly under flux and is constrained by the biochemical interactions, hydrodynamics, and nutrient availability in the vicinity of the biofilm. As the biofilm develops, the microorganisms within it remain in constant communication, a process known as quorum sensing (QS). This communication helps to further coordinate the developing biofilm's architecture, microbial growth rates, enzyme production, species interactions, toxin production, antimicrobial resistance, and bacterial virulence at the wound site. In addition, because of the many outside perturbations, the biofilm initially remains both structurally and metabolically heterogeneous.

Over weeks to months, a quasi-steady state evolve in the developing biofilm. As it becomes more complex, three-dimensional mushroom-shaped structures begin to develop (Stage 3) within *in vitro* biofilms. Adjoining microcolonies are connected by water channels that serve as a primitive circulatory system for delivery of nutrients and removal of wastes. The biofilm develops as a patchy network over the surface of the wound and may be only tens of micrometers in thickness. The host products that are available during biofilm formation act to scaffold the developing biofilm and also help to enhance colonization with more microorganisms. The climaxing microbiology formed in the developing biofilm will constitute ~10–20% of the biofilm's volume, with the rest being composed of EPS.[10]

To colonize new surfaces, microbial cells within the biofilm must detach and disseminate. Dispersal of microbes from the biofilm may occur by shedding, detachment, or shearing. Changes within the biofilm cause clumps containing thousands of microbes that reenter the exudate within the wound bed. These released cells contain the characteristics of the mother biofilm, thus retaining the characteristics of a biofilm before reforming on a new or existing surface.

The ability of the host to control the growth of microorganisms in a wound decreases as the biofilm community matures. Within a stable climax biofilm community, there are interactions between aerobic and anaerobic bacteria and yeasts. These likely increase the net pathogenic effect of these microorganisms, enhancing their potential to cause infection and delay healing. Mixed communities of organisms, in addition to having a direct effect on wound healing through the production of destructive enzymes and toxins, may indirectly affect healing by promoting a chronic inflammatory state.[11] This results in the release of free radicals

and numerous lytic enzymes, which could have a detrimental effect on cellular processes involved in wound healing. Proteases released from a number of microbes are known to adversely affect growth factors and other tissue proteins that are necessary for the wound healing process. The increased production of exudate that often accompanies the enlarging microbial load has been associated with the degradation of growth factors and matrix metalloproteinases (MMPs), which subsequently affects cell proliferation and wound healing.[12]

Evidence for biofilms in wounds

Biofilm evidence–animal studies. Many articles reported evidence of biofilms growing on foreign bodies in the wounds of animals in the early 1990s,[13] but it was not until 1996 that the first report presenting evidence of biofilm in a wound was published. In this study with a mouse model,[14] *Staphylococcus aureus* was inoculated onto a wound cut in the mouse's skin. In biopsies taken from the wound after 3–60 h following inoculation, *S. aureus* was found around the wounds, with clusters of cells (characteristic of a biofilm) evident after only 6 h. The authors reported fibril-like structures around *S. aureus* cells after only an hour and that *S. aureus* was enclosed in a membrane-like structure at 3 h. Clearly, this represented EPS, as it stained positively with ruthenium red. Thus, the authors confirmed that *S. aureus* formed biofilms in the dermal and subcutaneous tissues.

In 2000, Rashid *et al.*[15] observed biofilms on a murine burn. Later, Serralta *et al.*[16] discovered evidence of a potential biofilm on an experimental porcine acute wound. The investigators made partial thickness wounds on the backs of three pigs and challenged them with *Pseudomonas aeruginosa*. Coverslips were placed over the wounds and after 72 h, nonadherent bacteria were dislodged by flushing. Staining with Congo red revealed an amorphous EPS matrix surrounding the bacteria, indicating that biofilms formed in wounds. In a more recent study, Davis *et al.*[17] used a porcine partial-thickness wound model to demonstrate the ability of *S. aureus* to form biofilms within the wound environment. They inoculated experimental wounds with a *S. aureus* wound isolate and after 48 h observed microcolonies encased in an ECM on the surface of the wounds using light microscopy, scanning electron microscopy (SEM), and epi-fluorescence microscopy. Furthermore, antimicrobial studies revealed that these biofilm communities possessed increased antimicrobial resistance when compared with their planktonic counterparts.

In 2007, Schaber *et al.*[18] demonstrated the biofilm forming capacity of *P. aeruginosa* in a thermally injured mouse model. *P. aeruginosa* formed biofilms within 8 h of infection, suggesting that biofilms contribute to bacterial colonization in acute infections as well as in chronic infections. A range of visualization techniques, including confocal scanning laser microscopy, transmission electron microscopy, SEM, and fluorescence microscopy were used to identify biofilm formation *in situ* in tissue sections of infected wounds. This study followed the earlier study by Rumbaugh *et al.*,[19] which demonstrated the rapid proliferation of a *P. aeruginosa* strain in mice with full-thickness, third-degree scald burns.

A further study by Schierle *et al.*[20] used a murine cutaneous wound system to demonstrate that *S. aureus* or *Staphylococcus epidermidis* biofilms delayed reepithelialization of the wound. Biofilms were characterized as aggregates or microcolonies of bacteria. Later, Kanno *et al.*[21] developed full-thickness wounds on the backs of rats and inoculated them with *P. aeruginosa* carrying the green fluorescent protein gene. Taking the histological immunohistochemistry measurements at various intervals over 7 days, they found that biofilms had developed within 8 h. The biofilms were confirmed by fluorescent microscopy as microcolonies of cells.

The use of experimental diabetic mice and rats in the study of impaired wound healing is common, given that many wound healing parameters are defective in these animal models. Zhao *et al.*[22] created a reproducible chronic wound model in diabetic mice by placing 6 mm punch biopsy wounds on the dorsal surface of the mice, then challenging the defects with *P. aeruginosa* (PA01) biofilms 2 days post wounding. None of the biofilm-challenged wounds closed, demonstrating that biofilm significantly delayed wound healing compared with the control non-biofilm-infected wounds.

Gurjala *et al.*[23] created dermal punch wounds in rabbit ears, to which they applied green fluorescent protein labeled *S. aureus*. Using epi-flourscence and SEM, the *S. aureus* was observed to form biofilms within 24 h after inoculation, as characterized by evidence of microcolonies. Measuring serum markers confirmed that the biofilm created a low-grade inflammatory response with the biofilm, impairing epithelial migration and granulation tissue ingrowth, thereby impairing wound healing. Seth *et al.*[24] also used a rabbit ear model to investigate the effects of biofilms on healing by comparing wounds inoculated with *P. aeruginosa*

with those left uninfected. The uninfected controls healed quicker than the *P. aeruginosa* infected wounds, which were confirmed to be in a biofilm by the presence of microcolonies or aggregates of cells, as shown with SEM. Seth *et al.*[25] also used a rabbit ear model to study the effect of *Klebsiella pneumoniae* on wound healing, confirming biofilm as cellular aggregates by SEM. Later, Seth *et al.*[26] developed dermal punch wounds in white rabbit ears and inoculated them with *K. pneumoniae, S. aureus,* or *P. aeruginosa*. They determined virulence of the biofilm using histological and inflammatory markers and used SEM and bacterial counts to visualize the biofilm and determine its viability. Wound healing in those infected with *P. aeruginosa* biofilm was impaired compared with uninfected wounds. Furthermore, they reported that an EPS-deficient *P. aeruginosa* strain had less of an adverse effect on healing than the wild strain. Thus, different bacteria possess different levels of biofilm virulence, with the EPS component of the biofilm forming an integral part of the biofilm's pathogenicity.

Roche *et al.*[27] studied the development of biofilms on partial-thickness murine wounds infected with methicillin-resistant *S. aureus* (MRSA). Dense biofilm aggregates developed at the wound surface within 24 h following inoculation, as confirmed by microscopy. A further study by Roche *et al.*[28] used a porcine model to determine the effect of MRSA on full-thickness wounds. By taking wound biopsies, they noted that passaged strains resulted in a greater delay in healing compared with the parent MRSA. Both strains had evidence of biofilms, identified as dense microcolonies using microscopic techniques. The passaged MRSA resulted in more frequent bacterial colonies, which occurred in a concatenated structure.

Zhao *et al.*[29] further confirmed the presence of biofilms and their effect on wound healing using infection with a *P. aeruginosa* PA01 strain in a db/db mouse model. *Pseudomonas* biofilms were transferred onto a 2 day old wound that was created on the dorsal surface of mice, and none of the biofilm-infected wounds healed after 4 weeks. However, after 6 weeks 64% of the biofilm-infected wounds healed and all biofilm-infected wounds healed by 8 weeks. Although biofilms delayed wound healing, the wounds did eventually heal.

Watters *et al.*,[30] using an *in vivo* diabetic mouse chronic wound model, investigated the effect of a *P. aeruginosa* biofilm on healing. As noted by others, *P. aeruginosa* biofilm impaired healing. Of note, they found that the prevalence density of the *P. aeruginosa* biofilm increased when the mice were treated with insulin, suggesting that the diabetic wound environment may promote the formation of a biofilm.

Nguyen *et al.*[31] evaluated host responses in biofilm-infected wounds using the TallyHo mouse model of type 2 diabetes. They noted that these diabetic biofilm-containing wounds had significantly less *TLP* 2, *TLR* 4, interleukin-1 beta, and tumor necrosis factor-alpha than wild type wounds with biofilm and less neutrophil oxidative burst activity.

Thompson *et al.*[32] reported on an excisional murine wound model to investigate the effect of a multidrug resistant *Acinetobacter baumannii*. A 6 mm diameter full-thickness wound was created and inoculated with *A. baumannii*. Amongst bioburden and other tests, the wound was assessed for biofilms using SEM and PNA-FISH. In a study designed to explore the role of QS and *P. aeruginosa* biofilm in a pressure ulcer model in rats, Nakagami *et al.*[33] found that day 3 postwounding, the biofilm formation was immature in QS-deficient strains, with a lack of dense bacterial aggregates and EPS. The immature biofilm did, however, induce inflammation in the early development phase. Asada *et al.*,[34] using *P. aeruginosa* to study wound colonization (biofilms) and wound infections in 6 month old rats, also concluded that biofilms delayed wound healing. Trostrup *et al.*[35] developed a chronic *P. aeruginosa* biofilm infection model in C3H/HeN and BALB/c mice. They induced third-degree thermal lesions with full-thickness skin necrosis and infected them with *P. aeruginosa*. PNA-FISH and DAPI staining revealed bacteria within a cluster formation, identified as biofilm. Furthermore, the *P. aeruginosa* biofilm induced local inflammation.

Given the polymicrobial nature of nonhealing wounds, it is important to gain an understanding of the development of mixed species biofilms and the ways in which they affect wound healing. Seth *et al.*[24] applied 6 mm dermal punch wounds in rabbit ears and inoculated them with *S. aureus* and *P. aeruginosa* PA01. The presence of biofilm and evidence of co-localization of both species encased within an EPS at multiple sites was noted using SEM. The polymicrobial biofilm impaired all wound healing when compared with the monospecies infection. This study demonstrated synergy between bacterial species within a single biofilm and the enhanced effect this has in reduced healing rates. Similarly, a recent study by Pastar *et al.*[36] demonstrated the synergistic effects of MRSA and *P. aeruginosa* biofilms in delaying reepithelialization of experimental porcine wounds. An earlier study by Dalton *et al.*,[37] using an *in vivo* poly-

microbial-infected biofilm wound model found that biofilms transplanted onto wounds of mice resulted in wound infection. Furthermore, the biofilms impaired wound healing more when four bacterial species were evident compared with a single species of bacteria. The researchers also observed that the polymicrobial wound infections displayed increased antimicrobial tolerance when compared with the single species. These data suggest the synergistic interactions between different bacterial species in wounds may contribute to a greater delay in healing.

In 2009, Freeman *et al.*[38] identified evidence of biofilms by analyzing biopsies taken from the wound tissue of two horses. The biofilms were characterized as microcolonies or aggregates of cells with evidence of EPS surrounding the cellular aggregates. Later, Westgate *et al.*[39] further described the presence of bacterial biofilms within tissue samples of 18 chronic equine wounds. Results revealed that 8 out of 13 tissue samples showed evidence of biofilms. Furthermore, bacteria cultured from chronic and acute wounds demonstrated significantly greater biofilm forming potential than bacteria isolated from uninjured skin. The equine wounds also demonstrated microbial diversity, with both gram-positive and gram-negative bacteria identified in bacterial clusters within the collected tissue samples.

Swanson *et al.*[40] first reported on bacterial biofilms in a chronic wound of a dog evaluated for two chronic nonhealing pressure wounds. Clinically, the wound bed contained mottled red and yellow granulation tissue. The initial treatment strategy included wound debridement, with the tissue sent for histologic evaluation and microbial culture. The dog received systemic antibiotics and the left wound was closed, whereas the right wound was packed with a calcium alginate rope with silver and treated with wound vacuum-assisted closure and debridement. Cultures of the wounds revealed *Staphylococcus intermedius, S. epidermidis*, and *Streptococcus canis*. Based on this experience, the authors recommended a multimodal treatment approach for biofilms utilizing debridement, systemic antibiotic therapy, and pressure offloading of the wound.

Overall, these animal studies, mostly using biofilms of *Staphylococcus* and *Pseudomonas* strains, highlight that the presence of biofilm in a skin wound inflicts a number of adverse effects upon the host. These include impairing the immune response, delaying epithelialization, and lessening development of granulation tissue. In particular, polymicrobial biofilms appear to be more pathogenic than monospecies biofilms and delay wound healing for longer periods of time.

Human–clinical studies. Initial studies published in 1985 of human wounds concerned the evidence of biofilms on wound sutures, not within the wound itself.[41] In 2005, Percival *et al.*[42] provided a review on the potential significance of biofilms in wounds, but it was not until 2008, a decade after reports in animals, that biofilms were clinically observed in human wounds.[43] James *et al.*[43] obtained specimens from chronic wounds and acute wounds. By utilizing both light and SEM techniques, they found markers for biofilm. Within 50 chronic wound samples analyzed, 30 (60%) were found to contain biofilm compared to only one of 16 acute wounds. However, as biofilms are patchy and not confluent on a surface, utilizing high-powered microscopes on small specimens is likely to result in biofilm not being identified.

In 2008, Kirketerp-Moller *et al.*[44] when examining wound specimens from 22 patients using the PNA-FISH method, found *P. aeruginosa* within the tissue samples aggregated as microcolonies imbedded in alginate, that is, a biofilm. In the same year Bjarnsholt *et al.*[45] analyzed sections of chronic wound using fluorescent *in situ* hybridization techniques and identified distinct microcolonies, confirming the basal structure of bacterial biofilms. In 2010, Kennedy *et al.*[46] found evidence of biofilms in the ulcerated areas of burn wounds using light and electron microscopy techniques. Microbiological studies also confirmed the polymicrobial nature of wound infections, which also supports biofilm presence. In chronic venous leg ulcers, Fazli *et al.*[47] used PNA-FISH and confocal laser scanning microscopy (CLSM) on biopsy samples to detect bacteria and large aggregates of cells. Utilizing inflammatory markers, the researchers concluded that *P. aeruginosa* biofilms lead to the influx of high numbers of neutrophils and biofilms may be one of the main factors leading to a persistent inflammatory response, with a resultant impairment of wound healing. Also in 2011, Neut *et al.*[48] presented two case studies of patients with nonhealing ulcers. CLSM analysis highlighted densely aggregated colonies of viable bacteria surrounded by EPS and host cell debris, providing evidence of biofilm formation in human diabetic wounds. A further study by Han *et al.*[49] demonstrated the high microbial diversity within chronic wounds. Epi-fluorescence microscopy revealed the presence of highly organized, thick, confluent biofilms and 47% of the specimens had significant biofilm coverage arranged as aggregating colonies of varying size.

Overall, the data derived from human clinical studies make clear that biofilms have an important adverse effect on wound healing. Despite this, more fundamental scientific studies are required to understand what biofilms are doing to the normal wound healing processes from a cellular and immunological perspective. In particular, we need more studies to understand why some wounds with biofilm growing in them heal and others do not. Difficulty remains, however, in conducting appropriate large randomized controlled trials on biofilms as it is only possible to view biofilms when biopsies are analyzed microscopically.

TAKE-HOME MESSAGE

- In wounds that become chronic, the normal process of healing is disrupted
- Delayed wound healing appears to be largely related to the presence of microorganisms growing in a biofilm
- Microorganisms growing as a biofilm are more resistant to host defenses and to antimicrobial therapy
- Further scientific and clinical evidence on the role biofilms play on wound healing are needed, in particular the role of the *pathogenic* biofilm

SUMMARY

Microorganisms routinely contaminate, colonize, and often infect wounds of all types. Wound infection and possibly high-level colonization appear to be major barriers to healing. Within the last 10 years, the presence and negative effects of biofilm in chronic wounds has been increasingly recognised.[50] Microorganisms in biofilms appear to enable the bacteria to better resist the effects of antimicrobial agents and host defenses in the wound environment. Furthermore, biofilms have been shown to induce an inflammatory reaction in the host and to upregulate many genes linked to MMPs, and decrease tissue inhibitors of MMPs. Notwithstanding the substantial understanding that has come with these initial studies on biofilms and the effects they have on wound healing, further scientific and clinical evidence on the role biofilms play on wound healing are needed, in particular the role of the *pathogenic* biofilms.

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ABOUT THE AUTHORS

Professor Steven L. Percival holds a PhD in microbiology and biofilms, a BSc in Applied Biological Sciences, Postgraduate Certificate in Education, diploma in Business Administration, an MSc in Public Health, and an MSc in Medical and Molecular Microbiology. Early in his career, Steven held R&D positions in the Department of Biotechnology, British Textile Technology Group (BTTG)Plc, followed then by 6 years as a senior university lecturer in medical microbiology and Head of the Biofilm Research Group and later the positions of Director of R&D and Chief Scientific Officer at Aseptica, Inc., and senior clinical fellowships at the Centers for Disease Control, Atlanta, and Leeds Teaching Hospitals Trust, Leeds. More recently, Steven held senior R&D management and innovation positions at Bristol Myers Squibb, ConvaTec, Advanced Medical Solutions Plc and held an honorary Professorship in the medical school at West Virginia University. In 2011, Steven joined Scapa Healthcare PLC as Vice President of Global Healthcare R&D and in 2012 was awarded the position of honorary Professor in the Institute of Ageing and Chronic Diseases and the Surface Science Research Centre at the University of Liverpool, United Kingdom. He has written over 300 scientific publications and conference proceedings and has authored and edited seven textbooks on microbiology, biofilms, and public health. Steven has provided over 100 presentations globally, and is an editor of the Journal of Medical Microbiology and associate editor of BMC Microbiology.

Sara M. McCarty gained her BSc in Biomedical Sciences in 2008 from the University of Chester, United Kingdom. Since then, Sara has undertaken positions as a research technician and enjoyed gaining experience within the field of dermatology. She is currently completing a PhD in the role of proteases in wound healing at the University of Liverpool, United Kingdom.

Benjamin A. Lipsky is currently Teaching Associate at Green Templeton College and affiliated with the University of Oxford Division of Medical Sciences, Visiting Professor of Medicine at the University of Geneva and Professor of Medicine Emeritus at the University of Washington. He trained in internal medicine and infectious diseases at the University of Washington (Seattle),

where he became Professor of Medicine. At the Veterans Administration Puget Sound, he served as Chair of Infection Control, Director of the Primary Care Clinic, and ran an Antibiotic and Wound Infection Research Clinic. Professor Lipsky has authored over 200 peer-reviewed research papers, as well as over 100 other medical papers and textbook chapters, and three books on infectious diseases. He has chaired the guideline committees on diabetic foot infections of both the Infectious Diseases Society of American and the International Working Group on the Diabetic Foot since their inception. He currently collaborates on research projects worldwide (mainly involving diabetic foot infections) and is helping to develop a clinical research program at the Hospital of the University of Geneva.

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Abbreviations and Acronyms

CLSM = confocal laser scanning microscopy
ECM = extracellular matrix
EPS = extracellular polymeric substance
MMP = matrix metalloproteinase
MRSA = methicillin-resistant *Staphylococcus aureus*
SEM = scanning electron microscopy