Biofilms and wounds: much ado about nothing?

Biofilms are communities of multiple species of adherent microorganisms that are embedded within a blanket of slime. Their existence in natural environments and in industrial situations is well known, but their association with chronic wounds is more recent. Implications of biofilms for wound management are uncertain because means of diagnosing biofilm infections in wounds are not yet well developed and effective treatment strategies are not established. This review will outline present knowledge about the role of biofilms in wounds and indicate the problems that need to be addressed.

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Biofilm control

It has long been understood that microbial species living in natural environments normally associate with surfaces where they collectively secrete sticky materials that help to maintain their position long enough to provide an opportunity to develop complex structured communities or biofilms. Unattached microbes, otherwise known as planktonic cells, are considered to represent the form by which dispersal to other suitable locations is ensured and new biofilms are established (Watnick and Kolter, 2000). Ever since microorganisms were demonstrated to be the causative agents of infectious diseases during the late nineteenth century, the isolation, identification and control of planktonic microorganisms derived from acute infections has dominated medical microbiology.

Although the existence of biofilms has been documented for more than a century, their clinical importance was largely unrecognised until their involvement in persistent infections was realised (Potera, 1999). Now, biofilm diseases are thought to affect more patients than the numbers affected by heart disease and cancer combined (Balaban, 2008). Since the nature of biofilms in wounds was first debated (Mertz, 2003), much has been learnt about their role and the topic has gained a central place in wound biology, even though many questions remain unanswered.

Nature of biofilms

Microorganisms are frequently described as being ubiquitously but unevenly distributed in natural environments. Their environmental distribution patterns are always influenced by chemical conditions such as nutrient availability and the concentrations of inhibitory substances, as well as physical conditions such as temperature, acidity or oxygen tension, and also biological factors, such as competition or predation. Often nutrients are in short supply and microbial growth is limited. Adsorption of nutrients to inert surfaces creates complex concentration gradients in natural habitats which attract free-living bacteria. Using laboratory models, it has been discovered that the initial adherence of microbial cells to a surface is reversible. However, irreversible adherence is an important step in the initiation of biofilm formation that allows microbial cells to grow and divide to form small aggregates or microcolonies (*Figure 1*). Attachment is a pre-requisite for biofilm formation. The transition from a microcolony to a mature biofilm depends on the ability of bacteria to communicate.

The production and recognition of chemical signals (quorum sensing molecules) allows not only one cell to communicate with another cell of the same species and thereby estimate the number of the cells of the same species within the vicinity, but also allows communication between different species. When the number of cells of the same species has reached a critical threshold (or quorum), biofilm formation is triggered by cells starting to produce a slimy matrix. They secrete extracellular polymeric substances (EPS) such as polysaccharides, proteins and nucleic acids that hold the cells together; clumps of cells embedded in EPS may continue to grow and divide to form an uneven mass with irregular, bulbous projections (*Figure 1*). Two types of cell-cell chemical signals exist in Gram-negative bacteria which are acylhomoserine molecules and autoinducer-2-molecules, whereas Gram-positive bacteria communicate via peptide molecules.

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A biofilm has been defined as 'a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface' (Costerton et al, 1999). Cells within mature biofilms exhibit physiological characteristics that are distinct from planktonic cells. This arises because as a biofilm matures, quorum sensing molecules mediate changes in the expression of some of the genes of the constituent cells, which leads to differentiation. Hence, constituent cells exhibit increased virulence, diminished growth rate, decreased susceptibility to antimicrobial agents and reduced susceptibility to host immunological responses. The physiological and biochemical characteristics of planktonic cells are therefore distinct to those of cells living within a mature biofilm. Whereas planktonic cells involved in acute infections usually respond to appropriate antibiotic therapy, biofilm infections do not.

Biofilm dispersal can occur when fragments become detached by shear forces and also when cells within the central region of a mature biofilm undergo cell death, leading to disaggregation, reversion to planktonic form and release (*Figure 1*). Shedding of single cells which immediately revert to planktonic form can also occur from the surface layers of a biofilm.

Impact of biofilms on humans

Biofilms impact significantly on human activity. The safe disposal of organic waste relies largely upon biofilms found within sewage farms. Similarly, industrial effluents are degraded by microbial communities existing as biofilms in effluent treatment plants. Some of the undesirable effects of biofilms are the corrosion of submerged metal surfaces in ships' hulls, oil rigs and piers. Also, biofilms formed on the inner surfaces of pipes reduce lumen capacity and impact on flow rates.

The role of biofilms in nature and disease has been reviewed (Costerton et al, 1995). Within the human body many members of the natural flora have the potential to form biofilms, yet adverse effects are rarely seen and protection against infection may result. One of the most extensively researched human biofilm is dental plaque.

Biofilms have been implicated in persistent human diseases, such as respiratory infections in cystic fibrosis patients, dental caries, gingivitis, periodontal disease, osteomyelitis, chronic prostatitis, otitis media, endocarditis, infectious kidney stones and Legionnaire's disease (Costerton et al, 1999). Biofilms most commonly linked to human disease involve indwelling medical devices, particularly catheters, prosthetic joints, stents, intra-uterine implants, heart valves and contact lenses. The bacteria most frequently isolated form such infections are coagulase negative staphylococci, but Gram-negative bacilli and *Candida* have also been associated with implant infections. Often these infections manifest weeks or months after insertion of the device, in patients who seem to have successfully overcome surgery. It is possible that their device was unwittingly contaminated during the occasion when it was inserted, and that slow growth of adherent contaminants may have preceded microbial dissemination and acute infection. Removal of the offending device is normally indicated in such infections.

Biofilms in wounds

Biofilms were discovered in healed surgical wounds when 15 sutures and 15 staples were examined by scanning electron microscopy, and cocci encased in extracellular material were observed to be attached to the intradermal surface of the closures (Gristina et al, 1985). *Staphylococcus epidermidis* was isolated from the specimens. Interestingly, these biofilms had elicited neither overt infection nor immunological host response and the colonised bacteria had not impeded healing.

Evidence that *Staphylococcus aureus* biofilms were formed in acute wounds was generated using murine models (Akiyama et al, 1993; 1994). Examination of this experimental model by electron microscopy and confocal laser scanning microscopy demonstrated that *S. aureus* produced glycocalyx under these conditions (Akiyama et al, 1996; 2002). Glycocalyx is a copious extracellular layer of polymeric material produced by bacterial cells when they are adherent. Similarly in a pig model when partial-thickness burns wounds were infected with *Pseudomonas aeruginosa*, adherent bacteria were found to be encased in a polymeric matrix (Serralta et al, 2001).

The ability of bacteria isolated from wounds to form biofilms in the laboratory has been demonstrated. *S. aureus* isolated from clinical specimens collected from patients with impetigo, furuncle and atopic dermatitis was shown to develop biofilm on coverslips within 72 hours when cultivated at 37°C in media containing plasma (Akiyama et al, 1997). Similarly, a culture of *P. aeruginosa* isolated from a burns patient attached to a glass slide within 10 hours, synthesised EPS and subsequently formed a biofilm (Harrison-Balestra et al, 2003). From these observations, inferences that biofilms might have existed in the respective patients have been made.

A landmark study has demonstrated the existence of biofilms in chronic wounds (James et al, 2008). Here, scanning electron microscopy and confocal laser scanning microscopy demonstrated unequivocally the presence of biofilm in 30 of 50 chronic wounds, and in only one of 16 acute wounds. This statistically significant association between chronic wounds and biofilm should not, however, be interpreted as the presence of biofilm in all chronic wounds, because the reasons for failure to heal depend on patient characteristics as well as microbial factors. Yet, an interesting concept to explain how biofilms contributes to wound chronicity has recently been proposed. It involves the synthesis of rhamnolipid by *P. aeruginosa* which impairs neutrophil function and prevents effective bacterial clearance (Bjarnsholt et al, 2008). Curiously, the deliberate release of planktonic bacteria from biofilm has been proposed to maintain an inflammatory response in wounds (Ngo et al, 2007; Wolcott et al, 2008).

Chronic wounds are normally characterised by elevated levels of pro-inflammatory cytokines, free radicals and proteases (such as macrophage derived MMPs 2 and 9, neutrophil-derived MMP 8 and elastase), together with a continual

presence of neutrophils and a deficit of activated macrophages (Tarnuzzer and Schultz, 1996). Since biofilms in wounds seem to perpetuate inflammation, it is easy to understand why they have been found more frequently in chronic wounds than in acute wounds. The biofilm concept helps to explain many of the clinical challenges that make wound care rather difficult and sometimes unpredictable.

Biofilm detection

The acceptance that biofilms exist in chronic wounds now attracts much interest from practitioners, who justifiably seek reliable detection and treatment methods. Alas, satisfactory solutions to these issues do not yet exist. The validated cultivation methods that are routinely employed to isolate and identify microbial species from clinical specimens in most conventional diagnostic laboratories do not support the production of glycocalyx, or the development of biofilms. Hence, isolated organisms grow only in planktonic form and therefore give no information about whether they existed within biofilms in the wound. Furthermore, the adherence of biofilm to host tissue, together with the possibility that biofilm may form in deep tissue, makes the use of swabs on wound surfaces an unreliable method of recovering biofilms.

The presence of slimy material within a wound should never be interpreted as the presence of biofilm, since the arrangement of microbial cells within that slime can only be discerned with high power magnification techniques rather than the naked eye. Slough in a wound is not an indicator of a biofilm, because slough can be relatively easily removed by conventional debridement, while biofilm cannot (Hurlow and Bowler, 2009).

At present, the detection of biofilm in wounds depends on the examination of biopsy tissue using sophisticated research techniques. As previously mentioned, scanning electron microscopy and confocal laser scanning microscopy are appropriate means to observe biofilm, but are usually costly, time-consuming and are unavailable in routine medical microbiology laboratories. The ability to recognise specific bacteria using peptide nucleic acid fluorescence *in situ* hybridization (FISH) with epifluorescence microscopy is another specialised approach that has been employed in a biofilm model (Malic et al, 2009).

Molecular characterisation of microbial communities contained in pooled biopsy samples by analysis of ribosomal RNA genes by several methods has been used to deduce the presence of biofilms in chronic wounds. *Staphylococcus, Pseudomonas, Peptoniphilus, Enterobacter, Stenotrophomonas, Finegoldia,* and *Serratia spp* predominated in chronic wounds (Dowd et al, 2008a). The bacteria detected varied with both the molecular technique utilised and the type of wound studied. Gram-negative facultative anaerobes predominated in the venous leg ulcer sample and anaerobes were scarce, whereas facultative and strict anaerobic Gram-positive cocci were most prevalent in diabetic ulcers and strict Gram-positive anaerobes dominated in pressure ulcers.

In unpooled biopsy specimens from 40 patients with diabetic foot ulcers, *Corynebacterium spp* were found to be the prominent bacterial species, with strict anaerobes ubiquitous and facultative bacteria also present (Dowd et al, 2008b). In specialised laboratories, these molecular techniques can yield valuable microbial characterisations within a working day, but routine laboratories are not able to offer this level of service. Although a wider variety of bacterial species is usually identified using molecular approaches compared to conventional cultivation techniques, it is the presence of DNA from diverse microbial species that is interpreted as the presence of a biofilm, rather than the demonstration of specific biofilm markers, or the detection of cells expressing biofilm phenotype. DNA from species present in low numbers will be detected. Whether those organisms were resident or transient members of the wound microbiota may not be necessarily discerned; DNA from both viable and recently non-viable cells will be detected. Molecular techniques are not without their limitations.

Another means to recognise biofilms in wounds was developed in rats by comparing the level of quorum sensing molecules detected in 12 pressure-induced ischaemic wounds infected with *P. aeruginosa* to that found in 12 uninfected wounds. A reporter assay was utilised in which a colour change in a test bacterial culture denoted the presence of bacterial quorum sensing molecules in wound tissue (Nakagami et al, 2008).

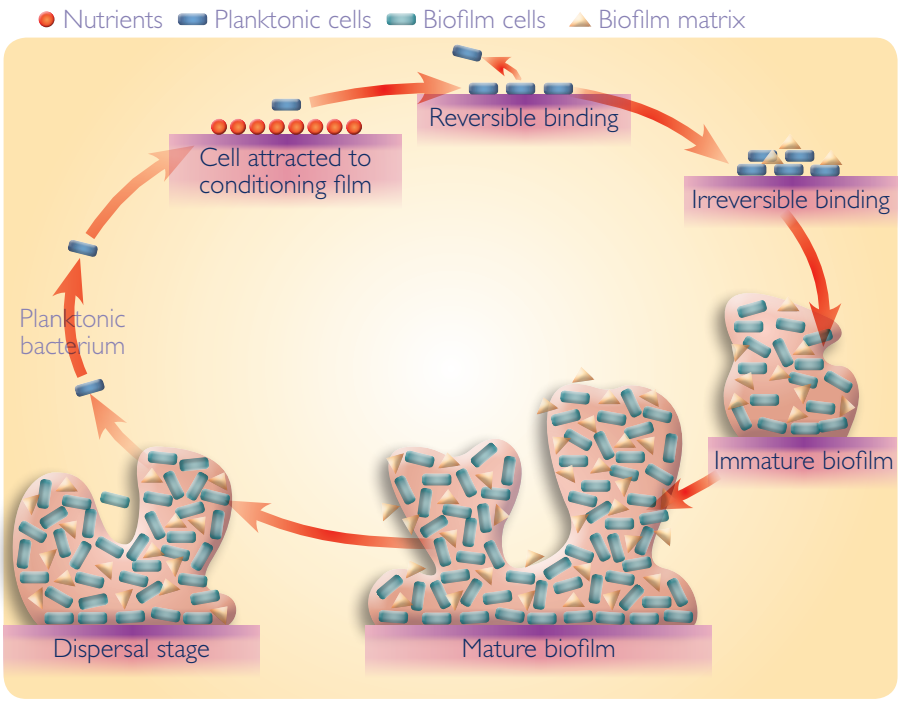

Figure 1. Formation of biofilm on solid surfaces.

A similar approach has been utilised to detect quorum sensing molecules in debridement specimens collected from chronic wounds (Rickard et al, 2009). The detection of quorum sensing molecules by chemical analysis using techniques such as thin layer chromatography, mass spectrometry and high-performance liquid chromatography (HPLC) are also possible. However, until simpler means of detecting biofilms are developed, the diagnosis of biofilm in wounds will be limited to research laboratories.

Biofilm control

The link between wound chronicity and biofilms has provided some valuable insight into the reasons why some wounds fail to heal within predicted times, but it has created a need to devise effective strategies to control biofilms. In established biofilms, microbial species grow at reduced rates and often exhibit decreased susceptibility to antimicrobial agents by factors up to 500 times (Costerton et al, 1995). The involvement of antibiotic-resistant strains in biofilms contributes further to treatment difficulties (Noiby et al, 2010).

Anti-biofilm measures can broadly be divided into those that aim to remove or disrupt biofilms, and those that aim to prevent them forming. Neither seems to be particularly effective and there is little clinical evidence to confirm the efficacy of either of these approaches. The first clinical study in which an anti-biofilm strategy was adopted in patients with critical limb ischaemia utilised sharp debridement, coupled with lactoferrin and xylitol, to restrict the availability of iron and to interfere with EPS formation, respectively (Wolcott and Rhoads, 2008). Biological debridement of wounds with maggots (Van der Plas et al, 2008), or disruption of EPS by enzymes (Donelli et al, 2007) have also been suggested as potential remedies for biofilms. Antimicrobial agents such as chlorhexidine, cadexomer iodine, hydrogen peroxide, octenidine, polyhexanide, povidone iodine and silver have also been evaluated *in vitro* with variable results (Kunisada et al, 1997; Akiyama et al, 2004; Johansen et al, 1997; Chaw et al, 2005; Bjarnsholt et al, 2007; Percival et al, 2007).

Honey has been proposed as a further anti-biofilm intervention because its most common sugar molecule (fructose) interferes with adherence of *P. aeruginosa* to host cells (Lerrer et al, 2007). Also, the inhibition of biofilms by honey *in vitro* has been reported (Alandejani et al, 2009; Merckoll et al, 2009; Okhiria et al, 2009).

The role of quorum sensing molecules in influencing some of the genes that contribute to enhanced virulence and biofilm formation has given rise to the idea that interventions that limit quorum sensing might be used to control biofilms. Assays for screening natural products for quorum sensing inhibitors have been developed (McLean et al, 2004; Rasmussen et al, 2005), and indicate that garlic inhibits quorum sensing in *P. aeruginosa*. A role in treating lung infections in cystic fibrosis patients has been suggested (Bjarnsholt et al, 2005). It is conceivable that chronic wounds might also be suitable targets for garlic extracts.

The development of biofilm models is providing a means to evaluate potential biofilm interventions (Sun et al, 2008; Thorn et al, 2009; Charles et al, 2009; Kanno et al, 2009; Malic et al, 2009), but clinical evidence will clearly be needed before effective anti-biofilm interventions will be accepted.

Future of biofilms in wounds

The burden that chronic wounds currently represent on medical resources and the prospect of increased numbers of chronic wounds in ageing populations is a major concern. The reasons why wounds fail to heal are varied and complex. Establishing an association between wound chronicity and the presence of biofilm has been an important advance in knowledge that has generated much interest and speculation within the wound healing arena. Not only are wound care specialists interested in learning about the nature of biofilms and the means to diagnose biofilm infections, but they need effective treatment strategies. At this time it is important to understand that routine biofilm detection methods are not yet available and that the development of reliable biofilm interventions is ongoing. Biofilms should neither be over-estimated nor under-estimated. Not all chronic wounds can be expected to be attributed to biofilms, so the development of appropriate anti-biofilm interventions will never eradicate all chronic wounds. Undoubtedly, effective treatments will be developed in due course, yet still wounds in some patients will continue to fail to heal. Until routine biofilm detection methods become readily available, knowing when to adopt an anti-biofilm strategy will be speculative. WUK

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Key points

- Although the existence of biofilms has been documented for more than a century, their clinical importance was largely unrecognised until their involvement in persistent infections was realised.
- Whereas planktonic cells involved in acute infections usually respond to appropriate antibiotic therapy, biofilm infections do not.
- The validated cultivation methods that are routinely employed to isolate and identify microbial species from clinical specimens in most conventional diagnostic laboratories do not demonstrate the presence of biofilms in wounds.

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