Biofilms, wound infection and the issue of control

Biofilms are surface-attached microbial communities with characteristic architecture and phenotypic and biochemical properties distinct from their planktonic counterparts. One of the best-known of these biofilm-specific properties is the development of antibiotic resistance that can be up to 1,000-fold greater than that of planktonic cells. Biofilms are not simply a diffusion barrier to antibiotics, but rather bacteria within these communities employ distinct mechanisms to resist the action of antimicrobial agents. The greater our understanding of the processes involved in biofilm formation, the greater the chance of developing remedies.

Rose Cooper and Olusola Okhiria

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There seems to be little doubt that biofilms have the potential to contribute to infection in wounds. In order to develop strategies to combat wound infections involving biofilms, a greater understanding of the way they are formed and persist is needed. This review will assess biofilm formation, and their role in human disease and infections. It will also discuss ways that biofilms — which are highly resistant to antibiotics — can be targeted and treated.

What is a biofilm?

Complex communities of microorganisms encased in slime and attached to surfaces are known as biofilms (Costerton et al, 1995). They usually comprise of several different microbial species embedded in sticky extracellular polymers that have been collectively secreted by constituent members. These structured, functionally coordinated communities form on a vast array of living and non-living surfaces and probably represent the most common form of existence for microbes in natural environments. Cultivation conditions routinely used in the microbiology laboratory do not, however, generally favour the production of extracellular slimes (known as glycocalyx) by microbes (Costerton et al, 1978) and so the occurrence of biofilms has been underestimated.

Rose Cooper is Principal Lecturer and Olusola Okhiria is a Research Student at the Centre for Biomedical Sciences, Cardiff School of Health Sciences, University of Wales Institute

Biofilm formation

Within the past 30 years the development of techniques that provide a means to see biofilms in their respective habitats, plus the development of conditions that support the formation and investigation of biofilms in the laboratory, have resulted in a greater understanding of biofilms. Surfaces and interfaces are important in biofilm formation because they facilitate the acquisition of nutrients. In aquatic environments nutrients are absorbed by surfaces to form a conditioning film with higher concentrations than in the surrounding bulk solution. Some surfaces (such as dead plants or animals) are themselves a source of nutrients.

Biofilms are differentiated communities that reflect complex interactions between microbial cells (Stoodley et al, 2002). Some of the species (such as *Pseudomonas*) that contribute to biofilms exist in two forms: planktonic (motile) and sessile (non-motile). Others (like *Staphylococci, Streptococci* and *Enterococci*) are non-motile. Planktonic cells swim and grow as single cells in suspension, whereas cells attracted to a surface can attach and become sessile. Adherence is always the first step towards biofilm development. Attachment (or coaggregation) between different species also leads to the formation of dense microbial aggregates in the early biofilm stages. Attachment is important because it generates intracellular signals that trigger the expression of specific genes essential for biofilm formation and leads to changed phenotypic characteristics (Fegan et al 1990; Li et al, 2001).

The synthesis and detection of chemical signals (auto-inducers) facilitates intra-species and inter-species communication, so that relative numbers can be evaluated and the expression of pertinent genes regulated. Cell-to-cell communication via auto-inducers is known as quorum sensing. Many auto-inducers have been discovered with both discrete and similar molecules in Gram positive and Gram negative bacteria; it is probable that many more auto-inducers have yet to be discovered.

Biofilms are diverse and dynamic hydrated structures, that constantly change and adapt to their environment. The developmental stages include reversible and irreversible attachments, followed by phases of maturation and dispersion. Following attachment, microbial cells begin to proliferate into small clusters. When a crucial number is exceeded (detected by quorum sensing), members excrete extracellular polymeric substances (EPS or matrix) and gradually develop into a biofilm (*Figure 1*) (Costerton et al, 1999; Sutherland, 2001), while continuing to attract other members — possibly different species of bacteria, fungi, or protozoa (Davey and O'Toole, 2000; O'Toole et al, 2000).

In a mature biofilm, cells embedded in EPS form three-dimensional bulbous, stalked structures that are interspersed with water channels. The water channels act as a crude transport system for the movement of nutrients and waste products (Stoodley et al, 1994) thereby protecting against starvation by nutrient depletion or inhibition due to the accumulation of toxic metabolites (Davies et al, 1998; Sutherland, 2001). Although cells within a biofilm can access nutrients and dispose of metabolites, growth rates are diminished. This confers reduced susceptibility to antimicrobial agents. Cells also exhibit decreased susceptibility to immunological defence mechanisms, particularly phagocytosis, and increased virulence. The close proximity of organisms within biofilms fosters exchange of genetic material via conjugation and transformation (Hausner and Wuertz, 1999; Wuertz et al, 2001), giving the opportunity for organisms with novel combinations of virulence genes and antibiotic resistance to evolve.

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Occurrence of biofilms

Biofilms are extensively distributed throughout the natural and industrial world, and their presence may lead to disastrous consequences. About a century ago the discovery of slimes attached to the hulls of ships and boats (now recognised as biofilms) was linked to corrosion. More recently biofilms have been implicated in the destruction of submerged structures such as oil rigs and piers. Degradation of oil and contamination in wood pulp and paper plants is attributed to biofilms of sulphur bacteria.

Biofilms within the lumens of pipelines slow the movement of fluids, cause structural damage by corrosion and present an increased risk of infection. Intermittent release of pathogens such as *Vibrio cholerae* or *Legionella pneumophila* in pipes in a water distribution system can result in human infection, yet the detection and treatment of such biofilms is problematic because adherent bacteria escape sample collection.

Cooling systems associated with ventilation and air conditioning in aeroplanes, hotels and offices can also support biofilms, and may be implicated in human infection if they are not properly maintained. In the home, biofilms form on the inner surfaces of waste pipes and the wet under-surface of plugs in sinks.

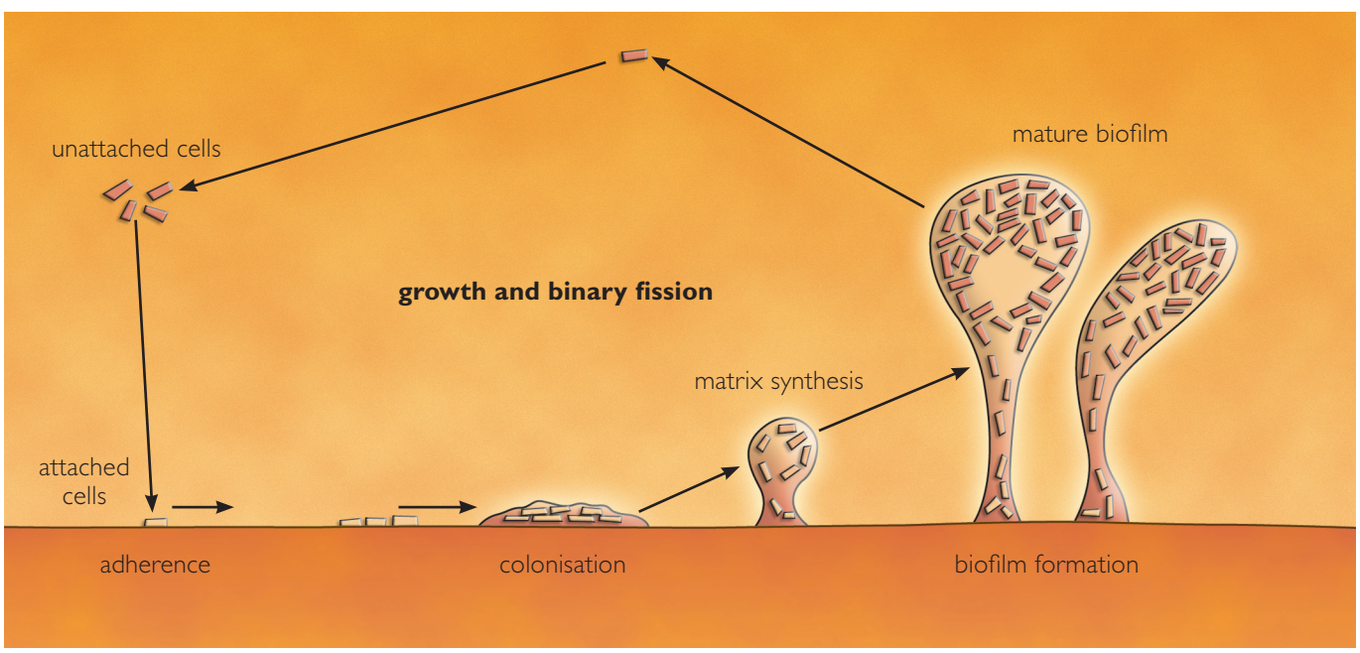

Figure 1. Biofilm formation.

In farming, biofilms have been implicated in animal diseases, such as chronic pneumonia in pigs and mastitis in cows. In plants they can cause Pierce's disease in grapes and citrus crops, and potato ring rot disease. Some biofilms are beneficial. Waste treatment facilities rely on biofilms to degrade organic matter and metal extraction by leaching in some mining processes makes use of biofilms.

Biofilms in humans

All surfaces of the body exposed to the external environment (skin, teeth, mouth, respiratory and gastrointestinal epithelia) support a population of sessile, commensal bacteria that possess the ability to form biofilms and yet infection rarely ensues. The majority of such surfaces are constantly being shed thereby minimising the opportunity of normal species to form biofilms. Slow shedding areas like the buccal cavity and the vagina can normally sustain biofilms without adverse effects.

Dental plaque is the biofilm that has received the most attention. It was first observed by simple light microscopy during the 17th century and is recognised to comprise more than 350 species of bacteria (Moore and Moore, 1994). Biofilms in the mouth, gut, vagina and wounds are not necessarily detrimental and may actually provide protection against infection (Reid et al, 2001).

Biofilms implicated in human disease

It has been estimated that 65% of human infections involve biofilms (Potera, 1999). However, acute infections that are readily treated with antibiotics are not considered to involve biofilms, unlike the majority of chronic infections in mildly compromised individuals that involve commensal or common environmental organisms (Costerton et al, 1999). The biofilms most frequently linked to human infection are associated with indwelling medical devices, particularly central venous catheters. Ingress of micro-organisms on either the exterior or interior surface is possible and infections can be localised to the insertion site or disseminated to cause bacteraemia, endocarditis or septic shock.

Staphylococcus epidermidis is the most common causative agent associated with implant infections (Rupp and Archer, 1994), but other staphylococcal species, enteric bacteria and *Candida albicans* have also been implicated. Other devices that have been implicated in biofilm infections include prosthetic heart valves, prosthetic orthopaedic implants, intra-uterine devices, contact lenses, and urinary catheters.

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Biofilms associated with implants are a major cause of nosocomial infection. Despite the sterile nature of implantable medical devices, inadvertent contamination during insertion followed by the adherence of microbes to biomaterials and biofilm initiation can result in unexpected postoperative infection weeks or months after surgery. Dehiscence at the incision site may be the first indication of the problem.

Infections in humans that are linked to biofilms but are not related to devices are typically chronic and difficult to resolve (Costerton et al, 1999). They include cystic fibrosis, infectious kidney stones, dental caries, periodontal disease, gingivitis, necrotising fasciitis, chronic prostatitis, osteomyelitis and otitis media (Costerton et al, 1999; Parsek and Singh, 2003).

Biofilms in wounds

The earliest indication that biofilms may be associated with wounds came from the electron microscopic examination of 15 sutures and 15 staples removed from healed surgical wounds (Gristina et al, 1985). Bacterial cells encased within extracellular material and adherent to the intradermal site of the closures were observed and *Staphylococcus epidermidis* was isolated from all of the specimens. These colonised bacteria had caused neither infection nor inflammation, demonstrating that biofilms in humans do not necessarily have a negative effect. The extracellular material surrounding the bacteria was assumed to have assisted persistence by protecting against the host's defence mechanisms (Gristina et al, 1985).

The prevalence of biofilms in human cutaneous wounds has not yet been established. One case report suggested the presence of a biofilm in a chronic leg ulcer that responded only to combined systemic and topical treatment (Boutli-Kasapidou et al, 2006) but the presence of bioflm structures was not confirmed by objective tests such as confocal microscopy.

Animal models have provided evidence of biofilm formation in acute wounds. In mice, for example, biofilms of *S. aureus* have been demonstrated on silk stitches inserted into skin (Akiayama et al, 1993) and also in skin inflammed by croton oil (Akiayama et al, 1994). Electron microscopy of incisions and damaged skin in neutropenic mice that had been inoculated with *S. aureus* revealed the presence of glycocalyx (Akiyama et al, 1996). Similarly, examination of damaged skin in both neutropenic and normal mice by confocal laser scanning microscopy confirmed that inoculated *S. aureus* produced microcolonies of cells embedded in glycocalyx (Akiyama et al, 2002). Partial-thickness wounds created in three pigs and challenged with *P. aeruginosa* were demonstrated to contain both adherent (EPS positive, hence biofilm producing) and non-adherent bacteria (Serralta et al, 2001).

The ability of a clinical isolate of *P. aeruginosa* derived from a burn to grow a biofilm in the laboratory within 10 hours illustrates the potential of wound inhabitants to form biofilm, albeit in vitro (Harrison-Balestra et al, 2003). Similarly, *S. aureus* cultures recovered from patients with impetigo, furuncle and atopic dermatitis produced biofilm on coverslips within 72 hours incubation at 37°C in the presence of plasma, and were deduced to be an inference of biofilms in vivo (Akiyama, et al, 1997).

The importance of biofilms in chronic wounds is not yet understood, although it has been proposed and discussed (Mertz, 2003). Images and data have been published on the website of Montana State University (2006) that suggest a statistically significant association between chronic wounds and biofilms, but none between biofilms and acute wounds. Using scanning electron microscopy and light microscopy of debrided material from 50 chronic wounds, biofilms were detected in 30 out of the 50, and in one of 16 acute wounds. Given the persistence of some wounds, and their unresponsiveness to antimicrobial agents, it is tempting to attribute chronicity to the presence of biofilms. Unequivocal evidence has yet to appear in peer-reviewed scientific journals.

Validated methods to detect biofilms in wounds do not yet exist. As stated above, routine investigation of clinical specimens by cultural methods will not normally support glycocalyx synthesis, but occasionally an isolate will simultaneously present as two distinct phenotypes on primary isolation that hint at diversification within the host — *P. aeruginosa*, for example, demonstrating non-mucoid (normal appearance) and mucoid (producing copious amounts of slimy alginate) colonies have been found in a patient with cystic fibrosis indicating biofilm formation (Lam et al, 1980).

The presence of slime in a wound does not conclusively indicate the presence of a biofilm because fibrin deposition is not exclusively mediated by micro-organisms. One indirect indicator of biofilms that has not been explored is the detection of quorum-sensing molecules in clinical specimens. Innovative molecular and imaging techniques are being developed to recognise biofilms in joint implants (Stoodley et al, 2005).

Biofilm control

The reduced growth rates of microbial cells within established biofilms confers reduced susceptibility to antimicrobial agents, which in turn contributes to persistence. Additionally, the existence of protected, inactive cells known as persister cells has been proposed (Spoering and Lewis, 2001) and a model to predict survival kinetics of the microbial cells has been formulated (Roberts and Stewart, 2005).

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Organisms residing in biofilms can be more than 500 times less susceptible to antibiotics than planktonic forms (Costerton et al, 1995). The infections associated with biofilms may appear to respond to systemic antibiotics because planktonic cells respond and symptoms are reduced, but the persistence of adherent cells leads to recurrent episodes of infection.

Two therapeutic approaches to coping with biofilms are possible: prevention and treatment. In the surgical arena the strict use of aseptic protocols and filtered laminar air in operating theatres has been shown to reduce the incidence of infection in prosthetic joint surgery (Lidwell et al, 1984). Prophylactic and peri-operative antibiotics and the incorporation of antibiotics into bone cement have proved beneficial (Bayston and Milner, 1982) but can increase the risk of selecting resistant organisms. Incorporation of antimicrobial agents into and onto implant materials has met with limited success. Catheters have been coated with antimicrobial agents such as antibiotics, antiseptics and silver and antimicrobial agents have also been impregnated into implant materials.

Many treatment methods have been proposed, but none seem ideal. Surgical debridement is thought to be essential for effective control (Costerton et al, 1995), so maggots would seem to offer advantages in removing biofilms from wounds. Enzymes have been used to treat biofilms on soft contact lenses (Johansen et al, 1997) and may be of value in treating wounds (Mertz, 2003).

Antimicrobial agents have not been found to be able to eliminate biofilms from human wounds and in vitro evidence to indicate potential efficacy is limited. Four antiseptics were tested in vitro against biofilms of *P. aeruginosa* and *Burkholderia cepacia* on Teflon chips. Results showed that 0.2% povidone-iodine effected a 6-log reduction in 10 minutes, whereas inhibition was not detected after 60 minutes exposure to 0.2% solutions of chlorhexidine gluconate, benzalkonium chloride or alkyldiaminoethylglycine hydrochloride (Kunisada et al, 1997). Sucrose in high concentration has induced adverse effects on immature *S. aureus* biofilms, especially in combination with other agents.

Biofilms cultivated on tissue culture coverslips were inhibited by levofloxacin or 10% povidone-iodine together with 70% sucrose and silver sulphadiazine or silver nitrate (Akiyama et al, 1998). In both of the above studies cell counts were used to monitor biofilm changes; another approach has been to observe

changes using microscopy. Confocal laser scanning microscopy was used to determine the effect of cadexomer iodine on *S. aureus* biofilms in vivo and in vitro (Akiayama et al, 2004). Bacterial cells surrounded by glycocalyx were located among cadexomer iodine beads, suggesting that biofilm structures were destroyed, glycocalyx was reduced by dehydration and that bacteria were killed.

Atomic force microscopy and scanning electron microscopy have been used to measure disruption of *S. epidermidis* biofilms by silver ions (Chaw et al, 2005). Whereas the viability of sessile cells was not affected by 60 minutes of contact with low concentrations of silver ions (50ppb), biofilm integrity was markedly impaired. Measurements showed that silver ion treatment reduced intermolecular forces in the EPS. The authors suggested that the binding of highly reactive silver ions to electron donor groups of matrix components prevented them forming electrostatic hydrogen bonds and divalent cation bridges that normally stabilise a biofilm matrix resulting in the destabilisation of the biofilm (Chaw et al, 2005). Although the concentration of silver ions used in this study was not inhibitory, the ability to disrupt biofilms may stimulate the development of novel strategies for clinical situations in the future.

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The principle of destabilising biofilms using an electric current has been suggested for *S. epidermidis* on surgical stainless steel (Van der Borden et al, 2004a). Newly adhered *Staphylococci* were stimulated to detach from surfaces by application of direct or block current >100 microamps, but a direct current was more effective than block current in disrupting a growing *S epidermidis* biofilm (Van der Borden et al, 2004b).

A different approach to disrupting *Pseudomonas* biofilms has recently been proposed, which relies on changing phenotype using lactoferrin (Singh et

al, 2002). Lactoferrin is a constituent of human secretions that is found in tears, mucus and human milk. It can prevent biofilm formation by sequestering iron (making it unable to react) and stimulating bacteria to adopt a specialised form of motility which precludes the formation of cell clusters and biofilms. Another option that has been considered is to search for molecules that interfere with cell-to-cell communication. The ability to prevent or confuse quorum sensing might stop the expression of biofilm and virulence genes. To date no such techniques are available for clinical use.

Conclusion

The existence of polymicrobial communities in wounds is not unexpected (Bowler et al, 2001). Although biofilms are not yet routinely characterised in human wounds, there seems to be little doubt that they have the potential to contribute to infections and persistence. Effective treatment strategies are essential and the better the understanding of the processes involved in biofilm formation, the greater the chance of developing appropriate remedies. There have been many developments in the past few years, but there is still some way to go. WUK

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Key Points

- Bacteria have evolved mechanisms for evading antimicrobials, one is the formation of a community which becomes attached to a surface — the biofilm.
- Biofilms usually comprise of several different microbial species embedded in sticky extracellular polymers that have been collectively secreted by constituent members.
- Biofilms have been associated with persistent infections in many tissues.
- Organisms residing in biofilms can be more than 500 times less susceptible to antibiotics than planktonic forms
- It is now believed that biofilms are present in many chronic wounds in humans.
- Evidence exists to show that some antimicrobial agents can disrupt biofilms in vitro and this forms the basis for treatment developments for critical colonisation of chronic wounds and/or infection.

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